PATHWAYS RESPONSIBLE FOR
APOPTOSIS IN CHICK CARDIOMYOCYTES

by
Jennifer Y. Kong

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Department of Experimental Medicine

The University of British Columbia
Vancouver, Canada

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ABSTRACT

The mechanisms responsible for apoptosis in the heart are currently being defined. The present study was designed to determine the roles of nuclear enzymes and signal transduction protein kinases in the development of apoptosis in chick embryo cardiomyocytes. Topoisomerase I was chosen as an example of a nuclear enzyme involved in apoptosis. Topoisomerase I is the enzyme responsible for relieving torsional stress in DNA replication and transcription. To determine whether inhibition of topoisomerase I would produce apoptosis in cardiomyocytes, the inhibitor camptothecin was used. Cardiomyocytes, obtained from 7 day old embryonic chick hearts, were treated with camptothecin and examined microscopically or their DNA was examined for fragmentation. Apoptotic cell death was produced by camptothecin as fluorescent microscopy with acridine orange demonstrated cardiomyocytes that were shrunken with cytoplasmic blebs and nuclear fragmentation. In contrast, untreated cells did not manifest these cellular alterations. Apoptosis was further substantiated by Hoescht 33258 dye stained cardiomyocytes that showed a strongly fluorescent nucleus which was undergoing disintegration. Cell death as quantitated by trypan blue exclusion showed that camptothecin, 10 μM, significantly increased cell death by 25.1±1.4% (+SEM). Cardiomyocytes were lysed and the DNA isolated and run on a 2% agarose gel. DNA laddering, indicated by fragments of approximately 200 bp or multiples, were found in camptothecin treated cells. DNA fragmentation was also observed quantitatively in camptothecin treated cells, as assessed by an enzyme linked immunosorbent assay (ELISA). Fragmented DNA was isolated from lysed cells and adsorbed onto a microtitre plate. Primary antibody specific for DNA histones was then added and subsequently treated with a horse-radish peroxidase linked-secondary antibody specific for DNA. The colorimetric results were reported relative to control. Camptothecin exposure (10μM) induced 1.5±0.5 fold more DNA fragmentation than control cells.

Alterations in intracellular calcium appeared to be a component of the mechanism of action of camptothecin-induced apoptosis. Ca\(^{2+}\) levels that can be decreased by the chelator EGTA reduced cell death induced by camptothecin, as demonstrated by membrane bleb formation, DNA fragmentation on agarose gel electrophoresis, and DNA fragmentation on the ELISA. Taurine, a free amino acid in many tissues which affects L-type and T-type Ca\(^{2+}\) channels, also reduced camptothecin-induced apoptotic morphology and DNA specific fragmentation, determined by ELISA. To further substantiate the role of calcium and to investigate the source of Ca\(^{2+}\) mediated topoisomerase-induced apoptosis, cardiomyocytes were exposed to thapsigargin, an inhibitor of sarcoplasmic and endoplasmic reticulum Ca\(^{2+}\)-ATPases which increases intracellular calcium. [Ca\(^{2+}\)], increased by thapsigargin exposure yielded greater DNA fragmentation, as assessed by ELISA, than camptothecin alone suggesting that increased [Ca\(^{2+}\)], induced apoptosis itself. With the caveat our use of agents that indirectly implicate the mechanism, these data show that apoptosis in cardiomyocytes is under regulatory control by DNA topoisomerase I and intracellular calcium modulates the pathway whereby topoisomerase I inhibition causes apoptosis.
To investigate the signal transduction mechanisms responsible for apoptosis, I investigated the role of serine/threonine kinases. Staurosporine, a potent serine/threonine kinase inhibitor, was used to investigate the role of kinase inhibition on the development of apoptosis. Staurosporine induced cell death in a dose and time dependent response to a maximal death of 40.9±6.3% at 1μM for 6h. DNA fragmentation, 2.8±1.2 fold that of control, determined by ELISA and electrophoretic separation was observed in staurosporine treated cardiomyocytes. Staurosporine-induced morphology, observed by acridine orange and NBD phallacidin staining, was distinct from usual apoptotic features: staurosporine induced cytoplasmic condensation resulting in dense vacuoles and a loss in volume. Staurosporine treatment failed to exhibit membrane blebbing and distinct nuclear disintegration.

Pretreatment by the Ca\textsuperscript{2+} chelator BAPTA blunted the apoptotic response of staurosporine exposure implicating Ca\textsuperscript{2+} in staurosporine-induced apoptosis. The activation of protein kinase C (PKC) by the phorbol ester PMA blocked staurosporine-induced cell death, morphology, and DNA fragmentation suggesting that the activation of PKC can reverse staurosporine-induced apoptosis. The addition of trophic factors such as insulin and EGF demonstrated a "rescue" pathway in staurosporine-induced apoptotic cardiomyocytes. In addition, de novo protein synthesis may relate to this rescue pathway.

To further investigate the signal transduction mechanisms responsible for apoptosis, the role of PKC was considered. The specific PKC inhibitor chelerythrine chloride was observed to induce cell death of 27.6±7.5% and DNA fragmentation (2.2±0.4 fold that of control) similar to staurosporine. However, chelerythrine exhibited usual apoptotic morphology contrasting staurosporine morphology. In addition, the apoptotic effects of chelerythrine are less potent than staurosporine suggesting that PKC alone is not responsible for staurosporine's apoptotic inducing abilities.
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<td>Ca²⁺</td>
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<td>[Ca²⁺]ᵢ</td>
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CHAPTER I

INTRODUCTION

Multicellular organisms have evolved from simpler organisms by having mechanisms to regulate proliferation, differentiation, aging and death. During the life of an organism, it becomes necessary for cells to be eliminated. Injured cells must be removed to prevent secondary damage that might occur to other cells when a cell break down. Cell death may come to targeted redundant cells to maintain a constant cell number. Similarly, transformed or infected cells must be removed to prevent tumorigenesis or subsequent infections of neighbouring cells. Cells no longer needed in embryonic development must also be eliminated at the correct developmental stage, such as in limb bud formation.

Three classes of cell death are currently recognized: programmed cell death (or apoptosis), necrosis, and non-apoptotic programmed cell death. *Apoptosis* is a functional type of cell death which is programmed genetically into every cell (Schwartz and Osborne 1993a). *Necrosis* is the form of death associated with structural and biochemical degradation of cell integrity due to accidental cellular damage. A type of cell death which doesn’t fall into either category has been labelled *non-apoptotic programmed cell death* (Schwartz and Osborne 1993a). Hence, a cell has different modalities of death.

The question arises: why do cells undergo 3 (or more) different types of death? The variety of types of death types may have evolved to meet demands other than simply to eliminate a cell. Cellular breakdown, seen in necrosis, encourages phagocytosis and reutilization of the cell constituents by other cells.
Unfortunately, this cellular recycling might also aid the spread of potentially infective virus or inflammatory agents, if the disassembled cell had been infected (Clem and Miller 1993, Takizawa et al 1993). Extensive DNA and RNA fragmentation seen in apoptosis may deactivate viral polynucleotides thus inhibiting the machinery needed for viral replication. However, this fragmentation may lead to the accumulation of these potentially harmful viral oligonucleotides (Clem and Miller 1993, Takizawa et al 1993). The early fragmentation of DNA in apoptosis may be particularly important in viral containment, since it could halt viral replication and possibly also inactivate already assembled DNA virus (Martz and Howell 1989). Thus, different types of death may function to deal with the containment and proper disposal of the injured/infected cell.

The most complicated mode of cell death is apoptosis. Apoptosis is an active process in which the cell participates in its own destruction utilizing a set of genetic factors and a complex network of enzymes (Schwartzman et al 1993, Wyllie 1992). Apoptosis may be considered to be a functional form of cell death and is important to the cell as an essential regulator in ontogenesis and tissue homeostasis (Binder and Hiddemann 1994). It occurs during embryogenesis, in parallel with the deletion of autoreactive T cells during thymic maturation, in senescence of neutrophil polymorphs following removal of specific growth factors, and in the presence of stimuli like tumour necrosis factor and glucocorticoids (Arends et al 1990, Sen 1992). Apoptosis can be induced by various chemicals, radiation, or cellular injuries, dependent on the cell type and dosage. Apoptosis is also induced
by cytotoxic T lymphocytes and natural killer cells (Sanderson 1981), ionizing radiation (Yamada and Ohyama 1988), and monoclonal antibodies like anti-Fas 13 (Yonehara et al 1989) and anti-APO-1 (Trauth et al 1989). Apoptosis is an important mode of cell death influencing the health of cells, tissues, and organs.

I) Morphology of cell death

A. Apoptosis

In most cases, apoptosis occurs in stages. Initially, an individual cell embedded in normal tissue loses contact with its neighbours. Chromatin within the nucleus condenses resulting in karyohexis (nuclear disintegration). The cell shrinks due to loss of cytoplasmic volume and condensation of cytoplasmic proteins occur. Most of the intracellular organelles remain intact, although there is condensation of the cytoplasm (Wyllie et al 1980, Gerschenson and Rotello 1992, Arends and Wyllie 1991). The second stage is characterized by membrane ruffling and blebbing (zeiosis) leading to cellular fragmentation and the formation of apoptotic bodies (blebs). The formation of blebs appears to involve disruption of cytoskeletal-membrane interactions (Orrenius et al 1992). Apoptotic bodies frequently contain whole organelles and nuclear remnants (Arends and Wyllie 1991, Williams et al 1974, Wyllie 1980). In the final stage, the neighbouring cells and macrophages phagocytose the cellular fragments for complete degradation of the cell. Apoptosis occurs without leakage of intracellular macromolecules therefore, it does not elicit any inflammatory response.

Chromatin condensation is associated with the activation of an endogenous, Ca$^{2+}$ and Mg$^{2+}$-dependent endonuclease which cleaves double
stranded DNA at the most accessible internucleosomal inner region, generating mono- and oligonucleosomes (Burgoyn et al 1974). In contrast, the DNA of the nucleosomes is tightly complexed to core histones H2A, H2B, H3 and H4 and is therefore protected from cleavage by the endonuclease (Stach et al 1979). The DNA fragments produced by endonucleases are discrete multiples of a 180 bp subunit, which are detected as a "DNA ladder" on agarose gels. Because DNA degradation precedes plasma membrane breakdown, there is an accumulation of mono- and oligonucleosomes in the cytoplasm (Duke and Cohen 1986).

An intact membrane is maintained in apoptotic development until its late stages. Even after the breakdown of apoptotic bodies, the membrane integrity is preserved. The difference in membrane function is considered to discriminate between necrosis and apoptosis (Wyllie 1980). In addition, there is no inflammatory reaction and organelle swelling in apoptosis, in contrast to necrosis (Wyllie 1980).

B. Necrosis

Necrosis is most commonly produced by an interruption of blood flow to an organ with resulting changes in cellular ion permeability leading to osmotic swelling and lysis (Jennings et al 1995). Necrosis is initiated by cellular damage that disrupts osmotic balance (Farber 1990). Ions, especially Ca$^{2+}$, then passively enter the cell resulting in swelling as water enters in response to the ion influx. Necrosis is characterized by dilation of the endoplasmic reticulum, increases in mitochondrial volume, amassing of nuclear chromatin, early membrane breakdown, and cell disintegration resulting in the release of lysosomal enzymes.
(Searle et al 1982, Kerr et al 1972). Necrosis is also characterized by the early breakdown of the intracellular energy supply (Farber 1990). This confirms that necrosis is a passive process that does not require active participation of the cell in its own death. The release of cellular components during cell lysis leads to an inflammatory response which causes secondary damage to neighbouring cells (Searle et al 1982, Kerr et al 1972, Wyllie 1980).

C. Non-apoptotic programmed cell death

Non-apoptotic programmed cell death encompasses cell death which cannot fit into either necrosis or apoptosis. Not all dying cells display the usual changes associated with apoptosis. For example, there have been reports which show cell death with ziosis, but no DNA fragmentation; or cells which undergo DNA fragmentation but do not show the typical morphological changes associated with apoptosis. For example, in the tobacco hawkmoth Manduca sexta, muscle is lost during metamorphosis so a functional form of cell death is needed. However, the muscle lost during metamorphosis does not display membrane blebbing, chromatin margination or the fragmentation of DNA (Schwartz et al 1993b, Schwartz and Truman 1983). A similar response is also seen in mammalian neurons that die during development (Chu-Wang and Oppenheim 1978, Clarke 1990). Ciliary neurons of the chick die during normal neurogenesis, yet the death appears to be non-apoptotic while the death induced by target removal appears to be apoptotic (Pilar and Landmesser 1976).

D. Biological implications of apoptosis

Programmed cell death is a functional form of elimination.
Only the cell that is selected for death is eliminated by its own cellular system responsible for the induction and control of apoptosis. Apoptosis may be a defense mechanism that is distinct from more traditionally accepted defense mechanisms such as DNA repair systems, antioxidant systems, heat shock proteins, and nuclear enzymes such as poly(ADP)ribose polymerase. It may also be the key mechanism for removal of damaged cells. Apoptosis is also involved in the removal of embryonic cells during embryonic morphogenesis. The correct functioning of apoptosis is critical to the phenotypic fidelity of multicellular organisms.

Defective controls of apoptosis maybe the etiology for some diseases. Apoptosis may lead to the cumulative breakdown of tissue and organ integrity seen with age, AIDS, and autoimmune diseases (Tomei et al 1994). Conversely, an inhibition of apoptotic mechanisms may be involved in the pathogenesis of cancers and degenerative diseases of the central nervous system (Carson and Ribeiro 1993, Collins and Lopez-Rivas 1993, Fesus et al 1991). Where apoptosis is a protective measure preventing malignant transformation and viral infection, a defective apoptotic mechanism can allow the uninhibited proliferation of these abnormal cells. Thus, the regulation of apoptosis is crucial.

