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THE ROLE OF CERAMIDE IN CELL DEATH

by

Nicos Karasavvas

A dissertation submitted to the Graduate Faculty in Biology
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy, The City University of New York

1997

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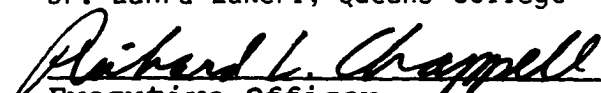
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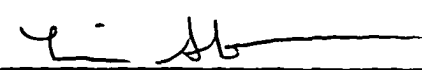
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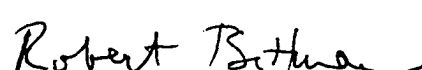
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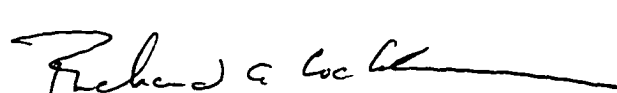

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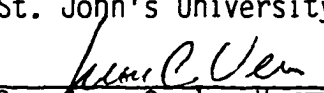
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Abstract

The Role of Ceramide in Apoptosis

by

Nicos Karasavvas

Adviser: Dr. Zahra Zakeri

Ceramide has emerged as a putative second messenger generated by stress signals and cytokines known to induce cell death. In the present study we investigated the role of ceramide in cell death. We first asked how structural modifications of ceramide could affect the induction of cell death. We showed that cell death induced by ceramide was stereospecific. The presence of the ceramide *trans*-4,5-double bond was required for cell death and its modification increased the potency of ceramide analogs to kill cells. The ceramide carbonyl group was not required for the induction of apoptosis. We established that the stereospecificity of ceramide was not mediated by the differential activation of ceramide targets or the generation of endogenous ceramide. Furthermore, we demonstrated that the overexpression of the cell death inhibitory bcl-2 gene protected only the cells that were treated with the naturally occurring ceramide

stereoisomer. Bcl-2 provided partial protection to cells treated with the other ceramide stereoisomers and analogs. To decipher the role of ceramide as a signaling molecule in cell death, we investigated its relationship to the induction of apoptosis by TNF- α , a cytokine speculated to kill cells via the generation of ceramide. We found substantial differences in the induction of cell death mediated by ceramide and TNF- α . Apoptosis promoted by TNF- α was rapid, irreversible and transduced within seconds. The ceramide levels in TNF- α -treated cells increased modestly and well after the cellular commitment to die. In contrast, exogenous ceramide killed cells after prolonged incubations and massive accumulations of intracellular ceramide. To study pathways involved in apoptosis induced by ceramide, we developed the ceramide resistant cell line UDtIN. Using phosphatase inhibitors we showed that ceramide cell killing depends on the activity of PP2A phosphatases whereas TNF- α cell killing is not. Taken together, our results suggest that ceramide and TNF- α kill cells via different pathways, suggesting that ceramide elevations are a consequence and not the cause of cell death under physiological conditions.

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I. INTRODUCTION

Prologue

1. Concepts of Cell Death

- A. Types of cell death
- B. Signals controlling cell death
- C. Genes that regulate cell death
- D. Second messengers in cell death

2. The Emergence of Ceramide as a Second Messenger

- A. Sphingolipid biosynthesis and the sphingomyelin cycle
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Prologue

Apoptosis (a term derived from the ancient Greek for "dropping off" or "falling off" of petals from flowers, or leaves from trees) is a physiological type of cell death by which cells are eliminated without inflicting an immunological response (Kerr, '65; '71; Kerr et al., '72). The realization of the importance of apoptosis in tissue homeostasis, disease and embryonic development led to an unprecedented effort to understand the biochemical pathways and mechanisms that govern its regulation. The activation of the apoptotic program is believed to be mediated by extracellular factors and conditions that transduce and propagate signaling events to the apoptotic machinery. Eventually, dying cells undergo proteolysis and nuclear fragmentation. Although considerable progress has been achieved in identifying extracellular factors, gene products and proteases involved in cell death, the identity of the second messengers and the signal transduction pathways mediating apoptosis are still elusive.

One pathway is mediated through ceramide. Ceramide is a sphingolipid belonging to a newly discovered class of second messengers and is postulated to be an important participant in the orchestrated transduction of signal(s) that lead to cell death. Ceramide is generated under stress conditions that are known to induce apoptosis, such as ionizing radiation, chemotherapeutic drugs and ultraviolet light; and by cytokines such as tumor necrosis factor alpha (TNF- α). This study will

focus on the importance of and requirement for ceramide in cell killing. To do so we will: a) investigate the stereospecificity of ceramide cell killing, b) examine the requirement for the ceramide double bond and carbonyl group in the activation of cell death, c) establish which of the known ceramide targets mediate the apoptotic signaling of ceramide, e) determine if ceramide and TNF- α kill cells via the same pathway and f) investigate the role of phosphatases in the cell death signaling pathway of ceramide and TNF- α .

1. Concepts of Cell Death

- A. Types of cell death
- B. Signals controlling cell death
- C. Genes that regulate cell death
- D. Second messengers in cell death

1. Concepts of Cell Death:

Cell death has been identified as a major physiological event, required during embryogenesis (Saunders, '66; Coucovanis et al., '95), homeostasis of adult tissues (Raff et al., 93; Guenette and Tenniswood, 95), regulation of cell number (Raff, '1992; Hakem et al., '96), and elimination of T lymphocytes (Cohen, '91; Killeen and Littman, 96). Uncontrolled cell growth in tumor development (Reed et al., '90; Rozengurt, 92; Carson and Ribeiro, '93; Mountz and Gause, '93) or excessive cell death in HIV infected T lymphocytes and neurons (Maayard et al., '92; Levine, 93; Gougeon and Montagnier, '93; Linnick et al., '93; Olson, '93) is deleterious to the host organism. Cell death plays a critical role in diseases transmitted by viruses such as HIV (Vaux et al., '94; Thompson, 95; Zhang et al., '96) and in the progression of tumor growth (Williams, '91; King et al., '96; Lu et al., '96). Understanding how growth factors and cytokines control the cellular cell death signalling cascades (Koury and Bondurant, '90; Yao and Cooper 95; Kinoshita et al., '95; Parijs and Abbas, '96) and the use of pharmacological interventions such as anticancer drugs, antibodies, trophic factors and protease inhibitors (Hickman, 92; Vincent et al., '92; Lindsay et al., '93; Sarin et al., '93; Cheng et al., '94; Karp and Broder, '94) may contribute significantly to the therapeutic control of excessive cell growth and death.

A. Types of cell death

Cell death is divided into three major subtypes (Schweichel and Merkers '73). The most well-known form is apoptosis or Type I. Apoptotic cell death occurs in discrete steps. Subsequent to the activation of the apoptotic program(s), the cytoplasm shrinks, and the nuclear chromatin condenses into defined dense masses. Although cell membranes form protuberances 'blebs', they remain intact and eventually surround organelles, cytoplasm, chromatin and other structures of the dying cells (Kerr et al., '72; Kerr, '72; Wyllie, '93). These fragments, called apoptotic bodies, eventually are phagocytosed (Bursch et al., '90; Sensibar et al., '90; Ellis et al., '91a; Zakeri and Ahuja, '94; Bennett et al., '95). The phagocytic cells are in close proximity to the apoptotic cells and express macrophage-specific antigens in their surface (Zakeri and Ahuja, '94). Concurrent with these later stages of apoptosis the chromatin is cleaved by nucleases into discrete fragments that can be detected as large (50 to 300 kilobase) DNA fragments. These large fragments subsequently are degraded into smaller 180 base pair fragments forming DNA ladders on agarose gels (Kerr and Harmon, '91; Neamati et al., '95).

Type II cell death is characterized by the expansion of the lysosomal system and the consequent consumption of the cytoplasm by lysosomes (Helminen and Ericsson, '70; Lockshin and Zakeri, '94; Halaby et al., '94), and a late internucleosomal DNA fragmentation (Zakeri et al., '93; Woo et

al., '94). Cells from metamorphosing or secretory tissues, such as the salivary gland of the tobacco hornworm *Manduca sexta* and mammary glands, which contain elaborate cytoplasm, are known to die by Type II cell death (Zakeri et al., '95). Type III or necrotic cell death is the result of a severe cellular injury, and is characterized by early loss of membrane integrity, mitochondrial swelling, loss of ion transport, initiation of an inflammatory response, release of cellular contents, and disintegration of the cell into fragments that are eventually no longer detected (Kerr et al., '72; Kerr and Harmon, '91; Schweichel and Marker, '73; Clarke, '90).

Concomitant with the morphological studies describing cell death, considerable effort has been devoted to: a) finding and characterizing genes that are associated with the cell death apparatus, b) determining how extracellular factors and conditions (such as cytokines, growth factors or cell damage) convey information to cells and activate or suppress the apoptotic apparatus, c) identifying the second messengers that are involved in the transduction of the apoptotic signals and the conditions under which these signals are generated, d) establishing which of the signal transduction pathways transmit and execute the apoptotic signaling and examine how different signaling pathways intercommunicate to decide whether cells should live or die, e) investigating how pharmacological interventions could promote or inhibit cell

death to control more effectively the outcome of various diseases.

B. Signals controlling cell death

Several growth factors initiate signaling cascades that suppress apoptosis (Collins et al., '94) and in most tissues cell survival appears to depend on growth factor signals provided by neighboring cells and tissues (Raff et al., '93). According to this theory, apoptosis is considered a default pathway. This is particularly apparent in hematopoietic cells in which deprivation of growth factors is associated with cell death (Collins et al., '94; Na et al., '96; Tong et al., '96). For instance, transforming growth factor beta 1 protects rheumatoid synovial cells from apoptosis mediated by Fas, a transmembrane protein initially identified as an autosomal recessive mutation, lymphoproliferation mutant gene (lpr), at the fas locus in mice, Kawakami et al., '96. Also, insulin-like growth factor inhibits cell death induced by interleukin-1 beta converting enzyme (Jung et al., '96) and interleukin-2 prevents cell death in IL-2 deprived activated T cells (Akbar et al., '96).

Intracellular signals associated with cellular lineage information can be decisive between life and death. For instance, in the absence of functional retinoblastoma family proteins (p107 and p130) neuroectoderm undergoes cell death (Slack et al., '95) and mutations in the D-mef2 gene, a

transcription factor expressed in differentiated muscles of *Drosophila* embryos, are associated with muscle survival (Ranganayakulu et al., '95). Cell death is also induced in T-cells and murine ovarian carcinoma cells exposed to radiation (Chen et al., '96; Thames et al., '96) or in T lymphocytes infected by viruses such as hepatitis B (Nakamura et al., '96). Cell to cell interactions during the formation of embryonic tissues are also coupled to the activation of cell death. Failure of glia cells in *Drosophila* to form axon contacts (Sonnenfeld and Jacobs, '95) or disruption of the cell adhesion contacts in chicken embryo fibroblasts (Crouch et al., '96) leads to apoptosis.

C. Genes that regulate cell death

The first evidence that genes are required for the induction of programmed cell death came from the work of Horvitz and his colleagues using the free-living nematode *Caenorhabditis elegans* (Ellis et al., '91b). During the embryonic development of *C. elegans* 131 of the 1090 somatic cells die by apoptosis. Mutational analysis of genes affecting the death of those cells identified 14 genes that were named ced (cell death abnormal) genes. Two of those genes, ced-3 and ced-4, are required for the elimination of all 131 somatic cells that are destined to die (Ellis and Horvitz, '86). These two genes are expressed in virtually all cells and mutations in either one results in prevention of cell death in all

somatic cells. Ced-3 has significant homology to the human interleukin-1 β -converting enzyme (ICE, Yuan et al., '93). ICE is a cysteine protease known to cleave pro-interleukin-1 β into active interleukin-1 β (Kostura et al., '89). ICE is a tetramer consisting of two large and two small subunits (20 and 10 kDa, respectively, Wilson et al., '94). Overexpression of ICE in cells promoted cell death (Yuan et al., '93). Furthermore activation of ICE, and apoptosis, has been demonstrated in neuronal and in mammary epithelial cells upon withdrawal of growth factors (Gagliardini et al., '94; Boudreau et al., '95). Molecular cloning and sequencing of ced-4 revealed a novel protein (Yuan and Horvitz, '92). Ced-4 encodes death preventing ced-4-long (ced-4L) and death promoting ced-4-short (ced-4S) transcripts (Shaham and Horvitz, '96). These authors suggest that the balance of the opposing effects of ced-4L and ced-4S are decisive for the induction or prevention of apoptosis.

Another ced gene, ced-9, is a negative regulator of cell death. Gain-of-function mutations of ced-9 inhibit cell death while loss of function mutations of ced-9 are lethal, due to excessive cell loss (Hengartner et al., '92). Ced-9 gene is homologous to the human oncogene bcl-2 (B-cell lymphoma) gene. Bcl-2, which has been identified as a chromosomal translocation t(14;18), is associated with follicular lymphoma (Bakhshi et al., '85; Tsujimoto et al., '85). In this translocation the bcl-2 gene moves from its normal position

and is transcriptionally activated. Bcl-2 is associated with inhibition of apoptosis (Vaux et al., '88; Miyashita and Reed, '92; Martin et al., '95; Karasavvas et al., '96b; Lu et al., '96), and oncogenesis (King et al., '96; Stoetzer et al., '96).

The bcl-2 gene belongs to an expanding family that includes members that can either promote or protect cells from apoptosis. The homology of the members of the bcl-2 family is within the conserved regions termed BH1, BH2 and BH3 (bcl-2 homology regions, Oltvai et al., '93). The members of the bcl-2 family that promote apoptosis are bad (Yang et al., '95), bak (Chittenden et al., '95), bax (Oltvai et al., '93), and bcl-x (Boise et al., '93). Bax protein forms heterodimers with bcl-2 and appears to inhibit the function of bcl-2 by protein-protein interactions (Oltvai et al., '93). Overproduction of the Bax protein induces cell death due to inactivation of the bcl-2 protein (Sato et al., '94). Bcl-x can be alternatively spliced to produce bcl-xs and bcl-xl. Bcl-xs overexpression induces apoptosis, whereas overexpression of bcl-xl protects from cell death (Boise et al., '93). Mcl-1, A1 and BRAG-1 are members of the bcl-2 family that are known to protect from cell death (Kozopas et al., '93; Lin et al., '93; Kroemer et al., '95; Das et al., '96). How the members of the bcl-2 family protect cells from death is still unknown.

Other gene products such as the adenovirus E1B (Hashimoto et al., '91; Debbas and White, '93), the baculovirus p35 (Clem

et al., '91; Sugimoto et al., '94) and the cowpox virus Crm A (Tewari and Dixit, '95; Gagliardini et al., '94) block apoptosis. Another group of genes, which includes p53 (Yonish-Rouach et al., '91; Aas et al., '96), c-myc (upon growth factor withdrawal) (Harrington et al., '94), the *Drosophila* reaper gene (White et al., '94), cell cycle-related kinases (Li et al., '95) and the transcription factors Nur77 and E2F (Woronicz et al., '94; Kowalik et al., '95), has been shown to trigger apoptosis.

Two members of the TNF superfamily, TNF- α and the Fas ligand (FasL), a transmembrane protein, are of particular importance, because their function is critical for the regulation of apoptosis in the immune system (Nagata and Golstein, 95; Parijs and Abbas, '96; Pfeffer et al., '93; Beutler, et al., '85). Mutation of FasL in mice (gld locus) is associated with a generalized lymphoproliferative disease, lymphadenopathy and autoimmune disease, (Roths et al., '84). FasL is associated with the elimination of self-reacting lymphocytes (preventing autoimmunity) and TNF- α is involved in the eradication of tumors and the mounting of an appropriate immune response against certain pathogens (Pfeffer et al., '93). Both cytokines are believed to exert their apoptosis inducing effects via the second messenger ceramide (Obeid et al., '93; Wright et al., '93; Verheij et al., '96; Cifone et al., '93; Tepper et al., '95; Gulbins et al., '94). In addition to cytokines, exposure of cells to various stressful

conditions or extracellular agents known to kill cells such as ionizing radiation, hydrogen peroxide, ultraviolet radiation, heat shock, retinoic acid, and daunorubicin or other chemotherapeutic agents (Verheij et al., '96; Kalen et al., '92; Bose et al., '95) induce generation of ceramide.

D. Second messengers in cell death

Second messengers are integral parts of signaling cascades that are defined by the fact that their generation unleashes events that translate into cellular responses. Previously established second messengers such as cAMP, fluxes of divalent ions such as Ca^{2+} and reactive oxygen species (ROS) were shown to be critical for the activation and propagation of cellular responses, but their role in signaling of cell death has been inconclusive. For instance, increase in $[\text{Ca}^{2+}]_i$ has been associated with cell cycle regulation, cell differentiation, and in some cases tumor promotion (Whitfield et al., '95), but the importance of Ca^{2+} in the propagation of the cell death signaling has been controversial. According to some studies, sustained increases in cytosolic free $[\text{Ca}^{2+}]_i$ by ionophores and pore-forming agents, was not sufficient to elicit apoptosis (Duke et al., '94), and in some cases increase of cytosolic $[\text{Ca}^{2+}]_i$ inhibited apoptotic cell death (Tong et al., '96; McConkey and Orrenius, '94). In other instances increase in $[\text{Ca}^{2+}]_i$ is correlated with cell death (Trump and Berezesky '95). It is worth mentioning that

elevations of $[Ca^{2+}]_i$ augment the activity of numerous catabolic enzymes, including the endonucleases associated with the fragmentation of DNA in apoptotic cells (McConkey et al., '94).

Similar observations were noted for cAMP. Agents that increase the levels of cAMP, such as the adenylyl cyclase activator forskolin and the cAMP analog dibutyryl-cAMP, inhibited death of neutrophils (Rossi et al., '95;). In other cases, treatment of leukemic T cells with forskolin or dibutyryl-cAMP (Kiefer et al., '95) or treatment of freshly isolated thymocytes with the antagonist of the cAMP pathway, Rp-cAMP (a diastereoisomer of adenosine 3',5'-(cyclic) phosphorothioate, Mentz et al., '95), induced apoptosis. In conclusion, depending on the cellular system and conditions, elevations of cAMP could either trigger or prevent apoptosis (McConkey and Orrenius, '94).

ROS, collectively, is another second messenger that has been implicated in the transduction of apoptosis. Several studies suggested that increases in reactive oxygen intermediates are responsible for apoptosis. For instance, transforming growth factor-beta (TGF-beta) induced cell killing in fetal hepatocytes (Sanchez et al., '96). Down's syndrome neuronal cell death (Busciglio and Yankner, '95) is speculated to be mediated by reactive oxygen species and cell death of thymocytes induced by glucocorticoids, radiation or cross-linking of the T cell receptor was inhibited under low

oxygen conditions (McLaughlin et al., '96). In addition, Veis et al., '93 suggested that bcl-2 prevents apoptosis by inhibiting the generation or action of ROS. Other studies using sympathetic neurons in culture showed that ROS production serves as a stress signal but not as a toxic agent that mediates cell death (Greenlund et al., '95). On the contrary, recent studies demonstrated that apoptosis can occur under low oxygen conditions where oxygen radical generation is inhibited or greatly reduced. For example, cell death promoted by staurosporin (a protein kinase inhibitor), anti-Fas antibodies and survival-factor deprivation, is not stopped under anaerobic conditions (Jacobson and Raff, '95). These studies demonstrate that ROS may not be required for the activation of cell death, in at least under some conditions. It is therefore unclear whether Ca^{2+} , cAMP or ROS play decisive roles in the execution of the cell death program.

A more recent candidate suspected to be critical in the execution of cell death is ceramide. Ceramide is a sphingolipid, a member of a newly discovered class of signal transduction molecules, that is generated (along with phosphocholine) by the hydrolysis of sphingomyelin. Neutral (magnesium-dependent and -independent) and acidic sphingomyelinases (Okazaki et al., '89; Okazaki et al., '94; Wiegmann et al., '94; Heller and Kronke, '94; Santana et al., '96) are enzymes localized in the plasma membrane, cytoplasm or endosomes and hydrolyze sphingomyelin under various

conditions to generate ceramide. Ceramide is suspected to play a pivotal role in the orchestration of cell death (Pushkareva et al., '95; Pronk et al., '96; Verheij et al., '96; Santana et al., '96; Obeid et al., '93; Karasavvas et al., '96a,b).

2. The Emergence of Ceramide as a Second Messenger

- A. Sphingolipid biosynthesis and the sphingomyelin cycle
- B. Elucidation of the physiological importance of ceramide
- C. Ceramide localization and targets

2. The Emergence of Ceramide as a Second Messenger:

Ceramide is considered a second messenger based on the following criteria: 1) Ceramide levels increase sharply in response to extracellular agents such as TNF- α and vitamin D₃ (Kim et al., '91; Okazaki et al., '90). 2) Exogenously added synthetic ceramide analogs (such as C₂- and C₈-ceramide) mimic some of the biological effects of TNF- α and vitamin D₃ (Obeid et al., '93; Okazaki et al., '90). 3) Ceramide has multiple cellular targets such as the ceramide-activated protein kinase (CAPK) (Mathias et al., '91) and ceramide-activated protein phosphatase (CAPP) (Dobrowsky and Hannun, '92). The physiological importance of ceramide in cellular homeostasis and metabolism is suggested by the discovery of multiple ceramide targets that are involved in cell growth, cell cycle, cell death, HIV replication, IL-6 production and differentiation (Ito and Horigome, '95; Olivera et al., '92; Dbaiho et al., '94; Jayadev et al., '95; Karasavvas et al., '96a,b; Rivas et al., '94; Laudererkind et al., '95; Okazaki et al., '90).

A. Sphingolipid biosynthesis and the sphingomyelin cycle

Sphingolipids are synthesized in the endoplasmic reticulum by the condensation of serine and palmitoyl-CoA to form 3-ketosphinganine (Merrill and Wang, '92) which is subsequently reduced to dihydrosphingosine. Further biosynthetic steps are required to synthesize ceramide,

glycosphingolipids and sphingomyelin (Merrill and Wang, '92). Sphingolipids are mostly transported to the plasma membrane, but are also localized in secretory vesicles, endosomes, lysosomes and other intracellular sites (Jeckel and Wieland, '93). Sphingolipids are recycled and remodeled by hydrolases (Hassler and Bell, '93).

