LITHIUM-INDUCED APOPTOSIS IN WIL-2 LYMPHOMA CELLS

BY

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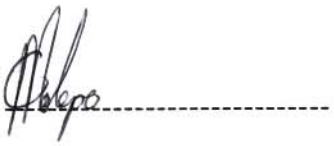
DECLARATION

I, Lefoka Calvyn Molepo, declare that the dissertation hereby submitted to the University of the North for the degree of Master of Science has not previously been submitted by me for a degree at this or any other University, that it is my own work in design and in execution, and that all material contained therein has been duly acknowledged.



Lefoka Calvyn Molepo

February 2004

DEDICATION

To my mom, Mosima Asnath "*Maphuti wa ga Somo*" Molepo. "Mothers never really die. I know that wherever you are, you are watching over me. May the good lord bless and keep you, till we meet again."

MAY YOUR SOUL REST IN PEACE

Robala ka khutšo Nare…

ACKNOWLEDGEMENTS

To the lord almighty, I'll never forget whose I am!!!

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ABBREVIATIONS

ActD- Actinomycin D

AIF- Apoptosis Inducing Factor

Apaf-1- Apoptosis Protease Activating Factor-1

BIR- Baculovirus IAP repeats

CART1- C-rich motif Associated with RING and TRAF domains 1

Caspases- Cysteinyl-ASPartate-acid-proteASES

CD- Cluster of Differentiation

CED- *Caenorhabditis elegans* Death gene

CrmA- Cytokine response modifier protein

Cyt C- Cytochrome c

DcR- Decoy Receptors

DED- Death Effector Domain

DFF- DNA Fragmentation Factor

DISC- Death-Inducing Signalling Complex

DMSO- Dimethyl Sulfoxide

DNA- Deoxyribose Nucleic Acid

DR- Death Receptors

DR3L- Death Receptor 3 Ligand

FADD- Fas Associated Death Domain

FasL- Fas Ligand

HRPO- Horseradish Peroxidase

IAPs- Inhibitor of Apoptosis Proteins

ICE- Interleukin-1-beta- Converting Enzyme

ICAD- Inhibitor of Caspase-Activated DNase

LARD- Lymphocyte Associated Receptor of Death

MRC- Mitochondrial Respiration Chains

NF-κβ- nuclear transcription factor-κβ

NIK- Nuclear factor- κβ Inducing Kinase

PARP- Poly (ADP-Ribose) Polymerase

PCR- Polymerase Chain Reaction

PMSF- Phenylmethylsulfonyl

RAIDD- RIP-Associated ICH-1/CED-3-homologous protein with DD

RIP- Receptor Interacting Protein

RNA- Ribose Nucleic Acids

SDS- Sodium Dodecyl Sulphate

Smac- (also known as **Diablo**, Direct IAP Binding protein with low pI)- Second mitochondria-derived activator of caspase

TBST- Tris-Buffered Saline with Tween

TNF-α- Tumor Necrosis Factor Alpha

TNF-R- Tumor Necrosis Factor Receptor

TRADD- TNF Receptor Associated Death Domain

TRAF- TNF Receptor Associated Factor

TRAIL- TNF-Related Apoptosis Inducing Ligand

TRAMP- TNF-receptor-Related Apoptosis-Mediating Protein

TUNEL- Terminal deoxynucleotidyl-Transferase-mediated dUTP Nick-End-Labeling

UV- Ultra Violet

v-FLIP- viral FLICE-inhibitory protein

XIAP- X-Linked Inhibitor of Apoptosis Proteins

ABSTRACT

Lithium, a drug used widely in the treatment of mood disorders, has been shown to induce apoptosis in various tumor cell lines. At concentrations below 10 mM, lithium is known to enhance proliferation of HL-60 cells, whereas at concentrations of 10 mM and above lithium was shown to be toxic to these cells. The mechanism by which lithium induces its cytotoxicity is still under investigation.

The objective of this study was to confirm the apoptotic effect of lithium on Wil-2 cells and elucidate possible mechanisms involved in the process. Lithium was found to be cytotoxic to the Wil-2 cells in a time and concentration dependent manner with concentrations as low as 1 mM inducing cell death. Its cytotoxicity was associated with apoptosis. The susceptibility of Wil-2 cells to undergo lithium-induced apoptosis was analyzed using terminal deoxynucleotidyl-transferase-mediated dUTP nick-end-labeling (TUNEL) and DNA ladder assays. Lithium induced DNA laddering into larger fragments in treated Wil-2 cells. TUNEL assay revealed that lithium caused DNA strand breaks, and therefore apoptosis.

Most forms of apoptosis are mediated by the activation and therefore proteolytic cleavage of caspases. Caspase activation during lithium-induced apoptosis was assessed by measuring the activities of caspase-3, caspase-8, and caspase-9. Significant caspase activities were observed in all the three caspases after 24 hours of treatment with increasing lithium concentrations, as compared with untreated control cells. Caspase-3 and caspase-8 activation was substantiated by Western immunoblot analysis of protein isolates from lithium treated cells. By 24 hours, caspase activation had occurred as demonstrated by the loss of pro-caspase-8, and the appearance of the processed subunit of caspase-3.

Apoptosis has been shown to be controlled by a number of cell cycle and apoptosis related proteins. In this study, the effects of lithium on the expression of Bax were investigated. Western blotting revealed that Bax, a protein that promotes apoptosis, was upregulated in Wil-2 cells exposed to lithium. As the concentration of lithium increased from 0 – 20 mM, the pro-apoptotic protein increased proportionally.

TNF-α, a cytokine shown to have cytotoxic effects on transformed cell lines, was also used in this study. The main aim was to determine if it can work in synergy with lithium in inducing apoptosis in Wil-2 cells. Previous studies with other cell lines have indicated that treatment with lithium and TNF-α led to induction of apoptosis in the cells. TNF-α on its own induced unremarkable cell death and apoptosis in Wil-2 cells. It failed to induce high molecular weight fragmentation of DNA, yet induced DNA strand breaks following TUNEL assay, indicating that the cells are sensitive to its cytotoxic effects. A combination of TNF-α and lithium enhanced cell death in Wil-2 cells compared to the extent of cell death caused by either lithium or TNF-α when used alone.

This led to the conclusion that lithium induced apoptosis in Wil-2 cells through a pathway that involves Bax and the activation of both caspase-3 and caspase-8. In addition, the study suggests that lithium and TNF-α can possibly work in synergy to enhance apoptosis in Wil-2 cells.

CHAPTER 1

1. INTRODUCTION

1.1. LITHIUM

Lithium, a group one alkali metal element, has been used in the treatment of mania for several decades (Birch *et al.*, 1999). Manic depression, characterized by dramatic swings in moods, can be effectively controlled by maintaining serum levels of lithium ion of ±1 mM (Berridge *et al.*, 1989). But, the underlying mechanism for lithium action in psychotherapy is still unclear.

Unfortunately, the administration of lithium is not without side effects. This may include nausea, diarrhea, polyuria, altered glomerular filtration rates, coma, death, and visual disturbances in patients prescribed with lithium for psychiatric disorders (Lydiard and Gelenberg, 1982; Armond, 1998). In addition, lithium has a narrow therapeutic window (Reisberg and Gershorn, 1979). Clinical effects of lithium occur at a minimum serum concentration of 0.4 mM, whereas at 1.5 mM and above, it is toxic (Julius and Breaner, 1987).

Other clinical uses of lithium have been suggested. Lithium has been shown to increase white blood cell production and initiate leukocytosis (reviewed by Becker and Gallicchio, 1997). It is finding potential roles in immunology, oncology, virology, dermatology, and in physiological measurements (Horrobin, 1990; Gallicchio *et al.*, 1994; Birch and Srinivasan, 1998).

Researchers have devised a variety of strategies in order to understand the mode of action of lithium effects. It was established that lithium causes a wide variety of metabolic effects leading to mitogenicity. It influences morphogenesis in Xenopus oocyte embryos and Drosophilla (Bursa and Gimlich, 1989), enhances the production of white blood cells in

patients receiving lithium therapy, specifically the production of granulocytes (reviewed in Becker and Gallicchio, 1997), and modulates the levels of cytokines which regulate processes such as cell differentiation and proliferation (Sokoloski *et al.*, 1993; Davies and Garrod, 1995).

The mode of lithium action has been investigated *in vitro* in cultured cells. Studies by Becker and Tyobeka (1990) have shown that lithium induces proliferation of HL-60 promyelocytic leukemia cells at low concentrations [0 - 5 mM]. Such proliferation capabilities have also been described for other cell culture systems and are prominent particularly in the haematopoietic system (Becker and Gallicchio, 1996; reviewed by Becker and Tyobeka, 1996).

Lithium has also been shown to be toxic to HL-60 promyelocytes. Concentrations of lithium above 10 mM were shown to be toxic to these cells (Becker and Tyobeka, 1996). It was further reported by Madiehe *et al.* (1995) that at 10 mM and above lithium induces cell death in HL-60's in a time and concentration dependent manner. On the other hand, lithium has also been shown to have neuroprotective activities in an neuronal environment (D'Mello *et al.*, 1994).

Lucas *et al.* (2000) have shown that Porcine Kidney epithelial cells (PK(15)) exposed to increasing concentrations of lithium undergo cell death in a dose dependent manner. Similar studies by Mampuru (2000) using bone marrow stromal (KLT2) cells, showed that exposure of these cells to 0 mM -10 mM lithium stimulated cell proliferation and, as with HL-60 cells, concentrations above 10 mM were cytotoxic.

More recently, lithium was shown to induce cell death in B-cell lymphomas, particularly Wil-2 and Raji cells, at concentrations as low as 2.5 mM (Mukhufi, 2000). However, the exact mechanism of lithium-induced apoptosis still remains to be elucidated.

1.2. TNF-α

Tumor Necrosis Factor-α is a 17 kD pleiotropic cytokine that is produced by a large number of cell types and mediates a wide variety of inflammatory and immunologic responses (Beutler and Cerami, 1988; Fiers, 1991). The biological actions of TNF-α have been studied for over a decade. These actions occur in many cell types and include inflammation, mitogenesis, differentiation and anti-tumor immunity (Sethi *et al.*, 2000).

Among all the known physiological inducers of apoptosis in mammalian cells, TNF-α is reported as the most potent and well studied. TNF-α exhibits different responses to a variety of cell types by killing some and inducing proliferation of others. This heterogeneity has been noticed even within the same cell types (Burow *et al.*, 1998). TNF-α is therefore an interesting example of activation of two conflicting pathways by a single cytokine. Many other members of the TNF-α superfamily also induce apoptosis, including lymphotoxin, Fas ligand, TRAIL (TNF-related apoptosis inducing ligand), and DR3L (Death receptor 3 ligand) (Rath and Aggarwal, 1999).

TNF-α exerts its effects by binding to two cell surface receptors, TNF-RI and TNF-RII. Both receptors are present on a wide variety of cell types (Vandenabeele *et al.*, 1995).

Prominent among the varied physiological effects of the cytokine TNF-α is its ability to act as a cytotoxin and induce apoptotic or necrotic cell death (Baker and Reddy, 1998). Although TNF-α cytotoxicity has been widely investigated in the context of its potential as an anti-neoplastic agent, some studies demonstrated that it may also induce death in normal tissue undergoing injury or inflammation (Bradham, 1998). TNF-α has cytotoxic effects on some transformed cells but the majority of human tumor cells and normal tissues are resistant to TNF-α induced cell death (Sugarman *et al.*, 1985; Wallach, 1997). Non-transformed cells, such as hepatocytes, are normally resistant to the TNF-α induced cytotoxicity. This resistance is thought to depend on the ability of TNF-α signalling to upregulate a protective cellular gene(s). This conclusion is based on the finding that inhibition of RNA synthesis by ActD or of protein synthesis by cyclohexamide sensitizes nonhepatic cells (Kull and Cuatrecasas,

1981) and hepatocytes (Leist *et al.*, 1997; Xu *et al.*, 1998) to TNF-α-induced cell death. Recent investigations have demonstrated that cellular activation of the transcription factor NF-κB is critical for the induction of resistance to TNF-α toxicity (Jones *et al.*, 2000).

TNF-α has direct cytotoxic effects on several transformed cells as well as several indirect effects on the host cells. These indirect effects limit the efficacy of TNF-α as an anticancer agent. Most tumors required high doses of TNF-α, which are quite toxic to the host cells. Early preclinical trials using TNF-α as an anti-tumor agent have thus been disappointing, mainly because the doses were too low to observe efficacy (Beyaert *et al.*, 1989).

Combination treatments that allow the use of lower TNF-α doses were seen as an alternative strategy. Lithium, well known for its use in manic depression, was one of the agents that were used in combination with TNF-α. Lithium proved to considerably increase the direct cytotoxic activity of TNF-α on tumor cells *in vitro* and *in vivo*, irrespective of the type of cell death (Beyaert *et al.*, 1989). More evidence came from *in vitro* cytotoxicity studies on murine fibroblasts. Neither TNF nor lithium alone was toxic to these cells within 3 days but their combination resulted in considerable cell death within 2 days of treatment (Beyaert *et al.*, 1991). Most recently Schotte *et al.* (2001) demonstrated that lithium sensitises rhabdomyosarcoma Kym37E4 cells to TNF-induced apoptosis in an NF-κβ independent way. Yet, the underlying mechanism for TNF/ lithium synergism is still unclear.

The question of how TNF-α signals are targeted to the different intracellular compartments is of fundamental biological significance. A study by Schutze *et al.* (1999) suggested a role of TNF-RI internalisation for the activation of TNF-RI death domain signalling including those leading to apoptosis. Wallach *et al.* (1998) also characterised the molecular basis of TNF-α and showed that it involves a group of adaptor proteins containing a conserved motif known as a death domain and a family of proteases known to play a central role in apoptosis (caspases) which themselves require proteolytic activation.

1.3. APOPTOSIS

Extensive research in the last few years has revealed that cell death, whether at the single cell level, the tissue/organ level, or the organism level, is as important to life as survival. It is a process of replacement, renewal, replenishment, regeneration, and revitalization of the tissues (Zimmermann *et al.*, 2001). Cell death keeps the cellular balance in check by ridding organisms of unwanted cells, ensuring normal development and protecting against tumor formation and viral infection. Yet increasingly, cell death is being implicated in a number of disorders, including cancers, autoimmune diseases, and neurodegenerative diseases (Nakagawa *et al.*, 2000; Sauter *et al.*, 2000; Johnston, 2002). Biologically cells die either by necrosis or by apoptosis (Figure 1.1).

Apoptosis is an essential physiological process of cell death that plays a pivotal role during normal development and throughout an organism's life. It occurs in response to a variety of physiological and pathological stimuli (Ekert and Vaux, 1997; Zimmermann, 2001) derived from either the extracellular or intracellular milieu (Wyllie, 1987). It is a biological suicide mechanism that is essential in physiological process such as maturation of the immune system, or development of the nervous system. The main characteristics are: organised lysis of DNA before actual cell death, the DNA material is collected within "apoptotic bodies", and cell shrinkage due to condensation of cytoplasm (Wyllie, 1987).

Cells undergoing apoptosis exhibit a series of characteristic morphological changes, including plasma membrane blebbing, cell body shrinkage, and formation of membrane-bound apoptotic bodies, which *in vivo* are quickly engulfed by neighboring healthy cells (Kerr *et al.*, 1972). Thus, during apoptosis, intracellular contents are not released and potentially harmful inflammatory responses are prevented. Apoptosis is also accompanied by certain biochemical changes, notably the appearance of discrete DNA fragments on conventional gel electrophoresis (due to cleavage between nucleosomes), the flipping of phosphatidylserine from the inner leaflet to the outer leaflet of the plasma membrane, and limited cleavage of various cellular proteins (Ekert and Vaux; 1997).

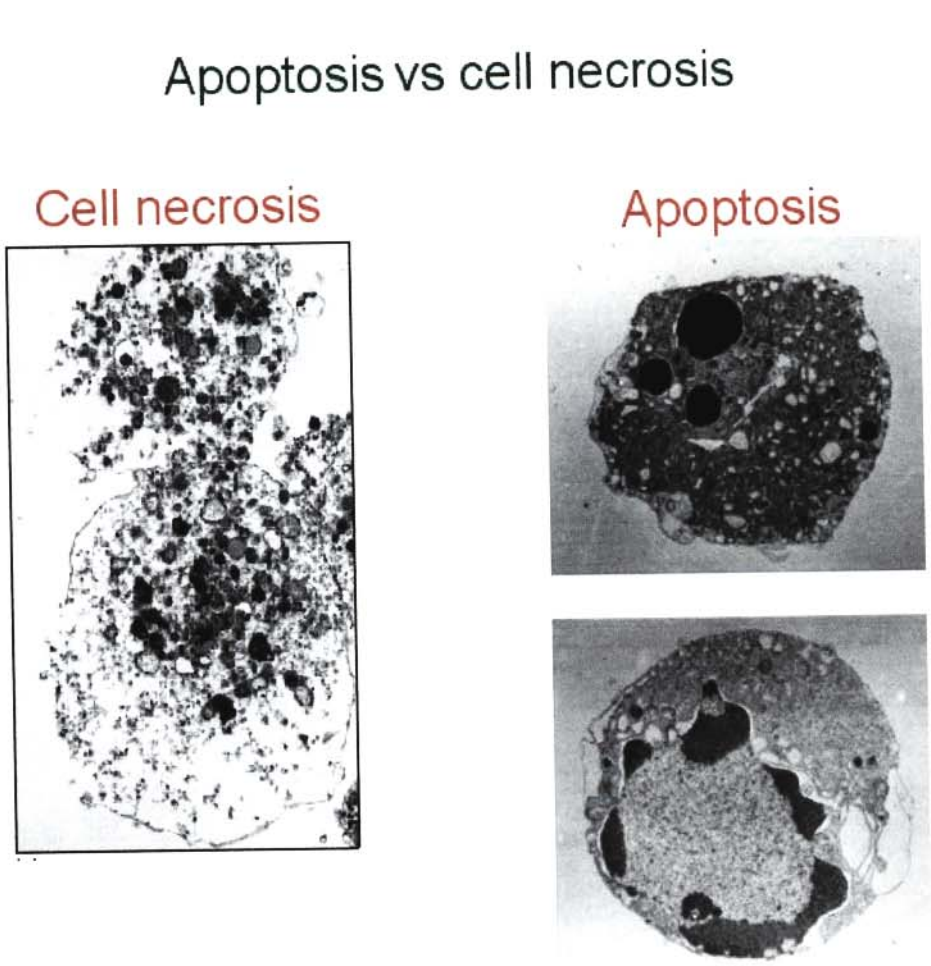


Figure 1.1: Modes of cell death: Cells dying through apoptosis or necrosis. (www.welc.cam.ac.uk/~jacksonlab/M7_Raff/sld002.htm, 2002)

The process of apoptosis may be divided into the following stages: the stimuli triggering a cell death response; the pathway by which the message is transduced to the cell; and the effector mechanism that implement the death programmed. A cell death response may be triggered by different stimulants but the pathways converge to the same evolutionally conserved effector mechanism, of which the effector molecules are a family of cysteine proteases. Upon activation the effector molecules directly or indirectly cause the morphological and biochemical changes characteristic of apoptosis (Ekert and Vaux, 1997).

1.3.1. ACTIVATION OF APOPTOSIS

In the last decade, much progress has been made towards elucidating the various signal transduction pathways that can ultimately lead to a cell's demise. Based on this information, many apoptotic cascades have been described, such as intrinsic and extrinsic, mitochondrial and death receptor (DR), p53-dependent and -independent, and caspase-dependent and -independent pathways in association with initiation, commitment, and execution phases. While such categorization is undoubtedly of value, it has become increasingly apparent that apoptosis is not a series of clearly defined pathways, but rather, a multitude of highly regulated, interconnected pathways (Ashe and Berry, 2003).

Two relatively well-characterised apoptotic pathways have been identified. The first pathway is mediated by death receptors, such as Fas or Tumor Necrosis Factor-α (Nagata, 1997). In the second pathway, Cyt C is released from the mitochondria to the cytoplasm in cells exposed to chemotherapeutic drugs, UV irradiation, growth factor withdrawal, or ligation of Fas and TNF receptors (Kluck *et al.*, 1997; Yang *et al.*, 1997; Bossy-Wetzel *et al.*, 1998).

Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific ligands. They play an important role in apoptosis and can activate a cascade of cysteine proteases within seconds of ligand binding. Induction of apoptosis via this mechanism is therefore very rapid. Death receptors belong to the tumour necrosis factor (TNF) gene superfamily and generally can have several functions other than initiating apoptosis (www.sghms.ac.uk/depts/Immunology/%7/apoptosis/receptors.html, 2001). The best characterised of the death receptors are CD95 (or Fas), TNFR-1 and the TRAIL (TNF-related apoptosis inducing ligand) receptors DR4 and DR5 (also called Apo2, TRAIL-R2) (Figure 1.2) (www.sghms.ac.uk/depts/immunology/~dash/apoptosis/tnfr, 2001). The ligands that activate these receptors are structurally related molecules that belong to the TNF gene superfamily (Ashkenazi, and Dixit, 1998).

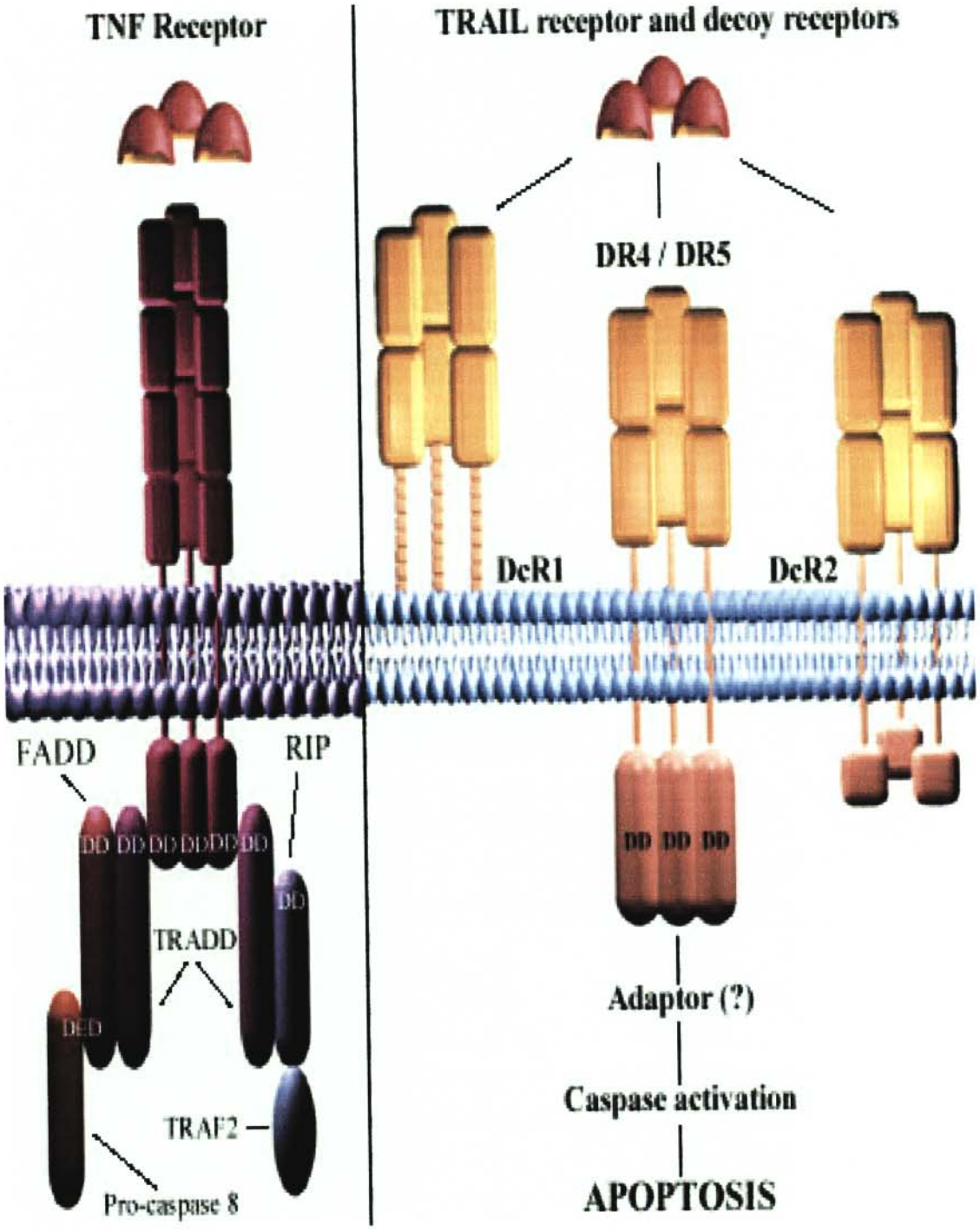


Figure 1.2: Activation of apoptosis through death receptors (www.sghms.ac.uk/depts/immunology/~dash/apoptosis/signaling.html, 2001).

Additional receptors are avian CAR1; death receptor 3 (DR3; also called Apo3, TRAMP, or LARD. The ligands that activate these receptors are structurally related molecules that belong to the TNF gene superfamily. CD95 ligand binds to CD95, and TNF and lymphotoxin bind to TNF-R1 (Ashkenazi and Dixit, 1998).

A protein-protein interaction motif, called a "death domain", is present in the intracellular portion of several TNF-R family members (e.g. CD95, TNF-R1, DR3) and is essential for apoptosis signalling by these receptors (reviewed by Ashkenazi and Dixit, 1998)

1.3.1.1. Fas Signalling/ Activation Pathway

Fas, also known as CD95, belong to a family of structurally related molecules and were the first member of this superfamily to be described in terms of its function in apoptosis (Schotte and Beyaert, 1999). Other members of this family include TNF receptors (TNF-R1 and -R2), CD40, and Apo-2 (Ekert and Vaux, 1997). Ligation of CD95 by CD95 ligand or treatment with an agonistic antibody results in receptor aggregation. The cytoplasmic domain of CD95 bears a motif termed "death domain" that upon ligation allows it to bind the death domain of cytoplasmic proteins FADD and RIP (Ekert and Vaux, 1997; Wallach *et al.*, 1998; Yeh *et al.*, 1998). FADD has a carboxyl terminal death domain and a "death effector domain" that allows it to associate with the caspases (Wallach *et al.*, 1997; Strasser and Newton, 1999), thereby promoting the processing and hence the activation of the caspases which then initiates cell death (Wallach, 1997; Wallach *et al.*, 1998).

1.3.1.2. TNF Signalling/ Activation Pathway

TNF, like CD95, belongs to an emerging family of structurally related molecules called the TNF receptor superfamily (Schultze-Osthoff *et al.*, 1998). TNF may signal through 2 receptors, which are TNF-R1, also called p55, and TNF-R2, also called p75 (Ekert and Vaux, 1997). Most of the biological activities of TNF including programmed cell death and antiviral activity are mediated by TNF-R1, while TNF-R2 has been demonstrated particularly in T- lymphocytes (Schultze-Osthoff *et al.*, 1998).

Binding of TNF-α to TNF-R1 induces Death-Inducing Signalling Complex (DISC) formation. TNF-induced DISC formation involves the recruitment of caspase-8, RIP, and FADD, but also recruits TNF receptor-associated death domain (Yuan, 1997). Upon ligation by appropriate stimulus, TNF-R1 death domains associate and bind to TRADD through its (TRADD's) death domain (Ashkenazi and Dixit, 1998). Association of TRADD leads to recruitment of FADD, which is thought to lie upstream of the caspase cascade and initiation of apoptosis (Figure 1.3) (Madge *et al.*, 1999). Also recruited by FADD are several signalling molecules such as the protein kinase RIP (Receptor-Interacting Protein) and TRAF-2 (TNF-R Associated Factor-2) leading to the activation of apoptotic suppressive proteins (Ashkenazi and Dixit, 1998; Madge *et al.*, 1999).

ACTIVATION PATHWAY

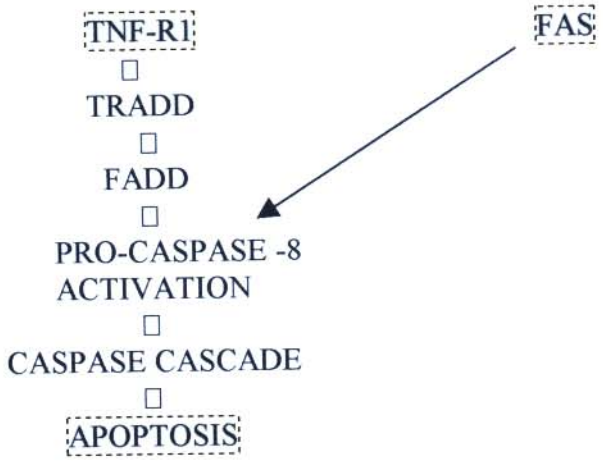


Figure 1.3: Proposed model of death receptor-mediated caspase-8 activation after TNF-α or Fas ligand stimulation (Schotte and Beyaert, 1999).

1.3.1.3. TRAIL signalling

TRAIL (TNF-related apoptosis inducing ligand) is a recently identified type II integral membrane protein belonging to the tumor necrosis factor (TNF) family that induces apoptosis in various tumor cell lines. Although it is a TNF family member, it has some notable differences from TNF and FasL. Unlike Fas, whose expression is limited to certain

tissues. TRAIL receptors are widely expressed, thus most tissues and cell types may be TRAIL targets (Wiley *et al.*, 1995; Pitti *et al.*, 1996). Hence, in contrast to FasL or agonistic Fas antibody, which induces massive liver damage when introduced systemically (Ogasawara *et al.*, 1993), TRAIL exhibited no detectable cytoxicity in mice (Walczak *et al.*, 1999) and monkeys (Ashkenazi *et al.*, 1999). TRAIL has a unique selectivity for triggering apoptosis in tumor cells and may be less active against normal cells (Wiley *et al.*, 1995; Pitti *et al.*, 1996; Kim *et al.*, 2001). This selectivity may be a function of the tissue expression profiles of DcR1 and DcR2, DcRs that inhibit the apoptotic signaling of TRAIL (Kim *et al.*, 2001). These features have focused considerable attention on TRAIL as a potential therapy to treat cancers.

In a number of ways, TRAIL is similar in action to CD95. Four DRs have been shown to bind specifically to TRAIL: DR4, DR5, DcR1, and DcR2 (Chaudhary *et al.*, 1997; Marsters *et al.*, 1997; Sheridan *et al.*, 1997). Binding of TRAIL to its receptors DR4 or DR5 triggers rapid apoptosis in many cells, however unlike CD95, its expression has been shown to be constitutive in many tissues. The DR4 and DR5 receptors contain death domains in their intracellular domain, but as yet no adapter molecule (such as FADD or TRADD) has been identified that associates with the receptor to initiate apoptosis. Work in FADD-deficient mice has indicated that FADD is not essential for triggering apoptosis via these receptors.

Since DR4 and DR5 mRNA has been shown to be expressed constitutively in several tissues, it has been suggested that there are mechanisms that protect cells from apoptosis. One possible mechanism of protection is based on a set of decoy receptors that compete for binding of TRAIL with the DR4 and DR5 receptors. The decoy receptors are called DcR1 and DcR2 (Figure 1.4). Both of these receptors are capable of competing with DR4 or DR5 receptors for binding to the ligand (TRAIL), however ligation of these receptors does not initiate apoptosis since DcR1 does not possess a cytoplasmic domain, while DcR2 has a truncated death domain lacking 4 out of 6 amino acids essential for recruiting adapter proteins (www.sghms.ac.uk/depts/immunology/~dash/images/trail.jpg, 2002). Selectivity may also be conferred by p53-mediated induction of DR5 (Kim *et al.*, 2001).

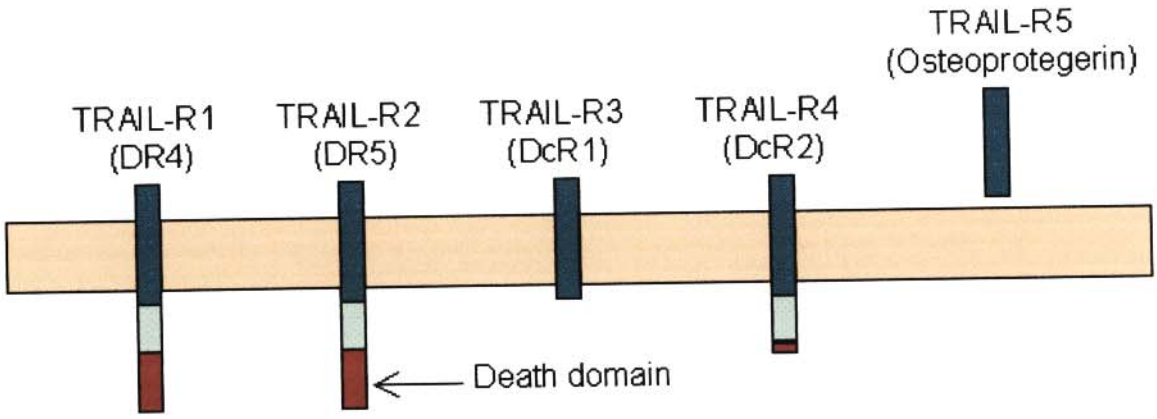


Figure 1.4: TRAIL receptor signaling.

Schematic drawing of trail receptors, which can be divided into two categories: **Death receptors:** TRAIL-R1 and TRAIL-R2 contain the death domain, capable of inducing apoptosis.

Decoy receptors: TRAIL-R3 and TRAIL-R5 lack the death domain while TRAIL-R4 contains a truncated non-functional death domain. These three receptors can bind to TRAIL, but cannot induce apoptosis. TRAIL-R5 is secreted to the extracellular fluid. All other receptors are transmembrane proteins (www.web-books.com/MoBio/Free/Ch6G1.htm, 2002).

Meng *et al.* (2000) demonstrated enhanced sensitivity to TRAIL following p53 expression; an effect correlated with DR5 expression. In accordance with this explanation, DR4 is also upregulated by p53; however, the anti-apoptotic DcRs are also induced by p53; thus, the specific role of p53 regulation of TRAIL receptors is questionable (Meng *et al.*, 2000; Guan *et al.*, 2001).

1.3.2. ADAPTOR PROTEINS

To date four adaptor proteins that take part in the signalling by TNF and Fas receptors have been identified. They are FADD, TRADD, TRAF, and RIP.

1.3.2.1. FADD

Activation of CD95 or TNF receptors by either CD95 ligand (CD95L or FasL) or TNF ligand, respectively, or treatment with an agonistic antibody results in receptor aggregation and the rapid recruitment of FADD (Fas-Associated Death Domain protein). The human FADD proteins contain 208 amino acids and have a predicted mass of 23 kD protein with a death domain (Boldin *et al.*, 1995; Zhang and Winoto, 1996). The interaction of FADD and Fas through their COOH-terminal death domains unmasks the NH-terminal death effector domain (DED) of FADD, allowing it to recruit caspase-8 to the Fas signalling complex and thereby activating a cysteine protease cascade leading to cell death (Muzio *et al.*, 1996).

FADD contains a DD that interacts with a similar domain on Fas and also contains a DED that binds to the DEDs of procaspase-8 (Ashkenazi and Dixit, 1998). Fas activation stimulates binding of the receptor's DD to the corresponding domain in FADD, which in turn recruits procaspase-8 by a homophilic interaction involving DEDs. Subsequent oligomerization then promotes procaspase-8 autoactivation (Muzio *et al.*, 1996; Boldin *et al.*, 1996; Ashekenazi and Dixit, 1998).

FADD couples the TNF-R1-TRADD complex to activation of caspase-8, thereby initiating apoptosis (Chinnaiyan *et al.*, 1996). Cells from FADD-knockout mice show resistance to TNF-induced apoptosis, demonstrating an obligatory role of FADD in this response (Yeh *et al.*, 1998).

1.3.2.2. TRADD

A similar death domain-containing protein, TRADD, binds to TNF-R1 (Hsu *et al.*, 1995). But unlike FADD/MORT1, TRADD does not contain a death effector domain, and its death domain is responsible for mediating apoptosis. TRADD is a 34 kD protein that interacts specifically with TNFR1 (Hsu *et al.*, 1995). The binding of TNF to TNF-R1 leads to the trimerization of TNF-R1 and the recruitment of TNF-R1-associated death domain protein (TRADD) into the receptor complex. Then TRADD serves as a platform to recruit other

proteins into the complex. Three of these adaptor proteins, FADD/MORT1 (Fas-associated death domain protein), TRAF2 (TNFR-associated factor 2), and RIP (the death domain kinase) have been shown to interact with TRADD directly (Hsu *et al.*, 1995; Rothe *et al.*, 1995).

This apparent discrepancy between FADD and TRADD was resolved by the finding that TRADD binds to FADD via interactions between their death domains (Hsu *et al.*, 1996b). These results suggest that Fas and TNF-R1 use FADD as a common signal transducer and share the signalling machinery downstream of FADD (Hsu *et al.*, 1996a).

1.3.2.3. TRAF

The TRAF family proteins, originally described by Goeddel *et al.* (Rothe *et al.*, 1994), are signal-transducing adapter proteins that interact with the cytosolic domains of tumor necrosis factor family receptors, linking them to downstream signalling pathways (Inoue *et al.*, 2000). Thus far, six TRAF family proteins have been described in mammals, TRAF1-6 (Sato *et al.*, 1995; Cao *et al.*, 1996; Ishida *et al.*, 1996; Nakano *et al.*, 1996). TRAF 1 and TRAF2 were initially identified based on their interaction with the cytoplasmic domain of TNFR2 (Rothe *et al.*, 1994). TRAF1 and TRAF2 form homo- and heteromeric complexes with each other. Importantly, TRAF2, but not TRAF1 binds directly to the cytoplasmic domain of TNFR2 (Rothe *et al.*, 1995). TRAF2, TRAF5, and TRAF6 are known to activate protein kinases NIK (Malinin *et al.*, 1997), and RIP2 (McCarthy and Dixit, 1998) that are involved in inducing the transcription factor NFκB. TRAF1 (but not TRAF6) was shown to be cleaved by certain caspases *in vitro* and during TNF-α- and Fas-induced-apoptosis *in vivo* (Leo *et al.*, 2000).

TRAF1 and TRAF2 were cloned by biochemical characterization of intracellular factors that are associated with TNFRII (Rothe *et al.*, 1994). TRAF3 (LAP-1, CD40bp, CRAF-1) was described independently as a cytoplasmic factor that interacts with CD40 and LMP-1 (Hu *et al.*, 1994; Cheng *et al.*, 1995; Mosialos *et al.*, 1995; Sato *et al.*, 1995). TRAF4 (CART1, C-rich motif associated with RING and TRAF domains 1) was isolated by differential screening of a cDNA library of lymph nodes that contained metastatic tumor cells (Régnier *et al.*,

1995). TRAF5 was identified by utilization of degenerate PCR primers that were homologous to a highly conserved region at the carboxy-terminal end of TRAF proteins (TRAF-C domain) and independently in a yeast two-hybrid screen (Ishida *et al.*, 1996; Nakano *et al.*, 1996). TRAF6 was isolated independently by the screening of an EST expression library and by utilizing CD40 as bait for a yeast two-hybrid screen (Cao *et al.*, 1996; Ishida *et al.*, 1996).

Several of the TRAFs play roles in regulating apoptosis (Baker and Reddy, 1998; Arch *et al.*, 1998). The best mechanism for apoptosis regulation by TRAFs is through NFkB, although contributions by JNK-activating kinases may also play a role in some circumstances (Wong *et al.*, 1999).

1.3.2.4. RIP (Receptor Interacting Protein)

RIP is an accessory protein with a death domain and a serine / threonine kinase activity which is involved in both induction and inhibition of apoptosis. It was originally identified as a Fas-binding protein, but preferentially binds to TRADD (Hsu *et al.*, 1996a). RIP can form a complex with CD95 and a second adaptor molecule referred to as RAIDD. This complex recruits proenzymatic caspase-2 (ICH-1), which thereby is converted into the active form and then triggers the caspase cascade.

In addition, RIP can also form a complex with TNF receptor 1 (TNFR1) and another second adaptor molecule referred to as TRADD. Since RIP binds only weakly to TNFR1 but strongly to TRADD, this adaptor molecule is probably required to recruit RIP to the TNFR1 complex. The functional relevance of the TNFR1 / TRADD / RIP complex for the induction and the regulation of apoptosis is not completely understood. There is compelling evidence that this complex can induce apoptosis in a way that does not require the kinase activity of RIP and which can be blocked by the caspase inhibitor CrmA. Hence, binding of TRADD and RIP to TNFR1 either directly or indirectly activates the caspase cascade. On the other hand, the same complex can activate NF-kappa β via another protein referred to as TRAF 2. This activation, which requires the kinase activity of RIP, protects against TNF-induced apoptosis by the subsequent expression of survival factors. How these different activities of

RIP, which have been defined in *in vitro* systems, relate to each other remains to be determined (Lin *et al*, 1999).