II) Nuclear pathways to apoptosis

Many pathways can lead to or control apoptosis. However, since apoptosis is a genetic program, it is rational to start investigation at the nuclear level. During apoptosis, the activity of nuclear enzymes may be altered so as to allow the induction or down-regulation of apoptotic death genes or cell survival genes such as p53 and bcl-2, respectively. Hence nuclear enzymes
responsible for gene activity would be an ideal locale to initiate the investigation into apoptosis.

A. Topoisomerases

Topoisomerases are the enzymes responsible for relieving the torsional stress caused by DNA unwinding in DNA synthesis and transcription. There are two major types: DNA topoisomerase I (topo I) and topoisomerase II (topo II). Topo I produces changes in DNA supercoiling by catalyzing the single-strand cutting of the DNA, thus allowing the rotation of a free end (relieving a supercoil) followed by the resealing of the nick (Mathews and van Holde 1990). Topo I is more active with negative than positive supercoils. Because the relaxation of DNA supercoils is energetically favourable, topo I does not require high energy cofactors. Topo II alters the supercoiling in order to facilitate chromosome folding and twisting and cuts both strands of one DNA double helix so that a neighbouring region of helix can pass through the cut ends, and eventually reseals the cut (Darnell et al 1986). Topoisomerases are reportedly involved in producing apoptosis (Tritton 1991, Walker et al 1991, Forbes et al 1992).

1. Inhibitors of topoisomerases I and II

Inhibitors of topo I and II were found to induce apoptosis in thymocytes (Onishi et al 1993), mature unstimulated lymphocytes (Roy et al 1992) and human lymphocytic leukemia cells (Rajotte et al 1992). Topo inhibitors were able to inhibit DNA fragmentation in target cells exposed to cytotoxic lymphocytes (Nishioka and Welsh 1992).

2. Camptothecin

The topo I inhibitor, camptothecin, is an alkaloid isolated
from the stem wood of *Camptotheca acuminata* (family Nyssaceae). Camptothecin (campto) has been reported to inhibit growth of experimental tumours in rodents (Hartwell and Abbott 1969) and to inhibit the synthesis of DNA and RNA in various cell lines (Horwitz et al 1971, Kessel 1971). Campto "poisons" topo I when the enzyme is associated with DNA during cleavage, increasing the steady-state concentration of topo I-DNA cleaved complexes, thus introducing high levels of transient enzyme-associated breaks in the genome (Froelich-Ammon and Osheroff 1995).

**III) Intracellular pathways to apoptosis**

An intracellular pathway must be in place to transduce signals from extracellular stimuli to the nucleus. In addition, the control for the cellular decision of which form of death program to implement (i.e. apoptosis vs. necrosis vs non-apoptotic programmed cell death) must be flexible enough to respond to a wide range of extracellular stimuli. Hence, protein kinases have been postulated as the mechanism, not only for signal transduction, but also for a cellular "rheostat control" that can respond to the degree of cellular damage and can implement the different forms of cell death.

Protein phosphorylation is performed by protein kinases. Protein kinases have been subclassified into identifiable groups, depending on the target amino acid residue where the phosphorylation occurs. Certain classes of kinases are predominant in different cellular effects. The serine/threonine kinase pathway is involved in the regulation of many processes pivotal for growth and differentiation (Nishizuka 1986). Hence, the investigation of serine/threonine kinases is an ideal pathway to perform primary
investigations into signal transduction pathways responsible for apoptosis.

The activity of protein kinases is regulated allosterically. In serine/threonine kinases, kinases are composed of regulatory and catalytic domains. In the kinase resting state, the regulatory domain keeps the catalytic part of the enzyme inactive. This inhibition is reversed when a second messenger or activator (eg. cAMP, cGMP, Ca\(^2\), DAG) binds to the regulatory domain (Tamaoki and Nakano 1990). Hence, there are regulators of kinase pathways which may control apoptosis.

A. Protein Kinase Inhibition

Apoptosis, like cell cycle and differentiation, is a complex process under dynamic regulation via a feedback control system. Thus, apoptosis may be controlled by proteins that mediate both the induction and inhibition processes. In addition, the inhibitory pathway may involve proteins that inhibit apoptosis, not only within the cell, but also in other cells through diffusible products and cell surface events.

1. Staurosporine

To first investigate the role of protein kinases in apoptosis, inhibitors are often used. Staurosporine was first identified from *Streptomyces* culture as an alkaloid with weak antibacterial and antifungal abilities (Omura et al 1977). However, staurosporine is a potent broad spectrum inhibitor of protein kinases. It potently inhibits PKA, PKC, cGMP dependent kinases, PTK, MLCK, and cdc2 kinase. Staurosporine inhibits the ATP binding site within the catalytic domain (C-terminal) of PKC while having no effect on the binding of phorbol esters to the regulatory domain (Ruegg and

B. Protein Kinase C

Protein kinase C (PKC) plays an important role as mediator between extracellular growth signals and gene expression and can be regarded as a decision point which switches cells into proliferation or self-elimination. It is instrumental in promoting gene expression directly or indirectly via different transcription factors.

The PKC family comprises at least 12 mammalian isoforms which differ in structure and enzymatic properties. PKC isoforms show different biochemical properties which occur at different proportions in different tissues, suggesting that the PKC isoforms can mediate different cellular functions. The members of the PKC family can be classified into three groups: classical isoforms (cPKCs α, β1/II, and γ); novel isoforms (nPKCs δ, ε, η(L), θ, and μ); and atypical isoforms (aPKCs ζ, ι, and λ) (Hug and Sarre 1993). The classical isoforms meet the original definition of PKC as a Ca²⁺- and phospholipid-dependent protein kinase. The novel isoforms lack
the region responsible for Ca\(^{2+}\) dependence, resulting in Ca\(^{2+}\) independence. The atypical forms according to Buchner (1994) are not able to bind and cannot be activated by phorbol esters, and are independent of Ca\(^{2+}\). In cardiac myocytes, PKC\(\alpha\), \(\beta_{II}\), and \(\zeta\) are located in the cytosol in non-stimulated cells and are translocated (hence activated) to the nuclear envelope after stimulation with PMA or norepinephrine (Disatnik et al 1994). In contrast, PKC\(\delta\) and \(\varepsilon\) are localized in the intranuclear area in resting cells according to Disatnik (1994). Upon stimulation PKC\(\delta\) is translocated to the nuclear envelope and PKC\(\varepsilon\) is translocated to myofibrils for further signal transduction effects (Disatnik et al 1994).

1. PKC activation

Upon cell receptor activation, phosphatidylinositol-4,5-biphosphate (PIP\(_2\)) is hydrolysed by phospholipase C into inositol-1,4,5-triphosphate (IP\(_3\)) and diacylglycerol (DAG). DAG activates PKC causing PKC to transduce the signal toward and within the cell nucleus. Signal transduction toward and within the cell nucleus can be mediated directly by PKC in several ways: a) PKC itself translocates to the cell nucleus; b) nuclear PKC is activated by a messenger from the cytoplasm reaching the cell nucleus; and c) nuclear PKC is activated by an activator generated in the nucleus, in the course of another signal cascade. Activated PKC can also translocate to the plasma membrane where PKC modulates ion channel activity (Dolphin 1990). Activated PKC phosphorylates components of the signal transduction system, including G-proteins and receptors (Dolphin 1990).

Physiological messengers reported to trigger nuclear translocation of PKC include insulin-like growth factor-1 (IGF-1),

Activated PKC phosphorylates components of the signal transduction system. PKC can phosphorylate a number of nuclear substrates (Buchner 1995), including the tumour suppressor protein p53 (Baudier et al 1992). Hence, PKC is activated by various agonists and during its activation, it translocates to other regions within the cell. Activated PKC can then phosphorylate components of signal transduction systems and nuclear substrates.

2. PKC involvement in apoptosis

To date, there are conflicting data on the role of PKC in apoptosis. Studies have shown PKC to both activate and block apoptosis (Lucas and Sanchez-Margalet 1995). The activation of PKC appears to induce apoptosis in HL-60 (Cotter et al 1992) and T-cell hybridoma cells (Jin et al 1992). Conversely, stimulation of the PKC pathway was able to prevent apoptosis induced by colchicine in chronic lymphocytic leukemia cells (Forbes et al 1992) or by glucocorticoids (Kanter et al 1984), Ca²⁺ ionophores (McConkey et al 1989a), and growth factor deprivation in immature thymocytes (Rodriguez-Tarduchy and Lopez-Rivas 1989). The use of PKC inhibitors, in concentrations that are below their toxicity limits,
reverts the suppression of apoptosis by IL-3 and GM-CSF, in hemopoietic cells (Rajotte et al 1992).

Phorbol esters are known tumour promoters which activate PKC, through the phosphorylation of cellular proteins and the induction of gene transcription (Greenberg and Ziff 1984). Phorbol esters induce as well as inhibit apoptosis (Cotter et al 1990, McConkey and Orrenius 1991b, Suzuki et al 1991, Tritton 1991, Forbes et al 1992, Terai et al 1991). In vascular endothelial cells, the phorbol ester TPA suppressed apoptosis without a requirement of any cooperative factors (Araki et al 1990b). In IL-2 dependent T lymphocytes, removal of IL-2 activates apoptosis, but activation of PKC by phorbol esters blocks the initiation of this death program (Rodriguez-Tarduchy and Lopez-Rivas 1989). Phorbol esters specifically inhibited the normal physiological process of apoptosis in cultures of C3H-10T1/2 (CL8) mouse fibroblasts (Tomei et al 1988). Thus, it has been speculated that phorbol esters inhibit the process of programmed cell death which may be initiated following cytotoxic stress.

The use of PMA is problematic because short time courses activate PKC whereas long time courses down-regulate PKC activity. Within each cell type, this can be complex and may in part reflect the nature of the findings above.

IV) The role of calcium ($Ca^{2+}$) in apoptosis

Because apoptosis involves $Ca^{2+}$ dependent kinases and endonucleases, calcium may have an important regulatory function in apoptosis (Trump and Berezesky 1992). Not only can increased levels of cytosolic calcium affect a signal transduction pathway leading to apoptosis, but it also can trigger secondary events resulting
from the activation of other Ca\(^{2+}\)-dependent enzymes culminating in cell death (El Alaoui et al 1992). Nuclear Ca\(^{2+}\) transport and the regulation of nuclear Ca\(^{2+}\)-dependent enzymes are involved during apoptosis in many cell types (Nicotera et al 1994, Nicotera et al 1992). An increase in cytosolic calcium occurs at a later stage of apoptosis in cells (Lennon et al 1992) suggesting that calcium influx might play different roles at different times during programmed cell death. Calcium has been implicated in apoptosis induced in specific cell systems by glucocorticoids (Cohen and Duke 1984, McConkey et al 1989a, McConkey et al 1989b), calcium ionophores (Ojcius et al 1991, Rodriguez-Tarduchy et al 1992, Rodriguez-Tarduchy G et al 1990), \(\gamma\)-irradiation (Story et al 1992), growth factors (Rodriguez-Tarduchy et al 1990), and hormone deprivation (Kyprianou et al 1988). Cell death was countered by application of Ca\(^{2+}\) chelating agents (Lockshin and Zakeri 1991, Bellomo et al 1992). As well, the transfections of genes encoding Ca\(^{2+}\) buffering proteins such as calbindin-D28K blocked cell death (Dowd et al 1992). However, apoptosis can be produced by factors which are independent of calcium, suggesting that calcium is involved in one of the many pathways leading to apoptosis (Bansal et al 1990, Alnemri and Litwack 1990).

There are conflicting data on the exact role of Ca\(^{2+}\) in the development of apoptosis. It has been proposed that loss of calcium homeostasis, rather than a sustained rise in \([\text{Ca}^{2+}]_i\), is a determining factor in cell death by apoptosis (Kluck et al 1994). However, because \([\text{Ca}^{2+}]_i\) is a major second-messenger molecule, increased \([\text{Ca}^{2+}]_i\) may also promote cell survival by stimulating a signal transduction pathway, possibly the same one stimulated by
trophic factors (Franklin and Johnson 1994). Hence, calcium may be involved in the development of apoptosis, either directly by affecting the activity of Ca$^{2+}$ dependent enzymes or indirectly via second messengers.

V) Growth factor and cell survival

Growth factors stimulate cell proliferation, involving new gene expression and protein synthesis (Pardee 1989). However, some factors may trigger specific signalling pathways which stimulate cell survival rather than cell proliferation. A pathway may be a control mechanism for apoptosis. For example, insulin-like growth factor I (IGF-I) promotes survival, but not proliferation in glial cells and bone marrow-derived IL3-dependent cell lines (Barres et al 1992, Rodriguez-Tarduchy et al 1992). In vascular endothelial cells, apoptosis is suppressed by fibroblast growth factor involving PKC activation (Araki et al 1990b). In addition, growth factor receptor (IL-3) occupancy can also control the decision of a cell to survive or proliferate in the presence of a given growth factor, as in the case of the IL-3 dependent cell line BAF3 (Collins et al 1994). On the other hand, trophic factor removal causes a cell to undergo apoptosis, as in the case of IL-3 withdrawal from BAF-3 cells (Rodriguez-Tarduchy et al 1990). Thus, growth factors can stimulate pathways responsible for cell proliferation and cell survival.