Sphingomyelin (*N*-acylsphingosine-1-phosphocholine) is a major structural component of the mammalian myelin sheath, plasma lipoproteins and cell membranes (White, '73; Barenholz and Thompson, '80; Merrill and Jones, '90). Sphingomyelin is comprised of a sphingoid base backbone (predominantly sphingosine), a fatty acid (in amide linkage at the second position of the sphingoid base) and a phosphocholine head group (Barenholz and Thompson, '80; Merrill and Wang, '92). Sphingomyelin is generally believed to be localized to the outer leaflet of the plasma membrane of most mammalian cells (Op den Kamp, '79; Barenholz and Thompson, '80; Venien and Le Grimellec, '88). Cellular biosynthesis of sphingomyelin is catalyzed by sphingomyelin synthase which transfers the phosphocholine group from phosphatidylcholine (PtdCho) to ceramide (Merrill and Jones, '90). Sphingomyelin is transported from the site of synthesis (Golgi apparatus and endosomes) to the plasma membrane via vesicular flow (Futerman et al., '90; Koval and Pagano, '91; Kallen et al., '94). Sphingomyelin hydrolysis is under the control of known signal transduction pathways and is activated by extracellular

signals (Okazaki et al., '90; Kim et al., '91; Rains et al., '93; Verheij et al., '96). Ceramide can be further hydrolyzed to produce sphingosine by ceramidases (Zhang et al., '90; Olivera and Spiegel, '93; Coroneos et al., '95).

The inhibition of protein kinase C (PKC) by sphingosine directed attention to sphingolipids and suggested that sphingolipids may function as second messengers (Hannun and Bell, '89). In the last few years, ceramide and sphingosine emerged as important signaling molecules that control cell differentiation, growth, proliferation, and death (Okazaki et al., '90; Kim et al., '91; Olivera and Spiegel, '93; Obeid et al., '93; Santana et al., '96). The hypothesis that sphingolipids could serve as second messengers, mediating the effects of extracellular signals, led to the search for agents that could induce sphingomyelin hydrolysis. In studies conducted in tissue culture cells vitamin D₃ induced sphingomyelin hydrolysis, ceramide generation and differentiation of human promyelocytic leukemia cells (HL-60, Okazaki et al., '89; Okazaki et al., '90). Since then, other factors such as γ -interferon (γ -IFN), TNF- α , interleukin 1 (IL-1) Fas, and neurotrophins were found to activate the sphingomyelinases that generate ceramide (Kim et al., '91; Mathias et al., '93; Cifone et al., '93; Dobrowsky et al., '95). The completion of the sphingomyelin cycle is achieved by transfer of a phosphocholine headgroup (from phosphatidylcholine) to ceramide forming sphingomyelin and

diacylglycerol (Hannun, '94).

B. Elucidation of the physiological importance of ceramide

Naturally occurring ceramides are very hydrophobic, poorly soluble lipids, and their use in tissue culture studies is limited. In order to investigate the biological activities of ceramide, more soluble ceramide analogs were synthesized. Such analogs include *N*-acetyl-sphingosine (C_2 -Cer), dihydro- C_2 -ceramide (DHC $_2$ -Cer, C_2 -Cer lacking the trans-4,5-double bond) and *N*-octanoyl-sphingosine (C_8 -Cer). These synthetic ceramide analogs have been used extensively to study the physiological function of ceramide (Hannun, '94; Jarvis et al., '94; Karasavvas et al., '95a,b).

Although ceramide analogs have been very useful in deciphering the sphingomyelin pathway, some of their properties differ from those of natural ceramide. Tritium-labeled C_2 -Cer is very poorly metabolized; after 24 hours of treatment approximately 80% was still intact within cells (Bielawska et al., '93). C_2 -Cer is not metabolized to sphingosine but a small percentage (2.8%) is converted to sphingomyelin (Okazaki et al., '90). Longer chain ceramide analogs such as C_8 -Cer behave differently. C_8 -Cer internalized by cells is metabolized to form the natural lipids glucosylceramide (GlcCer), ceramide and sphingosine (Abe et al., '92). Treatment of HL-60 cells with C_8 -Cer elevates the intracellular levels of ceramide as much as 57-fold (Younes et

al., '92).

Since sphingolipids are amphipathic molecules, it was essential to show that their activity was not due to nonspecific interactions with membrane proteins, or to detergent-like effects. The stereospecificity of ceramide was initially investigated by using *N*-acylphenylaminoalcohol analogs (Bielawska et al., '92), and later by the stereoisomers of C₂-Cer and DHC₂-Cer. C₂-Cer and DHC₂-Cer have an acyl group in the amide position (Bielawska et al., '93). The C₂-Cer stereoisomers were found to differ modestly in inhibiting cellular growth, activation of CAPP (a serine/threonine phosphatase belonging to the PP2A family), down-regulating the oncogene c-myc and inducing apoptosis in cells (Bielawska et al., '93; Wolff et al., '94). In addition the presence of the *trans*-4,5-double bond of DL-erythro-DHC₂-Cer was necessary for biological activity. A major concern about using C₂- and DHC₂-Cer stereoisomers was their poor metabolism, once internalized by cells (Okazaki et al., '90; Abe et al., '92; Bielawska et al., '93; Ridgway and Merriam, '95). The poor metabolism of the C₂-Cer analogs suggested that their biological activity might be distinct and functionally different from that of naturally occurring ceramide. To overcome this problem, the synthesis of ceramide analogs with longer amide chains was required (Jayadev et al., '95; Karasavvas et al., '96a,b).

C₈-Cer is a synthetic ceramide with a moderate size amide

chain that is soluble in tissue culture media, readily internalized by cells in culture and metabolized to generate natural sphingolipids such as sphingosine, ceramide and glycosylceramides (Ridgway and Merriam, '95). Since C₈-Cer was metabolized similarly to natural ceramide and therefore its use was more relevant for biological studies, the four stereoisomers of C₈-Cer, *N*-octanoyl-D-erythro-sphingosine (D-e-C₈-Cer), *N*-octanoyl-D-threo-sphingosine (D-t-C₈-Cer), *N*-octanoyl-L-erythro-sphingosine (L-e-C₈-Cer) and *N*-octanoyl-L-threo-sphingosine (L-t-C₈-Cer), the racemic compound *N*-octanoyl-DL-erythro-dihydrosphingosine (DL-e-DHC₈-Cer, DL-e-C₈-Cer which lacks the *trans*-4,5-double bond) and the novel compound *N*-octyl-D-erythro-sphingosine (D-e-C₈-Ceramine, in which the carbonyl group is replaced by a methylene group) were synthesized and tested for their ability to regulate cell death (Karasavvas et al., '96a). The C₈-Cer stereoisomers, unlike the C₂-Cer stereoisomers, showed stereospecificity in cell killing. Furthermore, DL-e-DHC₈-Cer had reduced biological activity in cell killing and the carbonyl group was not required in cell death (Karasavvas et al., '96a).

C. Ceramide localization and targets

The presence of sphingomyelin at different subcellular compartments implied that the location at which sphingomyelin is hydrolyzed could mediate different biological effects (Linardic and Hannun, 94; Andrieu et al., '95; Bettaieb et

al., '96). Sphingomyelin localized at the external leaflet of the plasma membrane (Das et al., '84) is hydrolyzed by a magnesium-dependent neutral sphingomyelinase in response to TNF- α , IL-1 β and ionizing radiation (Dressler et al., '92; Wiegmann et al., '94; Mathias et al., '93; Haimovitz-Friedman et al., '94). Other investigators claimed that ceramide generated by Fas and TNF at the endolysosomal compartments by acidic sphingomyelinases is required for apoptosis (Cifone et al., '93; Wiegmann et al., '94; Schütze et al., '92). Yet other studies suggested that the sphingomyelin pool hydrolyzed by TNF- α and interleukin-1 β in U937, HL-60, skin fibroblasts, and KG1a myeloblastic cells is localized in the inner leaflet of the plasma membrane (Linardic and Hannun, 94; Andrieu et al., '95; '96; Bettaieb et al., '96). As noted by Bettaieb et al., '96, reduced levels of sphingomyelin at the inner leaflet of the plasma membrane renders KG1a cells resistant to apoptosis induced TNF- α . Thus sphingomyelin distribution may profoundly influence the signaling cascades and apoptosis mediated by at least TNF and Fas.

The ceramide targets associated with the transduction of the apoptotic signaling are not known. One of the first ceramide targets to be characterized was the CAPK (Mathias et al., '91). CAPK is a 97 kDa plasma membrane bound protein kinase that shows phosphorylation preference for the sequence X-Ser/Thr-Leu-Pro-X. The phosphoacceptor site (serine or threonine) is amino-terminal to a proline residue and X's can

be any amino acid (Joseph et al., '93). CAPK is partly characterized (Mathias et al., '91; Liu et al., '94), and it has been shown to be activated by TNF- α and ceramide. Targets for CAPK include the EGFR (phosphorylated on threonine 669 which is the same site phosphorylated by the mitogen activated protein (MAP) kinases), the myelin basic protein (Liu et al., '94) and Raf. Raf kinases are enzymes that transduce the signaling of growth factors and their receptors. These signals are associated with transcriptional activation of growth-factor-activated signaling pathways and cell proliferation (Yao et al., '95). The consequence of phosphorylation of the above targets by CAPK is not yet clear. There are no reports establishing a connection between CAPK and activation of cell death. Studies by Karasavvas et al., '96a showed that the differential killing of the C₈-Cer stereoisomers is not associated with the differential activation of CAPK. Several studies hint that CAPK is not involved in apoptosis, but rather with inflammation (Yao et al., '95; Kolesnick and Golde, '94). According to these studies, ceramide generated by TNF- α activates CAPK which phosphorylates Raf on threonine 269. The active form of Raf initiates signaling through the MAPK cascade which leads to inflammation.

CAPP is a cytosolic phosphatase that is activated by natural and C₂ ceramide (Dobrowsky and Hannun, '92) and is a Ser/Thr phosphatase that belongs to the PP2A subfamily (Dobrowsky et al., '93). The members of the PP2A family are

heterotrimeric. The PP2A core dimer consists of a catalytic and a regulatory subunit that can associate with a third variable regulatory subunit, B-subunit, to form the holoenzyme (Mayer-Jaekel and Hemmings, '94). Ceramide activates only the holoenzyme form of CAPP implicating the requirement of the B-subunit for activation (Dobrowsky et al., '93). CAPP is potently inhibited by the phosphatase inhibitor okadaic acid (as are all members of the PP2A subfamily) with IC_{50} of 1-10 nM (Dobrowsky and Hannun, '92; Wolff et al., '94). Exogenous C_2 -ceramide and $TNF-\alpha$ (Kim et al., '91) down-regulate the oncogene c-myc. This inhibition is believed to be mediated by PP2A because inactivation of PP2A by okadaic acid reverses the inhibition (Kim et al., '91; Wolff et al., '94). The importance of PP2A in the regulation of cell death is not established. Studies by Karasavvas et al., 96a showed that inactivation of CAPP by okadaic acid does not abolish apoptosis in U937 cells.

Ceramide has been also shown to induce tyrosine phosphorylation of p42 mitogen-activated protein kinase (p42^{mapk}) (Raines et al., '93), dephosphorylate the retinoblastoma gene product (Dbaibo et al., '95), activate PKC ζ (an atypical form of PKC that is insensitive to DAG and phorbol esters) (Lozano et al., '94; Muller et al., '95), stimulate the activity of the guanine nucleotide exchange factor Vav (Gulbins et al., '94), induce cell cycle arrest and senescence (Jayadev et al., '95; Venable et al., '95) and

activate the stress-activated protein kinase SAPK/JNK (Verheij et al., '96). Lastly, a functional sphingomyelin pathway has been found in yeast. A ceramide analog, C₂-ceramide, inhibits growth and activates a yeast phosphatase (Fishbein et al., '93). The evolutionary conservation of the pathway reinforces the belief that sphingolipids play a critical role in cell growth, cell death and differentiation.

3. Evidence that Ceramide Plays an Important Role in Cell Death Signaling

- A. Ceramide is generated under conditions that lead to cell death
- B. Evidence that ceramide mediates the killing of members of the TNF superfamily
- C. The members of the TNF superfamily and cell death
- D. Elucidation of the signaling cascade involved in cell killing by TNF and Fas

3. Evidence that Ceramide Plays an Important Role in Cell

Death Signaling:

A. Ceramide is generated under conditions that lead to cell death

One of the most interesting and intriguing properties of ceramide is the induction of apoptosis in cells in culture and in vivo (Obeid et al., '93; Cifone et al., '94; Jarvis et al., '94; Bose et al., '95; Jayadev et al., '95; Tepper et al., '95; Karasavvas et al., '96a,b; Brugg et al., '96; Pronk et al., '96; Santana et al., '96). Ceramide generation was measured under various conditions that are known to lead to apoptosis. Indeed, ceramide levels were shown to increase in cells exposed to ionizing radiation (Haimovitz-Friedman et al., '94), withdrawal of serum (Jayadev et al., '95), ultraviolet-C radiation, heat shock (Verheij et al., '96), chemotherapeutic agents such as daunorubicin (Bose et al., '95) and oxidative stress (Jarvis et al., '95). Another indication of the importance of ceramide as a mediator of cell death, is the investigation of the cell death pathways in vivo models. Transgenic mice deficient in acidic sphingomyelinase and lymphoblasts from Niemann-Pick disease type A,B patients (a disease associated with inherited deficiency in acidic sphingomyelinase activity) were found to be resistant to apoptosis induced by ionizing radiation (Santana et al., '96). These studies suggest that the acidic sphingomyelinase was required for apoptosis. The mechanism(s) and the signaling

pathway(s) of cell death triggered by ceramide have not been elucidated. The activation of the SAPK/JNK (Verheij et al., '96) and Ras/Raf signal transduction pathway (Yao et al., '95) by ceramide suggests that these pathways may participate in the activation of cell death. It is also unclear whether known ceramide targets, such as CAPK and CAPP, are involved with the signalling that triggers cell death (Karasavvas et al., '96a). It is likely that the regulation of cellular homeostasis by ceramide is complex and multifactorial. This notion is supported by the fact that ceramide seems to be involved in various known signal transduction pathways and activates a cascade of various targets (Spiegel et al., '96; Hannun et al., '93; Kolesnick and Golde, '94). Ceramide is also hypothesized to control the apoptotic pathways of the two members of the TNF superfamily, TNF- α and Fas (Obeid et al., '93; Cifone et al., '94; Jarvis et al., '94; Cleveland and Ihle, '95; Heller and Krönke, '94; Pushkareva et al., '95; Santana et al., '96).

B. Evidence that ceramide mediates the killing of members of the TNF superfamily

The TNF superfamily and its receptors are considered as major components in the physiological regulation of apoptosis (Gill et al., '94; Rothstein, '96; Barinaga, '96; Smith et al., '94). Association of TNF- α with its receptor generates ceramide (Kim et al., '91; Schutze et al., '92) which is

speculated to mediate the cytotoxicity of TNF- α and diacylglycerol (DAG) via the hydrolysis of phosphatidylcholine by a phosphatidylcholine-specific phospholipase C (PC-PLC) (Schutze et al., '91). Addition of TNF- α (10-30 nM) to U937 cells results in sphingomyelin hydrolysis and elevation of ceramide, which reaches a peak (a 45% increase from control cells) in 10 to 15 min (Obeid et al., '93; Wiegmann et al., '94). Sphingomyelin concentrations and ceramide return to physiological levels within minutes suggesting that the apoptotic signals are transduced during the first minutes of exposure to TNF- α . Other investigators noted that ceramide concentrations stay elevated after TNF treatment (Verheij et al., '96; Wright et al., '96). In addition, exogenously added synthetic ceramides, which bypass the ligand-receptor interactions, are hypothesized to exert their effects and kill cells by triggering apoptosis in a manner which is indistinguishable from the one activated by the receptor-ligand interactions. Indeed, we and other investigators showed that synthetic ceramide analogs induce apoptosis in a variety of cells in culture (Obeid et al., '93; Jayadev et al., '94; Karasavvas et al., 96a,b) suggesting that apoptosis induced by TNF- α and Fas could be mediated by ceramide. This hypothesis is reinforced by the fact that other related lipids (oleic acid, dioctanoylglycerol and phorbol 12-myristate 13-acetate) and dihydroceramides failed to kill cells (Obeid et al., '93; Jarvis et al., '94).

C. The members of the TNF superfamily and cell death

Members of the TNF superfamily kill cells by signals transduced by receptor-ligand interactions. TNF- α is a potent cytokine which upon binding to its target receptor triggers an irreversible suicide response (Smith et al., '94; Heller and Krönke, '94; Baker and Reddy, '96). TNF- α , produced primarily by macrophages or monocytes, was originally characterized as a factor that caused necrosis in certain tumors in vivo (Carswell, et al., '75; Beutler, et al., '85). Recent studies implicate TNF- α as an important mediator of inflammation, autoimmune disease, septic shock, bone resorption (Beutler '91), cytotoxicity (Heller et al., '92) and apoptosis (Obeid et al., '93; Jarvis et al., '94; Verheij et al., '96). TNF- α binds to two cell surface receptors, TNFRI and TNFRII, (Gruss and Dower, '95; Beutler and Huffel, '94; Baker and Reddy, '96). The low affinity receptor is 55 kDa (p55 or TNFRI) and the high affinity receptor is 70-80 kDa (p75 or TNFRII) protein. The TNF receptors are glycoproteins with a single membrane spanning domain and are expressed on the surface of most cells. Most of the known responses of TNF are mediated by the activation of TNFRI (Wiegmann et al., '92). Knockout TNFRI transgenic mice confirmed the role of the TNFRI in the defense against pathogens such as *L. monocytogenes* infection (Pfeffer et al., '93).

Other members of the TNF family include LT- α , OX40L,

CD40L, CD27L, CD30L, 4-1BBL and LT- β (Gruss and Dower, '95). The homology of the family resides within 150 amino acids (cysteine-rich) at the C terminal region (Marsters et al., '92; Banner et al., '93). This region is involved in the ligand-receptor recognition. The members of the TNF receptor superfamily include TNFR-III, OX-40, 4-1BB, CD40, CD30, CD27, the poxvirus gene products PV-T2 and PV-A53R and the low affinity growth factor receptor p75 NGFR (Gruss and Dower, '95). The homology of these receptors is within the extracellular, ligand-binding, cysteine-rich domains (Banner et al., '93). The cytoplasmic region of the receptors does not show any apparent homology (Itoh et al., '91), except for some homology between TNFR1 and Fas. The lack of intracellular homology among the TNF receptor members suggests that they have different cytoplasmic targets and are coupled to diverse signal transduction pathways. The significance of Fas antigen was insinuated in 1989 when two independent groups isolated monoclonal antibodies that were shown to bind to a cell surface protein and induce apoptosis (Trauth et al., '89; Yonehara et al., '89). The interest in Fas was spurred when it was found that Fas can mediate cell death in different cell types (Watanabe-Fukunaga et al., '92a; Itoh et al., '91). Concomitant studies identified the Fas antigen to be the structural gene for *lpr* (a mutation caused by retrotransposon insertion in the Fas locus leading to abnormal transcription and reduced expression of Fas, Watanabe-Fukunaga et al.,

'92b). Mice homozygous at the *lpr* locus develop autoimmune diseases and adenopathy (Takahashi et al., '94; Wu et al., '93). The fact that Fas was shown to be associated with cell death and autoimmune diseases suggested its importance in the regulation and eradication of self reactive lymphocyte populations (Gill et al., '94; Nagata and Golstein, '95). Soon after the cloning of Fas, FasL was identified and, as expected, found to have homology to members of the TNF superfamily (Suda et al., '93).

Fas has structural homology not only with the extracellular portion of the TNF receptor, but also with the cytoplasmic part (Oehm et al., '92; Beutler and Hufel, '94; Cleveland and Ihle, 95). This suggested that these receptors may have converging apoptotic pathways and that some of the factors mediating apoptosis may be shared. This assumption prompted many laboratories to search for mechanistic and signaling relationships between TNFRI and Fas. Tartaglia et al., '93, and Itoh and Nagata, '93, identified a conserved region, a cytoplasmic domain of about 80 amino acids at the C-terminal end of Fas and TNFRI that was required for cell death and named it the death domain.

D. Elucidation of the signaling cascade involved in cell killing by TNF and Fas

Although the cell killing capabilities of TNF and Fas were well characterized (Obeid et al., '93; Jarvis et al.,

'94; Wright et al., '93; Verheij et al., '96; Zheng et al., '95; Breidler et al., '95; Katsikis et al., '95), the post-receptor signaling events that mediated apoptosis were elusive. TNFRI and Fas lack cytoplasmic catalytic domains, but both receptors have been reported to couple ligand binding to tyrosine and serine/threonine phosphorylation (Eischen et al., '94). The TNF signaling cascade began to unravel with the discovery of TRADD (TNF receptor-associated death domain protein). TRADD was the first protein identified to be associated with the death domain of the TNF receptor (Hsu et al., '95). The C-terminal 118 amino acids of TRADD form protein-protein interactions with the cell death domain of TNFRI. These interactions have been associated with the activation of the cell death pathway and the translocation of NF- κ B (a transcription factor activated by TNF- α , Hsu et al., '95). Cell death mediated by TRADD is inhibited by CrmA, a Cowpox viral gene that encodes a protease inhibitor for ICE. Unlike TRADD, NF- κ B translocation is not inhibited by CrmA suggesting that TRADD signaling for apoptosis is distinct from NF- κ B activation (Hsu et al., '95). Furthermore it implicates the importance of proteases in the execution of apoptosis in the TNF signal transduction pathway. Other cytoplasmic proteins recruited to the TNFRI include RIP (receptor-interacting protein) and FADD (Fas-associated death domain). Overexpression of these proteins induces apoptosis; they are all currently considered to be key players in the induction of

cell death (Stanger et al., '95; Chinnaiyan et al., '96). As in the case of TNFRI, Fas cytoplasmic domain recruits FADD and RIP which are believed to mediate the cytotoxic effects of Fas (Chinnaiyan et al., '95; Chinnaiyan et al., '96; Stanger et al., '95).

To establish a link between the death domain and the apoptotic cascade, it was necessary to verify the interactions between the factors associated with the death domain of TNFRI and Fas (such as TRADD and FADD) and the proteolytic machinery (activated during cell death). Earlier studies demonstrated that TNF and FasL induce the activation of members of the ICE family (Voelkel-Johnson et al., '95; Hasegawa et al., '96).

One of the first indications that proteases could be critical in cell death came from the studies on the nematode *C. elegans*. Ced-3 (one of the genes required for cell death in *C. elegans*) encodes a cysteine protease that exhibits significant homology to ICE (Yuan et al., '93). ICE is a member of a cysteine protease family that has been divided into three subgroups depending on sequence relationships (Chinnaiyan and Dixit, '96). ICE recognizes the sequence YVAD and hydrolyzes IL-1 β after the Asp residue (D) to form the 17.5 kDa active form of IL-1 β (Thornberry et al., '92). Other members of the family include Mch-2 and Mch-3 (Fernandes-Alnemri et al., '95a,b), Ich-1 (Wang et al., '94), Ich-2 (Kamens et al., '95), and CPP32 (Fernandes-Alnemri et al., '94). CPP32 recognizes the sequence DEVD and cleaves poly(ADP-

ribose) polymerase (Tewari et al., '95), whereas Mch-2 recognizes VENN and cleaves lamin (Orth et al., '96). Proteolytic hydrolysis of cellular targets, such as poly(ADP-ribose) polymerase (PARP), lamin B1, and β -actin (Lazebnik et al., '94; Neamati et al., '95; Kayalar et al., '95), is an important aspect in the execution of cell death.