1.3.3. APOPTOSIS EFFECTOR MECHANISMS

Diverse group of molecules are involved in the apoptosis pathway. One set of mediators implicated in apoptosis belong to the aspartate-specific cysteinyl proteases or **caspases** (Kothakota *et al.* 1997). Caspases, Cysteinyl-aspartate-acid-proteases, are a family of cysteine-dependent aspartate-directed proteases, that play critical roles in initiation and execution of apoptosis (Alnemri *et al.*, 1996).

Caspases exist as inactive proenzymes composed of a prodomain, a large protease subunit (which contains the active-site cysteine residue), and a small protease subunit. Initiators have long N-terminal prodomain that can couple them to extracellular receptors via adaptor molecules, allowing them to respond to the extracellular stimuli (Thornberry and Lazenbik, 1998). They are the key effector proteins that are produced in inactive forms and need to be cleaved at aspartate residues in order to be activated (Figure 1.5). Once activated they also cleave their substrate, which are either their own precursors or other cysteine proteases, at aspartate residues (Schotte and Beyaert, 1999; Stennicke and Salvesen, 1999; Wolf, 1999). Sequential activation of caspases plays a central role in the execution-phase of cell apoptosis.

A key to apoptosis is the discovery in many laboratories that, irrespective of the lethal stimulus, death results in the same apoptotic morphology that includes cell and organelle dismantling and packaging, DNA cleavage (to nucleosome-sized fragments), and caspase mediated cleavage of the same cellular proteins (Salvesen and Dixit, 1997).

A relationship between apoptosis and caspases was made in 1993 when Yuan *et al.* reported that caspase-1 is related to a *Caenorhabditis elegans* death gene, CED-3. It is their work that established proteases, particularly caspases, as effectors of apoptosis (Thornberry and Lazenbik, 1998).

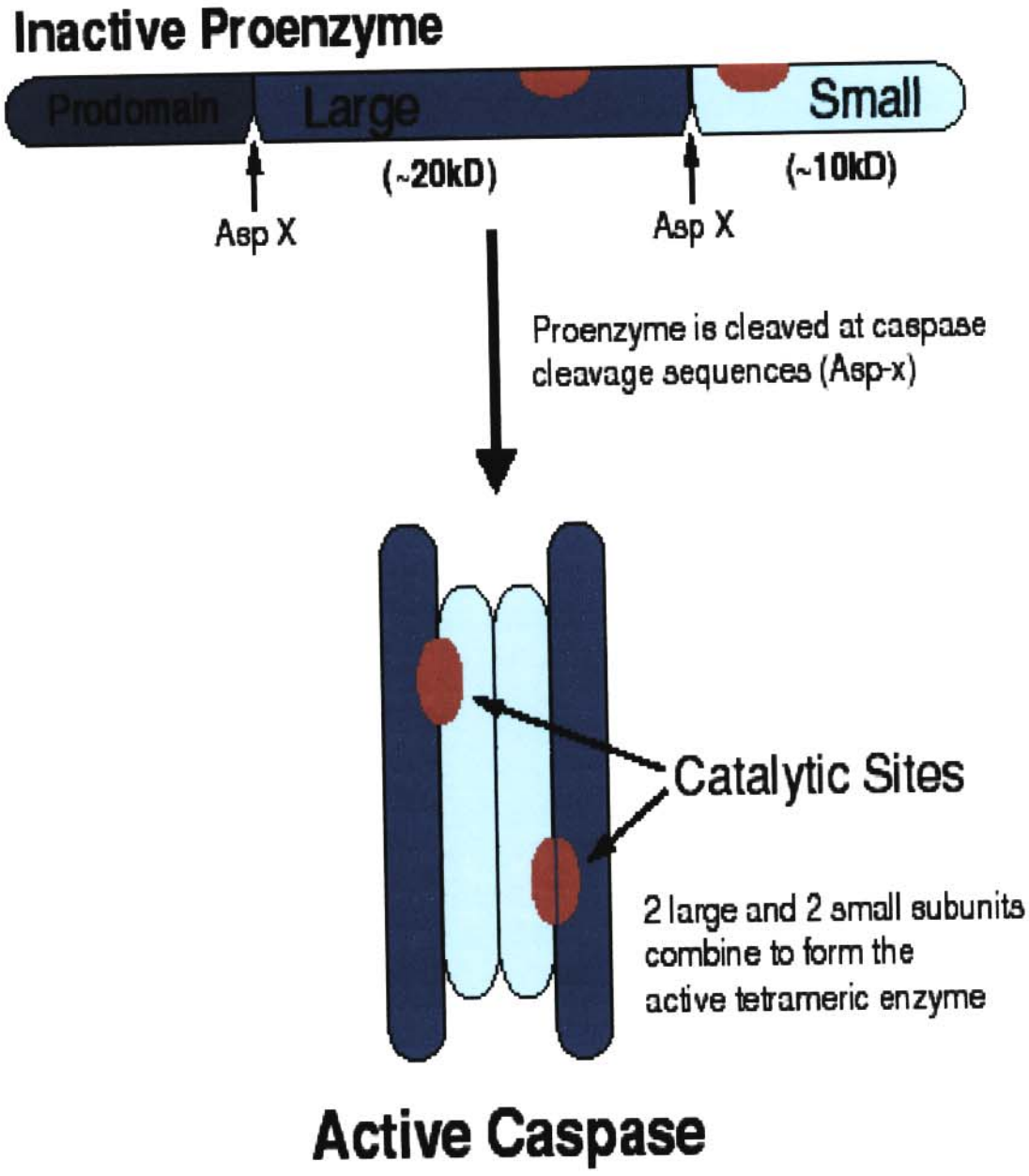


Figure 1.5: Caspase activation (http://cord.ubc.ca/~steeves /~chris/ cspstrfn.htm, 2001)

In no other area is progress more evident than in the field of apoptosis, where the basic framework of this process has been defined at the molecular level over a period of 10 years (Figure 1.6) (Thornberry, 1999). Following the latter's publication, advances have been made in the apoptotic field.

1989 * Identification of pro-IL-1B processing activity (Black *et al.*, 1989)

1990

1991

1992 * Purification and cloning of caspase-1, ICE/ interleukin- 1B converting enzyme (Cerretti *et al.*, 1992)

* First viral caspase inhibitor (CrmA) identified (Ray *et al.*, 1992)

1993 * CED-3 found to be closely related to caspase-1

1994 * Solution of first caspase three-dimensional structure

* Identification of first mammalian caspase homologue, Caspase-12

* Identification of first apoptotic caspase substrate, PARP.

1995 * First caspase knockout, caspase-1

* caspase-3 implicated as central mediator of apoptosis.

* Granzyme B found to activate caspases in CTL-mediated death

1996 * Caspase-3 knockout described; established caspases to be essential for mammalian apoptosis

* Fas/FADD/caspase-8 signalling pathway identified mammalian caspase inhibitor, XIAP

* Role of caspases in DNA degradation elucidated, DFF/CAD-ICAD

* Identification of first decoy caspase

1998 * Caspase-mediated cleavage of Bid established as link between Fas and mitochondrial signalling pathways

1999 * First identification of a caspase mutation in a human genetic disease, caspase-10

Figure 1.6: Milestones in caspase research (Thornberry, 1999).

Thus far, 14 caspase family members have been identified. Of these, eleven have been cloned in humans, with caspase-11, -12, and -14 only conclusively identified in the mouse (Van Den Craen *et al.*, 1997; Thornberry and Lazenbik, 1998; Slee *et al.*, 1999) (Table 1). Some of them, such as caspase-8, mediate apoptotic signals after the activation of death receptors. Others, such as caspase-9, are part of the apoptosome and play a role in signal transduction

after mitochondrial damage. Recently, an endoplasmic-reticulum-specific pathway of apoptosis has been described that is mediated by caspase-12 (Wieder *et al.*, 2001).

Table 1. The caspases (Ashe and Berry, 2003)

Caspase	Alternate name(s)	Physiological role	Human homolog
1	ICE	cytokine activation	yes
2	Nedd2, ICH-1	apoptosis initiator	yes
3	CPP32, Yama, apopain	apoptosis effector	yes
4	ICE_{rel}-II, TX, ICH-2	cytokine activation	yes
5	ICE_{rel}-III, TY	cytokine activation	yes
6	Mch2	apoptosis effector	yes
7	Mch3, ICE-LAP3, CMH-1	apoptosis effector	yes
8	Mch5, MACH, FLICE	apoptosis initiator	yes
9	ICE-LAP6, Mch6	apoptosis initiator	yes
10	Mch4	apoptosis initiator	yes
11	ICH-3	cytokine activation	no
12		cytokine activation	no
13	ERICE	cytokine activation	no
14	MICE	cytokine activation	no

Since some caspases sequentially process and activate others, a model has been proposed in which some caspases (such as caspase-8 and caspase-9) act as initiator or signalling proteases, while others (such as caspase-3 and caspase-7) act as effectors of apoptosis (Cohen, 1997). Activation of the caspase cascade requires activation of initiator caspase (procaspase-8) by binding to its specific co-factor FADD through the "death effector domain" (Thornberry, and Lazenbik, 1998).

1.3.3.1. Caspase 3

A member of this family, caspase-3 (CPP32, apopain, YAMA) has been identified as being a key mediator of apoptosis of mammalian cells. It consists of 17 kD and 11 kD subunits that are derived from a 32 kD proenzyme (procaspase-3) by cleavage at multiple aspartic acid sites (Fujita and Tsuruo, 1998).

Caspase-3 activation, a convergent event in apoptosis, is triggered by a variety of apoptotic stimuli. It is believed to play the role of executioner most downstream in the apoptotic pathway as it is commonly activated in cells by various death stimuli. Caspase-3 cleaves many cytoskeletal proteins, such as PARP and DNA fragmentation factor-45 (DFF-45) (Tewari *et al.*, 1995; Liu *et al.*, 1997). Two pathways lead to the activation of caspase-3 through release of cytochrome c and cleavage of caspase-9 and through ligation of death receptors by TNF and Fas ligand.

In the pathway stimulated by TNF-α and Fas ligand, pro-caspase-8 is recruited and activated by FADD. Caspase-8 then directly activates pro-caspase-3 (Yang *et al.*, 1998; Yamada *et al.*, 1999; Tang *et al.*, 2000).

1.3.3.2. Caspase-8

Caspase-8 (FLICE, MACH, Mch5), a 55 kD cytosolic protein, is a member of the family of ICE-like proteases. It is synthesized as an inactive proenzyme which is activated either by the prototypic CD 95 apoptosis pathway (through formation of a complex between CD 95, FADD and Caspase-8) or the TNFR1 pathway (formation of a complex between TNFR1, TRADD, FADD and caspase-8). Caspase-8 contains two effector domains (DED) which interact with the DED of FADD. Upon binding, proenzymatic caspase-8 is converted into its active form, a 20 kD subunit and a 10 kD subunit, which then induces apoptosis by triggering the caspase cascade (Muzio *et al.*, 1996).

1.3.4. MITOCHONDRIA

A lot of studies underline the importance of mitochondria in the regulation of apoptosis (Kroemer *et al.*, 1997a, Yang *et al.*, 1997). Changes in mitochondrial permeability, the release of mitochodrial proteins, and a decrease in the mitochondrial membrane potential are early events during apoptosis (Krippner *et al.*, 1996; Liu *et al.*, 1996), although not in all conditions (Tang *et al.*, 1998). Proapoptotic factors, such as Cyt C (Liu *et al.*, 1996; Yang *et al.*, 1997), procaspases -2, -3, and -9 (Mancini *et al.*, 1998; Susin *et al.*, 1999), Apoptosis

Inducing Factor (AIF) (Susin *et al.*, 1999), and second mitochondria-derived activator of caspases protein Smac (also known as Diablo) (Chai *et al.*, 2000; Du *et al.*, 2000; Srinivasula *et al.*, 2000, Verhagen *et al.*, 2000) are safely sequestered within the mitochondrial intermembrane space in non-apoptotic cells. Upon apoptotic challenge, however, a rapid release of these factors through the outer mitochondrial membrane into the cytoplasm signals the initiation of the apoptotic process. The mechanism of release of these factors into the cytoplasm remains unclear (Yin *et al.*, 2002).

Mitochondria have been implicated in TNF-α-induced cell death. After TNF-α treatment, mitochondria swell (Matthews, 1983) and mitochondrial respiration chains (MRC) are damaged (Higuchi *et al.*, 1992; Schulze-Osthof *et al.*, 1992) in the early phase of cell death. Because mitochondria are the source of ATP, damage to mitochondria will ultimately decrease cellular ATP levels leading to cell death. In addition to the passive cytotoxic effect of decreasing ATP levels, additional mechanisms may be directly involved in TNF-α-induced cytotoxicity.

Mitochondria play an important role in the regulation of cell death. The Bcl-2 group of proteins is located on the outer mitochondrial membrane and governs ion transport. The Bax protein resides in the cytosol. Upon receipt of an apoptotic signal Bax migrates and binds to the mitochondrial membrane inducing loss of selective ions. Due to changes of the mitochondrial membrane, there is release of cytochrome-c and apoptosis-inducing factor (AIF). AIF moves directly to the nucleus where it produces chromatin condensation and nuclear fragmentation. Cytochrome- c binds to Apoptosis protease activating factor-1 (Apaf-1) for necessary activation of procaspase-9. Activated caspase-9 activates downstream caspases, including procaspase-3, responsible for the cytosolic changes characteristic of apoptosis (Israel and Israel, 1999).

1.3.4.1. Cytochrome c release

Cytochrome c is a protein that is important to the process of creating cellular energy, the main function of mitochondria. The release of cytochrome c is part of the cascade of cellular

events that lead to apoptosis, or programmed cell death. When some trigger sets off the cycle that leads to apoptosis, cytochrome c appears outside the mitochondria within one hour. Cytochrome c is not a factor in necrosis.

Cytochrome c, which is usually present in the mitochondrial intermembrane space, is released into the cytosol following induction of apoptosis by many different stimuli including CD 95, TNF, and chemotherapeutic and DNA damaging agents (Liu *et al.*, 1996; Reed, 1997). The efflux of mitochondrial respiratory chain protein cytochrome c from mitochondria to cytosol seems to play an important role in the activation of cell death (Krippner *et al.*, 1996; Liu *et al.*, 1996).

1.3.5. CASPASE SUBSTRATES

Close to 100 caspase-sensitive substrates are broken down during apoptosis (Saraste and Pulkki, 2000; Wolf, 1999). Examples include PARP (poly (ADP-ribose) polymerase), which is a useful indicator of apoptosis, and lamins, structural proteins from the nuclear envelope (Cohen, 1997). Cell morphology is affected by cleavage of cytoskeletal proteins such as gelsolin, fodrin, and actin, leading to plasma membrane blebbing (Cohen, 1997; Saraste and Pulkki, 2000). Another key substrate is the Inhibitor of Caspase-Activated Dnase (ICAD), whose proteolysis promotes the endonuclease activity of CAD, and leads to apoptotic DNA fragmentation (Thornberry and Lazenbik, 1998; Wolf, 1999; Saraste and Pulkki, 2000).

Caspase-3, the most prevalent caspase in the cell, is ultimately responsible for the majority of the apoptotic effects, although it is supported by two others, caspase-6 and -7. Together, these three executioner caspases presumably cause the apoptotic phenotype by cleavage or degradation of several important substrates. Caspases inactivate proteins that protect living cells from apoptosis, and they contribute to cell death by direct disassembly of cell structures (Squier *et al.*, 1994). It is not known which caspase is responsible for cleavage of the diverse proteins such as PARP, lamin B, actin, and others under physiological conditions. Some caspases show overlapping specificities for some substrates (caspase-3 and caspase-7 both

cleave PARP), whereas caspase-6 is the only caspase known to cleave lamins (Wright *et al.*, 1994).

Poly (ADP-ribose) polymerase (PARP) is one of the principle substrates cleaved by caspases, primarily by caspase-3 and caspase-7 (Cohen, 1997). It is the best characterised proteolytic substrate of caspases, being cleaved during the execution of apoptosis in many systems. Intact PARP (116 kD) is cleaved to 24 kD and 89 kD, representing the N-terminal DNA-binding domain and the C-terminal catalytic domain of the enzyme, respectively.

Although many caspases, including caspase-2, -4, -6, -8, -9, and -10 can cleave PARP *in vitro* when added at high concentrations it appears that caspase-3 and caspase-7 are primarily responsible for PARP cleavage during apoptosis. Although the biological relevance of PARP is not clear, PARP cleavage is one of the most valuable indicators of apoptosis (Zeuner *et al.*, 2001).

1.3.5.1. Other caspase substrates

High- and low-molecular weight DNA fragmentation is caused by the action of caspase-3 on a complex of Caspase-Activated DNase (CAD)/DNA Fragmentation Factor (DFF) 40, a nuclease, and iCAD/DFF45, its inhibitor (Liu *et al.*, 1997; Enari *et al.*, 1998). These DNA fragments can be detected as a ladder pattern in DNA electrophoresis or *in situ* in the TUNEL assay (TdT-mediated dUTP Nick-End Labelling assay) (Saraste and Pulkki, 2000). In non-apoptotic cells, CAD (Caspase-Activated Deoxyribonuclease) is present as an inactive complex with iCAD. During apoptosis, caspase-3 cleaves the inhibitor, allowing the nuclease to cut the chromatin.

Blebbing is orchestrated via the cleavage and activation of gelsolin (Kothakota *et al.*, 1997), p21-activated kinase-2 (Lee *et al.*, 1997; Rudel and Bokoch, 1997), and most likely through cleavage of fodrin (Martin *et al.*, 1995) to dissociate the plasma membrane from the cytoskeleton. The result is probably an effect of microfilament tension and local release, since depolymerization of actin prevents blebbing (Cotter *et al.*, 1992).

The biochemical hallmark of apoptosis in many cells is the formation of distinct DNA fragments of oligonucleosomal size (180-200 bp). The oligonucleosomal DNA fragments result from a systemic degradation of genomic DNA forming a ladder pattern after agarose gel electrophoresis.

Although this kind of DNA fragmentation has been very widely observed in apoptosis, exceptions do exist. Bortner *et al.* (1995) have reported other types of DNA fragmentation in the prescence or absence of the characteristics internucleosomal DNA cleavage pattern. Three types of DNA fragmerntation occurring during apoptosis can thus be distinguished: internucleosomal DNA cleavage; fragmentation into large 50-300 kbp length; and single stranded cleavage.

However, some cells die by apoptosis without the characteristics internucleosomal DNA cleavage (Cohen *et al.*, 1992).

1.3.6. APOPTOSIS TRANSDUCING PROTEINS

There is compelling evidence that apoptosis is closely related to the altered expression of tumor oncogenes (Hockenberry *et al.*, 1990; Yonish-Rouach *et al.* 1991), Bcl-2 and related cytoplasmic proteins (Adams and Cory, 1998).

1.3.6.1. The Bcl-2 superfamily

The Bcl-2 family of proteins are central regulators of the intracellular apoptotic signalling cascades. They include both anti-apoptotic proteins, such as Bcl-2, Bcl-X_L, Bcl-w, Mcl-1, and pro-apoptotic proteins, such as Bax, Bak, Bok/Mtd, Bcl-Xs, Bid, Bad, Nik/Nbk, and Bik (Kroemer, 1997b; Reed, 1997). Some are most similar to Bcl-2 and promote cell survival by inhibiting adaptors needed for activation of the proteases (caspases) that dismantle the cell (Colombel *et al.*, 1993; Adams and Cory, 1998).

Members of the Bcl-2 family of intracellular proteins are essential mediators of cell survival and apoptosis. Both anti- and pro-apoptotic family members have been characterized and their classification is related to the presence or absence of Bcl-2 homology (BH) domains. Four BH domains have been described: BH1, BH2, BH3, and BH4. Bcl-2 and Bcl-X_L, both containing all four BH domains, possess established roles in the inhibition of apoptosis, although their exact mechanism remains elusive. It has been proposed that antiapoptotic Bcl-2 family members inhibit apoptosis by antagonizing the actions of proapoptotic family members. This antagonism is proposed to occur upstream of apoptosis-related mitochondrial alterations. Two subfamilies of proapoptotic Bcl-2 family members have been identified; the bax family (Bax, Bok, and Bak), containing BH1, BH2, and BH3, and the BH3-only family (Bid, Bim, Bik, Bad, Bmf, Hrk, Noxa, and PUMA). Similar to the anti-apoptotic family members, the exact mechanism of action of pro-apoptotic Bcl-2 family members is uncertain. In a number of systems, these proteins have been demonstrated to be essential for the completion of apoptotic programs (Lindsten *et al.*, 2000; Wei *et al.*, 2001; Yin *et al.*, 2002).