VI) de novo protein synthesis and apoptosis

Apoptosis requires de novo protein synthesis to initiate and regulate the suicide program. A current hypothesis suggests that the cells committed to undergo apoptosis promote the suicide cascade by synthesizing messenger/effecter molecules of cell death
(Wright et al 1992, Walker et al 1991, Kung et al 1990, Barry et al 1990, Thakkar and Potten 1992). The opposite has also been postulated (Robertson et al 1993, Manchester et al 1993): the death program is constitutively expressed and has to be continuously counterbalanced by the synthesis of suppressor molecules. Cycloheximide and actinomycin D, inhibitors of protein and RNA synthesis, can both induce and inhibit apoptosis (Gong et al 1993, Perreault and Lemieux 1993) suggesting that de novo protein synthesis is integral to the induction/inhibition of apoptosis.

VII) Genes and their products in apoptosis

Apoptosis requires the induction of a novel genetic programme and nuclear oncoproteins with transcriptional regulatory activity. Products of the c-fos, c-jun, c-myc, bcl-2, and p53 genes may work independently or cooperatively to determine the fate of the cell.

A. c-Fos and c-Jun

c-Fos and c-jun are nuclear proteins which interact to form the transcriptional complex AP-1. Once dimerized, they can interact with polymerase II and initiate transcription. In normal conditions, when its partner is present, overexpression of either gene is sufficient to increase cell division (Kato et al 1992). However, in lymphoid cells, the expression of proto-oncogenes c-fos and c-jun is induced during apoptosis (Colotta et al 1992). EGF treatment of human breast cancer cells, overexpressed with EGF receptors, was associated with an 18 fold induction of c-fos and a 16 fold induction of c-jun during the development of apoptosis (Armstrong et al 1994). This suggests an active role for fos and jun in programmed cell death.
B. Bcl-2

Bcl-2 is a family of gene products also including bax, bad, and bcl-x. Bcl-2 gene product has been implicated in apoptosis as a survival factor. Bcl-2 expressed in transgenic mice enhanced cell survival (McDonnell et al 1989). Bcl-2 protects a wide range of cells from triggers which would otherwise induce apoptosis, such as camptothecin (Zhong et al 1993, Mah et al 1993). However, bcl-2 does not inhibit T-cell receptor mediated apoptosis (Vaux et al 1992).

Bax, a protein with extensive homology to bcl-2, counteracts the death-repressing activity of bcl-2 (Oltvai et al 1993). The two proteins are able to compete with one another in forming homo- or heterodimers. The actual susceptibility to death might be determined by the ratio of Bax to bcl-2.

C. c-Myc


D. p53

p53 tumour suppressor gene product participates in the induction of apoptosis in myeloid leukemic cells and colon cancer

VIII) Potential significance of cardiac apoptosis

Cell growth and death in many cell types are in equilibrium during the normal cell state, but under diseased conditions, this equilibrium is shifted (Bright and Khar 1994). Apoptosis parallels cells undergoing proliferation or differentiation. Cardiac hypertrophy is often considered analogous to cell growth in non-cardiac cells because cardiomyocytes are terminally differentiated and cannot divide; thus they respond to various stimuli by increases in cell size. Hypertension-induced cardiac hypertrophy is often followed by the development of heart failure. It is suspected that apoptosis may be involved in the development of heart failure after the development of cardiac hypertrophy (Bing 1995).

A. Chick embryo ventricular myocytes culture model

The use of a chick embryo culture allows us to observe the biochemistry of the cardiomyocyte without the influence of systemic vasculature and circulating hormones. Furthermore, use of this cardiomyocyte culture eliminates the potential action of stimulation of cell surface receptors on the coronary and systemic vasculature. In addition, the use of a cell culture permits the observation of apoptosis without phagocytosis, in contrast to tissue studies.
B. Histogenesis of the Embryonic Chick Ventricular Myocardium

During heart development in the chick embryo, prospective heart-forming mesoderm cells migrate on an endoderm substratum, eventually forming two vesicles on either side of the developing foregut. The two heart rudiments are brought together in the ventral midline and they fuse in an anterior-to-posterior direction. The inner lining becomes the endocardium and the outer layer becomes the myocardium. This occurs at the 25th and 30th hour of incubation in the chick embryo.

The newly formed heart tube twists into a looped structure with specific chambers. This twisting may be caused by the migration of sheets of cells and by changes in cell shape. The first region formed after fusion is the truncus, or conus arteriosus, which leads to the ventricle. Next to form is the atrium and the last part to form is the sinus venosus, the heart chamber that receives venous blood. The heart tube bends to form an S shape. The heart begins to beat just after the paired heart rudiments begin to fuse (Oppenheimer and Lefevre 1989).

C. Ventricular myocytes of the seven day old embryo

Ventricular myocytes of the seven day old embryo contain many myofibrils, but they often still lack alignment. Whereas myofibrils insert into intercalated discs nearly at right angles in the mature heart, the random fibrils of these embryonic myocytes often insert at an acute angle. Similar configurations have been seen as late as the tenth day of development (Noble and Cocchi 1990).

The monolayer culture of beating seven day embryonic chick myocytes is an appropriate model for the investigation of
signalling in cardiomyocytes during apoptosis. Our studies were performed on 4th to 7th incubation day using confluent monolayers of spontaneously beating myocytes. Previous studies by this lab showed that the proportion of myocytes at this time was at least 90% as verified by the proportion of cells showing spontaneous contraction or displaying muscle specific markers on immunohistologic examination.

IX) Objectives and hypotheses with their rationale

I hypothesize that cardiomyocytes are capable of undergoing apoptosis. There are multiple potential factors and pathways that might be involved. Because apoptosis involves a genetic mechanism, an ideal location for apoptotic control is the nucleus. The control point might be associated with gene transcription since "death genes" must be activated during apoptosis. Topoisomerase I is an ideal example of a nuclear control point for apoptosis.

Some mechanism must be in place to transmit the signal from the extracellular environment to the nucleus. Hence, I hypothesize that some signalling mechanisms must be in place to allow the cell to respond to extracellular stimuli. I hypothesize that the PKC signalling pathway may be a part of apoptotic control, because PKC is a mediator between extracellular growth signals and gene expression and can be regarded as a decision point which switches cells into proliferation or self-elimination.

Because apoptosis involves Ca\(^{2+}\)-dependent enzymes in its program, I hypothesize that Ca\(^{2+}\) can modify the development of apoptosis. I speculate that a decrease in Ca\(^{2+}\) delays or abolishes the apoptotic program.
Growth factors may stimulate a pathway which can cause either cell proliferation or cell survival. I hypothesize that growth factors will be able to modulate the development of apoptosis.

*De novo* protein synthesis is required for apoptosis, either to produce "death" or cytoprotective proteins. I hypothesize that *de novo* protein synthesis will be observed during treatment with different agents inducing apoptosis.

My specific objectives to test my hypothesis and their rationale are summarized as follows:

1. \textbf{To establish that apoptosis occurs in cardiomyocytes}

   Because the death of a cardiomyocyte would compromise the terminally differentiated heart, one might speculate that cardiomyocytes would not have a genetically programmed machinery for cell suicide. However, one must consider the need for programmed cell death should a situation arise when the elimination of one cell is necessary for the good of the heart. Hence, I will attempt to establish whether apoptosis occurs in cardiomyocytes.

2. \textbf{To establish the role of topoisomerase I in the development of apoptosis}

   The nucleus is a prime location to establish the control mechanisms of apoptosis. Topo I is an essential enzyme involved in DNA replication and transcription. It is logical that topo I inhibition would put the cell's genomic DNA in a damaged state, since the machinery for replication and protein synthesis are halted. Hence, the role of topo I will be investigated to observe the effect of nuclear enzyme activity on the genetic program of apoptosis, recognizing that conclusions based on topo I are from the use of the topo I inhibitor camptothecin.
3. **To establish the role of protein kinases in apoptosis**

   Protein kinases are postulated to be part of the transduction pathway responsible for apoptosis. Staurosporine has been documented as a broad inhibitor of protein kinases and has been shown to induce apoptosis in some cell lines. I propose to implicate kinases in the development of apoptosis by using staurosporine. Conclusions of the role of protein kinases are based on the indirect evidence provided by staurosporine usage.

4. **To implicate protein kinase C in apoptosis**

   A specific activator (PMA) and inhibitor (chelerythrine) for PKC will be used to examine the role of PKC in apoptosis. The results from PKC will then be compared with staurosporine to determine whether PKC is the kinase responsible for staurosporine's results. Conclusions on the role of PKC is based on the used of the PKC inhibitor chelerythrine and the PKC activator PMA.

5. **To establish the role of calcium in apoptosis**

   Based on the Ca$^{2+}$ dependency of enzymes involved in apoptosis, I will explore the potential role of Ca$^{2+}$ in apoptosis. The role of Ca$^{2+}$ in apoptosis induced by either inhibition of topo I or protein kinase signal transduction will be determined using Ca$^{2+}$ chelators and ionophores to alter the level of cellular Ca$^{2+}$.

6. **To establish the role of growth factors in apoptosis**

   Growth factors have been implicated in the stimulation of cell proliferation and cell survival in other cellular models. I will explore the potential pathway stimulated by growth factor addition (insulin and EGF) as a means of delaying or abolishing apoptosis.
7. To establish *de novo* protein synthesis in apoptosis in cardiomyocytes

Apoptosis requires *de novo* protein synthesis. It is unclear whether this protein synthesis would produce either death proteins or cytoprotective proteins. I will determine the level of protein synthesis that occurs during apoptosis in cardiomyocytes, as well as any specific protein synthesized in apoptotic cells.
CHAPTER II
MATERIALS AND METHODS

1. Isolation of Chick Embryonic Cardiac Cells

Monolayer culture of beating 7 day embryonic chick ventricular cells were prepared using previously described methods (Rabkin and Sunga 1987). Fertilized White Leghorn eggs were incubated in an automatic incubator for 7 days at 37.8°C and 87% humidity. Hearts were then removed from the 7 day chick embryos under sterile conditions in a tissue culture hood. Ventricles were isolated from atria and used for further culturing. Ventricles were cut into 0.5 mm fragments under dissecting microscope and disaggregation was carried out by 5 minute digestions in 0.005% trypsin, 0.1% bovine serum albumin, and DNAse (1x10⁻⁷ Dornase units/mL) in the balanced salt solution DMS8 at 37.8°C. DMS8 is composed of the following (in mM): NaCl 170, KCl 5.4, NaH₂PO₄ 4.3, Na₂HPO₄ 1, dextrose 5.6. This procedure was repeated 5 times, each time with replacement of disaggregation solution. After 5 digestions, the digestes were diluted 5 fold in culture medium 818A. 818A was composed of Medium 199 (20%), fetal calf serum (6%), antibiotic-antimycotic (10 000 units/mL penicillin G sodium, 10 000 µg/mL streptomycin sulfate, and 25 µg/mL amphotericin B) (1%), and the balanced salt solution DBSK (73%). DBSK contains (in g/L): NaCl 6.8; MgSO₄ 7H₂O 0.2; NaH₂PO₄ H₂O 0.13; dextrose 1; NaHCO₃ 2.2. The disaggregated cells were centrifuged for 3 minutes at 1 000xg. Cells were plated into either 35 or 100-mm culture plates at 1x10⁶ cells per culture dish or 4.5x10⁶ cells per culture dish, respectively. Cultures were incubated in a humidified 5% CO₂-95% air atmosphere at 37°C. Studies were performed on the 4th to 7th incubation day using
confluent monolayers of spontaneously beating myocytes (Rabkin 1993, Rabkin 1989). The proportion of myocytes at this time was at least 90% as verified by the proportion of cells showing spontaneous contraction or displaying muscle specific markers on immunohistologic examination.

2. Microscopy

a) Cell viability assay - Trypan Blue

To assess cell viability, cardiomyocytes were grown on a coverslip in a 35mm petri dish. After 72 h in culture, drug agents or their diluent was added to the media. At predetermined times coverslips were removed, stained with 0.4% trypan blue and examined microscopically in a haemocytometer to determine the number of cells that were or were not stained by trypan blue. Dead cells retain the blue dye whereas live cells do not. Trypan blue is a standard assay for measuring cell death, including cell death (Lee et al 1993).

b) Cell morphology - acridine orange

To examine cell morphology, cardiomyocytes, grown on coverslips, were treated with drug agents or their diluent. The medium was removed and cells were stained with acridine orange (100μg/mL) and dried. Cells were examined microscopically with a Zeiss (Standard 16) microscope using a fluorescent light source as previously described (Rabkin and Sunga 1987). Acridine orange is pH-sensitive fluorescent dye which fluoresces green at neutral pH and red at an acidic pH (approx. pH 5) (Nairn and Rolland 1980).

c) Cell morphology - Hoescht dye

Hoescht dyes are specific for DNA and therefore ideal for observing nuclear changes (Bester et al 1994, Yamamoto et al 1990).
To provide further examination of nuclear changes, cardiomyocytes grown on coverslips were fixed washed in cold PBS and then fixed in 3.7% paraformaldehyde for 10 min. The coverslips were briefly washed in cold PBS and then extracted with cold acetone for 4 min. The coverslips were then stained with Hoescht 33258 (Hoe 33258) for 20 min, washed briefly, and then mounted on PBS/glycerol (1:1). Cells were examined microscopically with a Zeiss (Standard 16) microscope using a fluorescent light source as previously described (Rabkin and Sunga 1987).

d) Cell morphology - NBD phallacidin

7-nitrobenz-2-oxa-1,3-diazole-(NBD) phallacidin is a fluorescent dye specific for F-actin and therefore ideal for observing cytoskeletal changes (Packman and Lichtman 1990).