ICE activity was found to be important in the induction of apoptosis through Fas and it involves CPP32 β (Enari et al., '95; Enari et al., '96). It was demonstrated that the activation of CPP32 is dependent on ICE (sequential activation of proteases) and cell death was inhibited by ICE and CPP32 inhibitors (Enari et al., '96). The latest member of the ICE/Ced-3 family is FLICE (FADD-like ICE). FLICE has homology to the ICE/Ced-3 family of cysteine proteases and FADD (Muzio et al., '96). Upon binding of FasL to Fas, FLICE is recruited to the cytoplasmic region of Fas and may propagate the apoptotic signals. This finding established a link between the receptor mediated signal and the activation of the proteolytic cascade in Fas mediated cell death. A similar association for the TNF death domain has not been established as yet.

II. RESULTS AND DISCUSSION

Aims

Chapter 1. Ceramide Stereospecificity and the Involvement of Known Ceramide Targets in the Execution of Cell Death

- A. Objective
- B. Materials and Methods
- C. Results
 - a. Kinetics of cell death induced by D-e-C₈-Cer.
 - b. Effect of DL-e-DHC₈ -Cer on DNA fragmentation.
 - c. Cell growth and DNA fragmentation induced by C₈-Cer stereoisomers and D-e-C₈-Ceramine.
 - d. Nuclear fragmentation of U937 cells treated with C₈-Cer stereoisomers and D-e-C₈-Ceramine.
 - e. Measurement of the endogenous levels of ceramide in treated cells.
 - f. Effects of C₈ -Cer stereoisomers and D-e-C₈-Ceramine on the activation of CAPK.
 - g. Okadaic acid fails to prevent cell death activated by ceramide treatment.
- D. Discussion: Ceramide stereospecificity and the role of ceramide targets in the induction of cell death.

Chapter 2. Bcl-2 Protects Cells From Apoptosis Induced by the D-e-C₈-Ceramine but not from more Potent Ceramide Analogs

- A. Objective
- B. Materials and Methods
- C. Results
 - a. Can bcl-2 overexpression protect cell death induced by D-e-C₈-ceramide?
 - b. Can bcl-2 overexpression protect cells from the more potent stereoisomers of C₈-Cer?
 - c. Does the conversion of the ceramide trans-4,5-double bond into *cis* or triple bond change the ability of ceramide to kill cells?
- D. Discussion: Bcl-2 protects cells from apoptosis induced by the natural ceramide stereoisomer only.

Chapter 3. Relationships of Apoptotic Signaling Mediated by Ceramide and TNF- α

- A. Objective
- B. Materials and methods
- C. Results
 - a. Kinetics of TNF- α and D-e-C₈ -Cer induced cell killing.
 - b. Reversibility of TNF- α and ceramide induced cell death in U937 cells.

- c. Exogenous ceramide does not change the time required by TNF- α to induce apoptosis in U937 cells.
- d. Low pH washes and trypsinization do not inhibit cell death promoted by exposure to TNF- α .
- D. Discussion: Evidence that ceramide and TNF- α kill cells via different pathways.

Chapter 4. Ceramide Resistance to Cell Killing is Phosphorylation Dependent. Evidence that Ceramide and TNF- α Kill Cells by Independent Mechanisms

- A. Objective
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- C. Results
 - a. UDtIN cells are resistant to C₆-Cer stereoisomers, analogs and TNF- α .
 - b. The resistance of UDtIN cells to killing is more specific to ceramide and TNF- α .
 - c. The resistance to ceramide in UDtIN cells is not dependent on the rate of internalization or degradation of endogenous ceramide.
 - d. UDtIN cells contain functional TNF receptors.
 - e. The resistance of UDtIN cells to ceramide cell killing depends on the activity of PP2A phosphatases.
 - f. Okadaic acid does not render UDtIN cells

sensitive to killing by TNF- α .

D. Discussion: Ceramide cell killing depends on the activity of PP2A phosphatases, but TNF- α does not. Evidence that ceramide and TNF- α kill cells via different pathways..

Conclusions

Aims:

Although compilation of data suggests that ceramide is important for the activation and execution of cell death, the mechanisms and the targets involved remain unresolved. To address the importance of ceramide in the activation of cell death we examined, a) how structural modifications of ceramide affect its ability to kill cells; b) the role of the known ceramide targets CAPK and CAPP in the apoptotic pathway of ceramide; c) the relationship between bcl-2 and ceramide; d) the relationship between ceramide generation and activation of cell death by TNF- α and e) the biology of cell death in the cell death resistant cell line UDtIN.

CHAPTER 1

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 - a. Kinetics of cell death induced by D-e-C₈-Cer.
 - b. Effect of DL-e-DHC₈-Cer on DNA fragmentation.
 - c. Cell growth and DNA fragmentation induced by C₈-Cer stereoisomers and D-e-C₈-Ceramine.
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 - e. Measurement of the endogenous levels of ceramide in treated cells.
 - f. Effects of C₈-Cer stereoisomers and D-e-C₈-Ceramine on the activation of CAPK.
 - e. Okadaic acid fails to prevent cell death activated by ceramide treatment.
- D. Discussion: Ceramide stereospecificity and the role of ceramide targets in the induction of cell death.

A. Objective:

Ceramide generated by members of the TNF superfamily (TNF- α and Fas) and various stress signals (UV light and ionizing radiation) is implicated in the activation of the apoptotic machinery (Obeid et al., '93; Jarvis et al., '94; Cifone et al., '93; Nagata and Golstein, '95; Verheij et al., '96; Haimovitz-Fredman et al., '94). However, we need to verify that ceramide is a bioactive molecule and that ceramide induced cell death is not due to non-specific or toxic interactions with certain biochemical pathways in cells. To investigate the role of ceramide in cell death, synthetic cell-permeable ceramide analogs were synthesized and tested for: a) their ability to cause cell death in tissue culture cells and b) their ability to activate cellular target proteins during cell death. Exogenously added, synthetic ceramides bypass the ligand receptor interaction and sphingomyelin hydrolysis.

A major concern about using C₂- and DHC₂-Cer stereoisomers was that they are poorly metabolized (Okazaki et al., '90; Abe et al., '92; Bielawska et al., '93; Ridgway and Merriam, '95). The poor metabolism of the C₂-Cer analogs suggested that their biological activity may be distinct and functionally different from that of naturally occurring ceramide. To overcome this problem, the synthesis of metabolizable ceramide analogs was required. *N*-Octanoyl-sphingosine (C₈-Cer) is a synthetic ceramide with a moderate size amide chain that was shown to be

soluble in tissue culture media, readily internalized and metabolized (to generate natural sphingolipids such as sphingosine, ceramide and glycosylceramide) by cells in culture (Ridgway and Merriam, '95). Since C₈-Cer was metabolized in a manner similar to natural ceramide (therefore being more relevant for biological studies), the four stereoisomers of C₈-Cer were synthesized, N-octanoyl-D-erythro-sphingosine (D-e-C₈-Cer), N-octanoyl-D-threo-sphingosine (D-t-C₈-Cer), N-octanoyl-L-erythro-sphingosine (L-e-C₈-Cer) and N-octanoyl-L-threo-sphingosine (L-t-C₈-Cer). In addition, the racemic compound N-octanoyl-DL-erythro-dihydrosphingosine (DL-e-DHC₈-ceramide, which lacks the trans-4,5-double bond) and the novel compound N-octyl-D-erythro-sphingosine (D-e-C₈-Ceramine, in which the carbonyl group was replaced by a methylene group) were synthesized in order to test the role of ceramide (and some of its targets) in the regulation of cell death. The above ceramide stereoisomers and analogs were used to assess: a) the stereospecificity of C₈-Cer stereoisomers in killing of hematopoietic cells, b) the necessity of the trans double bond in the induction of cell death, c) the requirement of the ceramide carbonyl group for apoptosis, d) the involvement of the CAPP and CAPK in the activation of cell death and e) the role of endogenous ceramide in mediating the effects of the C₈-Cer stereoisomers and analogs.

B. Materials and Methods:**a. Tissue culture cells and treatment:**

U937 cells (human histiocytic lymphoma) were purchased from American Type Culture Collection (ATCC). U937 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 U/ml penicillin and 100 µg/ml streptomycin (all tissue culture media reagents were purchased from Biofluids, Inc). The cultures were maintained under a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Before treatment, U937 cells were washed once in 1 X phosphate buffered saline (PBS, 0.85% NaCl, 0.02% KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and then resuspended (5×10^5 cells/ml) in RPMI containing 1 % FCS, antibiotics and glutamine. All of the ceramide analogs and okadaic acid (Calbiochem) were dissolved in ethanol and added to the cells as ethanolic solutions (final concentration of ethanol not exceeding 0.1%). Cells were incubated in the presence of ceramide stereoisomers or other analogs for specified times. At the end of the incubation, cells were pelleted and washed once with PBS (4 °C). Cell viability was determined by trypan blue exclusion (Miyashita and Reed, '93). Well-suspended U937 cells (50 µl) were mixed with 50 µl of 0.4% trypan blue (Sigma, 0.4% trypan blue in 1 X PBS, pH, 7.4) and incubated at room temperature for 5 min. Cells were viewed under a microscope and blue stained cells were considered non-viable.

b. Preparation of C₈-Cer stereoisomers and analogs:

The C₈-Cer stereoisomers and analogs were synthesized in the laboratory of Robert Bittman, Queens College of the City University of New York. The synthesis of the ceramide stereoisomers and analogs is described in detail in Karasavvas et al., '96a,b.

c. DNA isolation:

1. Isolation of low molecular weight DNA.

Treated cells (5×10^6) were lysed in 600 μ l lysis buffer (0.2% Triton X-100, 10 mM Tris-HCl, and 10 mM EDTA, pH 7.5). The cell lysate was incubated on ice for 15 min and then centrifuged at 4 °C at 12,000 x g for 20 min. The supernatant contained the low molecular weight DNA and the pellet contained the high molecular weight DNA. The supernatant, containing the low molecular weight DNA, was incubated for one hr with RNase (Boehringer-Mannheim, 100 μ g/ml) and then extracted twice with 600 μ l of phenol:chloroform:isoamyl alcohol (24:24:1) and once with 600 μ l of chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 300 mM NaCl and 2.5 volumes ethanol at -20 °C overnight. The next day the samples were centrifuged at 16,000 x g for 30 min and the DNA pellet was washed once with 70% ethanol, air dried, and suspended in 20 μ l of T.E buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

2. Isolation of high molecular weight DNA.

The pellet containing the high molecular weight DNA (from above) was resuspended in 500 μ l of sarcosyl buffer (10 mM EDTA, 100 μ g/ml of proteinase K (Boehringer-Mannheim) 0.5% N-lauroyl sarcosine sodium salt (Sigma) and 50 mM Tris) and then incubated at 50 °C for 12-16 hr. The DNA was phenol/chloroform extracted and precipitated with 300 mM NaCl and 2.5 volumes ethanol at 4 °C overnight. The DNA was spooled out into a new tube and stored at 4 °C.

d. Analysis of DNA fragmentation:

The degree of DNA fragmentation was analyzed by agarose gel electrophoresis (Sambrook et al., '89). Agarose was added to Boyer's buffer (50 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA and 18 mM NaCl, pH 8.1) at a final concentration of 2% and then microwaved until agarose was in solution. The agarose mixture was cooled to about 65 °C and then ethidium bromide (Sigma) was added (final concentration of 1 μ g/ml). The agarose was poured into a casting tray and allowed to solidify. Low molecular weight DNA, from equal numbers of cells, was electrophoresed (85 volts for 2 hr) on 2% agarose gels with running buffer (Boyer's buffer). Gels containing the DNA were placed on a UV light box (Foto/Prep I, Fotodyne), and DNA was visualized by ultraviolet radiation. Gels were photographed by a polaroid camera using a polaroid black and white film (665 pos/neg, iso 80/20°). The high molecular

weight DNA (15 μ l/well) from control and treated cells was loaded on a 0.7% agarose gel and electrophoresed as described above.

e. End-labeling of DNA:

For greater sensitivity in analyzing DNA fragmentation, DNA was end-labeled with [α - ^{32} P] ddATP (Amersham) prior to gel electrophoresis (Tilly et al., '91; Zakeri et al., '93). Low molecular weight DNA from 1.8×10^6 cells was incubated in the presence of 5 μ l of [α - ^{32} P] ddATP, 25 U of terminal transferase (Boehringer Mannheim) and 2.5 mM CoCl_2 for 1 hr at 37 $^{\circ}\text{C}$. The reaction was terminated by adding EDTA to a final concentration of 50 mM. The DNA was extracted with phenol:chloroform:isoamyl alcohol (24:24:1) and electrophoresed on a 2% agarose gel. Gels were dried under vacuum for 3 hr at 60 $^{\circ}\text{C}$, and then exposed to X-ray film (Kodak).

f. Densitometry:

Trans-illuminated autoradiograms and photographs of fluorescent gels were scanned with a Logitech scanner set at 200 DPI and 256 gray levels (1 byte/pixel). The resulting image was analyzed by the use of SigmaScan 1.20.09, inverting the image density in the case of fluorescence, and assigning areas by flood fill of contiguous pixels darker than background. Background was subtracted and the standards were evaluated by several techniques (area, $\Sigma(\text{intensity} * \text{pixel})$,

and average intensity of the lane). Under our conditions, the integral intensity of the images was linearly correlated with the amount of applied DNA (intensity = $0.049 \times \text{DNA} + 5.604$, $r^2 = 0.934$). We therefore used this calibration for our evaluations.

g. Staining of dead cells with the fluorochrome bis-benzimide:

The chromatin fragmentation of apoptotic cells was further assessed by staining the ceramide treated cells with the DNA fluorochrome bis-benzimide (Hoechst 33258, Sigma) as previously described (Oberhammer et al., '92). Cells (5×10^6) were exposed to C_8 -Cer stereoisomers or D-e- C_8 -Ceramine for the indicated times and then pelleted and washed once in PBS. Cells were suspended in 200 μl fixative (3% paraformaldehyde (Fisher) in PBS) and then incubated on ice for 5 min. The fixative was removed, and cells were washed once in PBS. Chromatin staining was achieved by incubating the cells for 25 min (at room temperature) in PBS containing 16 $\mu\text{g/ml}$ of bis-benzimide. An aliquot of 10 μl of cells was placed on a slide and the cells were examined using a Leitz fluorescence microscope (DMRB, fluorescent filter system, block A). Stained nuclei with condensed chromatin (supercondensed chromatin at the nuclear periphery), or nuclei that were fragmented into multiple smaller dense bodies were considered as apoptotic. Nuclei with uncondensed and evenly dispersed chromatin were considered as not apoptotic.

h. Ceramide-activated protein kinase (CAPK) assay:

1. Membrane preparations.

Membrane isolation and renaturation of the kinase activity was performed as described previously (Liu et al., '94). U937 cells were washed once in PBS and then incubated for 165 min (5×10^5 cells/ml) in 1% FCS media. At the end of the incubation the cells were pelleted and washed in ice cold PBS. Afterwards, 10^7 cells were homogenized with a Dounce homogenizer in 1 ml of homogenization buffer (25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Sigma), pH 7.4, 5 mM ethylene glycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic Acid (EGTA, Fisher), 50 mM NaF (Fisher), 10 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor and 1 μ g/ml pepstatin A; all protease inhibitors were purchased from Sigma). The homogenate was centrifuged at 1000 x g for 10 min and the supernatant was transferred into a tube and centrifuged again at 12000 x g for 7 min. The membranes were pelleted by ultracentrifugation of the supernatant at 250,000 X g for 30 min (Beckman table top ultracentrifuge model TL-100). The membrane pellets were solubilized in 40 μ l of Laemmli buffer (65 mM Tris pH 6.8, 10% glycerol, 257 mM β -mercaptoethanol (Sigma), 2% sodium dodecyl sulfate (SDS) and 3 μ g/ml bromophenol blue) and boiled for 5 min. Samples were stored (in 15 μ l aliquots) at -80 °C.

2. Protein quantitation of mebrane fractions.

Proteins were quantitated with the dot metric protein assay kit (Midwest Scientific). One μ l of membrane fractions was added to 4 μ l of buffer I, vortexed vigorously and then 1- μ l aliquots were applied to the test strip membranes provided in the kit. The test strips were allowed to air dry completely and then placed in 10 ml of fixer for 2 min. After the 2-min incubation, 800 μ l of a sensitizer solution was added to the fixer and the incubation was continued for an additional 2 min. The strips were then transfered to 10-ml developer solution and developed for 5 min in the dark. In the developer solution the protein spots stain dark purple. The diameter of protein spots was measured by a special ruler provided in the kit. The protein concentration was determined using bovine serum albumin (BSA) standards.

3. CAPK assay.

Equal amounts of protein (50 μ g) were loaded on 7.5% SDS-PAGE gel (polyacrylamide gel electrophoresis, 7.5% acrylamide, 0.2% bisacrylamide, 375 mM Tris, pH 8.8, 0.1% SDS) and electrophoresed in running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS, pH 8.3), at 15 mA for 2 hr. At the end of the electrophoresis the gel was washed (at room temperature) for one hr in wash buffer (50 mM Tris, pH 7.4, 5 mM β -mercaptoethanol) containing 20% 2-propanol, and once in wash buffer without 2-propanol (1 hr). Denaturation of proteins was

accomplished by incubating the gel in two changes of 6 M guanidine hydrochloride (Fisher) in wash buffer (1 hr each). Renaturation was achieved by incubating the gel overnight at 4 °C in wash buffer containing 0.04% Tween-20 (Fisher). The gel was then equilibrated at room temperature (10-15 min) in 10 ml kinase reaction buffer (25 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.5 mM EGTA and 5 mM NaF). After the equilibration, 25 µl of [γ -³²P]-dATP (Amersham, 3000 Ci/mMol) was added to the reaction buffer and then the gel was shaken on a rocking platform for 1 hr at room temperature. The reaction was stopped by washing the gel in water for 10 min. Finally the gel was washed in wash buffer (5% trichloroacetic acid and 1% sodium pyrophosphate, Fisher) for 3-4 hr. For autoradiography the gel was not dried, but it was placed in a cassette with screen and exposed for 24 hr at room temperature.

i. Ceramide labeling and quantitation by diacylglycerol (DG) kinase:

The endogenous level of ceramide was determined by the *E. coli* DG kinase assay (Preiss et al., '86). First, the cellular lipids were extracted and labeled by DG kinase in the presence of [γ -³²P]-dATP. The concentration of the radioactively labeled ceramide was compared to known ceramide standard concentrations (ceramide type III from bovine brain, Sigma).

Cells were washed in PBS and plated at a concentration of 5×10^5 cells/ml in media containing 1% FCS. After treatment

with the various compounds, cells were washed once in PBS and then transferred into 12 x 75 mm tissue culture glass tubes (3×10^6 cells/tube). The cells were lysed in 1 ml of kill solution (chloroform/methanol/1 N HCl (100/100/1, v/v/v), and then 270 μ l of buffered saline solution (BSS, 135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl_2 , 0.5 mM MgCl_2 , 5.6 mM glucose and 10 mM Hepes, pH 7.2), and 30 μ l of 100 mM EDTA were added to separate the organic phase. A 500 μ l aliquot of the lower organic phase was transferred into a 12 x 75 mm glass tube and then dried under a stream of N_2 until the last droplets of liquid evaporated.

Extracted total lipids were subjected to mild alkaline hydrolysis (0.1 M methanolic potassium hydroxide for 1 hr at 37 °C) to destroy glycerophospholipids. The organic phase was separated with chloroform (500 μ l), BSS (270 μ l) and EDTA (30 μ l), and 500 μ l were transferred and dried under N_2 . The lipids were solubilized by bath sonication (Branson B3 sonicator), for 2 min into 20 μ l of aqueous 7.5% *n*-octyl- β -D-glucopyranoside (Calbiochem), 5 mM cardiolipin (Avanti Polar-Lipids, from heart, disodium salt) and 1 mM diethylenetriaminepentaacetic acid (Sigma). To the solubilized lipids were added 50 μ l of 2 X reaction buffer (100 mM imidazole (Fisher), 100 mM NaCl, 2 mM EDTA, 25 mM MgCl_2 , pH 6.5), 2 μ l of 100 mM dithiothreitol (Fisher), 3.5 μ l of DG kinase (Calbiochem, 1 mg/ml), 1 μ l of [γ - ^{32}P] dATP (Amersham, 3000 Ci/mmol) and water to a total volume of 100 μ l. The

reaction was allowed to proceed at room temperature for 30 min, and then stopped by the addition of 1 ml kill solution. The organic phase was separated by the addition of 170 μ l of BSS, 30 μ l of 100 mM EDTA and then vortexing for few seconds. The lower organic phase (500 μ l) was dried under N_2 . Labeled ceramide was resuspended in 50 μ l of chloroform/methanol (1/1, v/v) and 40 μ l were resolved by thin-layer chromatography (TLC) The TLC plate (Whatman, 6 nanomolar particle size silica gel plates of 0.25 mm thickness, LK6D) was developed in chloroform/methanol/glacial acetic acid (65/15/5, v/v/v) until the solvent front was about 2.5 cm from the top. The TLC plates were subjected to autoradiography for 6-24 hr, depending on the intensity of the labeled ceramide bands, and the silica from the TLC plates containing the ceramide was scraped and quantitated by liquid-scintillation counting. Ceramide type III was labelled the same way as lipids extracted from cells and used as standard.

C. Results:

a. Kinetics of cell death induced by D-e-C₈-Cer.

We initially tested if the natural stereoisomer of ceramide, D-e-C₈-Cer, induced cell death in U937 cells. We examined the kinetics of induction of cell death by D-e-C₈-Cer (Fig. 1) by monitoring both cell viability and the appearance of nucleosomal DNA fragments. We did not detect any differences in the high molecular weight DNA between treated and untreated cells. In all experiments low molecular weight DNA, recovered from equal number of cells, was loaded in agarose gels. Since gels were loaded with low molecular DNA extracted from equal numbers of cells, increase in the DNA fragmentation reflected an increase in the number of cells undergoing apoptosis.