1.3.6.2. Bcl-2 protein

Bcl-2 (B-cell lymphocyte/leukemia-2) is suggested to play a fundamental role in the protection of cells from programmed cell death due to its expression in a variety of tissues, cancer cells in particular, resistant to a variety of anti-cancer drugs (Halder *et al.*, 1996). It is an integral intracellular membrane protein that inhibits programmed cell death and promotes viability without promoting cell proliferation (Vaux *et al.*, 1988).

bcl-2 is a human proto-oncogene located on chromosome 18. Its product is an integral membrane protein (called Bcl-2) located in the membranes of the endoplasmic reticulum (ER), nuclear envelope, and in the outer membranes of the mitochondria. The gene was discovered as the translocated locus in a B-cell leukemia (hence the name). This translocation is also found in some B-cell lymphomas. In the cancerous B cells, the portion of chromosome 18 containing the *Bcl-2* locus has undergone a reciprocal translocation with the portion of chromosome 14 containing the antibody heavy chain locus. This t(14;18)

translocation places the *Bcl-2* gene close to the heavy chain gene enhancer (Vaux *et al.*, 1988).

1.3.6.3. Bax protein

More distant relatives to Bcl-2 instead promote apoptosis, apparently through mechanisms that include displacement of the adapters from the anti-apoptotic proteins (Adams and Cory, 1998). One such protein is Bax (Bcl-2 Associated protein X), an anti-proliferative gene encoding a protein which triggers cells to undergo apoptosis (Halder *et al.*, 1996).

Bax, a 21 kD protein, is one of the members of the *bcl-2* gene family that promotes apoptosis by a mechanism that is not understood. However, it is suggested to execute its proapoptotic activity by inhibiting the antiapoptotic activity of Bcl-2 (Oltvai *et al.*, 1993). For instance, Bax interacts with Bcl-2, and the high level of Bax relative to Bcl-2 promotes cell death whereas an excess of Bcl-2 promotes survival (Chittenden *et al.*, 1995). It is this Bcl-2/Bax ratio that dictates whether a cell survives or dies after receipt of a potentially apoptotic stimulus (Oltvai and Korsmeyer, 1994).

Bax protein can induce both membrane potential dissipation and cytochrome c release, suggesting that Bax acts to destabilize mitochondrial membrane function (Rosse *et al.*, 1998). Some members of this Bcl family like Bcl-x_s are known to promote rather than inhibit the onset and progression of programmed cell death. Bak (Bcl-2 homologous antagonist/ killer), another member of the family, and Bad are also known as promoters of apoptosis (Chittenden *et al.*, 1995)

The Bcl-2 family members associate with each other suggesting their relationship to be antagonistic (Oltvai *et al.*, 1993). Worth noting is that the growing Bcl-2 family can somehow register diverse forms of intracellular damage, gauge whether other cells have provided a positive or negative stimulus, and integrate these competing signals to determine whether the cell is "to be or not to be" (Adams and Cory, 1998).

1.3.6.4. Other cytoplasmic proteins

In addition to Bcl-2 and Bax, a number of other gene products may play a role in programmed cell death. These include proteins that are important in protection from DNA damage and in the regulation of the cell cycle. Proteins such as p53 are found at elevated levels in various tumors and play a critical role in the cellular response to DNA damage. It shares properties with c-Myc in that they are both localised in the nucleus. But c-Myc is normally known for its ability to promote cell proliferation. (Khannaik *et al.*, 1994).

1.3.6.4.1. p53

p53 protein, a 53 kD protein, is a tumor suppressor normally maintained in abeyance at low levels (Evan and Littlewood, 1998; Israel and Israel, 1999). The protein p53 is critical in maintaining ordered proliferation, growth, and differentiation of normal cells (www.intouchlive.com/cancergenetics/p53.htm&3 (2002).

Two cellular responses to p53 activation are well described- growth arrest and apoptosis. The p53 protein either temporarily halts cell division so that the cell can repair altered DNA, or sends the cell to apoptotic death. p53 binds directly to DNA, recognizes DNA damage, and induces cell-cycle G1 and G2 growth arrest in attempting to repair the damaged DNA (Figure 1.7) (Evan and Littlewood, 1998). In response to apoptosis initiating signals the p53 protein becomes destabilized and its DNA binding activity increases allowing it to mediate cell cycle arrest or apoptosis (Burns and El-Deiry, 1999). Which of these two responses prevails seems to depend on the cell type, cell environment, and factors such as oncogene expression.

Moreover, overexpression of p53 induces apoptosis in some cell types. The mechanism by which p53 promotes apoptosis is more obscure, although studies indicate that it involves induction of specific target genes that differ from those implementing growth arrest (Hansen and Braithwaite, 1996). Examples of p53 targets implicated in apoptosis are the Bcl-2 antagonist Bax (Yin *et al.*, 1997) the insulin like growth factor binding protein-3 (IGFBP-3), and IGF-1 (Williams *et al.*, 2000).

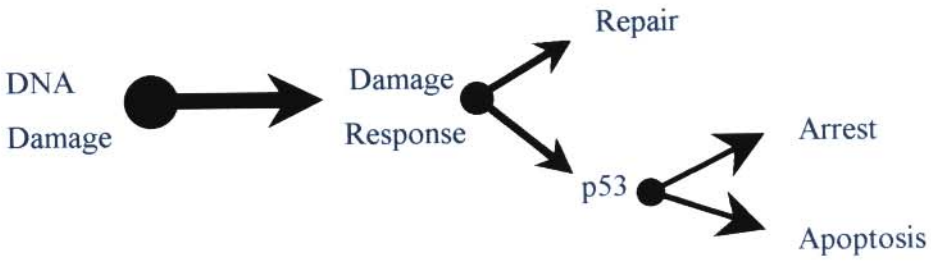


Figure 1.7: DNA damage response (Evan and Littlewood, 1998).

1.3.6.4.2. *c-Myc*

c-Myc is one of the first oncogenes demonstrated to have proapoptotic activity. It is one of the members of the family of related mammalian genes that encode the myc proteins. The myc family of oncogenes (*c-Myc, N-Myc, and L-Myc*) functions in the control of cell proliferation, differentiation, and tumorigenesis. Although it has long been recognized that *c-Myc's* positive effect on cell proliferation can contribute to cancer development, it is suspected that *c-Myc* has additional roles in the progression of malignancy (www.eurekalert.org/pub_releases/2002-09/cshl-sdr091302.php, 2002).

c-Myc is a double edged sword, being capable of driving a cell through the cycle under appropriate conditions or provoking cell death under sub-optimal conditions. The finding that *c-Myc* induces apoptosis in the absence of growth factors has been another key finding. Signals from the IGF-1 receptor counteract the c-myc to express its proliferative potential and explains the requirement for IGF-1 for growth of most cultured cells (Harrington *et al.*, 1994).

1.3.7. NATURAL CASPASE INHIBITORS

Cells also contain natural inhibitors of the caspases. These inhibitors of apoptosis proteins (IAPs) were first identified in baculovirus, but subsequently were found in human cells. At least five different mammalian IAPs — X-linked inhibitor of apoptosis (XIAP, hILP), c-IAP1 (HIAP2), c-IAP2 (HIAP1), neuronal IAP, and survivin — exhibit anti-apoptotic activity in cell culture (Liston *et al.*, 1996; Uren *et al.*, 1996; Ambrosini *et al.*, 1997; Deveraux *et al.*, 1997; Roy *et al.*, 1997; Deveraux *et al.*, 1998;Tamm *et al.*, 1998). The spectrum of apoptotic stimuli that are blocked by mammalian IAPs is broad and includes ligands and transducers of the tumor necrosis factor (TNF) family of receptors, pro-apoptotic members of the ced-9/Bcl-2 family, cytochrome c, and chemotherapeutic agents (LaCasse *et al.*, 1998). XIAP appears to have the broadest and strongest anti-apoptotic activity. XIAP, c-IAP1, and c-IAP2 are direct caspase inhibitors (Deveraux *et al.*, 1997; Roy *et al.*, 1997).

They all bind to and inhibit active caspase-3 and -7, and also procaspase-9, but not caspase-1, -6, -8, nor -10. The binding and inhibition of caspases by IAPs is mediated by baculovirus IAP repeats (BIR) domain(s) present within the IAP. BIR is a conserved sequence motif of ~ 70 amino acids that is repeated tandemly in a class of baculovirus proteins (e.g., Op-IAP). All mammalian IAPs have three BIR domains, except survivin, which has only a single BIR. Interestingly, in the case of XIAP, a 136 amino acid region spanning the middle BIR core was found to be necessary and sufficient for inhibition of caspase activity (Takahashi *et al.*, 1998). Another region within the IAP, the RING domain, acts as an ubiquitin ligase, promoting the degradation of the IAP itself (Yang *et al.*, 2000) and, presumably, any caspase bound to it. Near the RING finger of both c-IAP1 and c-IAP2 is a CARD domain, suggesting that these IAPs might directly or indirectly regulate the processing of caspases via CARD domain interactions. In this fashion, IAPs put the brakes on the apoptotic process by binding, inhibiting, and perhaps degrading caspases.

Because apoptosis and inflammatory responses are the major host defense mechanisms against viruses, it is not surprising that viruses employ inhibitors of caspases, the central components of the apoptotic machinery, to prolong the life of host cells for maximal viral

replication. These viral inhibitors may directly inhibit caspases, as exemplified by the cowpox virus protein CrmA (cytokine response modifier A) and baculovirus proteins p35 and IAPs, or inhibit caspase-adapter interactions, as examplified by v-FLIP (viral FLICE-inhibitory protein) (Tewari *et al.*, 1995).

1.3.7.1. CrmA and p35 IAPs

CrmA is a sepsin that directly targets the active site of mature caspases (Ray *et al.*, 1992). It has an active-site loop that is easily accessible to caspases. After being cleaved by a caspase, however, CrmA stays bound to the caspase and blocks the active site (Bertin *et al.*, 1996). The early experiments using this suicide substrate of caspases provide compelling evidence that caspases play a central role in apoptosis (Gagliardini *et al.*, 1994; Tewari *et al.*, 1995). CrmA is limited to the group I and group III caspases (except for caspase-6) and granzyme B (Zhou *et al.*, 1997). Similar to CrmA, the baculovirus protein p35 also targets mature caspases and serves as a suicide substrate (Bump *et al.*, 1995). The inhibition requires a substrate-like sequence containing Asp-Gln-Met-Asp-87-Gly that fits well with the caspase active site (Fisher *et al.*, 1999). p35 is a broad-spectrum caspase-specific inhibitor; it inhibits human caspase-1, -3, -6, -7, -8, and -10 (Zhou *et al.*, 1997). However, it does not inhibit granzyme B.

CPP32, a member of ICE family cysteine proteases, has emerged as one of the key proteases in spontaneous (Nicholson *et al.*, 1995), anti-Fas- (Schlegel *et al.*, 1996) and staurosporine-mediated apoptosis. Although TNF can activate CPP32-like protease (Fujita *et al.*, 1996), and overexpression of crm A that inhibits ICE family cysteine protease can inhibit TNF-induced cell death (Miura *et al.*, 1995), the involvement of CPP32 in TNF-induced apoptosis still needs intensive research.

1.3.7.2. Baculoviruses IAPs

In contrast to CrmA and p35, IAPs are not active-site-specific inhibitors, and their inhibition of apoptosis does not require cleavage by caspases. The baculovirus IAPs, Op-IAP and Cp-

IAP, were identified by their ability to functionally replace p35 (Crook *et al.*, 1993; Birnbaum *et al.*, 1994). While no cellular homologues of CrmA and p35 have been identified so far, the cellular homologues of IAPs constitute a major family of caspase regulators. The first human IAP, the neuronal apoptosis inhibitor protein, was cloned based on its frequent deletion in a neurodegenerative disorder, spinal muscular atrophy (Liston *et al.*, 1996). At least four other mammalian IAPs (XIAP/MIHA, c-IAP-1/MIHB, c-IAP-2/MIHC, and survivin) and two *Drosophila* IAPs (DIAP-1 and -2) have been identified (Deveraux *et al.*, 1997). Each IAP protein contains at least one but often two to three copies of the characteristic BIR sequence (baculovirus IAP repeat), which are required for their function. In addition, several IAPs, including IAP-1 and XIAP, also have a zinc ring domain that controls the degradation of these proteins.

Human XIAP and c-IAP-1 and -2 were found to inhibit mature caspase-3 and -7 (Roy *et al.*, 1997; Deveraux *et al.*, 1997; Deveraux *et al.*, 1998). However, the inhibitory effect is substantially weaker than that of CrmA and p35. While CrmA and p35 reach maximal inhibitory effect when present at an equal molar ratio to caspases, IAPs need to be in molar excess to achieve a reasonable level of apoptosis inhibition. The exact mechanism of caspase inhibition by IAPs is not fully understood. However, IAPs may target the early steps of caspase activation, as shown by the observation that IAPs block activation of *Drosophila* caspases *in vivo* and activation of mammalian procaspase-9 in a cell-free system (Deveraux *et al.*, 1998, Seshagiri and Miller, 1997). Evolutionarily ancient IAPs such as survivin may also be involved in cytokinesis and link caspase activation to cell cycle progression (Li *et al.*, 1998; Li *et al.*, 1999). The level and activity of IAPs may determine the sensitivity of cells to apoptotic stimuli, and both can be downregulated during apoptosis to ensure effective cell killing. For example, during thymocyte death, c-IAP-1 and x-IAP are degraded in proteosomes after auto-ubiquitination, which is catalyzed by the ubiquitin ligase activity of their zinc ring domains (Yang *et al.*, 2000). The pathway leading to this autoubiquitination remains to be determined. In addition, the activity of IAPs is regulated by the *Drosophila* cell death inducers Reaper, Grim, and HID and mammalian protein Smac/DIABLO.

1.3.7.3. v-FLIPs inhibitors

v-FLIPs represent another group of viral apoptotic inhibitors. v-FLIPs contain two DEDs that are similar to those in the N-terminal region of procaspase-8. They inhibit apoptosis mediated by death receptors through competition with pro-caspases for recruitment to the death receptor complex. Cellular homologues of v-FLIP have been identified, and they come as both a long form and a short form, termed c-FLIP$_L$ and c-FLIP$_S$, respectively. c-FLIP$_S$ is similar to v-FLIP and contains only the DEDs. Overexpression of this form inhibits apoptosis mediated by Fas and related death receptors (Boya *et al.*, 2001).

In contrast, c-FLIP$_L$ is strikingly similar to procaspase-8 and -10, comprising two NH_2-terminal DEDs and a COOH-terminal caspase-like domain. The role of c-FLIP$_L$ in apoptosis still remains controversial. Similar to c-FLIP$_S$, proteins derived from alternatively spliced caspase-2 and -9 transcripts also function as apoptotic inhibitors and are named caspase-2S and caspase-9S, respectively. Both inhibitors lack the large caspase subunit and may compete with the corresponding caspases for binding to adapters (Droin *et al.*, 2001).

1.3.7.4. Phosphorylation and Nitrosylation

As a major form of posttranslational modification, phosphorylation is also employed to modulate caspase activity. One example is the phosphorylation of caspase-9 by Akt (Cardone *et al.*, 1998), a serine-threonine protein kinase downstream of phosphatidylinositol 3-kinase, which is implicated in apoptosis suppression mediated by growth factor receptors. Phosphorylation of caspase-9 by Akt inhibits caspase activity *in vitro* and caspase activation *in vivo*. The phosphorylation occurs at a consensus substrate recognition sequence of Akt (RxRxxS/T), which is away from the enzymatic active site. The phosphorylation may affect assembly of the caspase tetramer. Alternatively, it may regulate caspase activity allosterically. However, this regulation is not conserved through evolution, because mouse caspase-9 does not contain the recognition site serine and is not phosphorylated by Akt (Fujita *et al.*, 1999).

Another way to modify caspases post-translationally is by *S*-nitrosylation. Nitric oxide (NO) and related molecules have been found to inhibit apoptosis. A study showed that in unstimulated human cells, the active site of endogenous procaspase-3 is *S*-nitrosylated, but during Fas-mediated apoptosis, it becomes denitrosylated. The denitrosylation enhances mature caspase-3 activity, although it does not affect procaspase-3 processing. The pathway that leads to denitrosylation has not been defined, nor is it clear whether other caspases are regulated by a similar mechanism. Nevertheless, this study suggests that caspase *S*-nitrosylation and denitrosylation is a dynamic process during apoptosis (Mannick et al, 1999).

1.4. LYMPHOMAS

The involvement of disrupted apoptosis has been evidenced in 1985 by Bakhshi and colleagues in the pathogenesis of some human tumors such as non-Hodgkin's Lymphomas (NHL), Burkitt's like lymphomas, especially follicular B-cell NHL. The dysfunction underlying NHL resistance to apoptosis was suggested to lie upstream of caspase-8, or could alternatively be influenced by anti-apoptotic regulators of the Bcl-2 family (Xerri *et al.*, 1999).

Burkitt's lymphoma is a solid tumor of the B-lymphocytes and is highly ranked as non-Hodgkin's lymphoma. The cells have a rapid doubling time and a starry appearance (Wright, 1997; Khanniak *et al.*, 1994). Burkitt's lymphoma cells contain the chromosomal abnormality t(8:14) involving the *c-myc* gene. The *c-myc* gene is translocated to a region of vigorous transcription, becomes overproduced, and may simply render the B lymphocytes cancerous (Kawanashi, 1993). Uncontrolled mitosis of these cells results in a clone of cancerous cells known as Burkitt's lymphoma (Wright, 1997).

1.4.1. WIL-2 CELL LINE

The Wil-2 cell line is a non-immunoglobulin secreting human B lymphocyte cell line originally isolated from the spleen of a caucasian male patient with hereditary spherocytic

anemia by Levy *et al.* in 1971. This cell line is useful in production of human monoclonal antibodies.

They have a rapid doubling time (Wright, 1997) and typically require a steady supply of nutrients and growth factors in the form of foetal calf serum. The cell line has for long been used as a model of experimental therapeutics in cancer.

Generally, human leukemia cell lines grow in suspension in culture flasks or in microtiter plates and are not adherent. Occasionally, some cells might attach loosely to the plastic; however, the adherence is significantly less pronounced than in fibroblast- or epithelial-like cells. Commonly, the cells can be detached easily by shaking the flask or by pippetting the cultured cells vigorously (Hay *et al.*, 1994).

1.5. AIMS AND OBJECTIVES

Lithium has been shown to induce cell death in B-lymphoma cells through apoptosis in a pathway that involves activation of caspases and some regulatory elements. Also, lithium-induced sensitization has been reported to be highly specific for TNF-induced cell death. Preliminary studies have implicated lithium in inducing apoptosis in these cell lines at concentrations as low as 2.5 mM. Yet the exact mechanism through which this cell death is executed still remains to be resolved.

This study is aimed at elucidating the mechanism through which lithium induces apoptosis in non-Hodgkin lymphoma cells (Wil-2 cells). The investigation focused on the activity of caspase-8 during lithium induced cell death, as well as the expression of apoptosis and cell regulatory proteins.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

The following equipment was used to carry out the experimental work:

* Coulter Counter (model Z1, **Coulter Electronics, Inc., Luton, UK**)
* CO_2 Incubator (NAPCO model, **Instrulab cc, Johannesburg, S.A**)
* Light Microscope (**Zeiss**)
* Fluorescence Microscope (**Zeiss**)
* Spectrophotometer (Genesys 5, **Milton Roy Company, USA**)
* Centrifuges (model GS-6R, GS-15R, **Beckmann Instruments Inc., Fullerton, USA**)
* Electrophoresis equipment (model # B1A, Model # B2, **Owl Scientific Inc**)
* UVP-Transilluminator (model TMW-20, **UVP, West Bury, NY, USA**)
* Image Analyser (Syngene, **Vacutec cc, South Africa**)
* Microtiter plate reader (model 550, **Bio-Rad Laboratories, Richmond, California**)

Chemicals of analytical grade, media and kits were purchased from the following companies:

American Type Cell Culture (ATCC), Rockville, USA):

* HL-60 cells, Wil-2 cells, and Jurkats cells.

Amersham Pharmacia Biotech, UK:

* ECL Western blotting detection reagent.

Bio-Rad Laboratories, Richmond, California:

* SDS

Boehringer Mannheim (Pty) LTD., Gauteng, S.A):

* *In situ* cell death detection kit.

BB Saarchem, Midrand, Gauteng:

* Acetic acid, Bromophemol blue, Chloroform, Comassie blue, Comassie brilliant blue G-250, Ponceau S, Sodium acetate, Sodium chloride, Sodium hydroxide.

CHEMICON International, Inc, Temecula, CA:

* Goat anti-rabbit IgG (H+L). HRP conjugated secondary antibody
* Rabbit anti-human PARP antibody

Fluka

* Acrylamide, Ethidium bromide, NP-40 substitute, Lithium chloride, and Trypan blue dye.