To examine for changes in actin, cardiomyocytes grown on coverslips were fixed washed in cold PBS and then fixed in 3.7% paraformaldehyde for 10 min. The coverslips were briefly washed in cold PBS and then extracted with cold acetone for 4 min. The coverslips were then stained with 1:10 dilution of NBD phallacidin in PBS for 1h, washed briefly, and then mounted on PBS/glycerol (1:1). Cells were examined microscopically with a Zeiss (Standard 16) microscope using a fluorescent light source as previously described (Rabkin and Sunga 1987).

3. DNA Fragmentation

The method described is a modification of the method described by Scheid (et al. 1995). Cardiomyocytes from the media and dish were isolated because dead cells become non-adherent and float into the medium. The medium was centrifuged at 200g and the resulting pellet processed concomitantly with the adherent cardiomyocytes.
isolated from the dish. Cardiomyocytes were washed with PBS, lysed (0.6% SDS, 10 mM EDTA), and the cells scraped from the dish with a rubber policeman. 5M NaCl was added, mixed gently, and incubated at 4°C for 16 h. Samples were centrifuged at 16 000g for 20 min. The supernatant was removed, treated with RNAse A, and incubated at 37°C for 30 min. Proteins were removed by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and the layers separated by centrifugation. The upper aqueous layer was removed and transferred to a fresh tube. 3M sodium acetate and 100% ethanol were added and the samples were incubated at -20°C for 10 min. The DNA was pelleted by 16 000g spin for 10 min and washed with 70% ethanol. The DNA was electrophoresed on a 2% agarose gel, stained with ethidium bromide, and destained with distilled water for 90 min.

4. DNA Fragmentation (Enzyme-linked immunosorbent assay, ELISA)

Cells plated into 35 mm culture dishes at 1x10⁶ cells per culture dish were grown for 4 days. Cells were washed twice with 1x PBS and then lysed. Meanwhile, antibodies specific for histones were coated onto a microtitre plate and washed. Lysates from lysed cardiomyocytes were centrifuged at 15 000g to pellet down whole cells, cell debris, and intact DNA. The supernatant containing fragmented DNA was added to the coated microtitre plate and allowed to react with the immobilized primary antibody. After several washings, the secondary antibody, anti-DNA-peroxidase, was added and incubated for 90 min. The excess antibodies were washed off and the substrate reaction solution was added. The colorimetric substrate (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) was measured at its absorbance wavelength of 405 nM.
5. \[^{35}\text{S}]\text{Methionine Incorporation and Preparation of Cell lysates}

The cardiomyocytes were cultured on 100 mm dishes in 818A medium containing 6% fetal calf serum for 4 days prior to the experiment. Samples of $1 \times 10^6$ isolated myocytes were rinsed twice in methionine-free DMEM and incubated in this medium for 1 h. Cells were labelled with 50 μCi/mL trans \[^{35}\text{S}]\text{methionine in DMEM for 2 h. Drugs were added for specific times and the reactions were stopped by removal of the radioactive media was removed and the cells washed twice with PBS. Cells were lysed with lysis buffer containing 137 mM NaCl, 20mM Tris pH 7.4, 1mM MgCl}_2, 1mM CaCl}_2, 1% Nonidet P-40, 1mM phenylmethylsulphonylfluoride (PMSF), and 2mM sodium orthovanadate. One millilitre of the lysis buffer was added to each dish, and the plates agitated for 20 minutes at 4°C. Thereafter, the cells and debris were scraped from the plates with a rubber policeman. The lysis buffer and cell debris were transferred to sterile 1.5mL eppendorf tubes. At this point, the samples are known as whole cell lysates. The samples were centrifuged at 600g for 10 min. The resulting supernatant and pellet are soluble protein and nuclear proteins, respectively. Some cytoskeletal proteins may pellet with the nuclear proteins. The supernatant was transferred to another eppendorf tube and the pellet resuspended in fresh lysis buffer. Aliquots of the supernatant and resuspended pellet were retained for protein quantitation by the Bradford assay. Aliquots reserved for polyacrylamide gel electrophoresis were resuspended in 4x electrophoresis sample loading buffer (125 mM Tris/HCl 6.8, 4% SDS, 10% β-mercaptoethanol, 20% glycerol, 0.01% bromophenol blue), boiled for 4 minutes, and stored overnight at 4°C.
6. [*^35^S]*Methionine Incorporation Assay

Five microlitre aliquots reserved from whole cell lysates were applied onto two 1 cm² squares of Whatman No. 3 filters and allowed to air dry. Filters were baked at 50°C for 10 min and then boiled in 10% trichloroacetic acid for 10 min. Filters were washed twice in water, ethanol, and acetone and then left to air dry. Filters were then placed in scintillation vials and 250 uL of 1N NaOH were added. The vials were left to incubate at 55°C for 30 min. Acetic acid and ACS counting solution were then added and left overnight in the dark. Vials were then counted on a program specific for ^14^C/^35^S energy levels.

7. Protein Determination

Protein content was assessed using the method of Bradford (Bradford 1976). Bradford reagent was purchased from Bio-Rad Canada Ltd (Mississauga, Ont). Bovine serum albumin was used as a standard.

8. SDS Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE of extracts was performed according to the method of Laemmli (1970) on 10% separating gels containing 5.6 mL resolving buffer, 16.9 mL 30% acrylamide/Bis, 150 µL 10% ammonium persulfate (freshly prepared), and 30 µL TEMED. Samples were dissolved in SDS gel loading buffer, boiled for 4 min, and equal amounts of protein loaded onto 4% stacking gels (6mL stacking buffer, 1.5mL 30% acrylamide/Bis, 50µL 10% ammonium persulfate, and 10µL TEMED) overlying the 10% polyacrylamide resolving gel. Following electrophoresis, gels were fixed with isopropanol:water:acetic acid (25:65:10) for 30 min and then fixed with AMPLIFY™ (Amersham Canada Ltd, Oakville Ont) for another 30 min. Gels were mounted onto
Whatman No 3 filter paper and dried.

9. **Radiolabelled Proteins**

$[^{35}\text{S}]$Methionine labelled protein gels were mounted, dried and autoradiographed on Kodak X-AR5 X-ray film using intensifier screens at $-70^\circ\text{C}$. Gels were exposed to Kodak X-AR5 film from 1 to 5 days.

10. **Drug pretreatment**

Experiments which require drug pretreatment before exposure to the inducting agent was performed by a 30 min. preincubation of the pretreatment agent before exposure to the inducing agent.

11. **Materials**

a) **Biochemicals**

All cell culture components were from Gibco/BRL Life Sciences (Burlington, Canada). DNase I was from Worthington Biochemicals (Freehold, New Jersey). Camptothecin, taurine, EGTA, BAPTA-AM, staurosporine, acridine orange, and Hoescht 33258 were from Sigma Chemicals Ltd (St. Louis, USA). Chelerythrine, and PMA, were from Calbiochem Ltd (La Jolla, Ca, USA). NBD phallacidin was purchased from Molecular Probes (Eugene, Oregon). Trypan blue was purchased from BDH Chemicals Ltd (Toronto, Canada). Components of the ELISA were from Boehringer Mannheim (Laval, Canada). All chemicals were purchased from Fischer Scientific (Ottawa, Canada).

b) **Radiochemicals**

$[^{35}\text{S}]$-Methionine cell labelling grade (1214 Ci/mmol specific activity) was from ICN Pharmaceuticals Inc (Irvine, California). $[^{14}\text{C}]$ labelled high molecular weight protein markers were from Amersham Canada (Oakville, Ont).
c) Pharmacology of drugs

i) Camptothecin

Camptothecin, is an alkaloid isolated from the wood of Camptotheca acuminata. Camptothecin "poisons" topo I when it is associated with DNA during cleavage. By poisoning topo I at this stage, the levels of topo I-DNA cleaved complexes are increased thus introducing high levels of transient enzyme-associated breaks in the genome (Froelich-Ammon and Osheroff 1995).

ii) Staurosporine

Staurosporine is a potent broad spectrum inhibitor of protein kinases, especially PKA and PKC. It has been shown to induce apoptosis in human malignant glioma cell lines.

iii) EGTA, or Ethyleneglycol-bis(β-aminoethyl)-N,N,N',N'-tetraacetic acid

EGTA is a chelating agent selective for Ca$^{2+}$. It induces apoptosis in pheochromocytoma (PC12) cells. EGTA chelates free extracellular calcium within the media thus preventing calcium entry into the cell. 1 mol of EGTA can chelate 2 moles of free Ca$^{2+}$. The effect of 1mM EGTA on 818A media, which contains 1.5mM Ca$^{2+}$, should chelate all free extracellular Ca$^{2+}$ resulting in an extracellular, Ca$^{2+}$-free environment.

iv) BAPTA-AM, or 1,2-bis-(o-Aminophenoxy)ethane-N,N,N',N'-tetraacetic acid

BAPTA is a Ca$^{2+}$ chelator with 105x greater affinity for Ca$^{2+}$ than for Mg$^{2+}$. BAPTA can enter the cell and through chemical reactions can trap free Ca$^{2+}$ ions (Chiamvimonvat 1995).

v) Thapsigargin

Thapsigargin is a sesquiterpene gamma-lactone which selectively inhibits the sarcoplasmic reticulum and endoplasmic
reticulum Ca\(^{2+}\)-dependent ATPase family of intracellular "Ca\(^{2+}\)-pumping" ATPases and produces a three to four fold elevation in the levels of [Ca\(^{2+}\)]\(_i\) (Furuya et al 1994).

vi) PMA, or Phorbol-12-myristate-13-acetate

PMA is an activator of PKC which also potentiates forskolin-induced cAMP formation. It has been shown to inhibit apoptosis induced by the Fas antigen, but induces apoptosis in HL-60 cells (Jarvis et al 1994).

vii) Chelerythrine chloride

Chelerythrine chloride is a potent and selective inhibitor of PKC. It acts at the diglyceride binding site within the PKC regulatory domain and is over 10x more potent than H-7. It has been shown to induce apoptotic DNA fragmentation and cell death in HL-60 (Jarvis et al 1994).

12. Methods of Statistical Analysis

The data are presented as the mean ± SEM within two standard deviations. Hypothesis testing used analysis of variance. The null hypothesis was rejected if the probability of a Type I error was less than 5% (p<0.05).
CHAPTER III

A. RESULTS OF TOPO I INHIBITION-MEDIATED APOPTOSIS

1. Cell Viability after exposure to camptothecin

To investigate the potential cytotoxicity of camptothecin in cardiomyocytes, cell viability was assayed by trypan blue exclusion (Fig 1). Camptothecin-induced cell death was apparent and occurred in a dose-dependent and time-dependent manner. After 6h of treatment, higher (50 μM) and lower 10 μM, camptothecin appeared to exert similar effects (Panel A). This time-dose was point chosen for further experiments. At 6 h of treatment, camptothecin produced dose dependent cell death with a plateau apparent for doses greater than 10μM. Camptothecin, 10μM, significantly (p<0.05) increased cell death by 25.1±1.4% (N=10) compared to control. Camptothecin concentrations higher than 50 μM could not be used as the drug was not very soluble.

2. Cell morphology after exposure to camptothecin

To investigate the morphological changes associated with apoptosis, cardiomyocytes were exposed to camptothecin and stained with Hoe 33258 or acridine orange (Fig 2). Hoe 33258 is a DNA minor groove-binding ligand and specifically binds to nuclei as it relaxes the suprahelical organization of DNA, leading to the formation of a B-like structure (Krishna et al 1993). Control cells, exposed to the diluent, showed a weakly stained, intact nucleus (Panel A). In contrast, camptothecin treated cells showed a strongly stained nucleus with evidence of nuclear disintegration (Panel B).
FIG 1. The effect of camptothecin on cell viability assessed by trypan blue exclusion assay. Results are reported as the difference in the percent of dead cells in camptothecin treated cardiomyocytes compared to control (diluent treated) cardiomyocytes. Panel A - Cardiomyocytes were treated with either 10 or 50 μM camptothecin for various times. The data are the mean±SEM of 3 to 13 experiments. Panel B - Cardiomyocytes were exposed to camptothecin, 1 or 10 or 50 μM for 6 hours. The data are the mean±SEM of 8 to 10 experiments.
FIG 2. The cell morphology of cardiomyocytes after camptothecin treatment. Representative cardiomyocytes exposed to 10 μM camptothecin (Panels B & D) or its diluent DMSO (control) (Panels A & C) for 6 h. Cells were stained with either Hoe 33258 (Panels A & B) or acridine orange (Panels C & D).
Acridine orange stained control cells showed an overall green appearing cytoplasm with an intact nucleus (Panel C). Cells exposed to camptothecin, however, demonstrated extensive membrane blebbing and nuclear disintegration (Panel D). The nuclei often stained red, consistent with alterations of cellular pH.

3. DNA fragmentation

To investigate potential changes to genomic DNA, cardiomyocytes were exposed to camptothecin and the DNA was isolated and electrophoresed on a 2% agarose gel (Fig 3). Camptothecin treated cardiomyocytes had DNA fragments of 180 bp or multiples of 180 bp (Lanes 1 and 3). In contrast, the control cells showed no DNA fragmentation or smearing (Lane 2).

To quantitate the extent of DNA fragmentation associated with apoptosis, cardiomyocytes were treated with camptothecin and processed by means of an enzyme-linked immunosorbent assay (ELISA) (Fig 4). Cells exposed to camptothecin had significantly (p<0.05) more DNA fragmentation. The amount of DNA fragmentation appeared to plateau after 10 μM camptothecin that produced 1.5±0.5 (+SEM) (N=8) fold more fragmentation signal than control.