U937 cells (5×10^5 /ml) were treated with 10 μ M D-e-C₈-Cer or ethanol vehicle for various times. DNA fragmentation was detected after 12 and 18 hr of incubation (Fig. 2A,B). Since by agarose gel electrophoresis and ethidium bromide staining we did not detect DNA fragmentation at earlier times, we employed the more sensitive DNA detection method of end-labeling. By this method we detected very low levels of DNA fragmentation as early as 3 h (Fig. 2C). When U937 cells were incubated with 10 μ M D-e-C₈-Cer, DNA fragmentation increased with time. Densitometric analysis showed a 12-fold elevation in the nucleosomal fragmentation in cells treated for 18 hr with D-e-C₈-Cer compared with the cells that were treated with

D-e-C₈-Cer for 3 hr (Fig 2C). However, cell viability, as determined by trypan blue exclusion, did not decrease significantly for at least 18 h in the D-e-C₈-Cer treated cells (Fig. 3A).

b. Effect of DL-e-DHC₈-Cer on DNA fragmentation.

Although some studies noted the absence of biological activity in the ceramide analog DL-e-DHC₂-Cer which lacks the trans-4,5-double band (Bielska et al., '93), other researchers found that dihydroceramides with longer amide chains (DHC₆-Cer) induced DNA fragmentation in HL-60 cells (Jarvis et al., '94). We therefore examined if the trans-4,5-double bond of DL-e-DHC₈-Cer (a ceramide analog of longer amide chain) is required for the induction of apoptosis. We examined the extent of DNA fragmentation in U937 cells treated for 18 hr with DL-e-DHC₈-Cer. The low molecular weight DNA was end-labeled. As indicated in Fig. 2C, there was an increase in DNA fragmentation in cells treated with 10 μ M of DL-e-DHC₈-Cer, relative to the ethanol control, but the degree of fragmentation was less than that induced by D-e-C₈-Cer. DNA fragmentation induced by DL-e-DHC₈-Cer is also detected by conventional agarose gel electrophoresis at 18 hr of incubation (Fig. 4A). DNA fragmentation induced by D-e-C₈-Cer is detected at earlier times (first detected at the 12 hr incubation by agarose gel electrophoresis, Fig. 2).

c. Cell growth and DNA fragmentation induced by C₈-Cer stereoisomers and D-e-C₈-Ceramine.

Cells were treated with the C₈-Cer stereoisomers and with D-e-C₈-Ceramine for 3 to 18 h. The viability of cells treated with the different compounds was time-dependent. Cells treated with D-e-C₈- and DL-e-DHC₈-Cer retained viability above 90% up to 18 h of incubation (Fig 3A). The rest of the C₈-Cer analogs caused varying and time dependent decreases in cell viability. The most severe reduction was detected in cells treated with D-e-C₈-Ceramine, where by 6 hr 50% of the cells were dead (Fig. 3A).

Low molecular weight DNA was isolated from equal numbers of cells and analyzed by ethidium bromide staining. After 3 hr of treatment there are no detectable differences in DNA fragmentation in any of the compounds (Fig. 4A). A 6 hr treatment with D-e-C₈-Ceramine, but not with any of the C₈-Cer stereoisomers, produced a substantial DNA fragmentation (Fig. 4A). When the time of incubation was subdivided (Fig. 4B), it was clear that DNA fragmentation peaked for D-e-C₈-Ceramine at 6 hr. By 18 h the apparent DNA fragmentation declined due to the elimination of the majority of cells by apoptosis. The threo stereoisomers (D-t-C₈- and L-t-C₈-Cer), demonstrated stereospecific induction of cell death (Fig. 4A). By 18 hr the DNA fragmentation induced by the D-t- and L-t-C₈-Cer stereoisomers was 5 to 6 fold higher than that produced by D-e-C₈-Cer (Fig. 3B).

d. Nuclear fragmentation of U937 cells treated with C₈-Cer stereoisomers.

Since agarose gel electrophoresis did not provide any information on the morphological changes of cells treated with the different ceramide stereoisomers, we used the DNA-specific fluorochrome bis-benzimide (Hoechst 33258) to stain nuclei and detect morphological changes that are characteristic of apoptotic cells. Such changes include compaction and margination of chromatin at the periphery of the nucleus and fragmentation of nuclei into multiple dense apoptotic bodies. As indicated in Fig. 5, nuclei from untreated U937 cells show diffuse staining of round nuclei with no chromatin condensation. Treatment with D-e-C₈-Cer or D-t-C₈-Cer (Fig. 5) resulted in condensed and fragmented nuclei (arrowheads) in proportion to the potency of the compounds to kill cells and induce DNA fragmentation as indicated by electrophoresis (Fig. 4).

e. Measurement of the endogenous levels of ceramide in treated cells.

To explore possible mechanisms by which the D- and L-threo stereoisomers of C₈-Cer and D-e-C₈-Ceramide kill cells, we tested the ability of these potent compounds to elevate the endogenous ceramide levels in U937 cells. Since *E. coli* DG kinase phosphorylates ceramide, the assay has been used extensively to estimate the content of ceramide in lipid

extracts (Preiss et al., '86; Younes et al., '92; Mathias et al., '93; Van Veldhoven et al., '95). We first determined which of the synthetic C₈-Cer analogs were substrates for the *E. coli* DG kinase. D-e-C₈-Ceramine was not a substrate for the DG kinase, and L-t-C₈-Cer and L-e-C₈-Cer were poorly phosphorylated (Fig. 6A). D-e-C₈-Cer as well as D-t-C₈-Cer and DL-e-DHC₈-Cer were good substrates.

To test if endogenous ceramide elevations contribute to the killing of cells treated with the synthetic ceramide analogs, we treated U937 cells with the nonsubstrate D-e-C₈-Ceramine and the poor substrate L-t-C₈-Cer for 3 h prior to the extraction of the lipids. The cellular lipids were extracted and labelled with DG kinase to estimate the ceramide content. Any elevation of ceramide in the extracted lipids would reflect the release of cellular ceramide due to the hydrolysis of sphingomyelin and glycosphingolipids. As seen in Fig. 6B, D-e-C₈-Ceramine fails completely to increase the endogenous levels of ceramide. L-t-C₈-Cer is a weak substrate for DG kinase, and a faint band is seen on the TLC plate. These results indicate that the powerful apoptotic properties of D-e-C₈-Ceramine and L-t-C₈-Cer are not attributable to the generation of endogenous cellular ceramide. In contrast, lipids extracted from cells treated with either D-e-C₈-Cer or D-t-C₈-Cer were heavily labeled (Fig. 6B). However, since D-e-C₈-Cer, D-t-C₈-Cer and cellular long-chain D-e-Ceramide are all phosphorylated by *E. coli* DG kinase, we cannot use this assay

for the measurement of the endogenous ceramide release by these compounds.

f. Effects of C₈-Cer stereoisomers and D-e-C₈-Ceramine on the activation of CAPK.

CAPK is a 97-kDa protein (Liu et al., '94) that is activated in A-431 and HL-60 cells by TNF and D-e-C₈-Cer (Mathias et al., '91; Liu et al., '94). Since CAPK may mediate the apoptotic signals of ceramide to other intracellular targets, we tested the ability of the different C₈-Cer stereoisomers and D-e-C₈-Ceramine to activate CAPK. U937 cells were treated for 30 min (Liu et al., '94) with 10 μ M of each of the following sphingolipids: D-e-C₈-Cer, D-t-C₈-Cer and D-e-C₈-Ceramine. Membrane proteins from treated cells were isolated and subjected to SDS-PAGE gel electrophoresis. The kinase activity was renatured and autophosphorylation was performed as described in Materials and Methods (Liu et al., '94).

We found that in the treated cells two protein bands (97 and a 110 kDa) were activated above the control (Fig. 7). In cells treated with 10 μ M D-e-C₈-Cer or D-t-C₈-Cer, the 97 kDa protein is approximately 2-fold higher than in the control, similar to earlier findings (Mathias et al., '91). In addition, we found that a 110 kDa protein, not reported previously, was differentially activated by D-e-C₈-Cer and D-t-C₈-Cer approximately 4-fold above the control. Therefore, D-

e- and D-t-C₈-Cer activated both the 97 and 110 kDa proteins above the control level, but there was no differential activation of either of the two proteins by D-e-C₈- vs. D-t-C₈-Cer. Activation of the 97 and 110 kDa proteins are also seen in cells treated with D-e-C₈-Ceramine. In conclusion, the C₈-Cer analogs activated CAPK, but in spite of the marked differences in the activity of these compounds to induce apoptosis, there was no differential activation of either the 97 or the 110 kDa protein in cells treated with any of the tested C₈-Cer analogs.

g. Okadaic acid fails to prevent cell death activated by ceramide treatment.

Another potential target of D-e-C₈-Cer is CAPP (Wolff et al., '94). CAPP, member of a family of serine/threonine protein phosphatases (PP2A), is potently inhibited by okadaic acid, a potent PP2A phosphatase inhibitor. Previous studies by Wolff et al., '94 showed that 10 nM of okadaic acid completely inhibit the activity of CAPP. The involvement of CAPP in the apoptotic pathway of ceramide is currently unknown, but if CAPP activation is associated with the propagation of the cell death signaling by ceramide, its inactivation by phosphatase inhibitors should prevent ceramide cell killing. Preliminary studies with U937 and the TNF- α resistant cell line U9-TR (developed from U937 cells) showed that inactivation of PP2A phosphatases with okadaic acid (and consequently CAPP)

augmented the DNA fragmentation in U937 and U9-TR cells treated with TNF- α (Wright et al., '93). If PP2A phosphatases were involved in the transduction of the apoptotic signalling of TNF- α , a cytokine speculated to mediate its cytotoxicity via the generation of ceramide, their inactivation with okadaic acid should have prevented, not increased, cell death.

To test if inactivation of PP2A phosphatases (including CAPP) protects U937 cells from apoptosis induced by D-e-C₈-Cer, we treated U937 cells with 10 μ M D-e-C₈-Cer and different concentrations of okadaic acid (0.1, 0.5, 1, 5 and 10 nM) for 18 hr. We did not detect any DNA fragmentation or cytotoxic effects in U937 cells exposed to okadaic acid alone (0.1-10 nM). Cells incubated in the presence of both okadaic acid (0.1-10 nM) and 10 μ M D-e-C₈-Cer showed DNA fragmentation (Fig 8). Since inhibition of CAPP by okadaic acid did not protect U937 cells from apoptosis triggered by D-e-C₈-Cer, we propose that the PP2A phosphatases may not be associated with the transmission of the cell death signalling by ceramide.

D. Discussion:

C₂-Cer and DHC₂-Cer stereoisomers were used to resolve the stereospecificity of ceramide. The four stereoisomers of C₂-Cer displayed only modest differences in inhibiting cell growth, inducing apoptosis, down-regulating c-myc and activating CAPP (Bielawska et al., '93; Wolff et al., '94). C₂-Cer (and its stereoisomers) have been used extensively despite the fact that they are poorly metabolized by cells in culture (Bielawska et al., '93; Ridgway et al., '95; Hannun et al., '93).

To analyze the biological properties of ceramide, we synthesized metabolizable C₈-Cer analogs with moderate size amide chain and tested their ability to induce apoptosis in U937 cells. We found that the *threo* stereoisomers dramatically and selectively increase internucleosomal DNA fragmentation in U937 cells. Thus the C₈-Cer stereoisomers showed stereospecificity with the *threo* stereoisomers being several-fold more potent than the *erythro* in inducing apoptosis. The order of potency is as follows: D-*t*-C₈-Cer = L-*t*-C₈-Cer > L-*e*-C₈-Cer > D-*e*-C₈-Cer > DL-*e*-DHC₈-Cer. A conclusion drawn from our studies is that the relationship of stereochemistry to biological activity is different for the metabolizable ceramide analogs such as D-*e*-C₈-Cer and its stereoisomers than for C₂-Cer.

The importance of the 4,5-*trans*-carbon-carbon double bond in the cytotoxicity of short-chain ceramides is at present

controversial. D-e-DHC₆-Cer induced DNA fragmentation in HL-60 cells (Jarvis et al., '94). In other studies D-e-DHC₂-Cer was inactive whereas D-t-DHC₂-Cer was active (Bielawska et al., '93). In the present study, DL-e-DHC₈-Cer induced DNA fragmentation in U937 cells as assessed by the more sensitive technique of end-labeling. The absence of the double bond significantly reduced the toxicity of DL-e-DHC₈-Cer as compared to D-e-C₈-Cer, the natural occurring stereoisomer. The low toxicity of DL-e-DHC₈-Cer may reflect the latent introduction of the trans-4,5-double bond to form DL-e-C₈-Cer once the dihydroceramide is internalized by cells (Ridgway et al., '95).

D-e-C₈-Ceramine proved to be an important tool in deciphering the importance of the amide carbonyl group of ceramide in triggering apoptosis. At micromolar concentrations, this novel compound induced extensive cell death in U937 cells, as assessed by DNA fragmentation analysis and trypan blue exclusion assays (Fig. 3,4). Cells treated with D-e-C₈-Ceramine exhibited a decrease in viability as early as 3 hr, and by 6 hr the nucleosomal fragmentation was at its peak (Figs. 3,4). The difference between D-e-C₈-Cer and D-e-C₈-Ceramine is the substitution of the amide linkage by an amine linkage suggesting that the carbonyl group of ceramide is not required in triggering the apoptotic pathway; on the contrary, the presence of the carbonyl group extended the incubation period required to kill cells. The above studies do

not exclude the possibility that the difference in potency between D-e-C₈-Ceramine and the other compounds is due to the differential uptake and intracellular compartmentalization of ceramine.

The death signaling pathways and ceramide targets involved in apoptosis are presently unknown. To explain the high potency of the *threo* stereoisomers and D-e-C₈-Ceramine to kill cells, we tested the possibility that the highly potent *threo* stereoisomers and D-e-C₈-Ceramine exert their apoptotic activity by inducing sphingomyelin hydrolysis or glycolysis of glycosphingolipids. In this instance the potency of these compounds would depend on their ability to generate and increase the intracellular pools of ceramide. The failure of D-e-C₈-Ceramine and L-t-C₈-Cer to increase the intracellular levels of ceramide suggested that sphingomyelin and glycosphingolipid hydrolysis did not contribute to the potency of these compounds in cell killing.

Although D-e-C₈-Cer, D-t-C₈-Cer and D-e-C₈-Ceramine differed markedly in killing potencies, they equally activated CAPK, another known ceramide target. These compounds increased the autophosphorylation activity of a 97 and a 110 kDa enzymes several fold above the control cells, but there was no difference among them in activating these enzymes (Fig. 7). The increase of CAPK phosphorylation in the presence of D-e-C₈-Ceramine suggests that the ceramide carbonyl group is not essential for either the activation of apoptosis or the

activation of CAPK. Thus the stereospecificity or potency observed in U937 cell killing cannot be explained by a differentially enhanced CAPK autophosphorylation activity.

The role of CAPP in apoptosis has not been tested previously. CAPP is activated by ceramide and its involvement in the apoptotic pathway was therefore possible. If CAPP activation were essential in the mediation of cell death induced by ceramide, its inhibition by okadaic acid should have abolished apoptosis. Here we show that inactivation of CAPP by okadaic acid does not inhibit the activation of the apoptotic pathway by D-e-C₈-Cer (Fig. 8). These results suggest that activation of CAPP does not lead to cell death. The fact that the D-e-C₈-Cer targets CAPK and CAPP do not seem to be involved in cell killing suggests that ceramide may mediate other effects and signals other than to provoke cell killing.

The involvement of other known ceramide targets in the apoptotic signalling of ceramide was not examined. Alternative ceramide targets include an atypical protein kinase C isoform (PKC ζ) and the guanine nucleotide exchange factor Vav (Muller et al., '95; Gulbins et al., '94). The differential activation of these proteins by the different C₈-Cer stereoisomers and D-e-C₈-Ceramide has not yet been tested. Additionally, unidentified ceramide targets may play a significant role in the activation and execution of ceramide cell killing.

CHAPTER 2

Bcl-2 Protects Cells From Apoptosis Induced by the D-e-C₈-Ceramine but not from more Potent Ceramide Analogs

- A. Objective
- B. Materials and Methods
- C. Results
 - a. Can bcl-2 overexpression protect cell death induced by D-e-C₈-ceramide?
 - b. Can bcl-2 overexpression protect cells from the more potent stereoisomers of C₈-Cer?
 - c. Does the conversion of the ceramide trans-4,5-double bond into cis or triple bond change the ability of ceramide to kill cells?
- D. Discussion: Bcl-2 protects cells from apoptosis induced by the natural ceramide stereoisomer only.

A. Objective:

Bcl-2 oncogene was originally identified as a chromosomal translocation $t(14;18)(q32;q21)$ associated with non-Hodgkin's lymphomas (Tsujimoto et al., '85; Tsujimoto and Croce, '86). Bcl-2 is a negative regulator of cell death, for instance, it protects interleukin 3 (IL-3)-dependent cell lines from cell death induced upon growth factor deprivation (Vaux et al., '88) and extends the longevity of lymphoid cell populations (Cory, '95). Overexpression of the bcl-2 gene is associated with cell proliferation (Krajewski et al., '94), cell survival (Vaux et al., '88), oncogenesis (Lu et al., '96), and inhibition of apoptotic signals (Hockenbery et al., '90; Sentman et al., '91). In addition, the tumor suppressor gene p53 was found to eliminate malignant cells by down-regulating the expression level of bcl-2 (Chiou et al., '94) whereas the resistance of cells to chemotherapeutic agents (such as methotrexate) involves the enforced expression of bcl-2 (Miyashita and Reed, '93). Although in general the expression of bcl-2 is coupled to cell survival, apoptotic pathways that may not be blocked by bcl-2 (such as Fas) have been reported (Itoh et al., '93; Chiu et al., '95).

The importance of bcl-2 in mammalian cell survival was demonstrated by the development of homozygous bcl-2-null mice. These animals developed abnormalities that included polycystic kidneys, atrophy of the thymus and spleen, and accelerated loss of epithelial cells (Kamata et al., '95).

Presently the mechanism by which the bcl-2 family perturbs cell death is unknown, but it is thought to occur via complex interactions among the members of the bcl-2 family (Yang et al., '95). Bcl-2 forms heterodimers with Bax, another member of the bcl-2 family and these protein-protein interactions inhibit the activity of bcl-2 (Oltvai et al., '93). Overproduction of the Bax protein induces cell death due to the inactivation of the bcl-2 protein (Sato et al., '94).

Since bcl-2 has been shown to be critical in the inhibition of apoptosis under many conditions, we investigated if bcl-2 could protect cells from ceramide-induced cell death. Ceramide is considered to be a mediator of cell death in radiation treatment (Haimovitz-Friedman et al., '94) which is blocked by bcl-2 (Sentman et al., '91). Also, Chen et al. '95 showed that the mRNA levels of bcl-2 were down-regulated in radiation- and C₂-Cer-treated cells. These studies suggested that the pathways of ceramide cell killing and bcl-2 protection converge. Since there are considerable differences in the stereospecificity of C₂-Cer and C₈-Cer in cell killing, we tested whether cells overexpressing the bcl-2 oncogene (FL5.12-bcl-2 cells) could be killed by the C₈-Cer stereoisomers. We also investigated the requirement for the ceramide double bond in cell killing by using two new C₈-Cer analogs, D-e-C₈-*cis*-Δ⁴-Cer and D-e-C₈-4-yne-Cer. In D-e-C₈-*cis*-Δ⁴-Cer the *trans*-4,5-double bond was converted into a *cis*-4,5-double bond, and in D-e-C₈-4-yne-Cer the *trans*-4,5-double bond

was converted into a triple bond.

B. Materials and Methods:

The synthesis of the ceramide stereoisomers, analogs, DNA isolation and agarose gel electrophoresis is described in Chapter I.

a. Cell culture and treatment:

All tissue culture media and reagents used are the same as in Chapter I. FL5.12, FL5.12-bcl-2 and WEHI-3B cells were a gift from Dr. Hockenbery (Hockenbery et al., '90). FL5.12 (parent) cells are a murine IL-3-dependent pro-B lymphoid cell line. FL5.12-bcl-2 cells were developed by stably transfecting FL5.12 cells with the human bcl-2 gene. WEHI-3B is an IL-3 producing cell line; the supernatant from these cells was used to supply IL-3 to FL5.12 and FL5.12-bcl-2 cells. WEHI-3B cells were grown in Iscoves modified Dulbecco media (IMDM, Biofluids) supplemented with 5% FCS, antibiotics, 5×10^{-5} M β -mercaptoethanol (Sigma) and L-glutamine. The supernatant from confluent WEHI-3B cells was filtered through a 0.22 μ m filter, and then used as a source of IL-3. FL5.12 and FL5.12-bcl-2 cells were maintained in IMDM supplemented with 10% FCS, 10% conditioned media from WEHI-3B cells, 2 mM L-glutamine, 50 U/ml penicillin and 100 μ g/ml streptomycin. To maintain the vector containing the bcl-2 gene, the FL5.12-bcl-2 cell growth media was supplemented with 0.5 μ g/ml G418 (geneticin, GibcoBRL). The cell cultures were maintained under a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Before

treatment, FL5.12 and FL5.12-bcl2 cells were washed once with 1 X PBS, pH 7.4 and resuspended (5×10^5 cells/ml) in media containing 1% FCS, 0.5% conditioned media from WEHI-3B cells, antibiotics and glutamine. Ceramide analogs were dissolved in ethanol and added to the cell culture media as ethanolic solutions; the final concentration of ethanol did not exceed 0.1%. Cells were incubated in the presence of ceramide stereoisomers or other analogs for specified times. Cell viability was determined by trypan blue exclusion (Miyashita and Reed, '93).

C. Results:**a. Can bcl-2 overexpression protect cell death induced by D-e-C₈-ceramide?**

We first investigated the ability of bcl-2 to protect cells from apoptosis induced by D-e-C₈-Cer, the naturally occurring stereoisomer. Cell death was monitored by the appearance of DNA fragmentation ladders on 2% agarose gels. At the end of the incubation with the different ceramide compounds, the DNA from 5×10^6 cells was isolated and loaded on ethidium bromide stained agarose gels. Increases in the intensity of the fluorescing DNA fragments indicated an increase in the number of cells undergoing apoptosis.

FL5.12 and FL5.12-bcl-2 cells, 5×10^5 cells/ml were treated with 10 μ M D-e-C₈-Cer or ethanol vehicle for 3, 6, and 12 hr. FL5.12 cells exposed to D-e-C₈-Cer for 12 hr showed an increase in the level of DNA fragments (Fig. 9, last lane), but we failed to detect DNA fragmentation in FL5.12-bcl-2 cells treated with D-e-C₈-Cer for the same time (Fig. 9). There was no cell death in either cell lines treated with ethanol (ethanol control vehicle). These results showed that overexpression of the bcl-2 gene protects FL.12 cells from apoptosis promoted by the naturally occurring ceramide stereoisomer, D-e-C₈-Cer.

b. Can bcl-2 overexpression protect cells from the more potent stereoisomers of C₈-Cer?