Highveld Biologicals (PTY) LTD, Lydhurst, RSA:

* Foetal Bovine Serum (FBS), Penicillin & streptomycin antibiotic solution, RPMI-1640 medium

ICN biomedicals Inc, Aurora, Ohio:

* Triton-X 100, Tumor Necrosis Factor (Human Recombinant), Phenol

Merck Laboratory Supplies (Pty) LTD, Midrand, S. A):

* Absolute ethanol, DMSO, 2-Mercaptoethanol, Tween,

Pharmingen, USA:

* Purified mouse anti-human caspase-8 antibody
* Purified mouse anti-human TRADD antibody
* Polyclonal rabbit anti-human Bax
* Polyclonal rabbit anti-human caspase-3

R&D systems, Minneapolis, USA:

* Caspase-3, -8, and –9 colorimetric kits.

Sigma

* Aprotinin, Ethylenediaminotetraacetic acid (EDTA), Guanidine, N,N-methylene-*bis*-acrylamide, PhenylMethylSulphonylFluoride (PMSF), Proteinase K, RNAse A, Sodium orthovanadate.

Transduction Laboratories:

* Goat anti-mouse IgG: HRPO.

Whitehead Scientific (Pty) LTD, Brackenfell, USA:

* Molecular grade agarose

2.2. METHODS

2.2.1. Cell cultures

Wil-2 cells were maintained between 2-12 x 10^5 cells/ml in suspension culture in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37^0C in a humidified atmosphere.

2.2.1.1. Cell proliferation studies

Experimental cultures were cultured in 12 well culture plates at a cell density of 2 x 10^5 cells/ml in the absence and presence of drugs. The lithium concentrations used were 1.0, 2.5, 5.0, 10, 20, and 50 mM. Cell growth was monitored daily using a Z1 model Coulter Counter.

2.2.1.2. Cell viability assays

The viability of cells was determined using the trypan blue dye exclusion method. Briefly, cells were treated with specified concentrations of lithium and a 0.5 ml aliquot was stained with trypan blue dye. The number of cells taking up the dye (appearing blue) was determined

on a haemocytometer using a light microscope. Viability was expressed as percentage viable cells per 100 cells counted.

2.2.2. Analysis of DNA fragmentation by agarose gel electrophoresis

High molecular weight DNA was extracted using a modified method described by Bouffard and Momparler (1995). Briefly, after exposure to various lithium concentrations, cells were pelleted at 100 xg for 10 min. The medium was carefully removed and the pellets suspended in 20µl lysis buffer (10 mM EDTA, 50 mM Tris-HCl pH 8.0, containing 0.5% SDS (w/v) and 0.5 mg/ml proteinase K). After incubation for 1 hour at 50 ^{0}C, 10 µl DNase free RNase A (0.5 mg/ml) was added and incubation continued for another hour at 50 ^{0}C. The samples were heated to 70 ^{0}C and then 10 µl of 10 mM EDTA pH 8.0 containing 1% (w/v) low melting point agarose, 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose were added. The samples were then loaded into the wells of a dry 1.5% agarose gel and electrophoresed at 80 volts for 4-5 hrs in 1 x TBE pH 8.0 containing 0.5mg/ml ethidium bromide at 4 ^{0}C. The DNA fragments were visualized under UV illumination and photographed.

2.2.3. *In Situ* cell death detection (TUNEL assay)

Apoptosis was also assayed at single cell level using the In Situ Cell Death Detection Kit, Fluoroscein (Boehringer Mannheim). Briefly, cells were adjusted to 1 x 10^{7} cells/ml and cytospun onto slides at 500 xg. The slides were then washed twice in ice-cold PBS/ 1% BSA and fixed by addition of 100 µl 4% freshly prepared paraformaldehyde solution (pH 7.4). The slides were incubated for 30 minutes at room temperature. Following incubation cells were washed once with 200 µl of PBS and permeabilised by addition of 100µl permeabilisation solution (0.1 % Triton-X100 in 0.1% of sodium citrate) for 2 minutes. Cells were then washed twice in PBS and resuspended in 50 µl TUNEL reaction mixture for 60 minutes at 37 ^{0}C in a humidified atmosphere in the dark. Cells were washed and their DNA content analysed by fluorescence microscopy.

2.2.4. Western blotting

2.2.4.1. Protein Extraction

Wil-2 cells were treated as previously described and were incubated for 48 hours, washed by centrifugation with PBS and the cell pellet resuspended in lysis buffer (20 mM Tris, 137 mM NaCl, 1% NP-40, 10% Glycerol) with freshly prepared protease inhibitors (1 mM PMSF, 0.15 U/ml aprotinin, and 1 mM sodium orthovanadate). The cell suspension was centrifuged at 10000 xg and the supernatant used as total lysate. The amount of total protein in each extract was determined using the BCA protein assay. The absorbance was read at 562 nm and the protein concentration was calculated from the standard curve.

2.2.4.2. SDS-PAGE and electrotransfer of proteins

About 30 µg/ml proteins were suspended in a loading buffer (125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8). Samples were boiled for 2-5 minutes and loaded onto 12% SDS-polyacrylamide gel (deionized H_2O, 30% acrylamide, 1.5mM Tris-HCl, pH 8.8, 10% SDS, 10% ammonium persulfate, TEMED) and electrophoresed at 30 mA for 2h.

Following SDS-PAGE, separated proteins were transferred onto a nitrocellulose membrane according to the method of Towbin et al., (1979). The transfer was carried out at 200 mA for 2h in the transfer buffer (250 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3). The efficacy of transfer was assessed by staining nitrocellulose membrane with 0.1% Ponceau S in acetic acid and washing with H_2O.

2.2.4.3. Immunoblotting

For Western blotting, the membrane was blocked overnight with 20 ml TBST 3% non-fat milk solution at room temperature to block any non-specific binding sites. The nitrocellulose membrane was then washed three times in 20 ml TBST for 30 minutes. Thereafter the

nitrocellullose was incubated for 1 hour with mouse/rabbit anti-human primary antibody diluted 1 in 2000 in antibody dilution buffer (TBST, 0.5% non-fat milk). The membrane was washed three times in 20 ml TBST for 30 minutes. The membrane was then incubated with goat anti-mouse/rabbit secondary antibody enzyme conjugate in antibody dilution buffer. The secondary antibody used was horseradish peroxidase (HRPO) conjugate. The working dilution used was 1 in 5000. The membrane was then washed 3 times in TBST for 30 minutes.

2.2.4.4. Chemiluminiscent Detection

The membrane was developed using the SuperSignal West Pico chemiluminiscent substrate following the manufacturer's instructions (Amersham). The developed membrane was exposed to film in a development cassette for 1 minute. The film was then developed for 1 minute in the dark.

2.2.5. Measurement of Caspase activities

The enzymatic activity of caspases was determined in cells by colorimetric reaction according to the manufacturer's guide. The cells were lysed to collect intracellular contents and tested for protease activity by addition of caspase-specific peptides conjugated to p-nitroanilide. Peptide cleavage by caspases release p-nitroanilide, which was quantitated spectrophotometrically.

Briefly, untreated and treated cells (2×10^6) were harvested in a conical tube at 250 xg for 10 minutes. The supernatant was discarded and the cells lysed in buffer for 10 minutes on ice, and then centrifuged at 10 000 xg for 1 minute. The supernatant was transferred to a new tube and kept on ice. The protein content of the cell lysate was determined using a commercial BCA Protein Assay.

For the assay 50 µl of cell lysate was pipetted per well in a 96 well microtiter plate and 50µl of 2x reaction buffer added to it. To each reaction well 5µl of caspase colorimetric substrate

was added. The microtiter plate was then incubated for 1-2 hours at room temperature and was read at 415 nm.

2.2.6. Statistical Analysis.

Statistical analysis was performed using Student t-test with $p<0.05$ indicating significant difference. Results were analysed in this way by comparing treated and untreated samples.

CHAPTER 3

RESULTS

3.1. EFFECTS OF LITHIUM ON GROWTH OF WIL-2 CELLS

Lithium has been reported to inhibit the growth of HL-60 leukemia cells in a time and concentration dependent manner. Lithium concentrations above 10 mM were shown to be toxic to these cells, whereas concentrations below 10 mM accelerated proliferation of the HL-60 cells (Becker and Tyobeka, 1990; Madiehe *et al.*, 1995). It's this growth inhibitory effect of lithium that sparked an interest in determining its effects in other cell lines. More recent studies by Mukhufi *et al.* (2000) showed that lithium induce growth inhibitory effects on Wil-2 cells in a time and concentration dependent manner. To confirm this effect, Wil-2 cells were seeded at an initial density of 2 x 10^5 cells/ml, and incubated for 72 hours in the absence or presence of increasing lithium concentrations of up to 50 mM. The cell counts were taken daily at 24 hour intervals. It was clear from our results that lithium inhibited the growth of Wil-2 cells in a concentration and time dependent manner. As illustrated in Figure 3.1, when the concentration of lithium increased from 1 mM to 50 mM, there was an increased growth inhibition that seemed to be concentration dependent. Even lithium concentrations as low as 1.0 mM significantly inhibited growth of these cells.

3.2. EFFECTS OF LITHIUM ON VIABILITY OF WIL-2 CELLS.

The inhibition of growth, as shown above, prompted the investigation of the survival of lithium treated Wil-2 cells after exposure to lithium. The cells were treated with 1, 2.5, 5, 10, 20, and 50 mM lithium respectively. Their viability was assessed using the trypan blue dye exclusion method. Cells that stained blue with the dye were assumed dead, and those that excluded the dye were considered to be viable. After 72 hours lithium induced a significant loss in cell viability in cells treated with 50 mM lithium, with their viability decreasing to about 30% (Figure 3.2). Cell death was induced in a concentration and time dependent

manner. The inhibition of cell growth was associated with loss of cell viability as determined by trypan blue uptake.

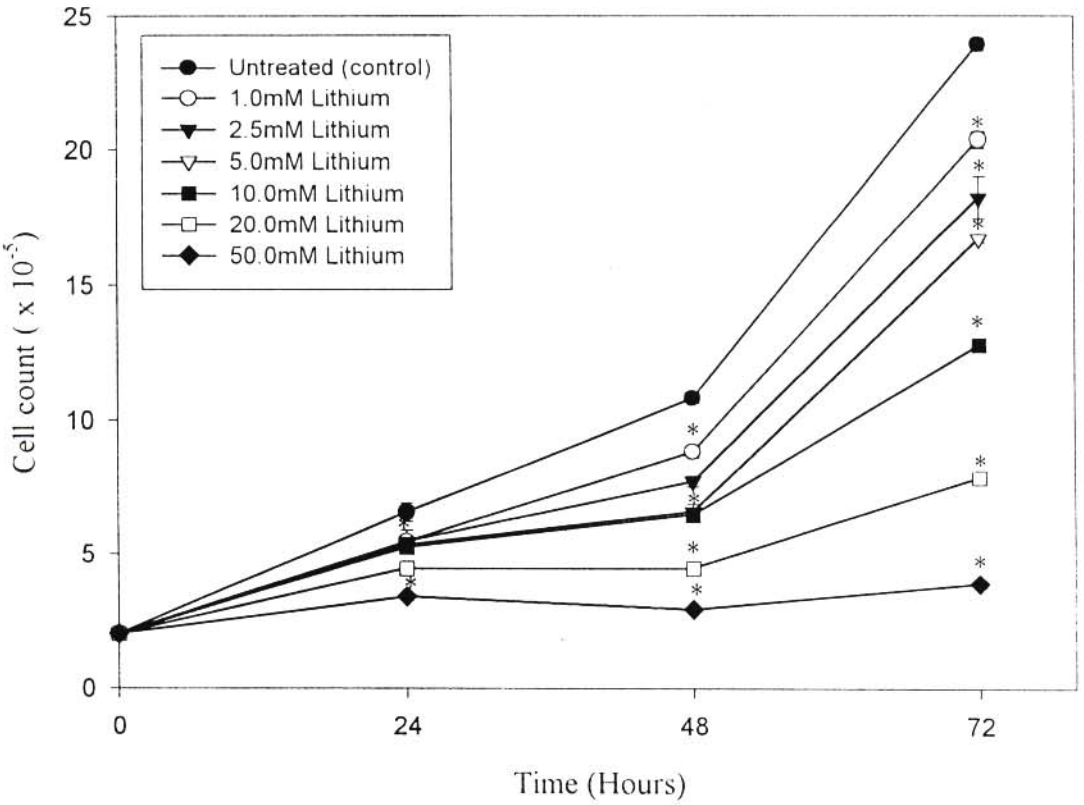


Figure 3.1. The effects of lithium on the growth of Wil-2 cells. Wil-2 cells were incubated with or without lithium for three days. The results represent the mean of three independent experiments, each done in duplicate. * $p<0.05$ when compared to control.

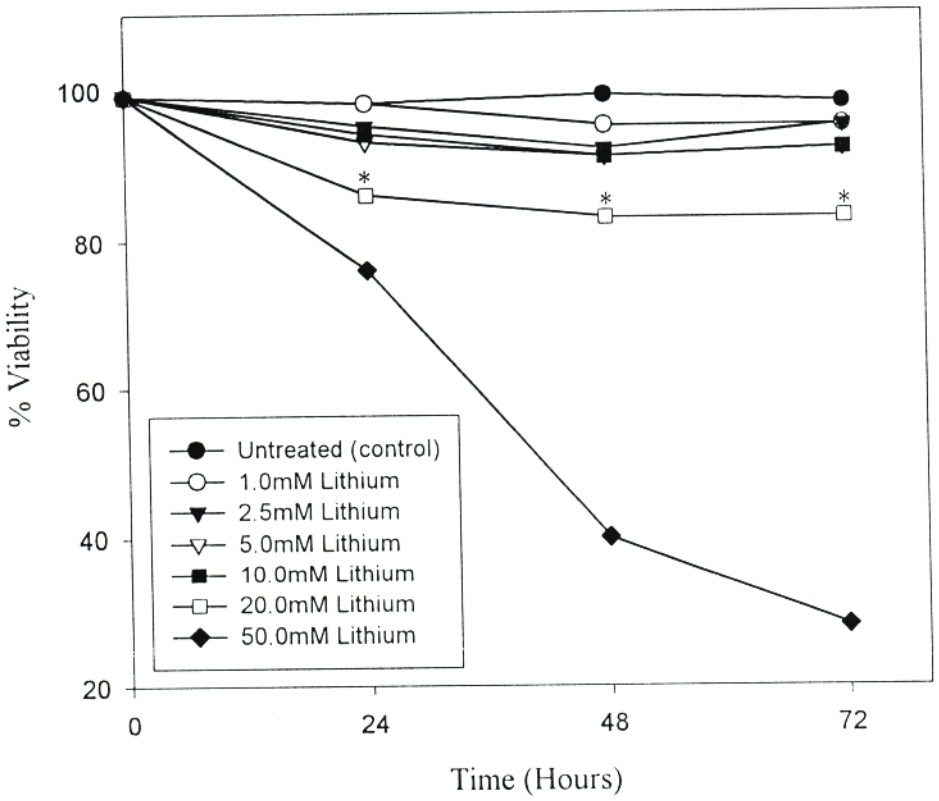
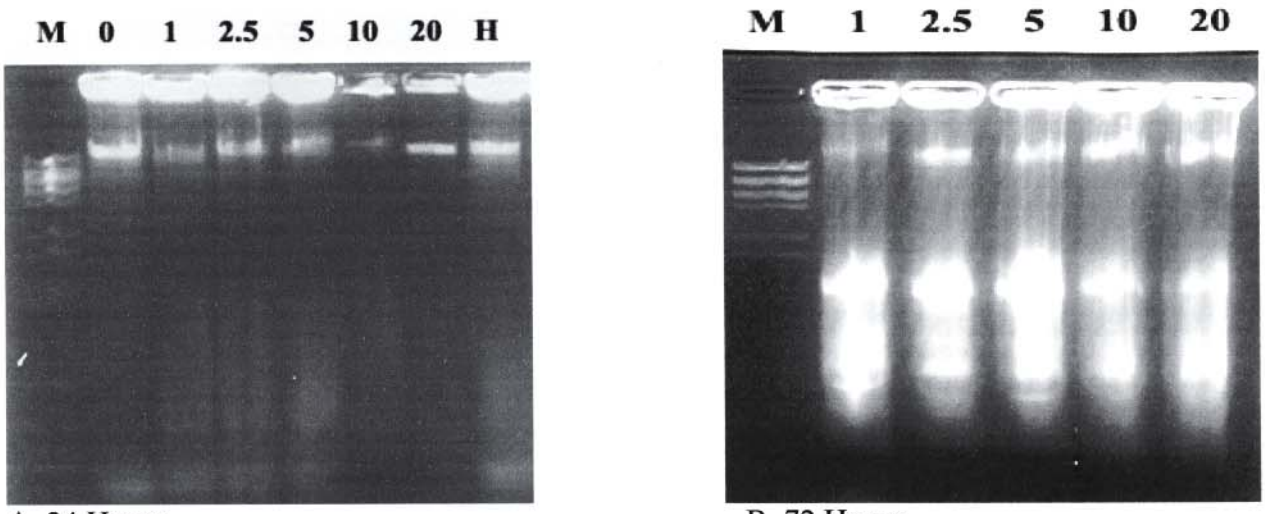


Figure 3.2: The effects of lithium on the viability of Wil-2 cells. Wil-2 cells were treated with increasing concentrations of lithium for three days. Viability was determined using the trypan blue dye exclusion method as outlined in materials and methods. The results are the mean of three independent experiments, each performed in duplicate. * $p<0.05$ when compared to controls.

3.3. INDUCTION OF DNA FRAGMENTATION BY LITHIUM

Dying cells do so either by apoptosis or necrosis. To investigate whether the decrease in growth and viability was due to apoptosis, cells were analysed using the DNA laddering assay. Apoptosis is accompanied by the appearance of discrete 200 bp DNA fragments on agarose gel electrophoresis, hence the assay. Wil-2 cells were reported to fragment their DNA into large fragments (Beletskya, 1997). Studies by Mukhufi (2000) confirmed this after the typical ladder observed with apoptosis, when cells fragment their DNA into internucleosomal sizes, could not be seen in lithium treated Wil-2 cells. The internucleosomal DNA remained intact for periods of up to 72 hours. However following the method by Bouffard and Mompaler (1995) apoptotic DNA fragmentation into larger fragments was evident. This confirms the earlier reports by Beletskya *et al.*, (1997) that Wil-2 cells fragments their DNA into larger fragments.

To examine this possibility, whole lysates of Wil-2 cells were prepared in low melting point agarose, following treatment with 0, 1, 2.5, 5, 10, and 20 mM lithium. As shown in Figure 3.3, Wil-2 cells fragmented their DNA into high molecular weight fragments in response to lithium treatment following 72 hour exposure. At 24 hours, there was no DNA laddering observed. Lithium concentration as low as 1.0 mM induced apoptotic DNA fragmentation in the treated cells after 72 hour treatment whilst the DNA of untreated cells remained intact over that period. HL-60 cells treated with 20 mM lithium, lane marked H, were subjected to electrophoresis as they were reported to fragment their DNA during apoptosis. The HL-60 did not show any DNA laddering. This could be due to the method not being sensitive enough or high molecular weight DNA laddering into larger fragments not occurring in lithium-induced apoptosis in HL-60 cells.



A. 24 Hours. B. 72 Hours.

Figure 3.3: DNA fragmentation in Wil-2 cells treated with lithium. Wil-2 cells were incubated for 24 hours (A) and 72 hours (B) with lithium. The cells were analysed for DNA fragmentation using a method that separates high molecular weight DNA on agarose gels. Lane marked with M represents lambda DNA digested with Hind 111. Lithium concentrations used are indicated at the top. Control cells (20mM lithium treated HL-60's) consistently did not show high molecular weight fragmentation (results not shown).

3.4. DNA STRAND BREAKS ANALYSIS BY TUNEL ASSAY

To further investigate whether the mode of cell death as indicated above could be apoptosis, a TUNEL assay was performed on Wil-2 cells treated and not treated with increasing concentrations of lithium for 24 hours. The assay is very sensitive and detects single strand breaks, in high molecular weight DNA, and double-stranded low molecular weight DNA fragments (mono- and oligonucleosomes). HL-60 promyelocytic leukemia cells treated with 20 mM lithium were used as positive control of apoptosis as it has already been documented in our laboratory that treating these cells with 10 mM and above of lithium culminate in cells undergoing pronounced apoptosis. As shown in figure 3.4 (C-H), fluorescein labels incorporated in nucleotide polymers were detected by fluorescence microscopy, thus indicating that lithium induced apoptotic cell death, with apoptosis noted in cells treated with concentrations as low as 1.0 mM. A concentration dependent increase in lithium-induced strand breaks was noted as the concentration of lithium increased from 0-20 mM.