4. Role of Ca²⁺ in camptothecin-induced cell death and apoptosis: EGTA

To evaluate the role of Ca²⁺ in camptothecin-induced apoptosis, EGTA, a Ca²⁺ chelator (Bers DM 1982), was co-incubated with camptothecin (Fig 5). Low EGTA concentrations were used to ensure cell viability. EGTA treatment reduced camptothecin-induced cell death as demonstrated by trypan blue exclusion (Panel A). EGTA
FIG 3. DNA fragmentation in cardiomyocytes exposed to camptothecin or DMSO. A representative gel of DNA isolated from cardiomyocytes and electrophoresed on 2% agarose. Lanes 1 & 3 – camptothecin 10 μM; Lane 2 – control; Lane 4 – 1 kb DNA ladder. All incubations were at 6 h.
FIG 4. DNA fragmentation quantitated by an enzyme-linked immunosorbent assay (ELISA). Cardiomyocytes were exposed to various concentrations of camptothecin for 6 h. Results are the sample absorbance at 405 nm and are expressed relative to control (DMSO treatment). The data are the mean±SEM (N=3 to 8).
FIG 5. The effect of EGTA on camptothecin-induced cell death. Panel A - Effect of various concentrations of EGTA on camptothecin-induced cell death assessed by trypan blue exclusion. All cardiomyocytes were pretreated with 0.01, 0.1, or 1.0 mM EGTA before 10μM camptothecin treatment (0μM). The bar graph represent the mean±SEM of 3-4 experiments. Panel B - Enzyme-linked immunosorbent assay of DNA fragmentation for cardiomyocytes treated with 10 μM camptothecin without (0μM) or with EGTA. The bar graph represent the mean±SEM of 3 to 5 separate experiments.
pretreatment also reduced DNA fragmentation as measured by the ELISA assay (Panel B).

5. **Role of Ca\(^{2+}\) in the morphologic changes of apoptosis in the cardiomyocyte**

To determine whether or not Ca\(^{2+}\) was involved in the apoptotic morphology produced by camptothecin, cardiomyocytes were exposed to camptothecin with EGTA pretreatment and examined microscopically (Fig 6, Panel A). EGTA treated cells did not show membrane blebbing after treatment with camptothecin. Cell death was still evident, consistent with the trypan blue data, however the absence of membrane blebbing was striking.

To further examine the role of intracellular Ca\(^{2+}\) in production of the cellular changes of apoptosis, cardiomyocytes exposed to camptothecin were pretreated with taurine (Panel B). Taurine is a free amino acid in plasma and many tissues such as heart, muscle, brain, and blood; it affects L-type and T-type Ca\(^{2+}\) channels in embryonic chick heart cells (Kaplan et al 1993, Satoh and Sperelakis 1993) and reduces intracellular calcium (Kaneko and Tsukamoto 1994). Taurine reduced camptothecin-induced apoptotic changes in cellular morphology. Red staining, associated with acidic pH and cellular damage, was seen in these cells suggesting cellular damage. However, the absence of membrane blebbing usually seen during apoptosis was apparent.

6. **Role of Ca\(^{2+}\) in camptothecin-induced DNA fragmentation**

To investigate the role of Ca\(^{2+}\) levels in camptothecin-induced DNA fragmentation, cardiomyocytes were exposed to camptothecin and
FIG 6. Cell morphology of cardiomyocytes exposed to camptothecin and pretreatment with EGTA or taurine. Cells were stained with acridine orange. Representative cardiomyocytes pretreated with 10 μM EGTA (Panel A) or 10 mM taurine (Panel B) before 10μM camptothecin exposure for 6h.
pretreatment with taurine (Fig 7). Taurine reduced DNA fragmentation in an apparent dose-dependent manner as the highest concentration of taurine showed the least amount of DNA laddering whereas the lower taurine concentration showed the greater camptothecin-induced DNA fragmentation.

To quantitate the effect of taurine on camptothecin-induced DNA fragmentation and to compare it to an agent that increases cellular calcium, cardiomyocytes were exposed to camptothecin and pretreated with taurine, or thapsigargin (Fig 8). Taurine protected the cardiomyocyte from DNA fragmentation induced by camptothecin. In contrast, thapsigargin significantly \((p<0.05)\) increased DNA fragmentation to levels greater than camptothecin.

7. **de novo protein synthesis during camptothecin-induced apoptosis**

Cardiomyocytes were exposed to camptothecin during \([^{35}S]\)methionine incorporation (Fig 9). New proteins synthesized were isolated and run on a SDS-PAGE. Panel A exhibits a 1, 2, 4h time course of de novo protein synthesis during exposure to 10\(\mu\)M camptothecin. Panel B exhibits the effect of cycloheximide, a known inhibitor of protein synthesis, on camptothecin de novo protein synthesis.

B. **Results of staurosporine-induced apoptosis**

1. **Cell Viability after exposure to staurosporine**

To investigate the potential cytotoxicity of staurosporine in cardiomyocytes, cell viability was assayed by trypan blue exclusion (Fig 10). Staurosporine-induced cell death was apparent and occurred in a dose-dependent (Panel A) and time-dependent manner
FIG 7. A representative gel of DNA isolated from cardiomyocytes and electrophoresed on 2% agarose. Cardiomyocytes were exposed to camptothecin and/or various concentrations of taurine. Because alive cardiomyocytes are adherent to the dish and cardiomyocytes which had died before the 6 h incubation would become dead and float in the media, the media was examined as well and termed as "media". Lane 1 - Control; Lane 2 - Control media; Lane 3 - 10 μM camptothecin; Lane 4 - camptothecin media; Lane 5 - 1 mM taurine + 10 μM camptothecin; Lane 6 - media for lane 5; Lane 7 - 10 mM taurine + 10 μM camptothecin; Lane 8 - media for lane 7; Lane 9 - 10 mM taurine; Lane 10 - media for Lane 9; Lane 11 - 1 kb DNA ladder. All incubations were at 6 h.
FIG 8. Bar graph showing DNA fragmentation quantitated by an enzyme-linked immunosorbent assay (ELISA). Cardiomyocytes were exposed to camptothecin and/or thapsigargin or taurine. Results are the sample absorbance at 405 nm and are expressed relative to control (DMSO treatment). The data are the mean±SEM (N=3). Cam = camptothecin 10μM, 6h; Th = thapsigargin 100nM, 6h; Th + Cam = thapsigargin + camptothecin; Ta 1mM = Taurine 1mM, 6h; Ta 10mM = Taurine 10mM, 6h. Ta + cam = taurine + camptothecin. (*=p<0.05)
FIG 9. de novo protein synthesis during camptothecin exposure. Cardiomyocytes were exposed to camptothecin during $^{35}$S-methionine incorporation. New proteins synthesized were isolated and run on SDS-PAGE. The gel was mounted, dried, and exposed. Panel A exhibits a 1, 2, and 4 h time courses of de novo protein synthesis during exposure to camptothecin. C=Control; CAM=camptothecin 10\mu M; MW reported as kDa; nuclear=nuclear protein fraction; soluble=soluble protein fraction.

Panel B exhibits the effect of cycloheximide on camptothecin de novo protein synthesis during 4h. C=Control; CM=Camptothecin 10\mu M; CY=cycloheximide 1\mu M; MW reported as kDa. First set of C,CM,CM+CY,CY (closest to MW standards)=nuclear protein fraction. Second set= soluble protein fraction.
At 6 h of treatment, staurosporine produced dose dependent cell death. Staurosporine, 1μM, significantly (p<0.05) increased cell death by 40.4±7.1% (N=7) compared to control. Staurosporine concentrations higher than 1 μM could not be used as a significant number of cardiomyocytes would die and float into the media, hence lost to trypan assay, before the incubation time was completed. Staurosporine exposure over time yielded a plateauing effect at times greater than 4h. Hence, the 6h time point and the 1μM dose were chosen for future experiments.

2. DNA fragmentation after exposure to staurosporine

To investigate potential changes to genomic DNA, cardiomyocytes were exposed to staurosporine and the DNA was isolated and assayed for fragmentation by ELISA (Figure 11). Staurosporine exposure induced DNA fragmentation in a time-dependent manner.

Cardiomyocytes were exposed to staurosporine and the DNA was isolated and assayed for fragmentation either by ELISA (Figure 12 - Panel A) or by electrophoretic separation (Panel B). Maximal DNA fragmentation yielded fragmentation 3.1±1.7 (N=8) fold more than control cells. Staurosporine treated cardiomyocytes yielded DNA fragments of 180 bp or multiples of 180 bp in a dose dependent manner. In contrast, the control cells showed no DNA fragmentation or smearing.

3. Cell morphology after exposure to staurosporine

To investigate the effects of staurosporine exposure to
FIG 10 - The effect of staurosporine on cell viability assessed by trypan blue exclusion assay. Results are reported as the difference in the percent of dead cells in staurosporine treated cardiomyocytes compared to control (diluent treated) cardiomyocytes. Panel A - Cardiomyocytes were exposed to staurosporine 1μM for various times. Panel B - Cardiomyocytes were treated with various concentrations of staurosporine for 6h. The data are represented by a log dose curve and are the mean±SEM of 4 to 8 experiments.
FIG 11 - Staurosporine induced DNA fragmentation quantitated by an enzyme-linked immunosorbent assay (ELISA). Cardiomyocytes were exposed to staurosporine 1μM for various times.
FIG 12 - Dose response of staurosporine-induced DNA fragmentation. Panel A - Cardiomyocytes were exposed to various concentrations of staurosporine for 6 h. Results are the sample absorbance at 405 nm and are expressed relative to control (DMSO treatment). The data are the mean±SEM (N=4 to 9). Panel B - Electrophoretic separation of staurosporine-induced DNA fragmentation. Lane 1 - DNA ladder; Lane 2 - staurosporine 1µM; Lane 3 - staurosporine 0.1µM; Lane 4 - staurosporine 0.01µM; Lane 5 - Control; performed at 6h.
cellular morphology, NBD phallacidin staining was performed (Fig 13). Control cell (Panel A) was compared to cells exposed to 1μM staurosporine for 6h. Panels B-D demonstrate the developmental changes to cellular morphology in response to staurosporine exposure. Cardiomyocytes exposed to staurosporine display unique morphology, dissimilar to the typical apoptotic changes. During the course of staurosporine exposure, cells appear to elongate due to the condensation of the cytoplasm and lose cell volume. NBD phallacidin staining exhibits an active reorganization of the cytoskeleton as the cell elongates and the cytoplasm condenses.

4. The effect of $\text{Ca}^{2+}$ on staurosporine-induced apoptosis

The role of $\text{Ca}^{2+}$ was elucidated by pretreating cardiomyocytes with EGTA or BAPTA before staurosporine addition (Fig 14). EGTA slightly reduced staurosporine-induced cell death, as assessed by trypan blue assay. BAPTA blunted staurosporine-induced cell death.

The effect of EGTA and BAPTA on staurosporine-induced DNA fragmentation was investigated by ELISA (Fig 15). Pretreatment with BAPTA significantly ($p=0.02$) reduced staurosporine-induced DNA fragmentation, as assayed by ELISA (Panel A).

Cellular morphology was assessed by acridine orange staining. (Fig 16). Staurosporine exposure (Panel B) is distinctly different than control cell (Panel A). The addition of BAPTA completely abolished staurosporine-induced morphology (Panel C). These results suggest that $\text{Ca}^{2+}$ is needed for staurosporine-induced cell death. Obviously, the more specific $[\text{Ca}^{2+}]_{i}$ chelation with BAPTA would be more effective than extracellular $\text{Ca}^{2+}$ chelation with EGTA.
FIG 13 - Cell morphology of representative cardiomyocytes exposed to staurosporine 1μM (Panels B-D) or its diluent (Panel A) for 6h and stained with NBD phallacidin.
FIG 14 - The effect of EGTA and BAPTA on staurosporine-induced cell death. Cardiomyocytes were exposed to staurosporine and/or various concentrations of either EGTA or BAPTA. Effect of EGTA or BAPTA on staurosporine-induced cell death assessed by trypan blue exclusion. The bar graph represent the mean±SEM of 3 to 4 experiments.
FIG 15 - The effect of EGTA and BAPTA on staurosporine-induced DNA fragmentation. Enzyme-linked immunosorbent assay of DNA fragmentation for cardiomyocytes pretreated with either EGTA or BAPTA before treatment with staurosporine (1μM). The bar graph represents the mean±SEM of 3 separate experiments. (*=p<0.05)
FIG 16 - Cell morphology of representative cardiomyocytes exposed to staurosporine 1μM (Panel B), its diluent (Panel A), BAPTA 10mM (Panel C), or PMA 10μM (Panel D) for 6h and stained with acridine orange.
5. Effect of PMA on staurosporine-induced apoptosis

The phorbol ester PMA was added concomitantly with staurosporine to elucidate the effect of active PKC on staurosporine-induced apoptosis (Figure 17). PMA prevented staurosporine-induced cell death (Panel A) and morphology (Fig 16, Panel D). In addition, this protection was time-dependent. However, PMA co-incubation yielded similar levels of DNA fragmentation, assessed by ELISA, as staurosporine alone (Panel B).

6. Effect of trophic factors, insulin and EGF on staurosporine-induced apoptosis

To investigate the role of trophic factor addition on staurosporine-induced apoptosis, insulin and EGF were added to cardiomyocytes preincubated with staurosporine (Fig 18). Insulin significantly (p=0.029) reduced staurosporine-induced death while the effects of EGF may be significant (p=0.057).