As we indicated in Chapter I, the C₈-Cer stereoisomers showed differential potencies in cell killing with the *threo* stereoisomers being several-fold more potent in cell killing. To examine if bcl-2 overexpression could protect cells from the more potent C₈-Cer stereoisomers and analogs, we treated FL5.12 and FL5.12-bcl-2 cells with 10 μ M D-e-C₈-Cer, D-t-C₈-Cer and L-t-C₈-Cer for 18 hr. The degree of apoptosis was assessed by agarose gel electrophoresis as in Chapter I. As in the case of U937 (Chapter I), the synthetic ceramide stereoisomers killed FL5.12 cells to various degrees with the D-e-C₈-Cer being the least potent (Fig. 10A). In contrast, FL5.12-bcl-2 cells exposed to the same C₈-Cer stereoisomers showed less death and a different hierarchy of sensitivity, with D-t-C₈-Cer being the most potent of the four ceramide stereoisomers (Fig. 10B). The above studies indicate that overexpression of bcl-2 protects cells efficiently from killing by the natural D-e-C₈-Cer stereoisomer, but less so from L-e-, D-t- and L-t-C₈-Cer. Additionally, L-e-C₈-Cer and D-t-C₈-Cer show equal potency in the generation of DNA fragments in FL5.12 cells (Fig. 10A) which is not true in the U937 cell line (L-e-C₈-Cer is less potent in cell killing than D-t-C₈-Cer (Chapter I, Fig. 4A).

c. Does the conversion of the ceramide *trans*-4,5-double bond into *cis* or triple bond change the ability of ceramide to kill cells?

Although the elimination of the ceramide *trans*-4,5-double bond reduced substantially the toxicity of ceramide (Chapter I, Fig. 2C), it was unknown if the conversion of the *trans* double bond into the *cis* or triple bond configuration had any effect on the biological activity of ceramide. Modification of the *trans*-4,5-double bond into *cis* or triple bond converted D-e-C₈-*cis*-Δ⁴-Cer and D-e-C₈-4-yne-Cer into potent cell killing compounds (Fig. 10A,B). Both FL5.12 and FL5.12-bcl-2 cells were susceptible to killing by D-e-C₈-*cis*-Δ⁴-Cer and D-e-C₈-4-yne-Cer, but the DNA fragmentation was more pronounced in the parent cell line (Fig. 10A,B). Thus bcl-2 overexpression provided some protection for FL5.12-bcl-2 cells, but the decrease in DNA fragmentation was not as significant as that for the other stereoisomers D-e-C₈-Cer and L-e-C₈-Cer (Fig. 10A,B). Another possible conclusion is that the several stereoisomers kill cells through different pathways.

D. Discussion:

In the present study we attempt to establish a relationship between ceramide cell killing and the protective role of bcl-2. We found that bcl-2 overexpression protects FL5.12 cells from apoptosis induced by the natural stereoisomer D-e-C₈-Cer (Figs. 9 and 10A,B). Bcl-2 was unable to block completely cell death induced by the more potent stereoisomers (D-t-C₈-Cer, L-t-C₈-Cer and L-e-C₈-Cer). We therefore speculated that the pathways of D-e-C₈-Cer-induced cell death signaling and bcl-2 protection converge.

Chen et al. '95 showed that C₂-Cer kills cells and decreases the expression of the bcl-2 mRNA, suggesting that the bcl-2 down-regulation is related to the activation of cell death. Our data support this interpretation. It is formally possible that the more potent stereoisomers (*threo*) activate apoptosis in FL5.12-bcl-2 by suppressing the expression of bcl-2 to greater extent than does D-e-C₈-Cer. It is more likely though that the more potent of the stereoisomers kill cells through different pathways.

Another interesting aspect of these studies is the time required by both U937 and FL5.12 cells to undergo apoptosis in the presence of D-e-C₈-Cer. As indicated in U937 (Fig. 2A, lane 3) and in FL5.12 (Fig. 9, last lane) cells treated with 10 μ M of D-e-C₈-Cer required 12 hr for the appearance of DNA fragmentation ladders by agarose gel electrophoresis. Thus, the kinetics of D-e-C₈-Cer are similar in both U937 and FL5.12

cells.

As indicated in Chapter I, the double bond was critical for the activation of cell death by ceramide. To resolve the importance of the ceramide *trans*-4,5-double bond in the activation of the apoptotic pathway, we synthesized the two novel compounds D-e-C₈-*cis*- Δ^4 -Cer and D-e-C₈-4-yne-Cer. The reduction of the double bond in DL-e-DH-C₈-Cer decreased the potency of the compound against U937 cells (Fig. 4A) and eliminated its toxicity against FL5.12 cells. Thus, the absence of the double bond is associated with the reduction or complete inactivation of the ability of ceramide to kill cells.

The conversion of the ceramide double bond into the *cis* configuration or to a triple bond affected the activation of cell death. Both FL5.12 and FL5.12-bcl-2 cells were susceptible to killing by D-e-C₈-*cis*- Δ^4 -Cer and D-e-C₈-4-yne-Cer (Fig. 10A,B). The intensity of the DNA fragmentation ladders in the FL5.12 cells was higher than that detected in the bcl-2 overexpressing cell line FL5.12-bcl-2 (Fig. 10A,B). Hence, overexpression of bcl-2 was not sufficient to protect completely from the toxicity of D-e-C₈-*cis*- Δ^4 -Cer and D-e-C₈-4-yne-Cer. We conclude that the mechanisms of killing by the ceramide stereoisomers and analogs differ at least in part, and that bcl-2 can protect against killing by the natural stereoisomer D-e-C₈-Cer but less well against the other compounds.

CHAPTER 3

Relationships of Apoptotic Signaling Mediated by Ceramide and TNF- α

- A. Objective
- B. Materials and methods
- C. Results
 - a. Kinetics of TNF- α and D-e-C₈-Cer induced cell killing.
 - b. Reversibility of TNF- α and ceramide induced cell death in U937 cells.
 - c. Exogenous ceramide does not change the time required by TNF- α to induce apoptosis in U937 cells.
 - d. Low pH washes and trypsinization do not inhibit cell death promoted by exposure to TNF- α .
- D. Discussion: Evidence that ceramide and TNF- α kill cells via different pathways.

A. Objective:

Several laboratories suggest that ceramide is the second messenger that transduces the apoptotic signaling under conditions that lead to cell death (Obeid et al., '93; Cifone et al., '93; Wiegmann et al., '94; Gill et al., '94; Jarvis et al., '94; Gulbins et al., '95; Verheij et al., '96). Although ceramide elevations have been associated with cell death signaling, it is still possible that the rise in ceramide is a consequence rather than a cause of apoptosis (Beyaert and Fiers, '94).

One of the cytokines that may kill cells via the generation of ceramide is TNF- α . The assumption that TNF- α cytotoxicity is mediated by ceramide is based on the following observations: a) treatment of cells with TNF- α generates ceramide (Obeid et al., '93; Jarvis et al., '94) b) exogenously added ceramides, which could bypass the TNF- α -TNF receptor complex signaling, kill cells (Obeid et al., '93; Jarvis et al., '94) and c) exogenous ceramide and TNF- α have common intracellular targets such as the stress-activated protein kinase, SAPK (Verheij et al., '96). Thus, cell death inflicted by TNF- α and exogenous ceramide is believed to be propagated via the same death pathway.

Since ceramide generated by TNF- α is considered the second messenger that kills cells, we examined the role of ceramide in the apoptotic pathway initiated by TNF- α . To do so we: a) compared the kinetics by which TNF- α and ceramide kill

cells, b) established the exposure required by ceramide and TNF- α to activate the apoptotic pathway, c) determined the incubation period needed by TNF- α to increase the endogenous levels of ceramide and measured how ceramide concentrations change over time in the presence of TNF- α , d) measured the internalization rate and the concentration of endogenous ceramide in cells treated with D-e-C₈-Cer and compared them to (c) above and e) determined whether ceramide generation precedes the activation of cell death in cells treated with TNF- α .

We found considerable differences in the way TNF- α and ceramide kill cells. Cells treated with TNF- α are committed early and irreversibly to the apoptotic pathway and the ceramide level in TNF- α -treated cells increases well after the commitment to cell death. On the other hand, exogenous ceramide increases the intracellular level of ceramide rapidly and significantly within minutes, but cellular commitment to cell death does not occur during the first 6 hr of incubation. We therefore suggest that a) ceramide may not be a second messenger in cell killing by TNF- α , b) ceramide elevations may be a consequence rather than a cause of apoptosis and c) exogenous ceramide and TNF- α may kill cells via alternative pathways.

B. Materials and Methods:

Protein quantitation, DNA extraction, agarose gel electrophoresis and ceramide labeling were performed as described in Chapter I and II.

a. Cell treatment and reversibility studies:

U937 cells were treated with 10 μ M ceramide (Biomol) or TNF- α (R&D systems) for different periods. At the end of the incubation period, the cells were lysed and lipids or DNA were extracted and processed as discussed previously (Chapter I).

For the ceramide reversibility experiments, U937 cells were plated in 1% FCS media and treated with 10 μ M D-e-C₈-Cer for 3 hr. At the end of the incubation, the cells were transferred into fresh media containing 10% or 1% FCS with or without 10 μ M D-e-C₈-Cer. The cells were incubated for an additional 15 hr (total incubation 18 hr) and then lysed in cell lysis buffer (Chapter I). To assess cell death and DNA fragmentation, the low molecular weight DNA was extracted and electrophoresed as described in Chapter I. U937 cells were treated with 10 ng/ml TNF- α for various times, washed in 1 X PBS and returned to TNF- α - free media (RPMI) supplemented with 10 or 1% FCS. The incubation was continued for a total of 2 hr and the low molecular weight DNA was extracted and electrophoresed as above to assess DNA fragmentation.

b. Determination of the fate of internalized D-e-C₈-Cer:

To determine the fate of ceramide internalized by U937 cells, two separate flasks of cells (5×10^6 cells/flask) were exposed to $10 \mu\text{M}$ D-e-C₈-Cer. The cells were incubated in the presence of ceramide for different times. At the end of the incubation period the cells of one of the two flasks were lysed, and the lipids were collected for the assessment of the endogenous levels of ceramide. The cells of the second flask were centrifuged, and returned into ceramide-free 1% FCS media and allowed to incubate for a total of up to 18 hr. At the end of this incubation the cells were washed in 1 X PBS and lysed. The endogenous level of ceramide was ascertained as described in Chapter I.

c. Acid wash and trypsinization of TNF- α treated cells:

To interrupt the propagation of the signals transduced by the TNF receptors we washed U937 cells in low pH 1 X PBS or proteolytically cleaved the TNF receptor with trypsin. Low pH (pH 3.0) washes, which disrupt receptor-ligand associations and stop the receptor signaling, were done as described previously (Okuda et al., '91). U937 cells (5×10^6) were exposed to 0.57 pM TNF- α for 2.5 min, washed with ice cold 1 X PBS and then the cell pellet was resuspended in 2 ml of ice cold 1 X PBS, pH 3 for 2 min on ice. The cells were washed twice in 1 X PBS and then returned in TNF-free media containing 1% FCS. The incubation was continued for a total of 2 hr. Control cells were carried through the same procedure,

but without TNF- α treatment. Low molecular weight DNA was extracted and electrophoresed as described in Chapter I.

Another mean of interrupting the TNF receptor signaling is by proteolytic cleavage of the receptor with proteases, such as trypsin. Trypsinization was performed as described previously (Chan and Aggarwal, '94). U937 cells (5×10^6) were exposed to 0.57 pM of TNF- α for 2.5 min and then washed in ice cold 1 X PBS. After the wash the cell pellet was suspended in 10 ml of FCS-free media containing 100 μ g/ml of trypsin (Gibco BRL). The cells were further incubated for a total of 2 hr. Cells not exposed to TNF- α were treated in a similar manner and used as controls.

C. Results:

a. Kinetics of TNF- α and D-e-C₈-Cer induced cell killing.

To elucidate the relationship of ceramide generation and TNF- α cell killing, we examined the time required by TNF- α and exogenous D-e-C₈-Cer to induce apoptosis in U937 cells. We postulated that if the ceramide generated by TNF- α mediates cell death, then addition of exogenous ceramide (so that endogenous levels of ceramide increase, bypassing the ligand receptor interactions) should activate the apoptotic pathway with timing similar to that of TNF- α .

We exposed U937 cells to 10 ng/ml of TNF- α for 30, 60, 90, 120, 180 and 360 min. At the end of the incubation period equal numbers of cells were lysed and the degree of apoptosis was assessed by agarose gel electrophoresis of DNA. DNA fragmentation ladders were first detected at 90 min (Fig. 11A). DNA fragmentation was thereafter detected in cells treated for 120, 180 and 360 min (Fig. 11A). In a similar analysis we examined the time required by D-e-C₈-Cer to kill cells. Contrary to the rapid induction of cell death by TNF- α , ceramide required incubation times that were substantially higher than those of TNF- α to kill cells. We detected nucleosomal fragments in cells treated with 10 μ M of D-e-C₈-Cer only after 12 and 18 hr (Fig. 11B). We did not detect nucleosomal fragmentation in cells treated with D-e-C₈-Cer for 3 and 6 hr (Fig. 11B). Our data above clearly demonstrate that the time required for cell killing is less for TNF- α than for

D-e-C₈-Cer.

A possible explanation for the differences in the kinetics of cell killing by TNF- α and D-e-C₈-Cer could be the rate of increase in the level of endogenous ceramide. TNF- α could increase the ceramide levels rapidly, whereas exogenously added ceramide might enter U937 cells more slowly. In this case, longer incubations of cells in the presence of D-e-C₈-Cer are required before the endogenous levels get to a point needed for cell killing. To examine this possibility, we measured the endogenous ceramide in cells treated with TNF- α and compared this level to the ceramide levels of cells treated with D-e-C₈-Cer. U937 cells were treated with 10 ng/ml of TNF- α for 7.5, 30, 60, 180 and 360 min. At the end of each incubation time the endogenous level of ceramide was measured as described in Chapter I. We detected a 2% increase from control (1 pmole/10⁶ cells) after 1 hr of incubation with TNF- α (Fig. 12 top right panel), 30 min before detecting DNA fragmentation (Fig. 11A). The ceramide continued to increase gradually and by the end of a 6 hr incubation time the ceramide levels increased 43% above the control (19 pmoles/10⁶ cells, Fig. 12 top right panel).

We next measured the rise in the endogenous ceramide in cells treated with D-e-C₈-Cer. U937 cells were treated with 10 μ M D-e-C₈-Cer for 2, 7.5, 15, 30, 60, and 180 min and at the end of each incubation time the ceramide level was quantitated as above. In D-e-C₈-Cer treated cells, ceramide

increases rapidly and significantly within the first 2 min of incubation to 45% increase (32 pmoles/ 10^6 cells) over the control (Fig. 12, top right and bottom panels). With a 15 and a 60 min incubation time the increase in ceramide was approximately 2- and 8-fold above the control (113 and 500 pmoles/ 10^6 cells), respectively (Fig. 12, top right and bottom panels). At the end of a 3-hr incubation, the levels rose 18-fold above the control and beyond the linear range of ceramide quantitation (50-2000 pmol, Veldhoven et al., '95). Hence the differences in killing time between ceramide and TNF- α could not be attributed to the rate of internalization of exogenously added ceramide.

b. Reversibility of TNF- α and ceramide induced cell death in U937 cells.

Previous authors have shown TNF- α to induce a transitory increase in the endogenous level of ceramide (Kim et al., '91; Obeid et al., '93; Wiegmann et al., '94) and have speculated that the generation of ceramide leads to the activation of cell death. If ceramide mediates the apoptotic signaling of TNF- α , then transient exposure of cells to D-e-C₈-Cer should increase the intracellular level of ceramide and trigger the activation of apoptosis.

Initially we asked if exposure of cells to D-e-C₈-Cer for 3 hr and an increase in the endogenous level of ceramide to 18-fold above control was adequate to activate the cell death

pathway. U937 cells (5×10^6) were incubated with $10 \mu\text{M}$ D-e- C_8 -Cer for 3 hr and then returned to ceramide-free media. The incubation was continued for a total of 18 hr and the cells were lysed and analyzed for DNA fragmentation ladders. We did not detect nucleosomal fragmentation in these cells (Fig. 13A, lane 5). DNA fragmentation and apoptosis were detected only in cells constantly exposed to $10 \mu\text{M}$ D-e- C_8 -Cer (Fig. 13A, lane 6). Furthermore, ceramide-induced cell death occurred only if cells were incubated in media that contained low serum (1% rather than 10%, Fig. 13A, lane 4). Since serum is a source of growth factors the above data suggest that ceramide cell killing is prevented by growth factors.

The above observations were restricted not only to cells treated with the D-e- C_8 -Cer stereoisomer. U937 cells treated with the much more potent stereoisomer D-t- C_8 -Cer for 3 hr and then returned to ceramide-free media for 15 more hours did not die (Fig. 14, last lane). We detected apoptosis and nucleosomal fragmentation only in cells that were continuously exposed to D-t- C_8 -Cer (Fig. 14, lane 5). Our results indicate that exposure of U937 cells to D-e- C_8 -Cer or D-t- C_8 -Cer for 3 hr, which increases the levels of endogenous ceramide to 18-fold over control, was not enough to trigger cell death, and a continuous exposure to these compounds was necessary for cell killing. Similar results were obtained from U937 cells treated with D-e- C_8 -Cer for 6 hr.

Treatment of cells with in TNF- α produced a different

result. When U937 cells (5×10^6) were treated for just 1 min with 10 ng/ml TNF- α and returned to TNF- α -free media containing 1% FCS for a total of 2 hr, DNA fragmentation and cell death were detected. (Fig. 13B). The induction of cell death by TNF- α was unaffected by high concentrations of FCS (10%) in the media suggesting that an increase in the growth factor concentration does not prevent cell killing initiated by TNF- α (Fig. 13C).

To determine what happens to accumulated ceramide once it is internalized, we exposed U937 cells to 10 μ M D-e-C₈-Cer for 3, 6, 12 and 18 hr. At the end of the incubation the cells were either lysed for determination of the endogenous level of ceramide or returned to ceramide-free media. Cells returned to ceramide-free media were kept in the incubator for a total of 18 hr, lysed and the endogenous level of ceramide was determined. The endogenous levels of ceramide in cells treated with D-e-C₈-Cer for 3, 6, 12 and 18 hr increased 18, 24, 30 and 22-fold above the control, respectively (Fig. 15, shaded bars). The endogenous levels of ceramide in cells treated with D-e-C₈-Cer for 3, 6 and 12 hr and then returned to ceramide-free media decreased to 2, 5 and 14-fold above the control, respectively. (A crude estimate is that $t_{1/2}$ for disappearance of ceramide is approximately 9 hr, indicating that ceramide is slowly degraded or modified into other sphingolipids, Fig. 15, white bars.) Thus continuing the incubation of cells initially treated with D-e-C₈-Cer in ceramide-free media decreases the

intracellular levels of ceramide significantly, reflecting the degradation or modification into other sphingolipids not phosphorylated by DG kinase within the cell. This decrease of ceramide (Fig. 15, from 18-fold to 2-fold above the control) is also associated with the lack of nucleosomal DNA ladders (Fig. 13A), suggesting that U937 cells can tolerate considerable increases in ceramide for hours without activating the apoptotic pathway.

c. Exogenous ceramide does not change the time required by TNF- α to induce apoptosis in U937 cells.

Although the above studies suggest that D-e-C₈-Cer and TNF- α kill U937 cells with different kinetics, we still detected ceramide elevations in TNF- α treated cells a 2% increase compared to the control levels detected at 1 hr post-treatment, and 30 min prior to the initiation of DNA fragmentation (Fig. 11,13). We therefore could not rule out the possibility that ceramide, despite its late generation, plays a role in TNF- α killing. To address the question of whether ceramide is necessary for the initiation of the TNF- α cell killing or, as appears from our findings, a secondary factor, we increased the cellular levels of ceramide prior to the addition of TNF- α . If ceramide is critical in the initiation of apoptosis by TNF- α , then providing U937 cells with ceramide prior to TNF- α exposure should decrease the time required for TNF- α cell killing.

U937 cells were either exposed to TNF- α alone, or pretreated with ceramide for 15 min (at which time ceramide levels increase to approximately 2-fold above the control, a 113 pmole increase and 112 pmoles above the 1 hr treated TNF- α cells) and then exposed to TNF- α . DNA was collected from cells treated with TNF- α for 30, 60, 90 and 120 min and apoptosis and cell death were assessed as previously described. Pretreatment of cells with D-e-C₈-Cer for 15 min prior to the addition of TNF- α did not alter the time needed for TNF- α to kill cells (Fig. 16). DNA fragmentation and apoptosis in cells receiving ceramide and TNF- α are detected after 90 and 120 min of incubation (Fig. 16, last 2 lanes) and it is indistinguishable from cells treated with TNF- α alone (Fig. 16, lanes 6,7). DNA fragmentation was not detected in cells exposed to TNF- α alone or to D-e-C₈-Cer and TNF- α for 30 and 60 min (Fig. 16, lanes 4,5 and 8,9, respectively). Thus accumulation of ceramide prior to the addition of TNF- α did not change the time needed by TNF- α to induce DNA fragmentation in U937 cells. A minimum of 90 min incubation was required for the induction of cell death. Therefore our results suggest that ceramide elevations are likely to be a result rather than a cause of TNF- α cell killing.

d. Low pH washes and trypsinization do not inhibit cell death promoted by exposure to TNF- α .

We demonstrated above that exposure of U937 cells to 10

ng/ml (0.57 nM) of TNF- α for just 1 min was sufficient to induce cell death. Based on these results we speculated that the apoptotic signaling initiated by TNF- α was an early and irreversible event. However, we could not exclude the possibility that complexes formed between TNF- α and its receptor remained intact after the washing with PBS. In that case, bound TNF- α could continue signaling, suggesting that the apoptotic signals may arise well after the initial receptor-ligand interactions. Other investigators have disrupted receptor-ligand signaling by washing cells in low pH buffers or by receptor proteolysis (Okuda et al., '91; Chan and Aggarwal, '94). Similarly, we exposed U937 cells to very low concentrations of TNF- α so that only a small fraction of the TNF- α receptors is occupied, followed by acid washes or trypsinization. We assumed that if the cell death signaling transduced by the TNF receptor is mediated at later times due to wash-resistant receptor-ligand complexes, then dissociation of the ligand-receptor complexes by acid washes and proteolytic cleavage of the TNF receptors could interrupt the apoptotic signaling cascade of TNF- α and prevent apoptosis.

We initially tested if transient exposure of U937 cells to a low concentration of TNF- α was adequate to promote cell death. Previous studies with U937 cells showed that a minimum of 10 pM TNF- α (a 17% TNF receptor occupancy) was necessary to translocate NF- κ B in U937 cells (Chan and Aggarwal, '94). U937 cells were treated with 0.57 pM TNF- α for 2.5 min, washed in

1 X PBS, returned to TNF- α -free media containing 1% FCS and incubated for a total of 2 hr. Even at this low TNF- α concentration (0.57 pM) we detected DNA fragmentation and apoptosis (Fig 17, lane 3). The induction of cell death by such low concentrations of TNF- α suggests that cell death signaling initiated by the TNF receptor is not only transmitted within seconds, but also a very small percentage of receptor occupancy is required.