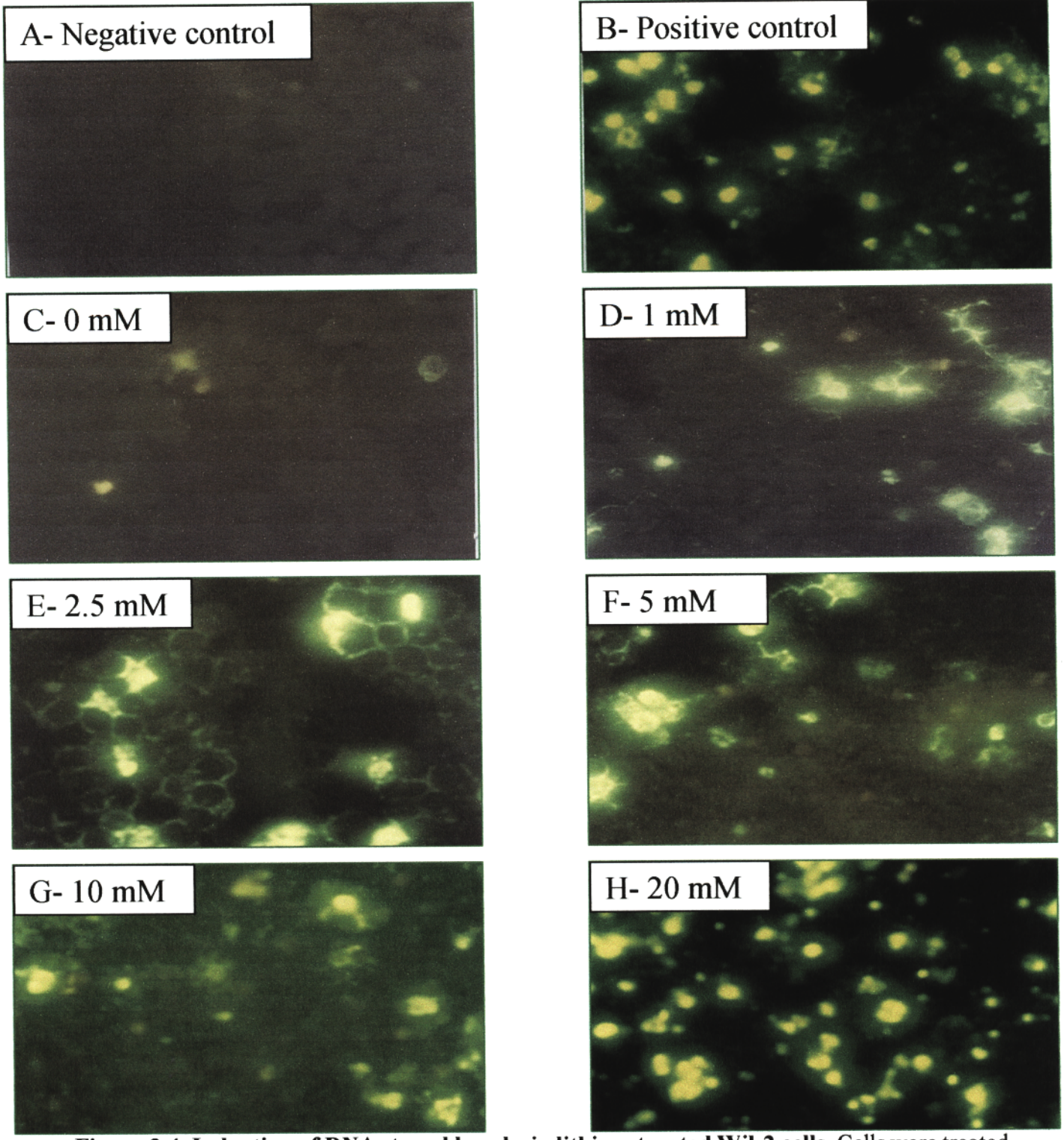


Figure 3.4. Induction of DNA strand breaks in lithium treated Wil-2 cells. Cells were treated for 24 hours with different concentrations of lithium and exposed to TUNEL mixture thereafter. **A**, Negative control (untreated cells). **B**, Positive control (HL-60 cells treated with 20 mM lithium. **C-H**, Wil-2 cells treated with 0, 1, 2.5, 5, 10, and 20 mM lithium respectively, at 10 x magnification.

3.5. CASPASE ACTIVITY

Key mediators that initiate and execute the apoptotic program are members of the caspase family whose members are believed to be essential for virtually all forms of apoptosis (Cohen, 1997). Caspase-3 and caspase-7 are involved in the execution of cells in response to many apoptotic stimuli including ligation of death receptors of the TNFR1 receptor family. However, these executioner caspases rely not directly on receptor ligation, but on the proteolytic activity of upstream initiator caspase-8, caspase-9 and caspase-10 (Boldin *et al.*, 1996; Stennicke and Salvesen, 1999). After association with TNFR1, TRADD and FADD, to form DISC (Death Inducing Signalling Complex) caspase-8 is activated. Active caspase-8 in turn activates pro-caspase 3 (Muzio *et al.*, 1996). Likewise the activation of caspase-9 through association with Apaf1 and cytochrome c activates pro-caspase-3, promoting the manifestation of some of the more classical features of apoptosis (Cardone *et al.*, 1998). Both caspase-8 and caspase-9 have been demonstrated to act *in vitro* on pro-caspase-3, whose activation culminates in apoptosis. Caspase-3 cleaves a variety of cellular molecules that contain the amino acid motif DEVD such as PARP, and a subunit of the DNA dependent protein kinase (Casciola-Rosen *et al.*, 1996)

To investigate whether an effector caspase (caspase-3), and initiator caspases (caspase-8 and -9) were involved in apoptosis induced in Wil-2 cells exposed to lithium; cells were cultured in the presence 0, 1, 2.5, 5, 10, and 20 mM of lithium for 24 hours. Cells were harvested, lysed, and their caspase activities were determined using the colorimetric protease activity kits for caspase-8, caspase-9 and caspase-3. The activity of each of these caspases was investigated by co-incubation of the specific tetra-peptide substrate included in the kit. As shown in Figure 3.5, Figure 3.6, and Figure 3.7 the activities of these caspases were elevated as the concentration of lithium increased, though not consistent, which suggest that caspases are involved in lithium induced apoptosis. Treated samples were in all respects significantly higher than the controls.

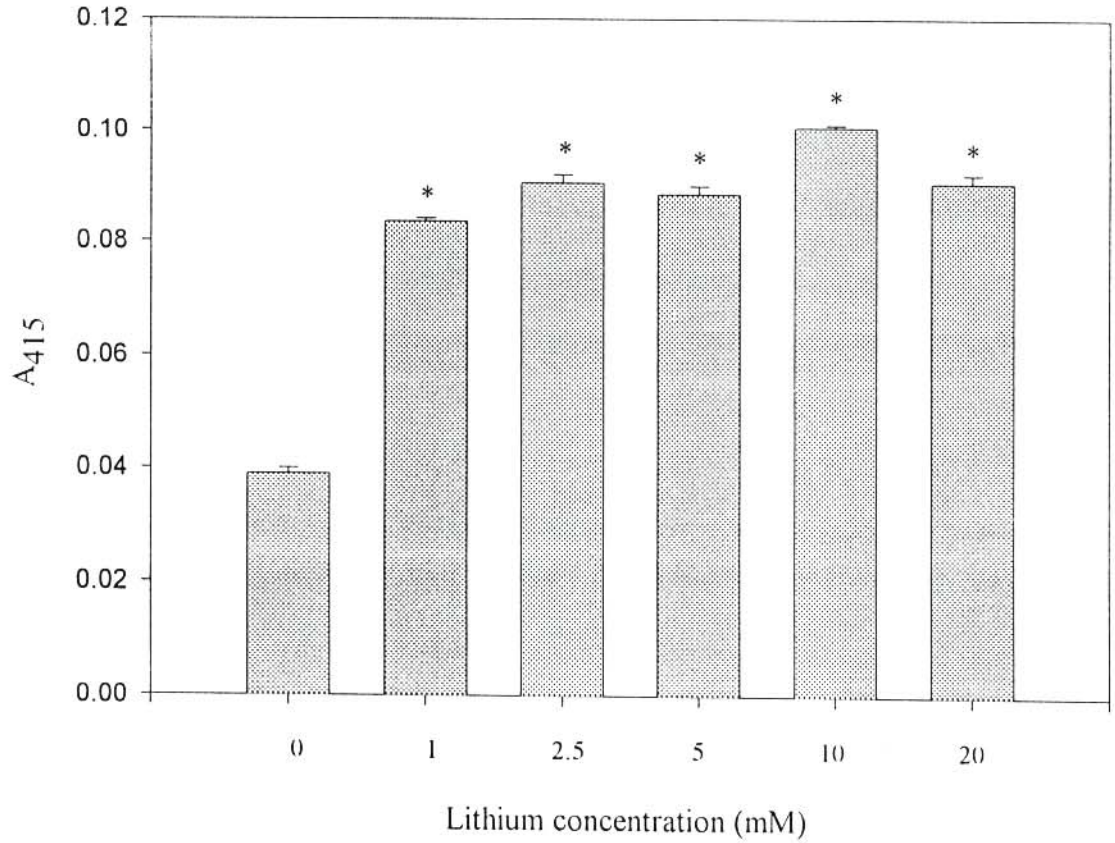


Figure 3.5: The effects of lithium on caspase-8 activity. Wil-2 cells were incubated for 24 hours in the presence or absence of lithium and assayed for caspase-8 activity as described in the materials and methods. The values represented are representative of three experiments each performed in duplicate. *The caspase-8 activities in treated cells were significantly ($p< 0.05$) higher than in the controls.

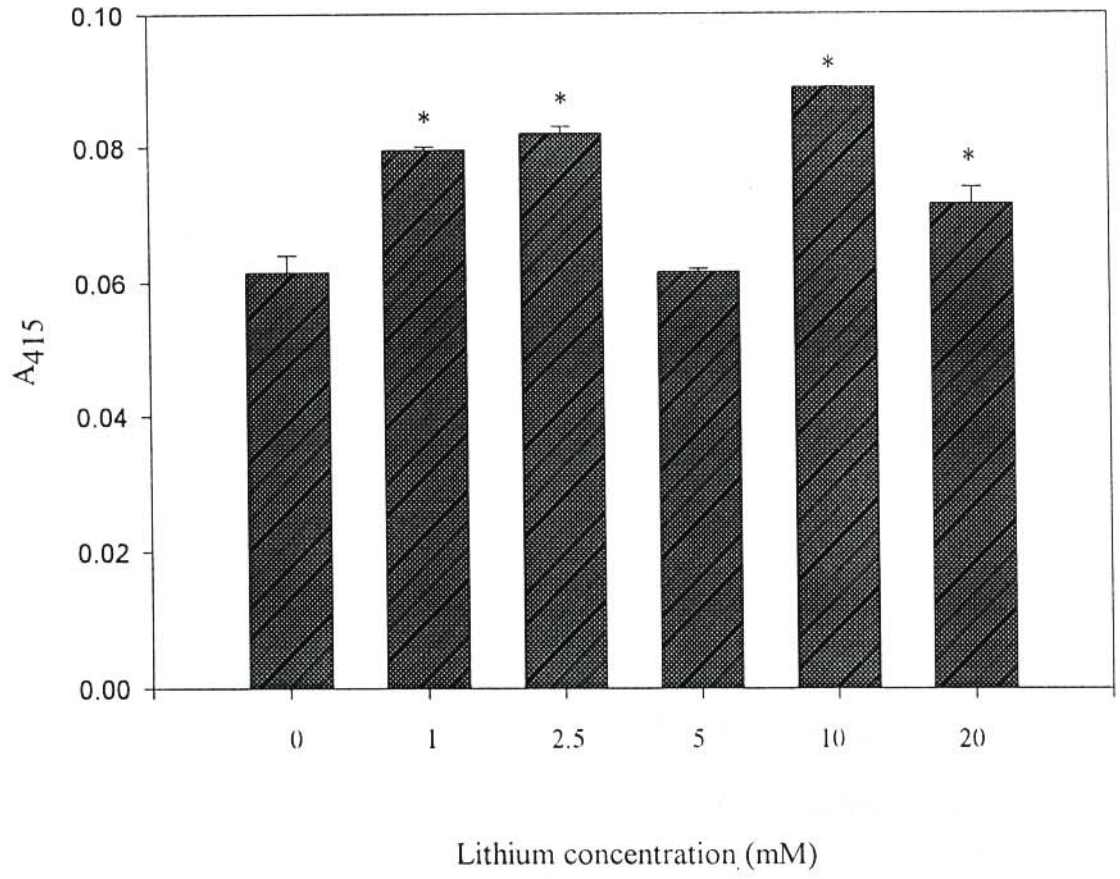


Figure 3.6: The effects of lithium on caspase-9 activity. Wil-2 cells were incubated for 24 hours in the presence or absence of lithium and assayed for caspase-9 activity as described in the materials and methods. The values represented are representative of three experiments each performed in duplicate. *The caspase-9 activities in treated cells were significantly ($p < 0.05$) higher than in control.

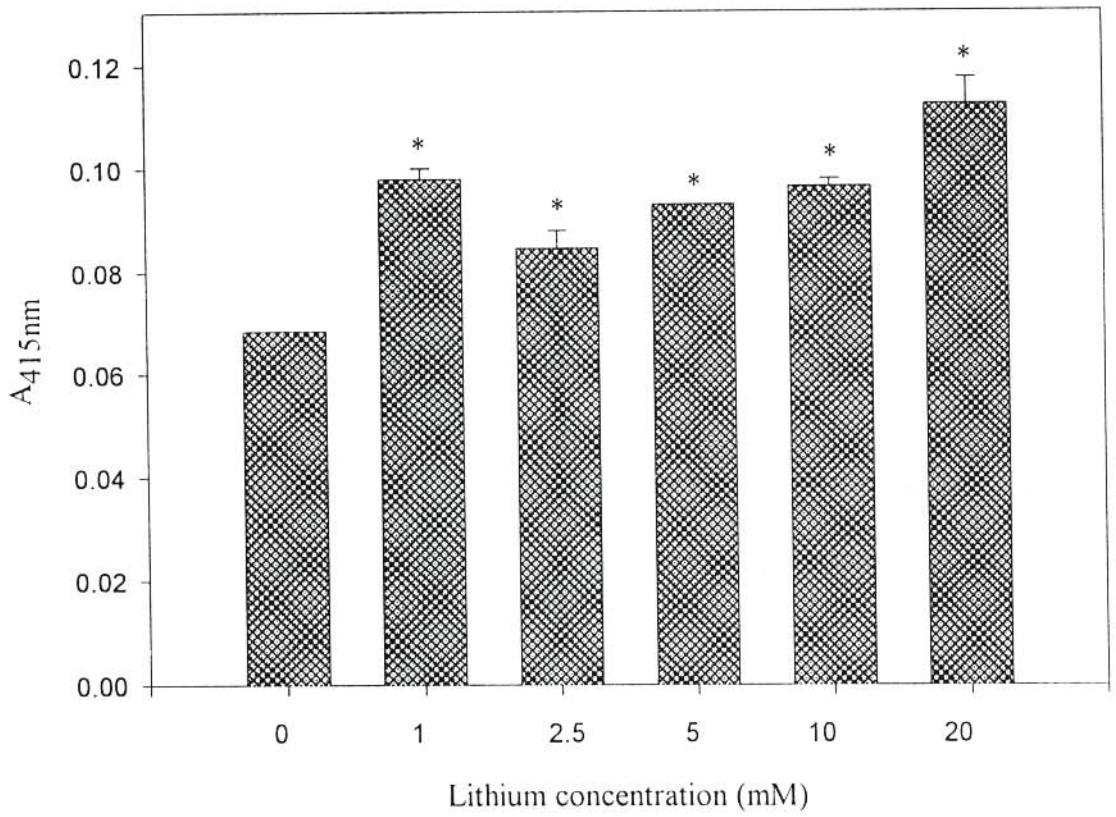


Figure 3.7: The effects of lithium on caspase-3 activity. Wil-2 cells were incubated for 24 hours in the presence or absence of lithium and assayed for caspase-3 activity as described in the materials and methods. The values represented are representative of three experiments each performed in duplicate. The caspase-3 activities in treated cells were significantly ($p < 0.05$) higher than in control.

3.6. PROTEIN ISOLATION AND WESTERN BLOT ANALYSIS

First it was necessary to determine the involvement and thereby the activation of caspases in Wil-2 cells. That was done by caspase activity assays, which revealed that caspases are involved in lithium-induced apoptosis in Wil-2 cells. The caspase activities were elevated following an increasing in lithium treatment. Caspases exist as inactive proenzymes and need to be cleaved at aspartate residues in order to be activated. The pro-form as well as the cleaved products could be detected through Western blot analysis. To investigate the cleavage, thereby activation of caspases, proteins were extracted from treated and untreated cells. Fifty μg of total protein lysates were electrophoresed on a 12% SDS PAGE-gel, figure 3.8, transferred onto a nitrocellulose membrane and then immunoblotted with different antibodies.

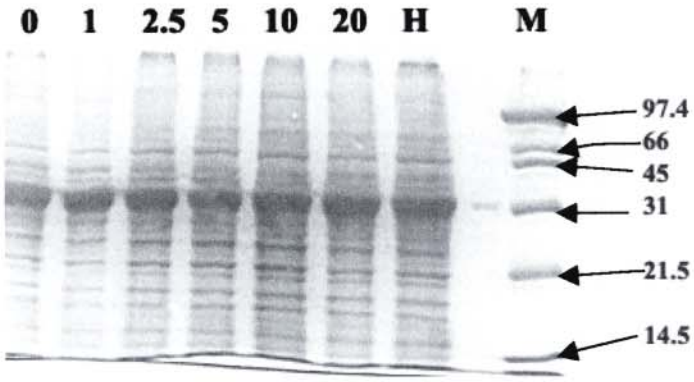


Figure 3.8: Typical electrophoretogram of total protein extract of Wil-2 cells treated with lithium. Wil-2 cells were treated with increasing concentrations of lithium for 24 hours and total proteins extracted as outlined in materials and methods. The protein extracts from control and lithium treated samples were electrophoresed on a 12 % SDS-PAGE-gel. Lane marked H represents 20 mM lithium treated HL-60 cells that were used as positive controls. Lane marked M represents the molecular weight protein marker. Lanes marked 0-20 represents the Wil-2 cells treated with the various lithium concentrations, respectively.

3.6.1. THE EFFECTS OF LITHIUM ON CASPASE EXPRESSION.

Studies by Varfolomeev *et al.* in 1998 showed that caspase-8 is essential for TNF- and FAS-mediated cell death: FAS and TNF could not induce cell death in the caspase-8 deficient mice. For Western immunoblots of caspase-8 and caspase-3, membranes were incubated with purified mouse anti-human caspase-8 primary antibodies and polyclonal rabbit anti-caspase-3 primary antibodies. Then Western immunoblotting was carried out as outlined in the materials and methods. Lithium was found to downregulate the expression of caspase-8 in a concentration dependent manner (Figure 3.9.B). The proform of caspase-8 disappeared in cell lysates as the concentration of lithium was increased. The untreated cells, which are the 0 mM lithium-treated Wil-2 cells and the control cells (Jurkat and HL-60's) did have a large 55 kD band, as expected from previous reports as they were not induced to undergo apoptosis. The immunoblot evidence of caspase-8 activation in lithium treated cells was further substantiated by proteolytic activation of caspase-3. Caspase-3 was activated efficiently as cleaved fragments of 17 kD could be detected (Figure 3.9.A). Caspase-3 consists of a 17 kD and 11 kD subunits that are derived from a 32 kD proform (procaspase-3) by cleavage at multiple aspartic acid sites (Fujita and Tsuruo, 1998).

Examination of cell lysates for proteolytic activation of caspases with Western blot analysis revealed that among control cells, the proforms of caspase-3 and -8 largely disappeared from cells in a concentration dependent manner, suggesting that they have been proteolytically activated.

3.7. BAX PROTEIN LEVELS IN LITHIUM TREATED CELLS.

Bax expression, a 21 kD protein that promotes apoptosis by inhibiting the anti-apoptotic activity of Bcl-2 (Oltvai *et al.*, 1993), was determined by western blotting. Since it is the Bcl-2/Bax ratio that dictates whether the cell dies or survives, the effects of lithium on the expression of Bax was investigated. Cell lysates were collected and processed at 24 hours. Western blot analysis was performed with antibodies against Bax. Lithium treatment lead to expression of pro-apoptotic Bax in treated cells in a concentration dependent manner (Figure

3.10). Therefore lithium probably induces apoptosis in the Wil-2 cells by up-regulation of the Bax protein.