To investigate the role of growth factors on staurosporine-induced DNA fragmentation, insulin and EGF were added to cardiomyocytes (Fig 19). Insulin and EGF addition blunted staurosporine-induced DNA fragmentation, as assessed by ELISA (Panel A) and electrophoretic separation (Panel B). These results suggest that the addition of EGF and insulin, to some extent, can blunt staurosporine-induced cell death. In addition, insulin preincubated before staurosporine treatment may activate some pathway which works in conjunction with staurosporine-
FIG 17 - The effect of PMA on staurosporine-induced cell death and DNA fragmentation. Cardiomyocytes were exposed to staurosporine and/or 10μM PMA for various times. Panel A - Effect of PMA on staurosporine-induced cell death assessed by trypan blue exclusion assay. The graph represents the mean±SEM of 3-5 experiments. Panel B - Enzyme-linked immunosorbent assay of DNA fragmentation for cardiomyocytes treated with staurosporine (1μM) and/or 10μM PMA. The bar graph represent the mean±SEM of 3-4 separate experiments.
FIG 18 - The effect of insulin and EGF on staurosporine-induced cell death. Cardiomyocytes were exposed to 1μM staurosporine and/or 100nM insulin or 50μg/mL EGF. Effect of insulin or EGF on staurosporine-induced cell death assessed by trypan blue exclusion. The graph represent the mean±SEM of 5-7 experiments.
FIG 19 - The effect of insulin and EGF on staurosporine-induced DNA fragmentation. Panel A - Enzyme-linked immunosorbent assay of DNA fragmentation for cardiomyocytes treated with staurosporine (1μM) and/or insulin or EGF. The bar graph represent the mean±SEM of 3-4 separate experiments. (*=p<0.05) Panel B - Electrophoretic separation of effect of insulin on staurosporine-induced DNA fragmentation. Lane 1 - Control; Lane 2 - Insulin 100nM; Lane 3 - media for lane 2; Lane 4 - Insulin 100nM + Staurosporine 1μM; Lane 5 - media for lane 4; Lane 6 - Staurosporine 1μM + Insulin 100nM; Lane 7 - media for lane 6; Lane 8 - Staurosporine 1μM; Lane 9 - DNA ladder; all performed at 6h.
induced DNA fragmentation which would account for the greater
degree of DNA fragmentation seen in Panel B.

7. **de novo protein synthesis**

To investigate the possibility that a cytoprotective protein
was synthesized during trophic factor rescue of staurosporine-
induced cell death, \[^{35}\text{S} \text{methionine incorporation studies were }
performed (Figure 20). New proteins were synthesized during
staurosporine exposure, compared to control, as demonstrated by \[^{35}\text{S-}
methionine incorporation assay (Panel A) and autoradiograph (Panel
B). Staurosporine treatment stimulates de novo protein synthesis
in cardiomyocytes compared to control. The autoradiograph shown is
a representative of 4 separate experiments.

8. **Role of PKC in staurosporine-induced cell death: comparison of
staurosporine and chelerythrine**

To elucidate whether staurosporine effects are mediated solely
by PKC, chelerythrine was used in experiments previously performed
with staurosporine and the results compared (Fig 21). Chelerythrine induced cell death, as assessed by trypan blue
exclusion assay, in a dose-dependent manner with a maximal death of
42.9±9.8% at 6h. This level of death was achieved at a
chelerythrine concentration over 10x greater than that of
staurosporine.

DNA fragmentation following chelerythrine exposure was
assessed by ELISA (Fig 22, Panel A) and electrophoretic separation
(PANEL B). Chelerythrine produced DNA fragmentation 2.2±0.4 fold
more than control and yielded DNA laddering (Panel B).
FIG 20 - *de novo* protein synthesis during staurosporine induced apoptosis. *De novo* protein synthesis was measured by incorporation rate assay (Panel A) or by PAGE (Panel B). **Panel A** - Relative $^{35}$S-methionine incorporation rate compared to control. Bar graph represents the mean±SEM of 4 separate experiments. **Panel B** - Newly synthesized proteins with $^{35}$S-Met incorporation were electrophoretically separated on a 10% polyacrylamide gel. The resulting autoradiograph is a representative of 4 separate experiments. C=control, S=Staurosporine 1uM, I=Insulin 100nM, nuclear=proteins isolated from the nuclear fraction, soluble=proteins isolated from the soluble fraction.
FIG 21 - The effect of chelerythrine on cell viability assessed by trypan blue exclusion assay. Cardiomyocytes were exposed to various concentrations of chelerythrine for 6h and cell viability assessed by trypan blue. Graph represents the mean±SEM of 3 separate experiments.
FIG 22 - The effect of chelerythrine on DNA fragmentation. Panel A - Enzyme-linked immunosorbent assay of DNA fragmentation for cardiomyocytes treated with various concentrations of chelerythrine for 6h. The data are the mean±SEM of 3 separate experiments. Panel B - Electrophoretic separation of DNA fragmented by chelerythrine treatment. Lane 1 - DNA ladder; Lane 2 - Control; Lane 3 - Chelerythrine 0.01μM; Lane 4 - Chelerythrine 0.1μM; Lane 5 - Chelerythrine 1μM; all performed at 6h.
Cardiomyocytes were exposed to chelerythrine and stained with either acridine orange (Fig 23- Panels A and B) or NBD phallacidin (Panels C and D). Chelerythrine exposed cardiomyocytes exhibited usual apoptotic morphology (Panels A and B) and less actin reorganization (Panels C and D) than staurosporine.

9. Role of Ca$^{2+}$ in chelerythrine-induced apoptosis

The effect of Ca$^{2+}$ on chelerythrine-induced DNA fragmentation was assessed by ELISA (Fig 24). Cardiomyocytes were pre-treated with either EGTA or BAPTA before exposure to 1µM chelerythrine for 6h. Both EGTA and BAPTA blunted chelerythrine-induced DNA fragmentation.

10. Role of growth factor in chelerythrine-induced apoptosis

The effect of growth factors on chelerythrine-induced DNA fragmentation was assessed by ELISA (Fig 25 Panel A) or electrophoretic separation (Panel B). Cardiomyocytes treated with 1µM chelerythrine and either insulin (100nM) or EGF (50µg/mL) for 6h. Both insulin and EGF slightly blunted DNA fragmentation induced by chelerythrine. However, insulin decreased the intensity of DNA laddering, when compared to chelerythrine alone (Panel B).
FIG 23 - The cell morphology of representative cardiomyocytes treated with chelerythrine. Representative cardiomyocytes exposed to 1 (Panels A' & C), 10 (Panel D), or 100μM (Panel B) chelerythrine for 6h. Cells were stained with either acridine orange (Panels A & B) or NBD phallacidin (Panels C & D).
FIG 24 - Effect of agents that alter [Ca\textsuperscript{2+}]\textsubscript{i} on chelerythrine-induced DNA fragmentation. Enzyme-linked immunosorbent assay of DNA fragmentation in cardiomyocytes pre-treated with either EGTA or BAPTA before exposure to 1μM chelerythrine for 6h. The bar graph represents the mean±SEM of 3 separate experiments.
FIG 25 - Effect of the growth factors insulin or EGF on chelerythrine-induced DNA fragmentation. Panel A - Effect of growth factors on chelerythrine-induced DNA fragmentation, as assessed by enzyme-linked immunosorbent assay of DNA fragmentation. Cardiomyocytes treated with 1μM chelerythrine and either insulin (100nM) or EGF (50μg/mL) for 6h. The bar graph represents the mean±SEM of 3 separate experiments. Panel B - Electrophoretic separation of DNA fragments. Lane 1 - DNA ladder; Lane 2 - Control; Lane 3 - Chelerythrine 1μM + Insulin 100nM; Lane 4 - Chelerythrine 1μM; all performed at 6h.
CHAPTER IV
DISCUSSION

I) Topoisomerase inhibition

Topo I inhibition had cytotoxic effects on cardiomyocytes as camptothecin, 10 µM, increased cell death by 25% in a 6 h time frame. Camptothecin produced cell shrinkage, membrane blebbing, and nuclear disintegration: all morphological changes characteristic of apoptosis. Furthermore, camptothecin induced DNA fragmentation producing small base pair fragments as demonstrated by DNA electrophoresis. Thus, camptothecin-induced cardiomyocyte death can be ascribed to apoptosis.

Camptothecin produced apoptosis in thymocytes, HL-60 and other cell lines (Lee et al 1994). In mouse thymocytes in primary culture, camptothecin produced a dose-dependent internucleosomal DNA cleavage which preceded cell death (Onishi et al 1993). Cells of the human promyelocytic HL-60 line, when treated with camptothecin, exhibited DNA cleavage (Hotz et al 1994) which correlated with electron microscopic changes in cell structure typical of apoptosis (Bertrand et al 1993, Solary et al 1993). This study demonstrates the ability of topo I inhibition with camptothecin to induce apoptosis in cardiomyocytes.

II) Involvement of intracellular calcium in camptothecin-induced apoptosis

Because apoptosis is an active process, I sought to investigate the role of Ca²⁺ in apoptosis in cardiomyocytes using different agents that have an impact on intracellular calcium ([Ca²⁺]ᵢ). EGTA and taurine reduce [Ca²⁺]ᵢ levels (Bers 1982, Kaneko and Tsukamoto 1994) while thapsigargin increases [Ca²⁺]ᵢ (Furuya et
al 1994). EGTA chelates $\text{Ca}^{2+}$ in the media (stoichiometrically, 1 mole of EGTA chelates 2 moles of $\text{Ca}^{2+}$), hence reducing $\text{Ca}^{2+}$ entry into the cell and $[\text{Ca}^{2+}]_i$.

A) EGTA and taurine

I suggest a role for calcium in camptothecin-induced apoptosis in cardiomyocytes, based on the ability of EGTA and taurine to blunt the development of apoptosis as manifested by less membrane blebbing and less DNA fragmentation in EGTA treated cells. Although EGTA produced a slight reduction in camptothecin-induced cardiomyocyte death, we found a dramatic effect of EGTA and taurine to prevent membrane bleb formation. Indeed few cardiomyocytes treated with EGTA or taurine showed bleb formation. Thus, the effect of EGTA or taurine was disproportionately greater for one of the morphologic features of apoptosis, namely bleb formation. This would support the findings in rabbit renal tubular cells that there is a $[\text{Ca}^{2+}]_i$ threshold for membrane bleb formation (Trump and Berezesky 1995).

Taurine, a free amino acid in plasma and many tissues such as heart, muscle, brain, and blood, affects calcium entry through L-type and T-type $\text{Ca}^{2+}$ channels in embryonic chick heart cells and decreases $[\text{Ca}^{2+}]_i$ (Kaplan et al 1993, Satoh and Sperelakis 1993). Taurine reduced DNA fragmentation and the morphologic changes of camptothecin-induced apoptosis in cardiomyocytes. To my knowledge, there have been no previous studies on the effect of taurine on apoptosis.

Our data suggest a role for taurine in the prevention of apoptosis in cardiomyocytes.

B) Thapsigargin
The proposal that $[\text{Ca}^{2+}]_i$ plays a role in camptothecin-induced apoptosis is further strengthened by the results of my experiments with thapsigargin. In the present study, thapsigargin co-incubated with camptothecin consistently yielded a higher DNA fragmentation signal on the ELISA assay than either thapsigargin or camptothecin alone. These results suggest that the accumulation of $[\text{Ca}^{2+}]_i$, by thapsigargin, plays a definite role in camptothecin-induced DNA fragmentation in cardiomyocytes.

Thapsigargin, a sesquiterpene gamma-lactone, selectively inhibits the sarcoplasmic reticulum and endoplasmic reticulum $\text{Ca}^{2+}$-dependent ATPase family of intracellular "Ca$^{2+}$-pumping" ATPases and produces a three to four fold elevation in the levels of $[\text{Ca}^{2+}]_i$ (Furuya et al 1994). Thapsigargin induces programmed cell death in androgen-independent prostatic cancer cells, which is critically dependent upon an adequate, sustained elevation in $[\text{Ca}^{2+}]_i$ (Furuya et al 1994). Part of the ability of Bcl-2 to suppress apoptosis may be attributed to its regulation of endoplasmic reticulum-associated $\text{Ca}^{2+}$ transport (Lam et al 1994). Thapsigargin increased $[\text{Ca}^{2+}]_i$ and subsequently induced morphologic features of apoptosis followed by internucleosomal DNA cleavage and cell death in cultured human hepatoma cells (Kaneko and Tsukamoto 1994), thymocytes and lymphoblastoid cell lines (Escargueil-Blanc et al 1994). In contrast, thapsigargin saved nerve growth factor-deprived rat sympathetic neurons from death (Lampe et al 1992).

The role of calcium in the development of apoptosis has been investigated in other cell lines and the results are controversial. The results of this study suggest that a decrease of $[\text{Ca}^{2+}]_i$, by EGTA or taurine, prevents camptothecin-induced apoptosis whereas an
increase of $[\text{Ca}^{2+}]_i$ induces apoptosis. My results are consistent with what was found in topoisomerase inhibition in HL-60 cells. In HL-60 cells, $[\text{Ca}^{2+}]_i$ plays an essential role in induction of apoptosis by VP-16, an epipodophyllotoxin derivative etoposide which inhibits DNA topo II. While the extracellular $\text{Ca}^{2+}$ chelator EGTA could not block the VP-16 induced DNA fragmentation, the $[\text{Ca}^{2+}]_i$ chelator BAPTA abolished both internucleosomal DNA fragmentation and the morphologic features of apoptosis (Yoshida et al 1993). Results from this study are also consistent with chick embryo spinal cord motoneuron cells. In this case, calcium loading with the ionophore A23187 stimulated and accelerated the physiological degeneration of motoneurons which will undergo apoptosis (Ciutat et al 1995).