To show that the cell death signaling transduced by TNF- α is mediated within the first min of the exposure, we investigated whether acid washes and trypsinization disrupting the receptor-ligand signaling could inhibit cell death signaling transduced by TNF- α . Acid washing was performed as described previously (Okuda et al., '91). U937 cells were treated with 0.57 pM of TNF- α for 2.5 min and washed with 1 X PBS pH 3.0 for 2 min. Subsequently the cells were washed with 1 X PBS pH 7.0, returned in media supplemented with 1% FCS and further incubated for a total of 2 hr. Cell death was assessed by DNA isolation and agarose gel electrophoresis. As shown in Fig. 17, lane 5 washing of cells in acidic conditions did not inhibit apoptosis transduced by TNF- α . In addition, incubation of U937 cells with 0.57 pM of TNF- α for 2.5 min followed by trypsinization (100 μ g/ml in serum-free media) and incubation in TNF- α free media for a total of 2 hr did not prevent cells from dying (Fig. 17, last lane). Therefore, acid washing and trypsinization did not inhibit apoptosis mediated by TNF- α .

The inability to reverse cell death by disrupting the receptor ligand complexes by acid washes and trypsinization strongly suggests that TNF- α treated cells (unlike ceramide treated cells) commit themselves to the apoptotic pathway within the first min of exposure to TNF- α .

D. Discussion:

Apoptosis triggered by TNF- α (Obeid et al., '93; Jarvis et al., '94), Fas antigen (Cifone et al., '93; Gulbins et al., 95), ionizing radiation, ultraviolet radiation, and heat shock (Verheij et al., '96) is hypothesized to be mediated by the second messenger ceramide. The data of these investigators suggest that the intracellular ceramide accumulation is associated with the receptor-ligand complex formation and the apoptotic signaling (Kolesnick et al., '94). Although there is little controversy over the generation of ceramide under these conditions, which lead to cell death, the involvement of ceramide in the execution of the apoptotic pathway is at least speculative (Beyaert and Fiers, '94; Higuchi et al., '96).

In the current study we investigated conditions by which ceramide and TNF- α kill cells to establish a relationship between use of ceramide, by TNF- α or internalization from exogenously provided pools, and activation of the apoptotic pathway. We hypothesized that if ceramide is the second messenger that mediates the cell death signaling of TNF- α , then exogenously added ceramide and TNF- α should require similar exposure times to kill cells. This was not the case (Fig. 11). U937 cells started to die within 90 min of exposure to TNF- α , whereas ceramide required at least 12 hr to kill. The difference could not be attributed to the slow internalization of the exogenously added ceramide (Fig. 12 upper right and bottom panels). Massive increases in

intracellular ceramide was not adequate to cause cell killing (Fig. 12). In contrast, U937 cells started to die after 90 min of exposure to TNF- α with the first ceramide elevations detected after 1 hr of treatment. Thus ceramide and TNF- α kill cells with different kinetics and this difference in cell killing is not governed by endogenous ceramide elevations. These observations strongly suggest that ceramide and TNF- α kill cells via independent pathways.

The ability of TNF- α to activate apoptosis at low concentrations and within the first 2 min of treatment, even when cells were acid-washed or exposed to trypsin (Fig. 17) suggests that cell death induced by TNF- α is a) transduced rapidly and within minutes of exposure to TNF- α , b) irreversible and c) effectuated by a very low receptor occupancy. The ability of TNF- α to transduce signals rapidly and at low concentrations is not restricted to the activation of apoptosis. Similarly, TNF- α was shown to translocate NF- κ B within a few minutes of exposure and at pM concentrations (Chan and Aggarwal, '94; Hohmann et al., '90; Chaturvedi et al., '94). It is therefore likely that the apoptotic signaling cascade mediated by TNF- α is transduced with the same rapidity as the signaling that translocates NF- κ B. More importantly, ceramide generation in U937 cells begins 1 hr post-treatment with TNF- α and well after the 2 min needed by cells to commit themselves to the apoptotic pathway indicating that ceramide generation is not required for the activation of cell death.

Another line of evidence supporting this idea is that the provision of D-e-C₈-Cer prior to the hydrolysis of the sphingomyelin pools by TNF- α did not alter the timing needed by TNF- α to kill cells (Fig. 16). Under these conditions availability of ceramide did not change the signal transduction cascades initiated by TNF- α .

Contrary to TNF- α , D-e-C₈-Cer failed to activate apoptosis even after prolonged incubations (3 hr, Fig. 13A,14). Continuous incubation with D-e-C₈-Cer and media containing low F.C.S concentrations (1%) was essential for the detection of DNA fragmentation ladders (Fig. 13A). Intracellular ceramide levels surpassed significantly the ceramide generated by TNF- α (Fig. 18), without activating apoptosis (Figs. 11 and 13). It is therefore possible that unlike TNF- α , cell death induced by D-e-C₈-Cer and the other stereoisomers to arises from toxic effects due to the sustained accumulation of unphysiologically high concentrations of ceramide within cells and the prolonged activation of ceramide targets such as the CAPK and CAPP. We found these observations intriguing because other investigators used much higher concentrations of synthetic ceramides (25-100 μ M, Yao et al., '95; Jayadev et al., '95; Verheij et al., '96) to establish the physiological role of natural ceramide within cells.

Several studies indicated that sphingomyelin pools are found in different subcellular compartments and their

selective hydrolysis has been associated with different cellular responses (Dressler et al., '92; Wiegmann et al., '94; Andrieu et al., '96). Interestingly, the location of the sphingomyelin pool hydrolyzed by TNF- α is controversial. Several investigators have proposed that the TNF- α -sensitive pool resides in the external leaflet of the plasma membrane (Chatterjee '94). Others proposed that the sphingomyelin pool hydrolyzed by TNF- α is localized in the endolysosomal compartments (Wiegmann et al., '94) or the inner leaflet of the plasma membrane (Linardic and Hannun, '94; Andrieu et al., '96). One could argue that exogenous ceramide kills cells with different kinetics, because of its differential accumulation in cellular compartments that are different from those generated by TNF- α . Studies with synthetic fluorescent ceramide analogs have shown ceramide to accumulate in the Golgi apparatus and sequentially to other membrane compartments (Pagano et al., '91), suggesting that cellular membranes are saturated quickly in the presence of exogenous ceramide. If TNF- α and other stress signals generate ceramide in subcellular compartments inaccessible to exogenous ceramide, then the use of exogenous ceramides to study the cell death mechanisms of TNF- α and other factors that lead to cell death may be irrelevant. Additionally, recent studies revealed that TNF- α -receptor complex recruits the cytoplasmic factors FADD and TRADD that may assist in the propagation of the cell death signaling to the apoptotic apparatus (Liu et

al., '96). The ability of exogenous ceramide to activate or recruit these factors in a way similar to TNF- α has not been confirmed. It is therefore possible that the differences in cell killing between TNF- α and exogenous ceramide to from the inability of exogenous ceramide to assemble the apoptotic apparatus alone and additional factors or conditions recruited by TNF- α and its receptors are required for the induction of cell death. Although this is an existing possibility requiring further investigation, our data strongly indicate that exogenous ceramide alone is not adequate to promote apoptosis with the same kinetics as TNF- α suggesting that ceramide and TNF- α may kill cells via alternative pathways.

CHAPTER 4

Ceramide Resistance to Cell Killing is Phosphorylation Dependent. Evidence that Ceramide and TNF- α Kill Cells by Independent Mechanisms

- A. Objective
- B. Materials and Methods
- C. Results
 - a. UDtIN cells are resistant to C₈-Cer stereoisomers, analogs and TNF- α .
 - b. The resistance of UDtIN cells to killing is more specific to ceramide and TNF- α .
 - c. The resistance to ceramide in UDtIN cells is not dependent on the rate of internalization or degradation of endogenous ceramide.
 - d. UDtIN cells contain functional TNF receptors.
 - e. The resistance to ceramide in UDtIN cells depends on the activity of PP2A phosphatases.
 - f. Okadaic acid does not render UDtIN cells sensitive to killing by TNF- α .
- D. Discussion: Ceramide cell killing depends on the activity of PP2A phosphatases, but TNF- α does not. Evidence that ceramide and TNF- α kill cells via different pathways.

Objective:

To elucidate the importance of ceramide in apoptosis, particularly its relationship to apoptosis induced by TNF- α , we established the cell death resistant cell line UDtIN by treating U937 cells with the potent C₈-Cer stereoisomer D-t-C₈-Cer. UDtIN cells proved to be resistant to killing by the C₈-Cer stereoisomers and analogs and TNF- α . The resistance of these cells to apoptosis induced by both ceramide and TNF- α makes them an important tool for the study of cellular pathways regulating cell death. Nevertheless, UDtIN were also partly resistant to cell death induced by high concentrations of ethanol (2.5%) and the protein synthesis inhibitor cycloheximide (50 μ g/ml). These observations suggest that UDtIN cells are generally resistant to a variety of stress conditions that lead to cell death.

We demonstrated that the resistance of UDtIN cells to ceramide was not attributable to the slow internalization or to the rapid degradation of internalized ceramide. Furthermore, we showed that the resistance of UDtIN cells to killing by TNF- α was not dependent on the absence or lack of function of TNF receptors, as indicated by the translocation of NF- κ B.

Previous studies have shown that ceramide activates a phosphatase (CAPP) belonging to the PP2A family of phosphatases. Since the importance of phosphatases in the activation of apoptosis is not well characterized, we asked

whether phosphatases play a role in the resistance of UDtIN cells to ceramide and TNF- α . We therefore inhibited the activity of PP2A and PP1 phosphatases and asked if this inhibition rendered UDtIN cells sensitive to killing by TNF- α and D-e-C₈-Cer. Sensitization of UDtIN cells to killing by both ceramide and TNF- α could indicate that ceramide and TNF- α kill cells via the same pathway. We used the phosphatase inhibitors okadaic acid (highly specific for PP2A), tautomycin (inhibits PP1 at lower concentrations than PP2A), and calyculin A (inhibits PP1 and PP2A equally). Inactivation of the PP2A phosphatase family by okadaic acid rendered UDtIN cells sensitive to killing by D-e-C₈-Cer but not by TNF- α . This indicates that the activity of PP2A and possibly CAPP protects UDtIN cells from killing by D-e-C₈-Cer but not to killing by TNF- α . Thus ceramide-induced cell death requires the inactivation of PP2A, but TNF- α induced cell death does not, suggesting that ceramide and TNF- α kill cells via independent pathways.

B. Materials and Methods:

Cell treatment, DNA isolation, electrophoresis, protein quantitation, lipid extraction and quantitation, fluorescence microscopy and the trypan blue exclusion assay were performed as in Chapter I.

a. Development of ceramide resistant cells (UDtIN):

U937 cells (5×10^6) were washed once in 1 X PBS, suspended in 1% FCS, and then treated once with 10 μ M of D-t-C₈-Cer for 24 hr. At the end of the incubation the cells were washed in 1 X PBS and then placed in culture media containing 10% FCS. Cells were grown in the above media for 2 weeks and then tested for their resistance to ceramide.

b. Cell treatment:

Cells were treated as described in Chapters I-III. The phosphatase inhibitors okadaic acid, tautomycin (Calbiochem), calyculin A (Gibco) and the protein synthesis inhibitor cycloheximide (Sigma) were added as ethanolic solutions.

*c. Mobility shift assay:*1. Cell treatment and isolation of the nuclear extracts.

The mobility shift assay was performed as described previously by Dignam et al., '83; and Müller et al., '95 with some modifications. Cells were washed in 1 X PBS, plated at 5×10^5 cells/ml in 1% FCS media with 10 ng/ml of TNF- α or 10 μ M

D-e-C₈-Cer for 60 min. At the end of the incubation, the cells were washed with ice cold 1 X PBS. All the following procedures were done on ice. The cells were resuspended in 400 μ l of buffer A (10 mM KCl, 10 mM HEPES, pH 9, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF (Sigma) and 2 μ g/ml each of the protease inhibitors leupeptin and aprotinin) and incubated on ice for 15 min. Cells were lysed by the addition of 25 μ l 10% Nonidet P-40 (Sigma) and incubated on ice for 2 min. The cell lysate was microfuged for 30 sec and the supernatant was discarded (cytoplasmic extracts). The nuclear pellet was suspended in 40 μ l of buffer B (400 mM NaCl, 20 mM HEPES pH 7.9, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 2 μ g/ml leupeptin and aprotinin) and then placed on a rotating platform for 20 min. The nuclear extracts in the supernatant were aliquoted (10 μ l/tube) in pre-chilled tubes and then stored at -70 °C.

2. Protein quantitation.

Nuclear proteins were quantitated as in Chapter I.

3. Labeling of the NF- κ B probe.

The two single-stranded oligonucleotides (5' to 3' TCG AGT CAG AGG GGA CTT TCC GAG ATC and 5' to 3' TCG AGA TCT CGG AAA GTC CCC TCT GAC) were purchased from Gibco BRL. The lyophilized oligonucleotides were reconstituted in T.E, pH 8.0 at a concentration of 1 μ g/ μ l. The two single-stranded oligonucleotides (20 μ g each) were mixed, boiled for 5 min,

and kept on the bench for 20 min to cool (allowing the single stranded oligonucleotides to reanneal into a double-stranded oligonucleotide). The oligonucleotide was stored at -20 °C. The reannealed double stranded oligonucleotide had single stranded overhangs that were filled by the Klenow fragment (Amersham). For the probe labeling, 50 ng of double stranded oligo (2 μ l) was mixed with 48 μ l of reaction buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, 35 μ M of dNTP, 2 μ M dATP, 10 μ l of [α -³²P] dATP (6000 Ci/mmol) and 2 U of Klenow fragment) and incubated at 30 °C for 15 min. The reaction was terminated by adding 2 μ l of 0.5 M EDTA. The labeled NF- κ B oligonucleotide was purified (from unincorporated [α -³²P] dATP) with a purification column (NucTrap purification columns, Stratagene) and 1 μ l (from 140 μ l of labeled probe) was used for scintillation counting to determine the amount of probe to be used (we used 10,000 CPM of labeled probe per tube).

4. Mobility shift assay.

For NF- κ B complex formation, 5 μ g of nuclear extracts were incubated with 1 μ g of poly(dI-dC) (Pharmacia, inhibits non specific binding of proteins to DNA), 10,000 CPM of labeled oligonucleotide from (c) above (usually 1 μ l of labelled NF- κ B probe is equal to 5-6,000 CPM or 35 pg) and 1% NP-40 in a total reaction volume of 20 μ l with binding buffer (25 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 10% glycerol). The incubation mixture was allowed to proceed at

room temperature for 20 min and then loaded on a non-denaturing acrylamide gel (4%).

5. Preparation of the non-denaturing acrylamide gel.

The gel mix was prepared with 8 ml of 5X Tris-glycine stock solution (250 mM Tris, 1.9 M glycine, 10 mM EDTA), 5.33 ml 30% acrylamide, 1 ml 2% bisacrylamide, 2 ml of 50% glycerol and 23.7 ml H₂O. The gel mix was vortexed briefly and then filtered through a 0.2- μ m filter. The gel was polymerized with 100 μ l of 30% ammonium persulfate (BioRad) and 34 μ l TEMED (Biorad). The gel was allowed to polymerize completely and then pre-run for 2 hr at 100 volts in a cold room (1X Tris-glycine stock solution was used as a running buffer). After the gel pre-run, the samples were loaded and the gel was electrophoresed for 2 hr at 35 mA (in a cold room). The gel was dried for 2 hr at 80 °C and then autoradiographed (at -75 °C for 24 hr with a fluorescent screen).

C. Results:**a. UDtIN cells are resistant to C₈-Cer stereoisomers, analogs and TNF- α .**

U937 cells were treated with 10 μ M of D-t-C₈-Cer for 24 hr, representing the time at which the majority of the cells were dead. Cells that survived the treatment were placed in ceramide-free media containing 10% FCS and allowed to grow. Morphologically, UDtIN cells appear to be similar to the parental cell line U937 (Fig. 19) although sometimes we detected "giant" cells (occasionally reaching 60 μ m in diameter or 60-200 X larger in volume, as compared to an average size cell of approximately 10-15 μ m in diameter, Fig. 19). Nuclear staining of UDtIN cells with the fluorochrome Hoechst 33258 (as described in Chapter I) revealed that the giant cells were syncytia with multiple and centrally localized nuclei (Fig. 5, arrowheads) surrounded by a single plasma membrane (Fig. 19). To confirm the resistance of UDtIN cells to ceramide cell killing U937 and UDtIN cells (5×10^6 cells/flask) were washed in 1X PBS, placed in media containing 1% FCS and treated with 10 μ M of D-e-C₈-Cer or D-t-C₈-Cer for 18 hr. The extent of cell death was assessed by DNA isolation and agarose gel electrophoresis, trypan blue exclusion and Hoechst 33258 staining. As anticipated, incubation of U937 cells for 18 hr in the presence of D-e-C₈-Cer and D-t-C₈-Cer resulted in cell death characterized by DNA fragmentation (Fig. 20A) and decrease in cell viability, as determined by

trypan blue staining. The viability of U937 cells treated with D-e-C₈-Cer and D-t-C₈-Cer was 87% and 50%, respectively, as compared to 95% detected in the control. However, UDtIN cells were resistant to both D-e-C₈-Cer and D-t-C₈-Cer stereoisomers (Fig. 20A, lanes 6 and 7) as indicated by the absence of DNA fragmentation ladders. Cell viability in all treated cells was 95% or higher. DNA fragmentation was not seen in the untreated (control) cells.

The amount of cell death in U937 and UDtIN cells was confirmed by the nuclear staining with Hoechst 33258 (Fig. 5). The nuclei of U937 cells treated with D-e-C₈-Cer and D-t-C₈-Cer bear the morphological changes that characterize apoptotic cells. The nuclei are fragmented into multiple small dense apoptotic bodies (Fig. 5, arrows). Untreated control cells show diffuse staining of oval nuclei with no chromatin condensation. The abundance of the apoptotic bodies in the photos reflects the intensity of the DNA ladders seen in the gel (Fig. 20A) and the decrease in cell viability by the trypan blue exclusion assay. In contrast to U937 cells, staining of UDtIN cells with Hoechst 33258 revealed no apoptotic bodies in either D-e-C₈-Cer and D-t-C₈-Cer treated cells (Fig. 5). The lack of apoptotic bodies by Hoechst 33258 staining and the high cell viability by the trypan blue exclusion assay is reflected by the absence of DNA ladders in the agarose gel (Fig. 20A, last two lanes).

We tested if UDtIN cells were resistant to the potent C₈-

Cer analogs D-e-C₈-Ceramine, D-e-C₈-cis-Δ4-Cer and D-e-C₈-4-yne-Cer. Treatment of the parent cell line (U937) with 10 μM D-e-C₈-Ceramine, D-e-C₈-cis-Δ4-Cer or D-e-C₈-4-yne-Cer for 6 hr induced cell death (Fig. 21A, lanes 7,10,13). Thus like D-e-C₈-Ceramine, D-e-C₈-cis-Δ4-Cer and D-e-C₈-4-yne-Cer kill U937 cells after 6 hr of treatment. DNA fragmentation ladders are also seen in cells incubated with D-e-C₈-Cer, D-e-C₈-cis-Δ4-Cer, D-e-C₈-4-yne-Cer and D-e-C₈-Ceramine for 18 hr (Fig. 21A, lanes 5,8,11,14, respectively). Although we did not detect a decrease in viability in any of the cells treated with the different compounds for the first 3 hr (viability was 90% or higher), we noticed a decrease in viability in U937 cells treated with D-e-C₈-Ceramine and D-e-C₈-4-yne-Cer for 6 hr (85%) reflecting the DNA fragmentation seen in the agarose gel (Fig. 21A). Cell viability decreased to various degrees for all U937 cells treated for 18 hr with the different ceramide compounds. Nevertheless, we did not detect DNA fragmentation or change in cell viability (Fig. 21B). The cell viability in UDtIN cells treated with any of the C₈-Cer analogs or stereoisomers for 3, 6 or 18 hr was 90% or higher.

b. The resistance of UDtIN cells to killing is more specific to ceramide and TNF-α.

To examine if the resistance to cell death was specific to ceramide-like compounds, we tested the response of these cells to other cell death inducers. We exposed the parental

U937 and UDtIN cells to 10 ng/ml TNF- α for 2 hr and assessed the degree of apoptosis by monitoring the appearance of nucleosomal DNA ladders and the presence of apoptotic bodies. U937 cells treated with TNF- α for 2 hr died by apoptosis indicated by the appearance of apoptotic bodies and DNA fragmentation ladders (Fig. 5 and Fig. 20B, lane 3). On the other hand, UDtIN cells showed resistance to killing by TNF- α and no nucleosomal DNA ladders were detected after 2 hr of exposure (Fig. 20B, last lane). This observation was confirmed by the lack of apoptotic bodies in Hoechst 33258 stained cells (Fig. 5) and the high cell viability of UDtIN cells (95% or higher). Thus in addition to killing by ceramide, UDtIN cells show resistance to cell killing by TNF- α .

We extended our analysis of the specificity of cell death resistance of UDtIN cells by examining the activation of cell death by two other cell death inducing compounds, ethanol and cycloheximide. U937 and UDtIN cells were treated with 50 μ g/ml cycloheximide or 2.5% ethanol for 3 and 18 hr. Cells treated with D-e-C₈-Cer and D-t-C₈-Cer were used as positive controls. DNA fragmentation and apoptosis were detected in U937 and UDtIN cells treated with 50 μ g/ml cycloheximide for 3 hr (Fig. 22A, lane 6 and last lane, respectively). We did not detect internucleosomal fragmentation in U937 or UDtIN cells treated with 2.5% ethanol, D-e-C₈-Cer or D-t-C₈-Cer for 3 hr. Incubation of U937 cells in the presence of cycloheximide, ethanol, D-e-C₈-Cer or D-t-C₈-Cer for 18 hr generated intense

DNA fragmentation ladders (Fig. 22B). Like U937, treatment of UDtIN cells with 2.5% ethanol or cycloheximide for 18 hr generated DNA fragmentation ladders (Fig. 22B, last two lanes), but as expected, UDtIN cells were resistant to D-e-C₈-Cer and D-t-C₈-Cer (Fig. 22B, lane 8 and 9).