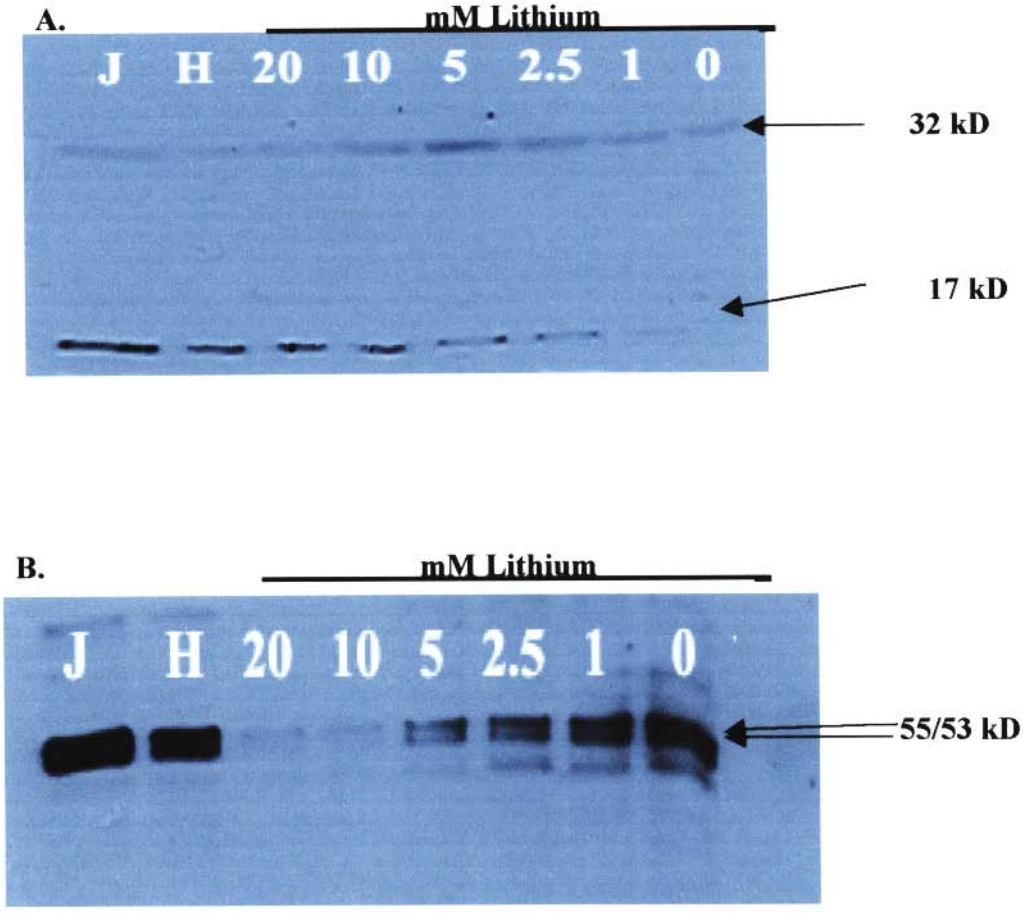


Figure 3.9: Caspase-3 and caspase-8 activation in response to lithium treatment. Wil-2 cells were treated for 24 hours with increasing lithium concentrations and protein extracts were isolated thereafter. The protein extracts were electrophoresed and transferred onto a nitrocellulose membrane. **(A)** The membrane was probed with polyclonal rabbit anti-human caspase-3 antibody. HL-60 cells (H) and Jurkats cells (J) treated with 20 mM lithium were used as positive controls. **(B)** The membrane was probed with rabbit anti-human caspase-8 antibody. Untreated HL-60 cells (H) and Jurkats cells (J) were used as controls.

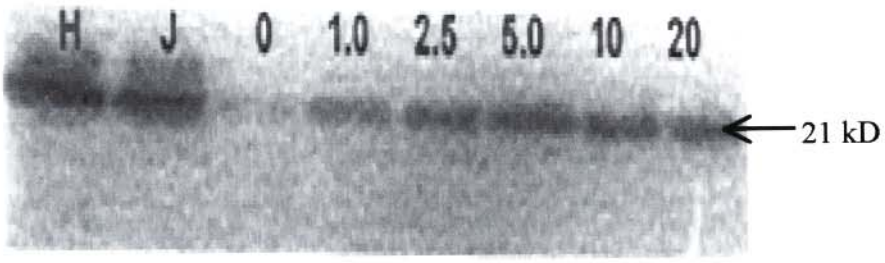


Figure 3.10. Western blot analysis of Bax expression. Wil-2 cells were treated with lithium for 24 hours following which cytosolic extracts were prepared for immunoblotting. The nitrocellulose membrane was probed with rabbit anti-human Bax antibody. The concentrations of lithium are shown at the top of the figure. Lane **H** represents HL-60 cells and **J** represents Jurkat cells treated with 20 mM lithium for 24 hours and served as positive controls.

3.8. EFFECTS OF TNF-α ON WIL-2 CELLS.

The biological action of TNF-α as previously documented includes anti-tumor activity (Sethi *et al.*, 2000). It is reported to have cytotoxic effects on some transformed cells with the majority of human tumors and normal cells being resistant to its killing effects (Wallach *et al.*, 1997). Those susceptible to its cytotoxic effects either undergo apoptotic or necrotic cell death (Baker and Reddy, 1998). Studies by Beyaert *et al.* (1989) showed that TNF-α cytotoxicity was considerably increased by lithium co-treatment *in vitro* and in animal studies, without significant side effects. To determine if lithium and TNF-α could synergistically act on the cells, it was necessary to first test the effects of TNF-α only on Wil-2 cells. Wil-2 cells were cultured in the presence of 0, 1, 10, 20, 50, and 100 ng/ml of TNF-α and the cell density determined daily for three days at 24 hour intervals, as described in materials and methods. TNF-α induced inhibition of Wil-2 cell proliferation within 72 hours

(Figure 3.11). At the same time, the viability of Wil-2 cells (Figure 3.12) decreased significantly ($p<0.05$) after 72 hours with treatment of 50 ng/ml TNF-α. Control cells (Wil-2 cells without lithium) maintained their viability throughout the incubation period.

3.9. ANALYSIS OF DNA FRAGMENTATION IN TNF-α TREATED CELLS.

Studies in our laboratory showed that lithium treated Wil-2 cells fragment their DNA into higher molecular weight DNA fragments (Mukhufi, 2000). The effects of TNF-α on the induction of DNA laddering were examined. Cells were treated with increasing concentrations of TNF-α over three days. Wil-2 cells treated with 20 mM lithium were used as positive controls as they were shown previously, and herein, to induce high molecular weight DNA fragmentation. The "typical laddering" observed in apoptosis could be clearly seen. TNF-α treated Wil-2 cells failed to induce high molecular weight DNA fragmentation, as shown in Figure 3.13. The immediate question that comes to mind is whether TNF-α induces low molecular weight (internucleosomal) DNA fragmentation? If yes, that would be an advantage, because then TNF-α, and lithium can have possible synergistic effects via two different pathways. Future work will focus on whether TNF-α induces internucleosomal DNA fragmentation.

In addition to the DNA laddering assay, a TUNEL assay was performed as described in materials and methods, and the micrographs of fluorescing cells taken after 24 hours of treatment. Contrary to the results observed with DNA laddering, there was fluorescence detected in TNF-α treated cells indicating DNA strand breakage (Figure 3.14). The fluorescence intensity was increasing as the TNF- α concentration increased.

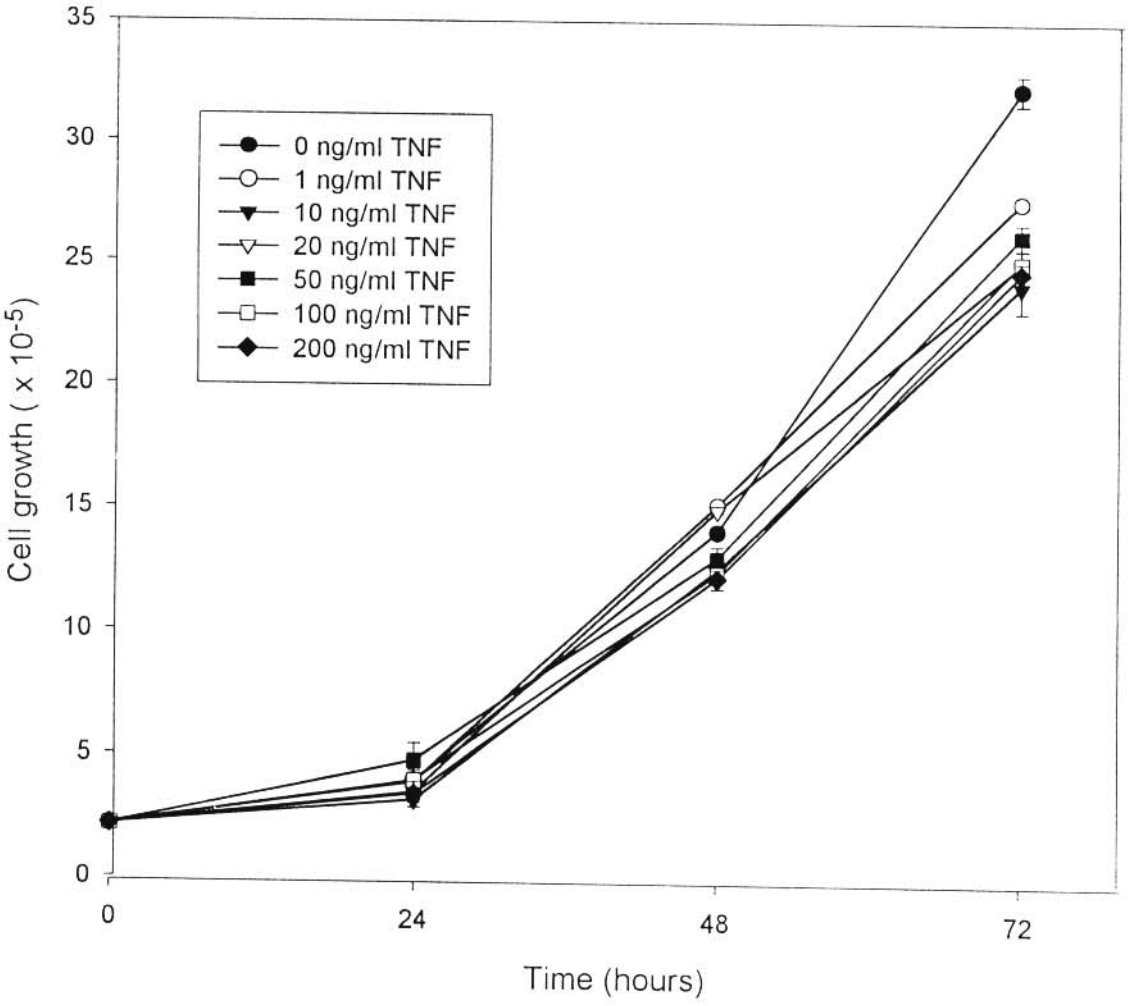


Figure 3.11: The effects of TNF on proliferation of Wil-2 cells. Wil-2 cells were treated with various concentrations of TNF for 72 hours. Cell counts were determined at 24, 48, and 72 hour intervals. The results represent the mean and standard deviation of three independent experiments done in duplicates.

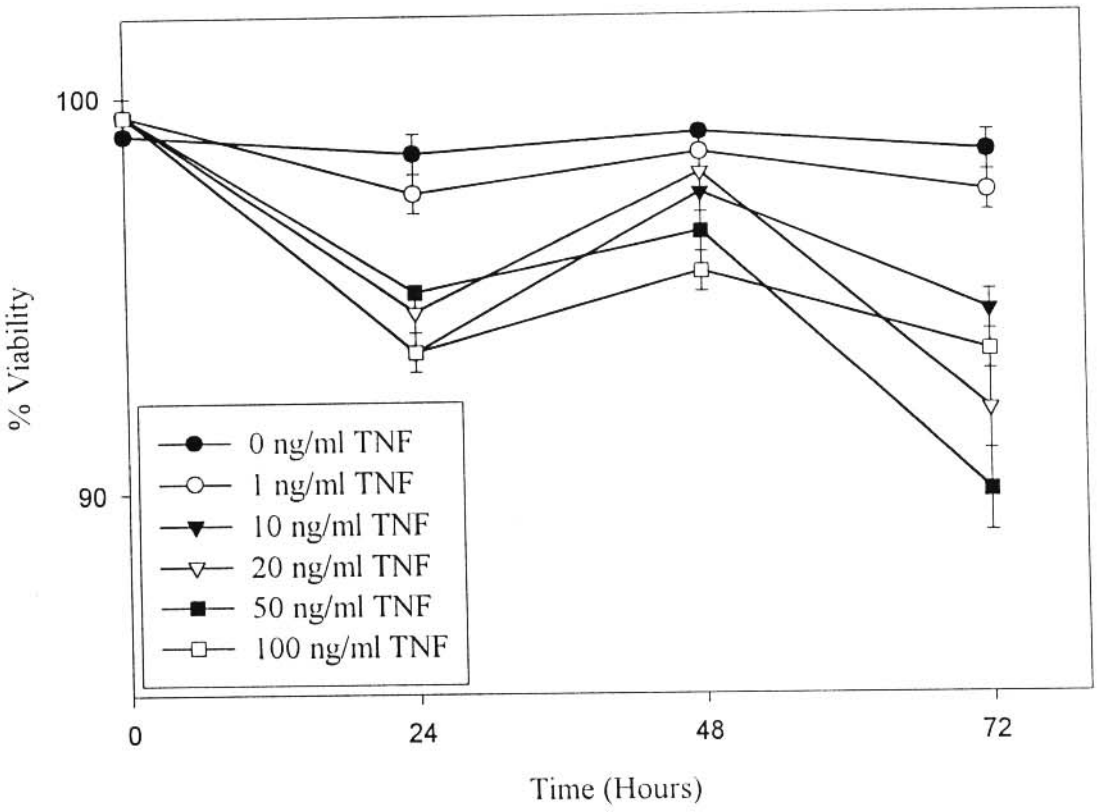


Figure 3.12: The effects of TNF on the viability of Wil-2 cells. Cells were incubated for three days in the presence of 0-100 ng/ml TNF as described in the materials and methods. Samples were taken daily at 24 hours intervals.

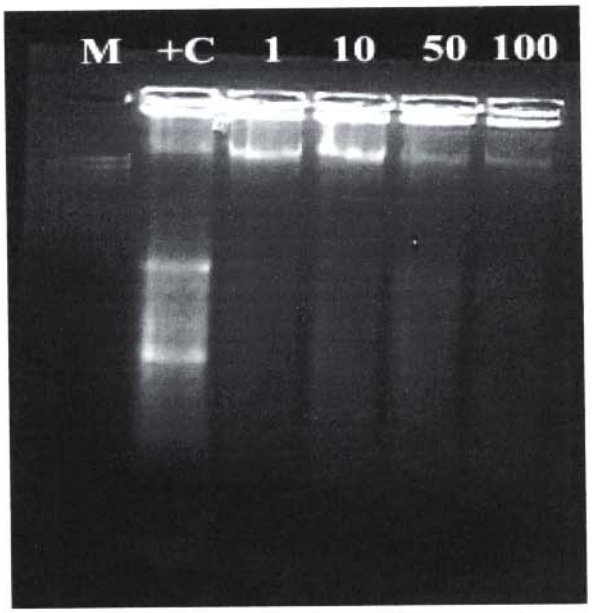


Figure 3.13: DNA fragmentation in TNF-α treated Wil-2 cells. Wil-2 cells were incubated for 72 hours with TNF-α. The cells were analyzed for DNA fragmentation using the method of Bouffard and Mompaler (1995), as outlined in Materials and methods. Lane marked **M** represents the molecular weight marker, lambda DNA digested with Hind III. Lane marked **+C** represents 20 mM lithium treated Wil-2 cells, which were used as a positive indication of apoptosis. TNF-α concentrations (ng/ml) used are indicated at the top of the figure.

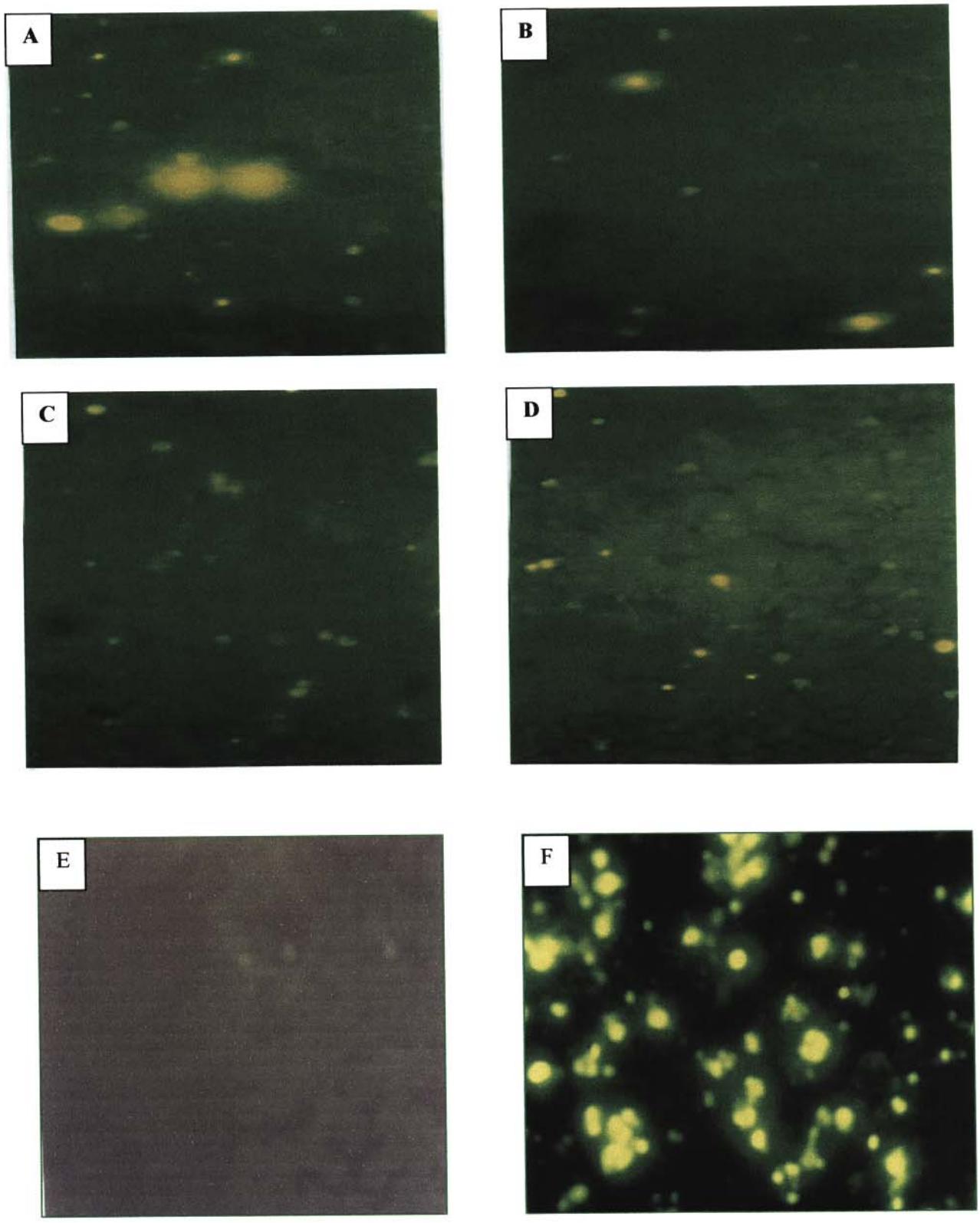


Figure 3.14. Analysis of DNA strand breaks in TNF-α treated Wil-2 cells. Cells were treated for 24 hours with (**A**) 1 ng/ml, (**B**) 10 ng/ml, (**C**) 50 ng/ml, and (**D**) 100 ng/ml TNF-α, (**E**) Negative control (Untreated), (**F**) Positive control (20 mM treated Wil-2 cells) and exposed to TUNEL. Appearance of apoptosis was confirmed under a fluorescent microscope.