There are conflicting data on the exact role of $\text{Ca}^{2+}$ in the development of apoptosis because apoptosis is not always preceded by a rise in the level of $[\text{Ca}^{2+}]_i$ and calcium chelators may produce as well as prevent apoptosis in cultured cells. For example, in thymocytes, some studies noted that calcium chelators had no detectable effect on camptothecin-induced apoptosis (Hotz et al 1994). In addition, it has been suggested that $[\text{Ca}^{2+}]_i$ does not correlate with apoptosis in thymocytes (Beaver and Waring 1994). In human leukemic cells, apoptosis is not sensitive to $\text{Ca}^{2+}$ levels (Fernandes and Cotter 1993). Hence, it has been proposed that loss of calcium homeostasis, rather than a sustained rise in $[\text{Ca}^{2+}]_i$, is a determining factor in cell death by apoptosis (Kluck et al 1994). However, because $[\text{Ca}^{2+}]_i$ is a major second-messenger molecule, increased $[\text{Ca}^{2+}]_i$ may also promote survival by stimulating a signal transduction pathway, possibly the same one stimulated by trophic
factors (Franklin and Johnson 1994). My data suggest a role for calcium in the apoptosis produced by topoisomerase I inhibition in cardiomyocytes as agents that reduce \([\text{Ca}^{2+}]_i\) decrease apoptosis, while those that increase \([\text{Ca}^{2+}]_i\) accentuate camptothecin-induced apoptosis.

III) Serine/threonine protein kinase inhibition with staurosporine

Staurosporine exposure induced cell death and DNA fragmentation, as evidenced by an increase in cell death by 41%, altered cellular morphology, and extensive DNA fragmentation (three times that of normal cells). Staurosporine-induced apoptosis was blunted by BAPTA and slightly reduced by PMA, insulin and EGF. These results suggest that serine/threonine kinase inhibition can cause apoptosis and that this mechanism involves Ca\(^{2+}\), PKC, and communication with signal transduction pathways stimulated by trophic factors.

A) Staurosporine induces DNA fragmentation and apoptosis

The data presented confirm the observations reported in other cell models that staurosporine increases cell death and yields extensive DNA fragmentation. Staurosporine has produced apoptosis in cell lines from different origins, such as HL-60 and lymphoma cell lines (Bertrand et al 1994). Staurosporine induces apoptosis in human malignant glioma cell lines (Couldwell et al 1994) and in lymphoma and mammary carcinoma cell lines (Shi et al 1994). In lymphocytes, PKC inhibition by staurosporine and polymyxin B induced apoptosis in a manner which was both time and dose dependent (Lucas et al 1994). Staurosporine induced a dose-dependent increase in DNA fragmentation in various cell lines, including Burkitt lymphoma, lung fibroblast, and small cell lung

B) Staurosporine-induced changes in cardiomyocyte morphology

Staurosporine caused cardiomyocytes to lose volume in a time and dose dependent manner. The most dramatic change was that the cytoplasm appears to be condensing into dense vacuoles, yet the cell membrane appeared to remain intact. The nucleus was relatively homogenized, but no definite membrane blebbing was observed. Because NBD phallacidin specifically stains actin, the use of this agent suggests that staurosporine causes cytoskeletal remodelling with dismantling of actin fibres.

These morphologic changes were induced by staurosporine but not by the specific PKC inhibitor chelerythrine. The shapes and elastic properties of cells are dictated by a cytoplasmic filamentous network composed largely of actin (Cortese et al 1989). Agents which disrupt microfilaments prevent the appearance of apoptotic bodies without affecting DNA fragmentation (Cotter et al 1992). Inactivation of PKC is known to decrease the rate of actin polymerization, while activators of PKC, like PMA, increase this rate (Phatak et al 1988). Staurosporine inhibits the formation of apoptotic bodies (Cotter et al 1992), supporting the theory that actin-containing filaments play a central role in apoptotic process (Cotter et al 1992). The unique morphology seen with staurosporine maybe closely linked to its action on the actin-containing filamentous network.

Although staurosporine exposure to cardiomyocytes did not
display the classic morphologic picture of apoptosis, one cannot conclude that staurosporine did not induce apoptosis. It is possible that staurosporine is unique to other apoptotic-inducing agents because staurosporine induces cytotoxicity and DNA fragmentation, as well as affecting the actin directly. Our experiments show that staurosporine produced DNA fragmentation similar to camptothecin. As a result, morphology induced by staurosporine is unique in cardiomyocytes compared to other apoptotic-inducing agents. In human lymphocytic leukemia MOLT-4 cells, staurosporine exposure induced ultrastructural changes, some typical of apoptotic cell death and some not (Falcieri et al 1993). Staurosporine exposure caused the formation of numerous homogeneously electron dense micronuclei, in addition to classical signs of apoptosis (Falcieri et al 1993). In HL-60 and Burkitt lymphoma cells, staurosporine exposure caused the chromatin to condense at the periphery of the nuclei and form dense micronuclear bodies. The cell volume was reduced and the plasma membrane remained well defined. Some organelles such as mitochondria remained intact during the early stages, while others including the endoplasmic reticulum and golgi apparatus appeared dilated (Bertrand et al 1994). In mixed mouse cortical cultures containing both neurons and glia, the neurons exposed to staurosporine did not undergo the cell body swelling typically induced by excitatory toxins, but rather gradual cell body shrinkage accompanied by chromatin condensation (Koh et al 1995). Hence, our results support the contention that staurosporine produces apoptosis and a morphology that is unique because of staurosporine's direct effect on actin.
Actin has been implicated in apoptotic development in other cell systems. Microfilaments play an important role in the formation of apoptotic bodies. The expression patterns of β-actin declines following induction of apoptosis by actinomycin D in HL-60 cells (Naora and Naora 1995). Similarly, in the intersegmental muscles of the tobacco hawkmoth Manduca sexta, expression of actin mRNA was greatly decreased when the cells were committed to die (Schwartz et al 1993). It is possible that the reduction of actin plays a role in the rapid dissolution of cells during apoptosis. The morphology induced by staurosporine showed a dissolution of the cardiomyocyte cytoskeleton, confirming the theory that actin reduction is a part of apoptotic development and the formation of apoptotic bodies.

C. The role of Ca\(^{2+}\) in staurosporine-induced apoptosis

Our study observed that a decrease in \([\text{Ca}^{2+}]_i\) blunted staurosporine-induced cell death, DNA fragmentation, and morphology. The role of Ca\(^{2+}\) in apoptosis has already been discussed in a previous section. Agents that reduce \([\text{Ca}^{2+}]_i\) decrease apoptosis, while those that increase \([\text{Ca}^{2+}]_i\) accentuate camptothecin-induced apoptosis. In a group I Burkitt lymphoma cell line, EGTA partially reduced apoptosis induced by anti-Ig or by Ca\(^{2+}\) ionophore (Knox et al 1992). Hence, the blunting action of calcium chelators on staurosporine-induced apoptosis supports this dissertation's hypothesis that Ca\(^{2+}\) is an important regulator of apoptosis, despite the apoptotic-inducing agent.

D. The role of the phorbol ester PMA on staurosporine-induced apoptosis

PMA blunted staurosporine-induced cytotoxicity as demonstrated
by the trypan blue exclusion assay and blunted apoptotic morphology, but had little effect on DNA fragmentation. These results will be discussed separately.

1) Inhibitory action of PMA on staurosporine-induced cytotoxicity and morphology

The blunting effect of PMA on staurosporine-induced apoptosis, which I observed in cardiomyocytes, has been reported in other cell systems. Tumour-promoting phorbol esters blunted or inhibited (time-dependent) spontaneous DNA fragmentation and cell death in chronic lymphocytic leukemia cells (McConkey et al 1991a). In some Burkitt's lymphoma cell lines, phorbol esters prevented apoptosis (Bonnefoy-Berard et al 1994). PMA suppressed apoptosis mediated by dexamethasone or IL-2 withdrawal in murine T cells exhibiting intermediate affinity IL-2 receptors (Gomez et al 1994). In lymphocytes, PKC inhibition by staurosporine and polymyxin B each induced apoptosis in both time and dose dependent manner, and was counteracted by PMA (Lucas et al 1994). Similar results were seen in freshly isolated rat hepatocytes (Sanchez et al 1992). These data suggest a role for PKC in cell survival during apoptosis.

2) Effect of PMA on staurosporine-induced DNA fragmentation

The results of this study suggest that by activating PKC by PMA, staurosporine-induced cell death and morphology was inhibited and that this inhibition was time-dependent in cardiomyocytes. However, PMA had little or no effect on staurosporine-induced DNA fragmentation. It should be noted that the concentrations and exposure times of PMA used in this study had been previously determined (in our lab) to stimulate PKC activity and not down-regulate PKC activity in chick embryonic cardiomyocytes:
Because PMA can inhibit staurosporine-induced cell death and morphology yet have no effect on DNA fragmentation, the results of this study suggest that either PKC acts in a dual role during apoptotic development or PKC activation by PMA cannot reverse apoptosis past the DNA fragmentation stage. I speculate that the cardiomyocyte, responding to extensive cell damage and death, induces the PKC signal transduction pathways to block or delay the onset of damage or death caused by staurosporine exposure. However, in the absence of cellular injury, an activated PKC can also induce apoptosis, as evidenced by our DNA fragmentation (ELISA) data. Hence, PKC is speculated to have a dual role in apoptosis.

a) The dual role of PKC in apoptosis

The conflicting PKC results reported in this study as well as in other cell lines may be attributed to a dual role for PKC in apoptosis. This dual role may be attributed to changes in PKC subspecies mRNA expression during the process of apoptosis. I speculate that during initial apoptotic induction, a specific PKC isoform is predominant in its functions to block the development of apoptosis. However, as apoptosis is an active mechanism requiring de novo protein synthesis, the gene for another PKC isoform may be activated during the course of apoptotic development. The result would be a shift of PKC isoform dominancy and hence, a shift in the role of PKC (Lin et al 1995). The newly dominant PKC isoform could theoretically be ineffective in the late apoptotic stage of DNA fragmentation. This hypothesis might explain why PMA appeared to be ineffective in staurosporine-induced DNA fragmentation as seen in this study.
The search for the potential dominant PKC isoforms has only recently begun. Preliminary studies in immature thymocytes suggested that glucocorticoid-induced apoptosis selectively induced an increase in Ca\(^{2+}\)-independent PKC activity. PKCe translocated from the cytosolic fraction to the particulate fraction upon glucocorticoid treatment, suggesting the selective activation of PKCe through de novo synthesis of macromolecules (Iwata et al 1994). PMA’s inability to reverse DNA fragmentation observed in this study may be attributed to the different roles of the different PKC isoforms during apoptotic development.

Another explanation for why PMA does not inhibit staurosporine-induced DNA fragmentation is that long exposures to phorbol esters down regulation PKC (Wu et al 1992). Down-regulation of PKC or its activity decreases phosphorylation of one or more substrates for PKC which are essential for preventing apoptosis. In a T-cell hybridoma cell line, PKCa down-regulation by phorbol ester treatment abolished activation-induced cell death (Young et al 1987). The degree of PKC down-regulation, caused by increased degradation of PKC (Young et al 1987), correlated well with the degree of cell death abolishment, suggesting that PKC activation represents an essential step in the molecular mechanisms underlying cell death (Jin et al 1992).

IV) PKC inhibition and apoptosis

The PKC inhibitor chelerythrine produced apoptosis. Chelerythrine inhibits PKC in the regulatory domain, thus making chelerythrine inhibition specific for PKC, as opposed to staurosporine which inhibits at the catalytic domain making it a general kinase inhibitor. Our results suggest that an active PKC,
produced by PMA, is involved in cell survival. PMA (hence, activated PKC) blunting staurosporine-induced apoptosis supports this hypothesis. Induction of apoptosis by PKC inhibition with chelerythrine also supports the hypothesis that an active PKC is involved in the prevention of apoptosis.

A. Effect of PKC inhibition on apoptosis

The data confirm the hypothesis, from other cell models, that inhibition of PKC induces apoptosis. Chelerythrine triggered apoptosis in the Group I Burkitt's lymphoma cell line BL60 and B104 lymphoma cell line (Bonnefoy-Berard et al 1994). In non-transformed rat coronary vascular smooth muscle cells, treatment with calphostin C, a PKC inhibitor, induced usual apoptotic morphology as well as a decline in bcl-2 expression, but not classical apoptotic DNA degradation into nucleosomal fragments (Leszczynski et al 1994). Chelerythrine produced concentration-dependent increases in DNA fragmentation and typical apoptotic morphology in HL-60 cells (Jarvis et al 1994). Conversely, PKC inhibition has also been shown to prevent apoptosis. In mouse thymocytes, PKC inhibition, by the inhibitor 1-(5-isoquinolinylsulfonyl)-2-methylpiperasine dihydrochloride, prevented apoptosis and PMA potentiated radiation-induced apoptosis. However, the PKC activators initiated apoptosis in mouse but not in rat thymocytes suggesting that the role of PKC varies among cell models (Shaposhnikova et al 1994).

B. PKC activation in apoptosis

The role of PKC in cardiomyocytes can be interpreted in two ways: a) the addition of apoptotic inducers stimulates the PKC pathway as a step for cell survival; or b) the cell, in order to
survive, requires a level of PKC activation to fulfill the cell rescue process. The first alternative implies that apoptotic agents directly activate PKC or at least induce hydrolysis of PIP$_2$ to produce IP$_3$ and DAG which would then activate PKC; the activated PKC in turn would then activate the survival pathway needed. The second alternative implies that cell survival requires an independent process of cell stimulation at least to the point where PKC is activated to start the program leading to cell survival. This last possibility appears especially attractive in relation to the postulated role of trophic/mitogenic factors rescuing the cell from apoptosis.