Although we detected DNA fragmentation ladders and cell death in UDtIN cells treated with cycloheximide and ethanol, the degree of fragmentation was substantially less intense compared to one seen in the parental U937 cell line (Fig. 22A and B). The DNA fragmentation ladders from UDtIN cells treated with cycloheximide for 3 hr (Fig. 22A, last lane) and 18 hr (Fig. 22B, last lane), or ethanol for 18 hr (Fig. 22B, lane 10) were considerably less intense than those seen in the U937 cells (Fig. 22A, lane 6 and Fig. 22B, lanes 5 and 6, respectively). The above results are supported by viability tests with the trypan blue exclusion assay. A decrease in viability was generally characterized by an increase in the intensity of the DNA fragmentation ladders. Cell viability by trypan blue indicated that U937 cells were substantially more sensitive to both cycloheximide and ethanol. Although we did not observe any viability changes in U937 cells treated with cycloheximide for 3 hr, we noticed the appearance of apoptotic bodies that were trypan blue impermeable. By 6 hr the viability of U937 cells treated with cycloheximide dropped to 60% and after 18 hr we did not detect any live cells. We did not notice any viability changes in U937 cells treated with

the other compounds for 6 hr, but at 18 hr the viability of cells treated with 2.5% ethanol, 10 μ M D-t-C₈-Cer or D-e-C₈-Cer decreased to 0, 50 and 87%, respectively. The viability of the untreated (control) U937 cells was 96%. In contrast to U937 cells, the trypan blue exclusion assay did not indicate any viability changes in UDtIN cells treated with the different compounds for the first 3 hr. By 6 hr we noticed the appearance of trypan blue impermeable apoptotic bodies in UDtIN cells treated with cycloheximide. Although we did not detect viability changes at earlier times with any of the compounds tested, the viability of UDtIN cells treated with cycloheximide or ethanol declined to 70% at the 18 hr treatment time point (as compared to 0% in the parental U937 cell line). No changes in viability were detected in the control and ceramide treated UDtIN cells (the viability was 95% or higher). The above results indicated that UDtIN cells are generally resistant to killing, although we detected better protection from ceramide- and TNF- α -induced cell death.

c. The resistance to ceramide in UDtIN cells is not dependent on the rate of internalization or degradation of endogenous ceramide.

Having established that UDtIN cells are resistant to ceramide analogs and TNF- α , we began a series of investigations to determine mechanisms and signaling pathways that might control apoptosis. We first asked if the resistance

of UDtIN was due to a reduced or absent rate of internalization of exogenously added ceramide. Since low intracellular accumulation of ceramide could reduce cytotoxicity and cell death, we measured the concentrations of internalized ceramide over time. UDtIN cells were treated with 10 μ M D-e-C₈-Cer for 2.5, 7.5, 15, 30, 60 and 360 min and at the end of each incubation period the cells were lysed and the endogenous level of ceramide was determined. The ceramide levels of UDtIN cells exposed to 10 μ M D-e-C₈-Cer increased significantly within several minutes (Fig. 23) and in a manner similar to the parental cell line U937. Exposure of 3×10^6 UDtIN cells to ceramide for 7.5, 15, 30 and 60 min increased the endogenous ceramide levels 200, 400, 750 and 2000 pmoles above the control, respectively (Fig. 23), whereas exposure of the parental U937 cell line for the same time increased the endogenous level of ceramide 160, 360, 770 and 1175 pmoles, respectively. A 3 hr incubation increased the ceramide levels above the linear range of quantitative labeling for both cell lines. Thus the rate of ceramide internalization does not appear to be the factor that contributes to the resistance UDtIN cell to killing by ceramide.

We next examined if the resistance of UDtIN cells was dependent on the rapid degradation of internalized ceramide. Rapid ceramide breakdown could alleviate the stress exerted by the high ceramide concentrations, preventing the activation of the apoptotic pathway. UDtIN cells were washed in 1 X PBS,

suspended in media supplemented with 1% FCS and returned into two sets of flasks (6×10^6 cells/flask). In the first set of flasks the cells were exposed to $10 \mu\text{M}$ D-e- C_8 -Cer for 3, 6, 12 or 18 hr and at the end of the incubation the cells were washed in 1X PBS, lysed and the endogenous level of ceramide was measured as above. The cells in the second set of flasks were treated as in the first set, but at the end of the 3, 6 or 12 hr incubation the cells were not lysed but returned to ceramide-free media containing 1 % FCS. These cells were incubated further for a total of 18 hr and lysed, and the endogenous level of ceramide was measured. A decrease in ceramide between the end of the first and the end of the second incubation reflected the amount of ceramide that has been degraded or modified into other sphingolipids such as glycosceramides or sphingosine.

Incubation of UDtIN cells in the presence of ceramide for 3, 6, 12 and 18 hr increased the endogenous ceramide levels 9, 13, 14 and 12 fold above the untreated UDtIN cells (control), respectively (Fig. 24, black bars). The endogenous levels of ceramide in UDtIN cells treated with D-e- C_8 -Cer for 3, 6 and 12 hr and then returned to ceramide-free media for 15, 12 and 6 hr decrease from 9 to 3.3, 13 to 4.5 and 14 to 7.5 fold above the control, respectively (a crude estimate is that $t_{1/2}$ for disappearance of ceramide is approximately 9 hr, suggesting that intracellular ceramide is modified or degraded slowly, Fig. 24). The above studies showed that return of

UDtIN cells to ceramide-free media and continuation of the incubation reduces the ceramide levels considerably, but that substantial supraphysiological amounts of intracellular ceramide were still present. Therefore, the resistance of UDtIN cells is not dependent on alterations in the ability to take up or degrade ceramide.

d. UDtIN cells contain functional TNF receptors.

Since we found UDtIN cells to be resistant to not only ceramide but also to TNF- α , we investigated whether the resistance to TNF- α was due to the absence or lack of function of the TNF receptors. We assessed the presence of functional TNF receptors by examining whether TNF- α can translocate NF- κ B in UDtIN cells. NF- κ B is a transcription factor that resides in the cytoplasm (inactive form) and is associated with an inhibitory protein (I- κ B, Baeuerle, '91; Gosh and Baltimore, '90). It was shown previously that binding of TNF- α to its receptors induces activation and translocation of NF- κ B to the nucleus (Chan and Aggarwal, '94; Müller et al., '95) in U937 cells.

To test the functionality of the TNF receptors, we treated UDtIN cells with 10 ng/ml of TNF- α for 60 min. At the end of the incubation period the cells were lysed and the nuclei were collected. Nuclear proteins were isolated and tested for the presence of NF- κ B by the mobility gel shift assay. Translocation of NF- κ B to the nucleus is an indication

that UDtIN cells have functional TNF receptors.

In nuclear extracts prepared from untreated (control) cells we detected 3 retarded bands that represent complexes formed by the ^{32}P labelled oligonucleotide and NF- κ B (Fig. 25, lane 2). The same 3 bands are also seen in UDtIN cells treated with TNF- α for 60 min, but in addition we detected an inducible NF- κ B band (Fig. 25, arrow). The inducible NF- κ B band shows that TNF receptors in UDtIN cells are both present and functional. To show that the retarded bands we detected on the gel were specific to complexes formed between the ^{32}P labelled oligonucleotide and NF- κ B, we incubated nuclear extracts isolated from cells treated with TNF- α and excess unlabelled probe. The abolishment of the retarded bands indicated that the bands detected on the gel are NF- κ B specific (Fig. 25, lane 4). We did not detect NF- κ B related bands in the lane that did not contain nuclear extracts (Fig. 25, first lane).

Since ceramide has been implicated as an important mediator of the apoptotic signaling of TNF- α , we tested whether ceramide could translocate NF- κ B in a manner similar to TNF- α in UDtIN cells. UDtIN cells were treated with 10 μM D-e-C₈-Cer for 60 min and at the end of the incubation the nuclear extracts were isolated as described above. As indicated in Fig. 25, last lane, D-e-C₈-Cer did not translocate NF- κ B, suggesting that NF- κ B translocation induced by TNF- α is not mediated by ceramide. Nuclear translocation of

NF- κ B was also detected in the parent cell line U937 treated with 10 ng/ml TNF- α (not shown). As in the case of UDtIN cells, D-e-C₈-Cer failed to translocate NF- κ B in U937 cells. Taken together, our results suggest that the resistance of UDtIN cells to TNF- α killing is not due to the absence of TNF receptors and that ceramide does not induce the translocation of NF- κ B.

e. The resistance of UDtIN cells to ceramide cell killing depends on the activity of PP2A phosphatases.

Having shown that UDtIN cells are not resistant to cell killing due to the lack of TNF receptors or to the poor internalization or rapid degradation of D-e-C₈-Cer, we analyzed other pathways that could be involved in rendering UDtIN cells resistant to cell killing. Since ceramide activates a protein phosphatase (CAPP), and we developed the UDtIN cell line by treating U937 cells with the ceramide stereoisomer D-t-C₈-Cer, we postulated that the resistance of UDtIN cells might involve biochemical pathways controlled by proteins that are ceramide targets, such as the CAPP.

Previous studies showed that ceramide activates a cytosolic serine/threonine phosphatase (CAPP) belonging to the PP2A family (Dobrowsky and Hannun, '92; Dobrowsky et al., '93; Wolff et al., '94). CAPP is completely inhibited by 10 nM of okadaic acid, a specific inhibitor of PP2A phosphatases, (Dobrowsky and Hannun, '92). The involvement of phosphatases,

particularly CAPP, in cell death has not been established. Preliminary studies suggested that CAPP may not be involved in the transduction of the apoptotic signaling by ceramide (Karasavvas et al., '96a), because inhibition of the PP2A phosphatases and consequently CAPP did not prevent cell killing promoted by D-e-C₈-Cer in U937 cells. In addition, PP2A phosphatase activity had been shown to protect cells from apoptosis triggered by TNF- α (Wright et al., '93). To elucidate further the role of PP2A phosphatases in apoptosis and particularly their involvement in the resistance of UDtIN cells, we investigated whether the ceramide and TNF- α resistance of UDtIN cells was dependent on the activity of PP2A phosphatases.

We exposed cells to okadaic acid alone or okadaic acid and 10 μ M D-e-C₈-Cer for 18 hr. The degree of cell death was assessed by agarose gel electrophoresis. We did not detect DNA fragmentation in UDtIN cells treated with 0.1, 1.0 and 15 nM okadaic acid alone or 10 μ M D-e-C₈-Cer alone (Fig. 26A). We also did not observe DNA fragmentation in UDtIN cells exposed to 0.1, 0.5, 1.0, 5.0 or 10 nM of okadaic acid and 10 μ M D-e-C₈-Cer (Fig. 26A). However, DNA fragmentation and cell death was seen when UDtIN cells were incubated with both 15 nM okadaic acid and 10 μ M D-e-C₈-Cer (Fig. 26A, last lane, Table 1) suggesting that PP2A phosphatases play a protective role in UDtIN cells and their inhibition sensitized these cells to killing by D-e-C₈-Cer. As previously reported, PP2A

phosphatases, including CAPP, are very sensitive to okadaic acid (IC_{50} 0.2 nM, Haystead et al., '89) and are completely inactivated in the presence of 10 μ M okadaic acid (Cohen et al., '90; Wolff et al., '94). Since PP1 phosphatases are less sensitive to okadaic acid (IC_{50} 20 nM, Haystead et al., '89) and are completely inhibited by 1 μ M okadaic acid (Cohen et al., '90), PP1 phosphatases are less likely to be involved in the resistance of UDtIN cells.

To further examine if the sensitization of UDtIN cells to killing by D-e-C₈-Cer is controlled by the activity of PP2A phosphatases, we used two additional phosphatase inhibitors, tautomycin and calyculin A. Tautomycin, unlike okadaic acid, has higher specificity for PP1 phosphatases (Mackintosh and Klumpp, '90; Hori et al., '91) and inhibits completely PP1 at 3 nM whereas PP2A requires 30 nM for complete inhibition (Mackintosh and Klumpp, '90). Calyculin A inhibits PP1 and PP2A with similar potencies at IC_{50} 1-2 nM, (Ishihara et al., '89). Unlike okadaic acid, tautomycin was not effective in rendering UDtIN cells sensitive to killing by D-e-C₈-Cer. In fact incubation of cells with 40 and 80 nM tautomycin, which completely inhibit PP1, did not render UDtIN cells sensitive to killing by D-e-C₈-Cer, as indicated by the absence of DNA fragmentation ladders (Fig. 27A, lanes 3 and 4, Table 1). Incubation of UDtIN cells in the presence of 160 nM tautomycin induced DNA fragmentation in the absence of D-e-C₈-Cer (Fig. 27A, last lane, Table 1) suggesting that inhibition of both

PP1 and PP2A phosphatases is lethal to UDtIN cells. The inability of tautomycin to render UDtIN cells sensitive to killing by D-e-C₈-Cer at concentrations that completely inhibit PP1 indicates that PP1 phosphatases may not be involved in the resistance of UDtIN cells to killing by ceramide.

We found UDtIN cells to be very sensitive to killing by calyculin A alone. Treatment of cells with 0.5 nM calyculin A alone (Fig. 27B, lane 5) or with 0.5 nM calyculin A and 10 μ M of D-e-C₈-Cer did not kill UDtIN cells (Fig. 27B, lane 4,). Incubation of cells in the presence of 1.0 and 1.5 nM calyculin A was cytotoxic by itself and induced cell death in the absence of D-e-C₈-Cer (Fig 27B, lanes 6 and 8, Table 1). Treatment of UDtIN cells with 1.0 or 1.5 nM calyculin A and 10 μ M D-e-C₈-Cer caused massive cell death. By 18 hr of incubation the majority of cells were eliminated and the DNA fragmentation ladders appear very faint (Fig. 27B, lanes 7 and 9). As in the case of tautomycin, incubation of UDtIN cells with calyculin A at concentrations that inhibit both PP1 and PP2A phosphatases (1.0 and 1.5 nM, Table 1) induces cell death in the absence of ceramide (Table 1).

Table 1

Cells	Medium	Inhi-bition	Result	Conclusion
UDtIN	Oka (0.1-15 nM)	PP2A	No DNA Fragmen- tation	PP2A not toxic to cells
UDtIN	Cer(10 μ M) or TNF- α (10 ng/ml)	----	No DNA Fragmen- tation	UDtIN resistant to killing
UDtIN	Oka(15 nM) & Cer(10 μ M)	PP2A	DNA Fragmen- tation	PP2A involved
UDtIN	Oka(15 nM) & TNF- α (10 ng/ml)	PP2A	No DNA Fragmen- tation	PP2A not involved
UDtIN	Taut(40 nM or 80 nM) & Cer(10 μ M)	PP1	No DNA Fragmen- tation	PP1 not involved
UDtIN	Taut (160 nM)	PP1 & PP2A	DNA Fragmen- tation	Inhibition of PP1 & PP2A is lethal
UDtIN	Cla (0.5-1.5 nM)	PP1 & PP2A	DNA Fragmen- tation	Inhibition of PP1 & PP2A is lethal
UDtIN	Cla (1-1.5 nM) & Cer(10 μ M)	PP1 & PP2A	DNA Fragmen- tation	Inhibition of PP1 & PP2A is lethal

f. Okadaic acid does not render UDtIN cells sensitive to killing by TNF- α .

Since we have shown that UDtIN cells exposed to okadaic acid are killed by D-e-C₈-Cer, we tested whether exposure of UDtIN cells to okadaic acid could promote cell killing by TNF- α . U937 and UDtIN cells were treated with 15 nM okadaic acid, or 10 ng/ml TNF- α or both 15 nM okadaic acid and 10 ng/ml TNF- α for 2 hr. At the end of the incubation the DNA was extracted and apoptosis was assessed as described in Chapter I. Untreated and okadaic acid treated U937 and UDtIN cells did not show DNA fragmentation and apoptosis (Fig. 26B, lanes 2,3, 6,7). U937 cells treated with TNF- α alone or TNF- α and okadaic acid underwent extensive DNA fragmentation (Fig. 26B, lane 4,5, respectively). In contrast to the results obtained with U937 cells, we did not detect DNA fragmentation in UDtIN cells exposed to TNF- α alone or to both TNF- α and okadaic acid (Fig. 26B, lanes 8 and 9, Table 1). Thus inactivation of PP2A phosphatases with okadaic acid did not render UDtIN cells sensitive to killing by TNF- α . Since we found calyculin A and tautomycin to be cytotoxic in the absence of ceramide we did not use them with TNF- α .

D. Discussion:

Although evidence from other laboratories suggests that ceramide is a second messenger required for the activation of cell death under stress conditions, the biochemical pathways and the ceramide targets mediating these effects are still elusive. To understand better the cell death signaling of ceramide and its relationship to cell death induced by TNF- α , we developed the cell death resistant cell line UDtIN. Although UDtIN cells were selected from a population of U937 cells that survived a 24 hr treatment with D-t-C₈-Cer, UDtIN cells proved to be resistant to killing by D-e-C₈-Cer, D-t-C₈-Cer, and the very potent ceramide analogs D-e-C₈-Ceramine, D-e-C₈-cis- Δ 4-Cer, D-e-C₈-4-yne-Cer, TNF- α and partly resistant to high concentrations of ethanol and cycloheximide. Our data suggest that UDtIN cells are resistant to cell death in general and not specifically to ceramide. Furthermore we demonstrated that the resistance of UDtIN cells to apoptosis was not due to the slow internalization (Fig. 23), or degradation of endogenous ceramide (Fig. 24) or to the absence or lack of functional TNF receptors (Fig. 25). Interestingly, bcl-2 overexpression in FL5.12-bcl-2 cells prevented cell death induced by D-e-C₈-Cer, but provided only partial protection from the other C₈-Cer analogs and stereoisomers (Karasavvas et al., '96b). We showed that UDtIN cells are resistant to all ceramide stereoisomers and analogs tested, suggesting that the resistance of the UDtIN cells may not

involve the overexpression of bcl-2.

To elucidate possible pathways involved in the resistance of UDtIN cells, we asked if PP2A phosphatases play any role in prevention of cell death promoted by ceramide or TNF- α . We showed that inhibition of the serine/threonine PP2A phosphatases by the highly specific PP2A phosphatase inhibitor okadaic acid rendered UDtIN cells sensitive to killing by D- ϵ -C₈-Cer but not to killing by TNF α that the activity of CAPP is required for the survival of UDtIN cells under stressful conditions, such as high concentrations of ceramide. Since ceramide generation has been mostly associated with the promotion and activation of cell death, our finding that the ceramide target CAPP may protect rather than promote cell killing was quite intriguing. In previous studies we showed that CAPK, another protein that is activated by ceramide, is not involved in the differential killing of cells by the *threo* stereoisomers (Karasavvas et al., '96a). Since there is evidence that the ceramide targets CAPP and CAPK may not be involved in apoptosis induced by ceramide (Karasavvas et al., '96a), it is likely that ceramide plays an intricate role in the physiological regulation of cells and activation of ceramide targets may not necessarily lead to apoptosis. For instance, ceramide has been shown to play a role in cellular senescence (Venable et al., '95) and cell cycle arrest (Jayadev et al., '95) suggesting that the apoptotic pathway is one of several that can be activated by ceramide (Pushkareva

et al., '95).

Our data suggest that PP1 phosphatases are not likely to be involved in apoptosis. Inhibition of PP1 by tautomycin did not sensitize UDtIN cells to killing by D-e-C₈-Cer, suggesting that PP1 phosphatases are not involved in the death resistance of the UDtIN cells. Inhibition of both PP1 and PP2A by high concentrations of tautomycin or calyculin A was lethal to UDtIN cells in the absence of D-e-C₈-Cer, indicating that the functional activity of both phosphatases is needed for cell survival.

The translocation of NF- κ B by both ceramide and TNF- α has been controversial. Several studies indicated that ceramide and TNF- α translocate NF- κ B (Wiegmann et al., '94; Müller et al., '95; Antwerp et al., '96) raising the possibility that the apoptotic signaling of ceramide and TNF- α could be mediated by NF- κ B. Still other studies showed that ceramide did not promote NF- κ B translocation (Betts et al., '94) and that translocation of NF- κ B by TNF- α can occur without sphingomyelin hydrolysis and generation of ceramide (Higuchi et al., '96). In this study we showed that although TNF- α induced NF- κ B translocation in both U937 and UDtIN cells, D-e-C₈-Cer did not suggesting that ceramide may not, mediate the cellular effects of NF- κ B activation.

Conclusions:

In the last few years ceramide has emerged as an important second messenger involved in the propagation of the cell death signaling of cytokines and stress conditions that lead to apoptosis. In this study we tested this hypothesis by investigating the role of ceramide in the induction of cell death in the leukemic cell line U937. We initiated our studies by asking if ceramide can kill cells and how the structural modifications of ceramide affected its ability to induce apoptosis. We showed that exogenously added ceramide killed cells stereospecifically and the presence of the *trans* double bond proved to be necessary for ceramide to kill cells. Modification of the double bond into the *cis* configuration or conversion into a triple bond increased the killing potency of ceramide, suggesting that the presence and integrity of the *trans*-4,5-double bond is important for the promotion of cell death. In addition, we showed that the carbonyl group of ceramide is not required to induce cell killing. All these results were supportive of the hypothesis that ceramide is a bioactive molecule that could be important for the propagation of cell death signaling. Another line of evidence supporting this speculation was that cells overexpressing the oncogene *bcl-2* did not die when treated with the natural stereoisomer of ceramide suggesting that the two pathways converge and are therefore likely to intercommunicate under conditions in which cells make decisions between life and death.