3.10. TNF-α-SENSITIZING EFFECT OF LITHIUM

To investigate whether lithium and TNF-α can work in synergy to induce apoptosis in Wil-2 cells, the cells were treated with TNF-α 1 hour prior to lithium treatment. Previous studies have shown that KYM37E4 cells co-treated with TNF-α and lithium die in apoptotic ways (Schotte *et al.*, 2001). Co-treatment with lithium in that study was found to drastically increase the cytotoxic effect of TNF-α in that cell line. Figure 3.15(A) and Figure 3.15(B) show the effect of cells treated with TNF-α, lithium, and TNF-α plus lithium. In both cases, it was evident that TNF-α can really sensitize cells to the cytotoxic effects of lithium. Therefore the combination of TNF-α and lithium is quite efficient in inhibiting the growth of Wil-2 cells.

3.11. EFFECTS OF TNF-α AND LITHIUM ON THE INDUCTION OF APOPTOSIS

From Figure 15(A) and Figure 15(B), it was evident that co-treatment of Wil-2 cells with TNF-α and lithium leads to a greater decrease of proliferation, compared to incubation with TNF-α or lithium alone. This was accompanied by reduced cell viability (data not shown) suggesting that co-treatment with lithium was not only inhibiting growth, but was also cytotoxic to these cells. To determine whether the co-treatment will as with the individual drugs induced apoptosis, Wil-2 cells were co-treated with TNF-α and lithium for 24 hours after which the TUNEL assay was performed as outlined in the manufacturer's protocol. As with the other results, it was found that the drastic decline in cell counts was associated with apoptosis (Figure 3.16). It was also worth noting that as the dosage of TNF-α increased, so did the fluorescence, and therefore apoptotic cells. All cells treated with co-treated with TNF-α and lithium showed more response than those treated with lithium or TNF-α alone.

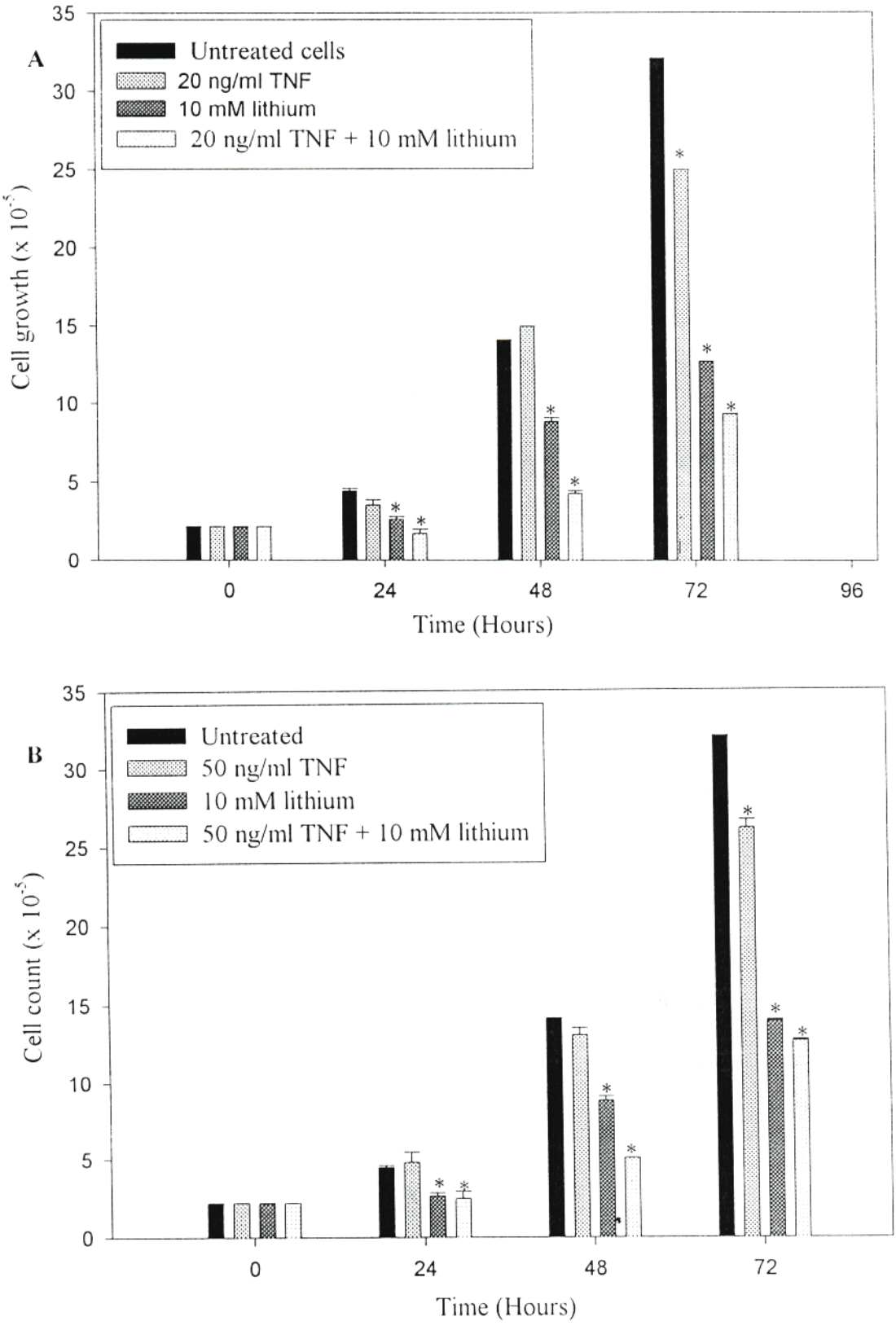


Figure 3.15: Combined effects of lithium and TNF on Wil-2 cells. Wil-2 cells treated with **(A)** TNF alone (20 ng/ml), lithium alone 10 mM, and TNF (20 ng/ml) plus lithium (10 mM). **(B)** Wil-2 cells were treated with TNF alone (50 ng/ml), lithium alone (10 mM), and TNF (50 ng/ml) plus lithium (10 mM). TNF was added 1 hour prior to lithium treatment. The results are the mean of three independent experiments, each done in duplicate. *$p<0.05$ when compared to controls.

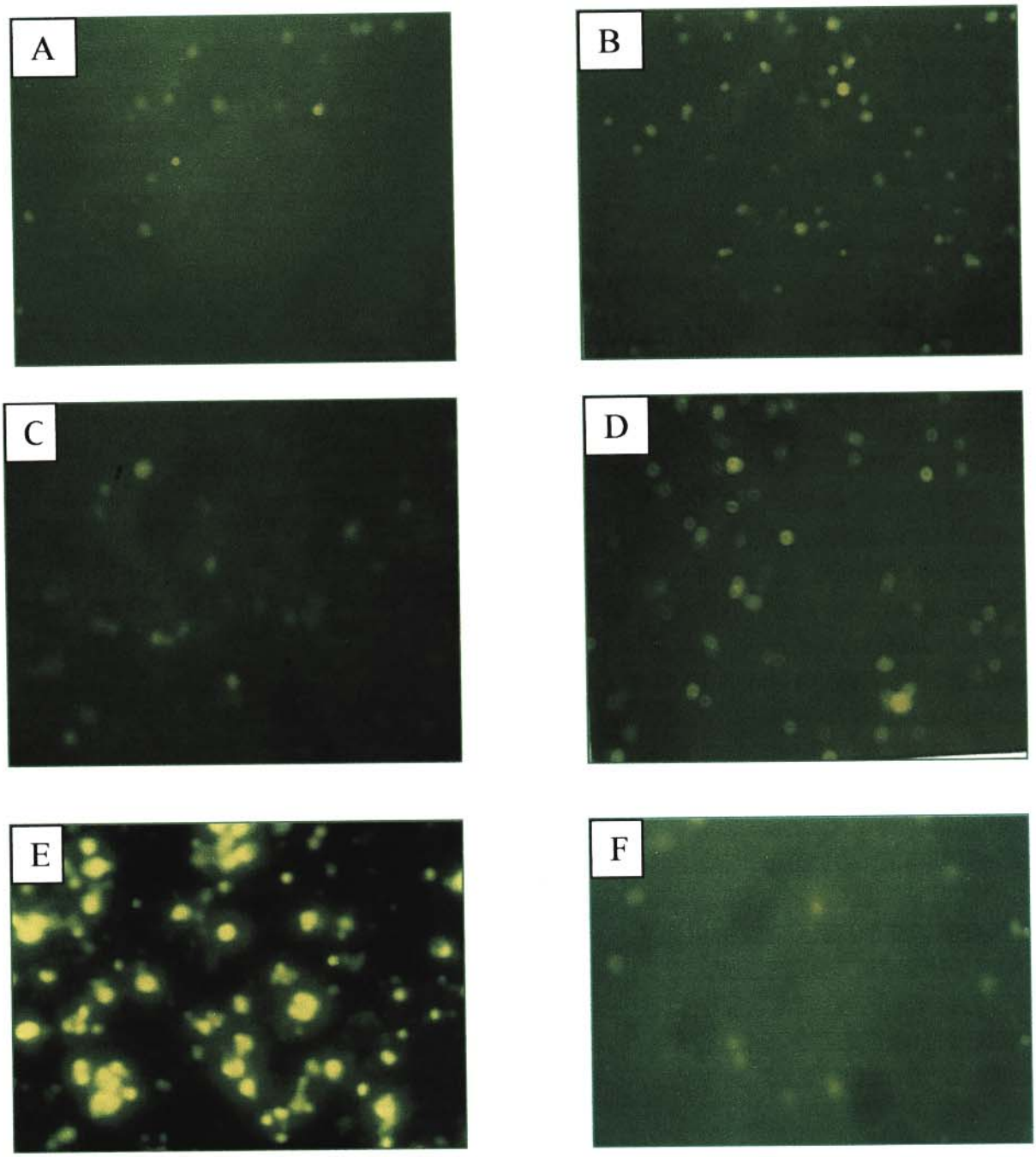


Figure 3.16. The effects of TNF-α and lithium co-treatment on the induction of apoptosis in Wil-2 cells. Wil-2 cells were pre-treated for 1 hour with TNF-α and stimulated with lithium, then subjected to TUNEL assay after 24 hours. Wil-2 cells were treated with 10 ng/ml and 20 ng/ml of TNF-α (**A** and **B** respectively), and subsequently co-treated with 2.5 mM lithium; with 10 ng/ml and 20 ng/ml of TNF-α (**C** and **D** respectively), and subsequently co-treated with 5 mM lithium. **E** represents 20 mM lithium treated HL-60 cells (positive control); **F** represents 2.5 mM lithium treated Wil-2 cells.

CHAPTER 4

DISCUSSION AND CONCLUSION

Lithium, a drug known to have neuroprotective effects in a number of model systems, has been used in clinically for the treatment of mood disorders for several decades (Lu *et al.*, 1999; Birch *et al.*, 1999). It has drawn overwhelming research interest in that the mechanism underlying its therapeutic efficacy is still not clearly understood. A major drawback in lithium therapy is its narrow therapeutic range with in *vivo* fatality at concentrations above 1.5 mM (Reisberg and Gershorn, 1979). Lithium has been shown clinically to increase the production of white blood cells in patients receiving its therapy (Becker and Tyobeka, 1990).

During the past fourteen years studies in our laboratory have shown that lithium affects the growth of HL-60 cells. A number of studies showed that lithium stimulates growth of HL-60 cells and various cells *in vitro*. At concentrations below 10 mM, lithium induced proliferation of HL-60 cells, whereas above this concentration it was toxic (Becker and Tyobeka, 1990). The cytotoxicity was associated with apoptosis, exerted in a concentration- and time-dependent manner (Madiehe *et al.*, 1995). The first report of lithium induced apoptosis in cell cultures was by D'Mello *et al.* (1994), who in their study found that 10 mM lithium induced apoptosis in immature granule cells. The effects of lithium were further investigated, in our laboratory, on two lymphoblastic leukemia cells of human origin, Wil-2 NS and Raji cells. Contrary to the findings with HL-60 cells, lithium was in this respect found to inhibit the growth of the two cell lines in a time and concentration dependent manner, but no enhancement of proliferation was detected. In the Wil-2 cells even concentrations as low as 2.5 mM induced the apoptotic cell death (Mukhufi, 2000).

In this study lithium was found to inhibit proliferation in Wil-2 cells in a time and concentration dependent manner, with concentrations of 1.0 mM being cytotoxic to these cells. Viability studies using the trypan blue dye exclusion method showed that lithium induces cell death in the Wil-2 cells, associated with the reduction in proliferation.

To determine the mode of cell death, which could either be apoptosis or necrosis, cells were subjected to DNA laddering and TUNEL assays. In apoptosis cell death is programmed whilst necrosis is a passive degenerative process without precise regulation. The main characteristics of apoptosis, distinguishing it from necrosis, are: organised lysis of DNA before actual cell death, formation of "apoptotic bodies", and cell shrinkage due to condensation of cytoplasm (Wyllie, 1987). The hallmark of apoptosis, which is internucleosomal DNA fragmentation, has been widely observed in dying cells. Studies by Belenskya *et al.* (1997) showed that Wil-2 cells do not fragment their DNA into internucleosomal sizes, but larger fragments. This observation was confirmed in our laboratory by Mukhufi (2000) in an attempt to elucidate the mechanism of lithium action in Wil-2 cells. In the present studies, lithium induced apoptosis in Wil-2 cells, with concentrations as low as 1.0 mM inducing DNA fragmentation into high molecular weight fragments in a time dependent manner. In cells treated for 24 hours, there were no detectable DNA fragments, characteristic of apoptosis, which indicates that the cell death was time dependent. The loss of viability was therefore attributed to cell death by apoptosis. The present study therefore supports previous findings that Wil-2 cells fragment their DNA into larger sizes in a concentration and time dependent manner.

To further investigate the high molecular weight DNA fragmentation results, Wil-2 cells were subjected to TUNEL assay, which detects and quantifies apoptosis at single cell level based on the labelling of DNA strand breaks by addition of fluorescent dUTP. The assay detects strand breaks irrespective of the size. Lithium concentrations as low as 1.0 mM induced apoptotic DNA strand breaks. Worth noting is the sensitivity of the assay as it was able to detect fragmentation of DNA within 24 hours. This, as reported by Mukhufi (2000), shows that Wil-2 cells are more responsive to lithium induction of apoptosis than the HL-60 promyelocytes that requires lithium to be above 10 mM before apoptosis can be induced (Madiehe *et al.*, 1995).

Apoptotic death results in the same morphological changes, that includes cell and organelle dismantling and packaging, DNA cleavage (to nucleosome-sized fragments), and caspase mediated cleavage of the same cellular proteins, irrespective of the lethal stimulus (Salvesen

and Dixit, 1997). Caspases, a family of cysteine-dependent aspartate-directed proteases, have been thought to play a central role in apoptosis (Alnemri *et al.*, 1996). They exist in the cell cytoplasm as zymogens, which upon stimulation are processed into their active forms either through autocatalysis or by other caspases.

Caspase-3 is probably the best understood of the mammalian caspases in terms of its specificity and roles in apoptosis. Recent progress has generally confirmed the notion of multiple, complex death pathways that converge on common events which appear to require caspase-3 in all cell types so far examined (Porter and Janicke, 1999). Once caspase-3 is activated, downstream death substrates are cleaved irrespective of the involvement of cytochrome c (Reed, 1997).

Previous studies have established that etoposide-induced apoptosis in intact HL-60 cells is accompanied by selective activation of the pro-caspase species present in these cells. In this study the involvement of these caspases in Wil-2 cells induced to undergo apoptosis after treatment with lithium was investigated. The caspase-3, caspase-8, and caspase-9 cleavage activities were determined on Wil-2 cells after 24 hours following lithium treatment. The caspase-8 activity was determined by its ability to cleave a peptide substrate IETD-pNA, and caspase-3 activity by its ability to cleave a peptide substrate (DEVD-pNA) to release a colorimetric substrate pNA. An increased and early involvement of caspases was observed for caspase-8 and caspase-3. Caspase-9 activities were also significantly higher than those of untreated cells but were not as prominent.

To confirm whether the increased lithium-induced apoptosis in Wil-2 cells is associated with increased caspase activation, the kinetics of activation of caspase-3 and caspase-8 were analysed. The caspase -8 and caspase-3 cleavage activities were determined on Wil-2 cells at 24 hours following lithium treatment using Western blotting. Processing of pro-caspase-8 to the active enzyme was demonstrated, which showed the loss of the 55 kD precursor with an increase in lithium concentrations. The loss of a 32 kD caspase-3 precursor and the appearance of a 17 kD subunit was also unmistakable. The loss of the pro-forms of caspase-8

and caspase-3, and the appearance of the smaller active subunits undoubtedly shows that caspase are involved in lithium-induced apoptosis in Wil-2 cells.

Apoptosis has been shown to be regulated by a number of cell cycle and apoptosis related proteins. Several reports have been published that implicate the involvement of Bax in apoptosis. Bax, a Bcl-2 related family member, has been shown to accelerate apoptosis. It can induce both membrane potential dissipation and cytochrome c release, suggesting that Bax acts to destabilize mitochondrial membrane function (Rosse *et al.*, 1998). Suppression of survival signals is the result of a complex formation between Bcl-2 or Bcl-X_L and apoptosis inducing proteins like Bax. In this study the involvement of Bax in lithium induced apoptosis was investigated. Cells treated for 24 hours with lithium were analysed by Western blot, as described. Bax expression was upregulated by an increase in the lithium concentration. Thus it appears that lithium induces apoptosis in a pathway that involves Bax. Evidence supporting Bax involvement in lithium induced apoptosis was previously reported by Mampuru (2000), in murine stromal cells, wherein semiquantitave RT-PCR analyses of Bax mRNA levels stayed constant following treatment with various concentrations of lithium. Similar studies by Chen and Chuang (1999) on cerebellar granule cells showed that treatment of these cells with lithium chloride induces a concentration-dependent down-regulation of Bax (Chen R-W,. and Chuang D-M, 1999). These contradictory results might be due to the authors using a different cell line (primary cultures of neuronal cells), and a longer treatment period than that used in this study.

TNF-α is recognised as a naturally occurring cytokine that orchestrates many cellular responses, in many cell types, including induction of apoptosis. There have been attempts to use it as a drug, but they have not proven successful clinically (Sethi *et al.*, 2000). The therapeutic application of TNF-α in cancer treatment is limited by the fact that many tumour cells are only weakly sensitive to its direct action. Pre-clinical trials using TNF-α as an anti-tumour agent in early years has thus been disappointing, mainly because the doses were too low to observe efficacy (Beyaert *et al.*, 1989). In this study, Wil-2 cells were found to undergo apoptotic cell death induced by TNF-α. Yet, only lithium treated cells, which served as positive control showed DNA laddering into high molecular weight DNA fragments. The

cytokine was able to inhibit a significant fraction of treated Wil-2 cells from proliferating. There was also a small, but significant decrease in viability. TUNEL assay showed that only a small population was susceptible to DNA cleavage induced by TNF-α. Even doses as high as 100 μg/ml were not enough to induce significant cell death. TNF-α therefore, failed to induce pronounced apoptosis contrary to lithium.

To overcome this problem of TNF-α resistance, as well as to be able to use lower doses of TNF-α with less side effects, agents that specifically increase the sensitivity of tumour cells to TNF-α might be employed (Schotte *et al.*, 2001). Studies by Beyaert and colleagues have since 1989 been able to show that lithium can considerably increase the direct effect of TNF-α on tumour cells, both *in vivo* and *in vitro*. However, the underlying mechanism has remained largely unknown. Several lines of evidence have indeed confirmed the sensitizing effect of lithium in TNF-α treated cells. This study showed that Wil-2 cells pre-treated with TNF-α can be sensitised to lithium effects as shown by the reduction in proliferation. The Wil-2 cells responded more to the combined treatment than they did to any of the drugs used individually. The effect of TNF-α on Wil-2 cells was therefore increased by lithium. This was confirmed using TUNEL assay where co-treated cells showed more fluorescence than cells treated with individual drugs.

In conclusion, the results suggest that lithium induces apoptosis in Wil-2 cells with concentrations as low as 1.0 mM. It was evident that caspases play a role in the process of apoptosis. This lithium-induced apoptosis is thought to be caspase-dependent The pro-apoptotic protein Bax was upregulated by treatment of the cells with lithium. Therefore the use of this drug may be important in the therapy of B-cell lymphomas. The results also show that TNF-α alone induce programmed cell death but not as sufficient as in lithium induced apoptosis. Lithium was shown to be more effective when working in synergy with TNF-α, with treated cells dying through apoptosis. As lithium has been used for many years in the clinic to treat manic depression, the TNF-α /lithium sensitizing effect is intriguing and merits further experiments aimed to understand the underlying mechanism. In summary, the mechanism of lithium-induced apoptosis in Wil-2 cells was found to involve the activation of caspase-3 and caspase-8, as well as the up-regulation of Bax.

CHAPTER 5

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