C. Sustained activity of PKC during apoptosis

The sustained PKC activity necessary to blunt or abolish an apoptotic induction signal must be put into context. Sustained PKC activity in a cell, minutes to hours after signal initiation, is attributed to the level of DAG which increases with a relatively slow onset (Nishizuka 1992). A second wave of DAG appears after the first wave produced during PIP$_2$ hydrolysis disappears (Nishizuka 1995). Hence, active PKC can counteract apoptotic induction by its sustained activity due to waves of DAG activation of PKC.

D. PKC inhibitor efficacy: a comparison between staurosporine and chelerythrine

In the present study, the general kinase inhibitor staurosporine and the specific PKC inhibitor chelerythrine were compared. Staurosporine was more potent than chelerythrine since chelerythrine required a greater concentration than staurosporine to yield its maximal apoptotic effect. It is possible that
staurosporine and chelerythrine are not equipotent in cardiomyocytes. However, these results suggest that staurosporine mediates its apoptotic effects by inhibiting other protein kinases in addition to inhibiting PKC. Furthermore, chelerythrine-induced apoptosis was similar in magnitude to staurosporine's results, suggesting that PKC is active in apoptosis.

1. Variable staurosporine potencies

Staurosporine's potency for apoptotic induction is not seen consistently in other cell models. Effective concentrations and times of exposure of staurosporine vary depending on the cell model and, as a result, staurosporine-induction of apoptosis can vary. In HL-60 cells, staurosporine concentrations known to achieve maximal inhibition of PKC failed to induce DNA fragmentation (Jarvis et al 1994). However, staurosporine promoted fragmentation at considerably higher concentrations (>4x $K_i$ where $K_i=0.7nM$ for PKC) (Jarvis et al 1994). A long (24h) exposure of staurosporine to human glioma cell lines failed to induce DNA fragmentation at concentrations generally used to achieve maximal inhibition of enzyme activity; however, higher concentrations of staurosporine promoted DNA fragmentation (Ikemoto et al 1995).

2) Catalytic vs Regulatory Domain in PKC

Considering that staurosporine is more potent than chelerythrine, yet less potent than calphostin C, in producing apoptosis (Jarvis et al 1994), one must consider the differences in these inhibitors of PKC. As mentioned, calphostin C inhibits PKC at the regulatory domain whereas staurosporine inhibits PKC at the catalytic domain. It is tempting to speculate that apoptosis induced by PKC inhibition is determined more dominantly at the
regulatory domain than the catalytic.

V) Cell survival program during the development of apoptosis

Ca\(^{2+}\) chelators, PMA, and trophic factors such as insulin and EGF blunted apoptosis induced by kinase inhibition (staurosporine and chelerythrine) in cardiomyocytes. In addition, de novo protein synthesis occurred during staurosporine exposure with and without growth factor rescue, fueling speculation on the existence of a death/survival protein. This phenomenon has been reported in other cell systems. Evidence that growth factor-induced cell survival can occur independently of proliferative signals (Hamilton et al 1990, Rodriguez-Tarduchy et al 1990) supports the view that cell survival may be separately controlled by growth factors.

The theory of a "second window of protection" has been postulated in relation to myocardial ischemic injury. Briefly, after the myocardium receives its initial sublethal stress (eg. ischemia), the biochemistry of the cardiomyocyte adapts by increased synthesis of cytoprotective proteins (eg. stress proteins and/or endogenous anti-oxidants). By the time the second stress signal arrives, the myocardium is either protected, or has at least enhanced its tolerance. This preconditioning can sometimes abolish or delay the results of the second injury, depending on the time course (Yellon and Baxter 1995). It is therefore reasonable to speculate that this second window of protection can be related to apoptotic induction.

Based on the PMA blunting actions on staurosporine-induced cell death, PKC may be involved in a survival program. PKC may be one of the cytoprotective kinases involved in the second window theory. The activation of PKC isotypes, mediated by different
effector mechanisms, may function to counteract apoptotic induction. Hence, when PKC is inhibited by an inhibitor such as staurosporine or chelerythrine, apoptosis results because there is no functioning survival program. The second window of protection has been reported to be inhibited by chelerythrine given concurrently with the preconditioning insult (Baxter and Yellon 1994) supporting the theory that PKC is involved in cell protection from apoptosis.

The role of IGF-1 as a survival factor has been demonstrated previously in cells derived from the nervous system where it is required for maintenance of cultured cells and protection from direct injury (D'Mello et al 1993). IGF inhibited apoptosis of several IL-3 dependent cell lines when IL-3 was removed (Rodriguez-Tarduchy et al 1992). The addition of insulin-like growth factor-I markedly inhibited etoposide-induced apoptosis in BALB/c3T3 cells. IGF-I was not mitogenic in the presence of etoposide. In addition, IGF-I had no effect on etoposide-induced apoptosis that had a targeted disruption of the IGF-I receptor gene (Sell et al 1995). These results demonstrate an important role for the IGF-I receptor as an inhibitor of apoptosis, independent of its mitogenic actions (Sell et al 1995). IGF-I is relatively weak in preventing apoptosis in cells with low levels of insulin receptors. IGF-I may function as a survival factor in response to diverse agents suggesting that it blocks a common late intracellular apoptosis pathway.

Similar results with EGF were seen in human breast cancer cells which lack estrogen receptors. In this situation, the treatment of these breast cancer cells with EGF led to the inhibition of cell proliferation, DNA fragmentation, and the
development of apoptotic morphology. These results suggest that EGF can blunt apoptosis (Armstrong et al. 1994).

Other growth factors may also protect against apoptosis, depending on the cellular model. Basic fibroblast growth factor (bFGF) was found to protect bovine aortic endothelial cells against radiation-induced apoptosis (Haimovitz-Friedman et al. 1991). Translocation, hence activation, of PKC α from the cytoplasm to the membrane was observed immediately after bFGF addition, suggesting a role for activated PKC. Similarly, the phorbol ester TPA mimicked the radioprotective effect of bFGF. PKC inhibition counteracted the radioprotective effect of bFGF, as did the depletion of PKC by long exposure to high doses of TPA. These results suggest that active PKC prevents radiation-induced apoptosis and mimics the rescuing mechanism seen during bFGF addition (Haimovitz-Friedman et al. 1994).

VI) de novo protein synthesis

New protein synthesis was found during exposure to staurosporine. This data supports the observation that de novo protein synthesis must occur during the active program of apoptosis. The presence of newly synthesized proteins supports the theory of death/cytoprotective proteins. De novo synthesis of a PKC isoform could be stimulated by the induction of apoptosis, consistent with my speculation that PKC is integral to the mediation of apoptosis. Based on the molecular weight of newly synthesized protein during 35S incorporation, I speculate that PKC-related kinase protein is synthesized during apoptotic induction due to its size (120 kDa) (Palmer and Parker 1995). I speculate
that this PKC-related protein is responsible for the inhibition of apoptosis seen during PMA, insulin, and EGF treatment after staurosporine exposure. I also speculate that higher molecular weight proteins seen during de novo protein synthesis may be EGF receptor (180kDa) or the insulin-receptor substrate (160kDa) (Kawase T et al 1995). New synthesis of these growth factor receptors would support the theory of growth factor receptor occupancy mediating the cytoprotective pathway induced by insulin and EGF. Further experiments must be performed to elucidate the identity of these newly synthesized proteins.

VII) Why "normal" cells remain during apoptosis

No matter what the cell line or inducing agent, 100% cell death due to apoptosis is never seen. Resistance to apoptosis was observed in other cell lines (McConkey et al 1991a, Ido et al 1987, Cohen and Duke 1984) as well as in our chick embryo cardiomyocytes. These cells may lack endonuclease, have alterations in PKC, lack an auxiliary protein required for activation of the endonuclease, or have enhanced efflux of drugs, as in multidrug resistance. This theory may account for the failure of an absolute apoptotic response (100% cells dead).

VIII) Protein kinase signal transduction pathway in determining the form of cell death

To reconsider the previous question of why one cell line has different forms of cell death, we must ponder the mechanism that must be responsible for choosing which death process to activate. In light of our data on signal transduction pathways in apoptosis, we speculate that an altered protein phosphorylation may trigger distinct pathways leading to different types of death in one cell
line. In the promyelocytic leukemia (IPC-81) cell line, different cell death programs were initiated based on the target protein and degree of phosphorylation of cAMP dependent kinases (Gjertsen et al 1994). In cardiomyocytes, PKC may be a kinase that is responsible for the decision of which cellular death program to implement in response to a cellular insult.

IX) Why not investigate PKC in camptothecin-induced apoptosis?

The roles of protein kinase pathways in camptothecin-induced apoptosis were not performed in this study. The rationale for this decision was based on results of Bertrand R (et al 1993) The authors reported that in HL-60 cells, apoptosis was induced by camptothecin but kinase activators (TPA) and inhibitors (staurosporine) had no effect on camptothecin-induced apoptosis. However, most of the intracellular signalling modulators were able to induce DNA fragmentation in HL-60 cells by themselves, suggesting that even though modulation of signalling pathways was unable to prevent camptothecin-induced apoptosis, their deregulation could induce apoptosis in HL-60 cells. Hence, we decided to investigate kinase and topoisomerase pathways individually and to correlate any conferring data. Given that camptothecin is known to induce apoptosis, I was able to determine the cell morphology of apoptosis and compare it to other agent-induced morphologies (staurosporine, chelerythrine).

X) Limitations of the study

The study was conducted in embryonic chick cardiomyocytes in culture, so the type of cardiomyocytes and their conditions limit the implications of the study. The embryonic nature of the cell allows us to only extrapolate what might occur in adult tissue.
These cells in culture allow apoptosis to be readily and clearly demonstrated perhaps more than detection of DNA fragments in situ using the terminal deoxynucleotidyl transferase (TDT)-mediated dUTP nick end labelling (TUNEL) assay. In some organs, TUNEL failed to discriminate between apoptosis, necrosis and autolytic cell death (Grasl-Kraupp et al 1995). Another strength of the cardiomyocytes used in the present study is the similarity of the chick and human bcl-2 gene, which protects from apoptosis (Eguchi et al 1992, Boise et al 1995). Furthermore, chick heart has a considerable amount of bcl-2 (Eguchi et al 1992).
CHAPTER V

SUMMARY AND CONCLUSIONS

Topo I inhibition by camptothecin produced apoptosis in cardiomyocytes, evidenced by an increase in cell death, apoptotic cell morphology, DNA laddering, and the quantitative measurement of DNA fragmentation. Decreased [Ca\(^{2+}\)]\(_i\) by taurine and EGTA protected cardiomyocyte viability and reduced DNA fragmentation induced by topo I. Membrane bleb formation was almost completely prevented by EGTA or taurine. A role for [Ca\(^{2+}\)]\(_i\) in camptothecin-induced apoptosis was further supported by the finding that thapsigargin, which increases [Ca\(^{2+}\)]\(_i\), accentuated the DNA fragmentation produced by camptothecin. Calcium may function to render chromatin more susceptible to the action of an endonuclease (Evans 1993).

I speculate that topo I is an active and integral enzyme in the normal heart and that changes to topo I activity are a determinant of apoptosis in cardiomyocytes.

Protein kinase inhibition by staurosporine produced apoptosis in cardiomyocytes, evidenced by an increase in cell death, unique apoptotic cell morphology, DNA laddering, and the quantitative measurement of DNA fragmentation. BAPTA protected cardiomyocytes from cell death and reduced staurosporine-induced DNA fragmentation. Membrane bleb formation was almost completely prevented by BAPTA addition suggesting a role for [Ca\(^{2+}\)]\(_i\) in staurosporine-induced apoptosis.

Staurosporine-induced apoptosis in cardiomyocytes was blunted but not abolished by the activation of PKC via the phorbol ester PMA. PMA addition blunted staurosporine-induced apoptotic morphology, but not DNA fragmentation. This suggests that PKC is
involved in the development of apoptosis.

Staurosporine-induced unique morphologic changes in cardiomyocytes. To my knowledge there are no similar morphologic changes produced by agents that produce cell death of cardiomyocytes. The effect of staurosporine may be attributable to its direct effect on actin. In contrast, chelerythrine yielded morphology similar to usual changes of apoptosis. Because the morphology yielded by staurosporine and chelerythrine are distinct, I speculate that staurosporine is inhibiting other kinases besides PKC to produce its unique morphology.

The addition of the growth factors insulin and EGF blunted staurosporine-induced apoptotic effects. In addition, de novo protein synthesis was observed during staurosporine exposure may be affected by insulin or EGF. These results suggest that the pathways stimulated by agonists at the insulin or EGF receptor work against the development of apoptosis, implying a possible cellular rescue program.

Two inhibitors of PKC, chelerythrine and staurosporine produced cardiomyocyte death implicating PKC in the development of apoptosis. As chelerythrine did not produce the same morphologic changes as staurosporine, PKC alone is not responsible for all of staurosporine's effect on apoptotic induction.

The data in this thesis demonstrate that apoptosis does indeed occur in cardiomyocytes. Apoptosis was produced by the inhibition of topoisomerase I, serine/threonine kinases, and protein kinase C. The induction of apoptosis was reversed by interventions that decrease intracellular calcium. The activation of PKC by PMA and the addition of the growth factors, insulin and EGF, blunted
apoptosis induced by kinase inhibition, implicating PKC in the control of apoptosis in cardiomyocytes.
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