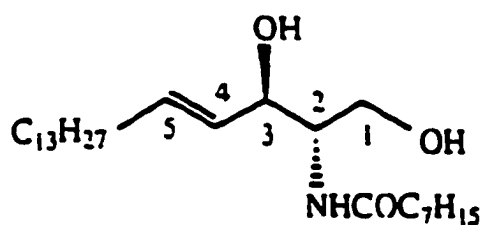
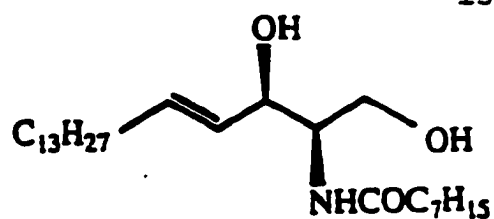
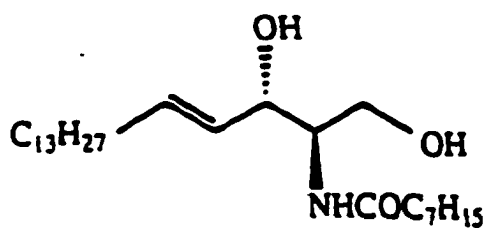
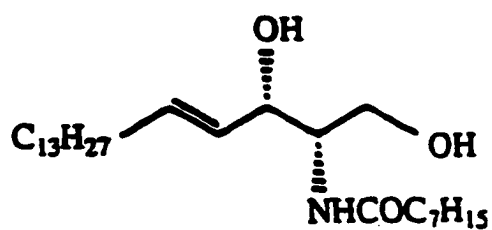
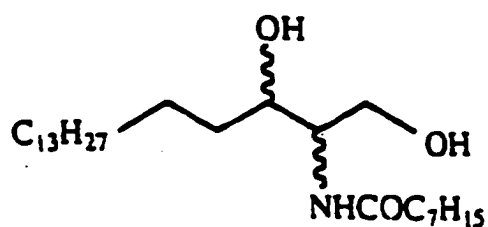
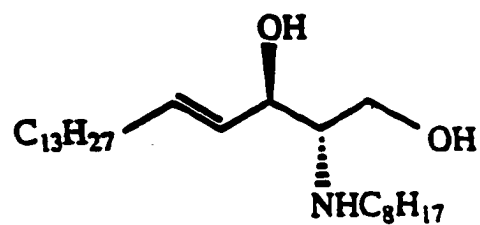
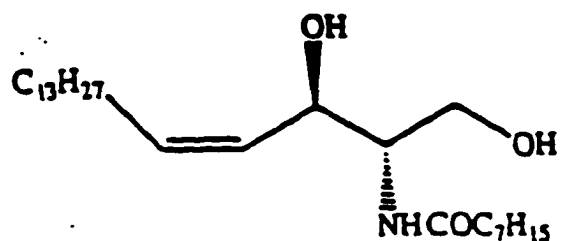
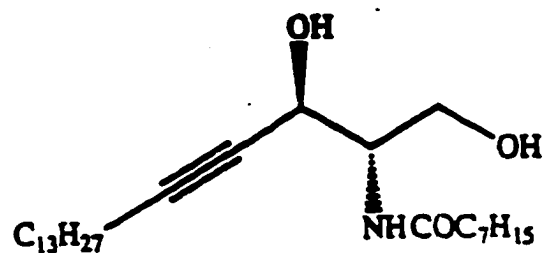
Although generation of ceramide has been reported to increase under conditions that lead to apoptosis, the requirement for ceramide in the activation of cell death has not been established. To establish the role of ceramide in the activation of cell death, we examined the relationship of ceramide generation to the induction of cell death by TNF- α , a cytokine that is speculated to activate apoptosis in the target cells via the generation of ceramide. Our data clearly show that an increase of intracellular ceramide alone is not adequate to kill cells. Furthermore, ceramide and TNF- α killed cells with different kinetics and mechanisms, suggesting that ceramide and TNF- α are likely to kill cells via different pathways. In this case, ceramide generation by TNF- α , and other apoptosis inducing compounds and stress conditions, might not be the cause but the consequence of apoptosis.

From these studies an important question arises, If ceramide is not the second messenger activating apoptosis, then why is generated under stress conditions and what is its role after generation? The identification of many ceramide targets such as CAPK, CAPP, PKC ζ and the retinoblastoma gene product (Rb) suggests that ceramide may be vital for the cellular homeostasis during stress. As we demonstrate, the two ceramide targets CAPK and CAPP are unlikely to be involved in apoptosis. In fact, we showed that CAPP might be involved in cell survival rather than cell killing. This finding is paradoxical and intriguing because ceramide has been mostly

associated with cell death and not with cell survival. Our studies clearly show that a careful analysis is required for the elucidation of the role of ceramide under stress conditions.

PART III. FIGURES AND FIGURE LEGENDS

Fig. 1. Structures of C₈-Cer stereoisomers, DL-e-DHC₈-Cer, D-e-C₈-Ceramine, D-e-C₈-cis- Δ^4 -Cer and D-e-C₈-4-yne-Cer.

D-*e*-C₈-CeramideD-*t*-C₈-CeramideL-*e*-C₈-CeramideL-*t*-C₈-CeramideDL-*e*-DHC₈-CeramideD-*e*-C₈-CeramineD-*e*-C₈-*cis*-Δ⁴-CeramideD-*e*-C₈-4-yne-Ceramide

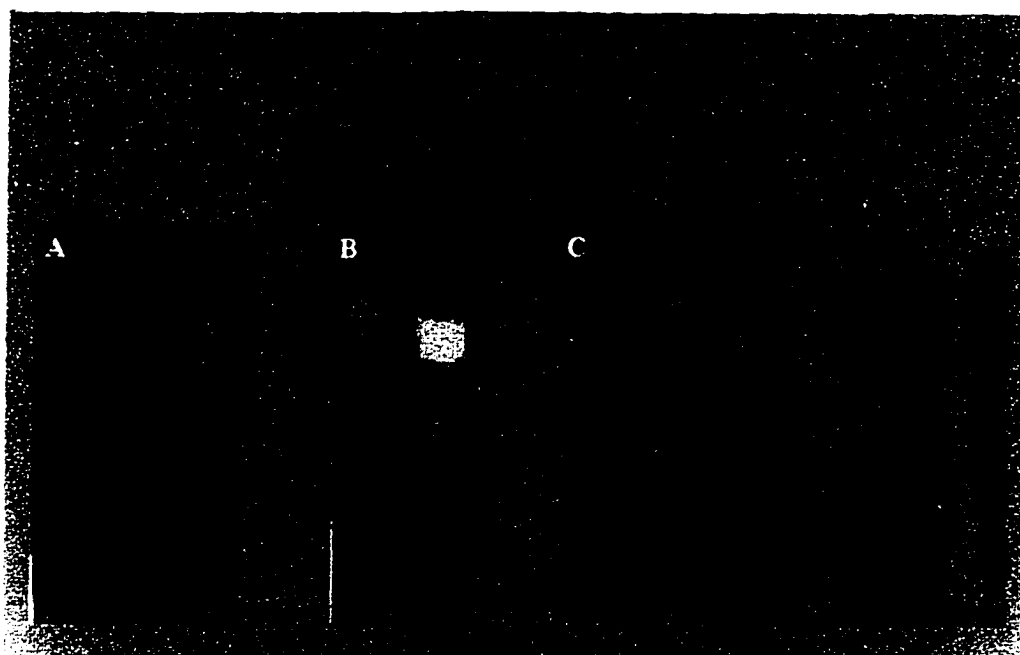
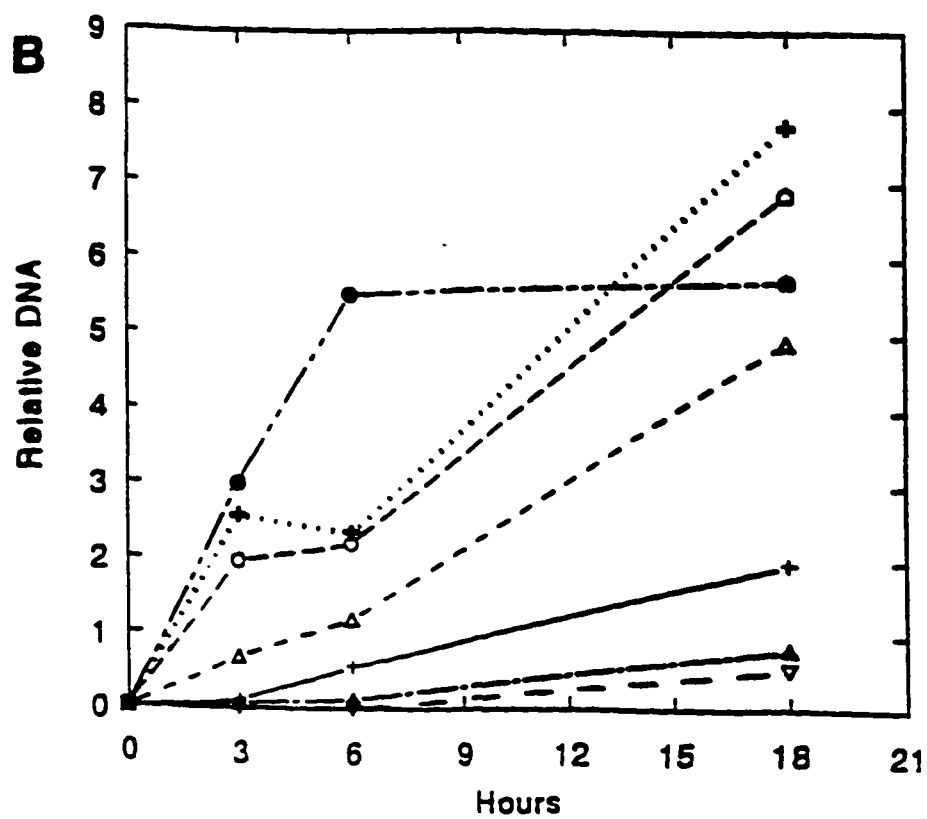
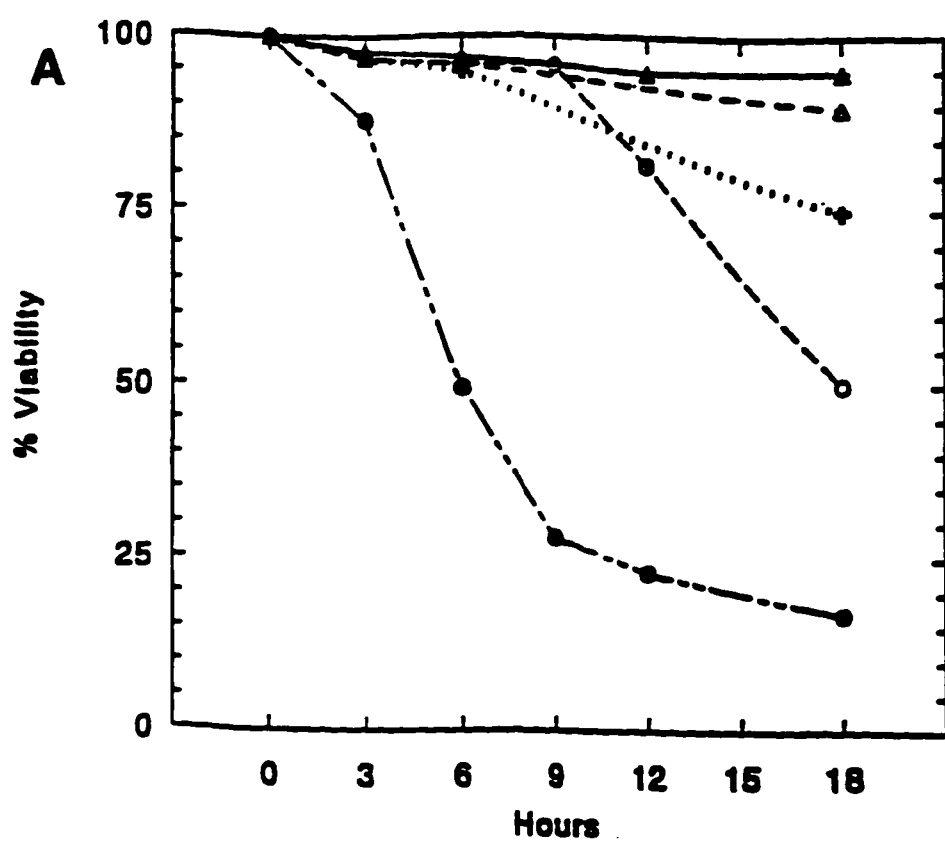


Fig. 2. Quantitation of DNA fragmentation induced by D-e-C₈-Cer and DL-e-DHC₈-Cer.

Low molecular weight DNA was extracted from 8.2×10^6 cells treated with ceramide analogs. Panel A: Agarose gel electrophoresis of U937 cells treated with $10 \mu\text{M}$ D-e-C₈-Cer or DL-e-DHC₈-Cer for 12 hr. Panel B: Agarose gel electrophoresis of cells treated with $10 \mu\text{M}$ D-e-C₈-Cer for 18 hr. Panel C: End-labeling of low molecular weight DNA extracted from 1.8×10^6 cells treated with $10 \mu\text{M}$ D-e-C₈-Cer for 3, 12 or 18 hr (from left to right, respectively) or with $10 \mu\text{M}$ DL-e-DHC₈-Cer for 18 hr (last lane). Control cells were treated with ethanol (0.1% final volume) for 3, 12 or 18 hr (from left to right, respectively).

Fig. 3. Cell viability and densitometric quantitation of DNA fragments induced by C₈-ceramide analogs at different time points.

Panel A: Cell viability of U937 cells as assessed by trypan blue exclusion. Cells were exposed to the different compounds from 3 to 18 hr. The curves of D-e-C₈-Cer and DL-e-DHC₈-Cer overlap. Panel B: Densitometric analysis of DNA on agarose gels extracted from cells treated for 3, 6 and 18 hr with the different C₈-Cer analogs (EtOH control = -- ∇ --; D-e-C₈-Cer = --- + ---; L-e-C₈-Cer = --- Δ ---; D-t-C₈-Cer = --- o ---; L-t-C₈-Cer = --- + ---; D-e-C₈-Ceramine = --- - - - - - • - ; DL-e-DHC₈-Cer = --- - Δ ---).



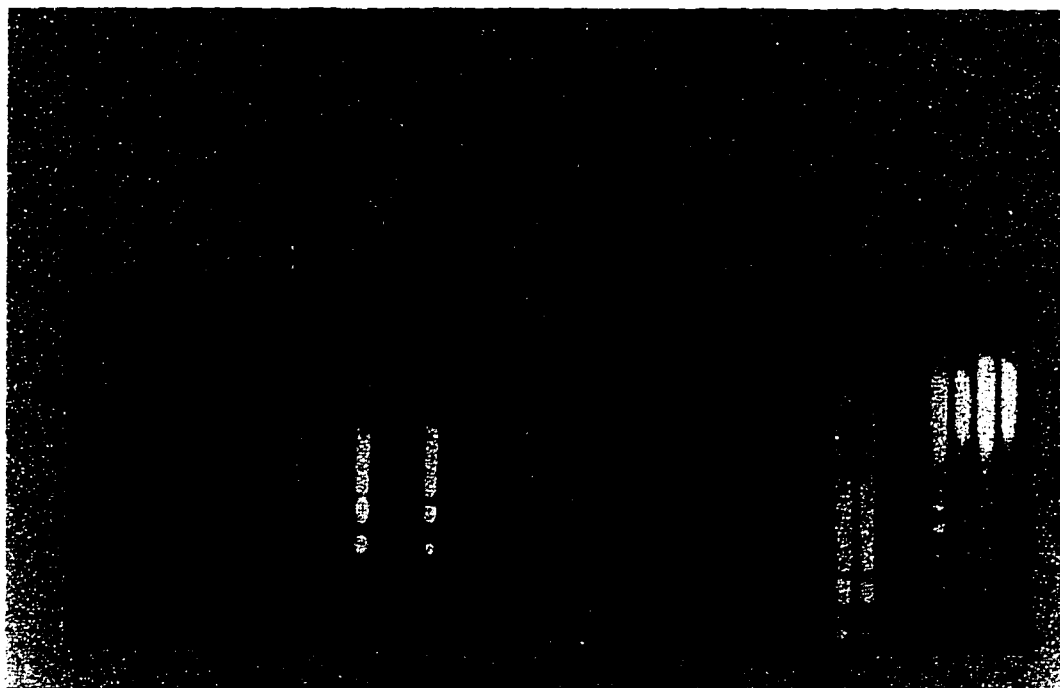


Fig. 4. Time course of DNA fragmentation induced by C₈-Cer stereoisomers, DL- ϵ -DHC₈-Cer and D- ϵ -C₈-Ceramine.

Low molecular weight DNA extracted from 8.2×10^6 cells treated with C₈-Cer analogs for 3 to 18 hr. DNA was collected at the times indicated and analyzed by 2% agarose gel electrophoresis and ethidium bromide staining. Panel A: For each C₈-Cer analog there are three lanes, representing DNA collected at 3, 6 and 18 hr of treatment. Panel B: For each compound there are 5 lanes, representing DNA collected at 3, 6, 9, 12 and 18 hr of treatment. MW: molecular mass markers, in base pairs. DNA from (control) cells treated with ethanol (0.1% final volume) is also shown.

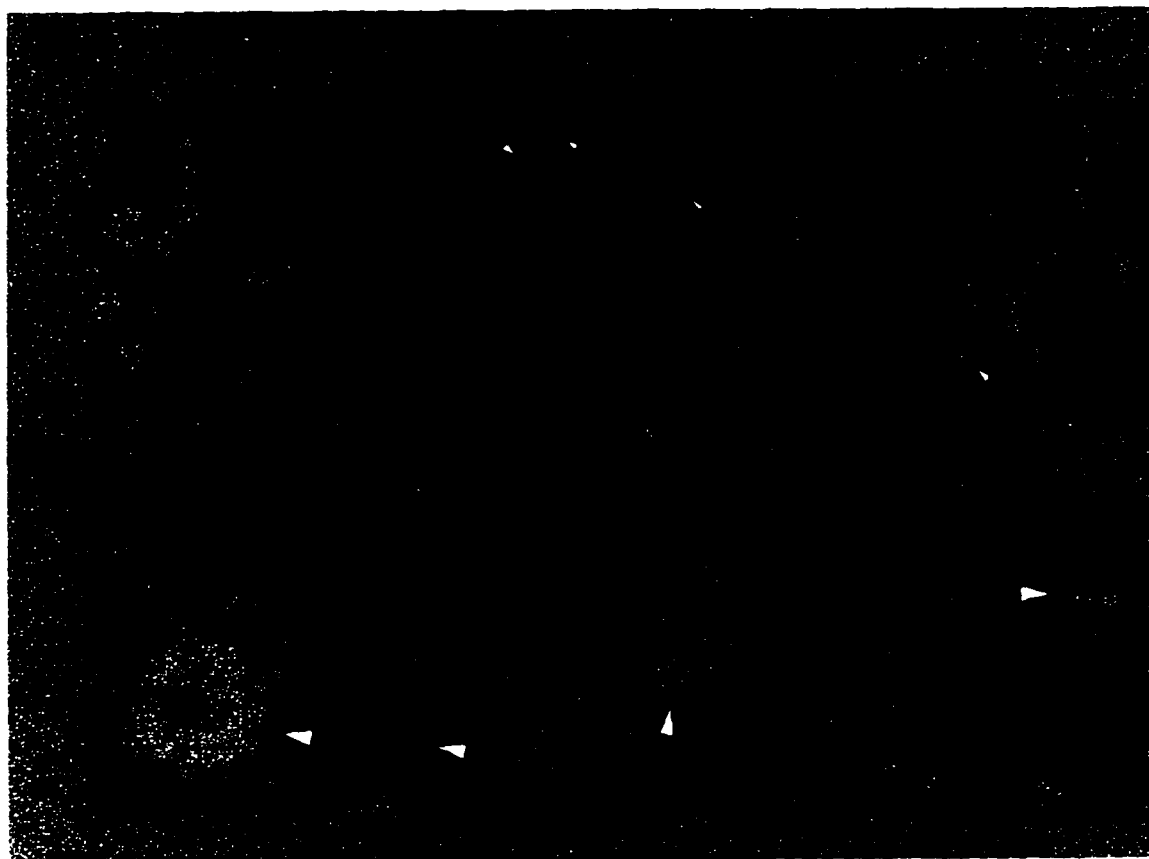


Fig. 5. Fluorescence microscopy of U937 and UDtIN cells stained with Hoechst 33258.

U937 and UDtIN cells were treated with 10 μ M D-e-C₈-Cer or D-t-C₈-Cer for 18 hr or with 10 ng/ml TNF- α for 2 hr and stained with the DNA-binding fluorochrome Hoechst 33258 (as in Materials and Methods). Top 4 panels from left to right, stained nuclei of untreated (control) and U937 cells treated with D-e-C₈-Cer, D-t-C₈-Cer or TNF- α , respectively. Arrows indicate apoptotic bodies (fragmented nuclei). Bottom 4 panels from left to right, stained nuclei of untreated (control) and UDtIN cells treated with D-e-C₈-Cer, D-t-C₈-Cer or TNF- α , respectively. Arrowheads indicate multinucleated cells. The magnification is 400X.

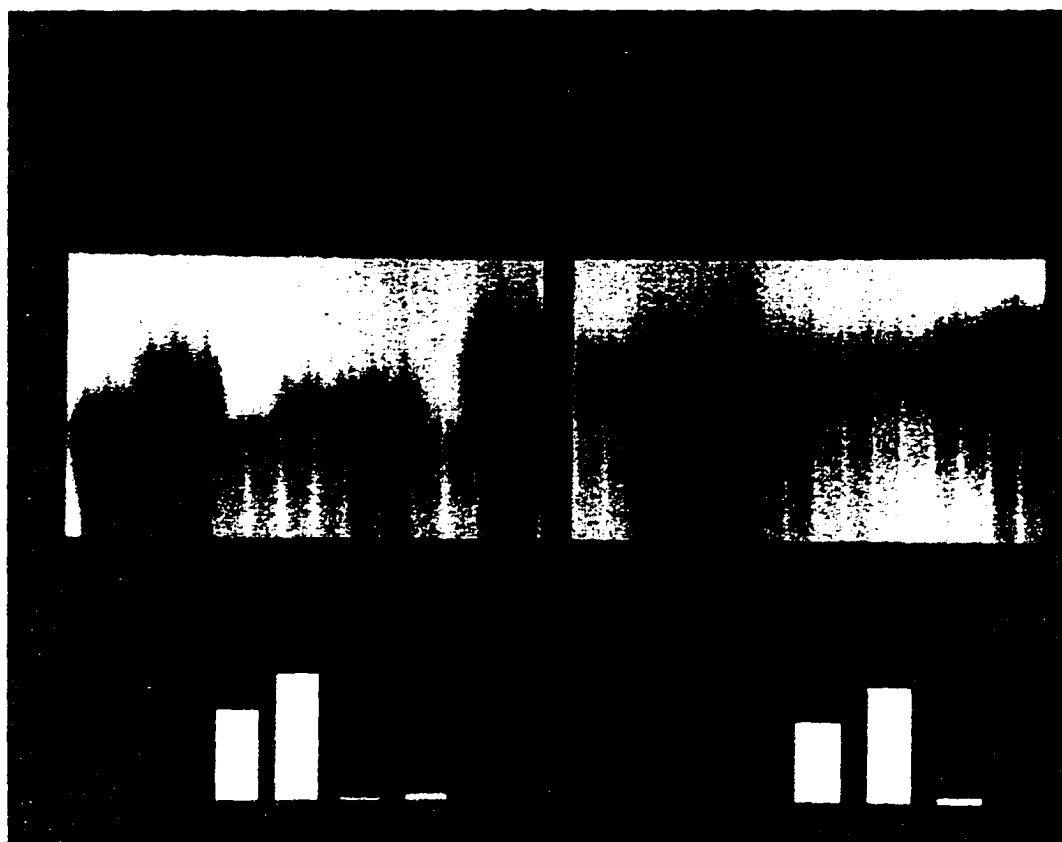


Fig. 6. Lipid labeling by DG kinase for determination of endogenous ceramide release by C₆-ceramide analogs.

Panel A: 750 pmoles of the different ceramide analogs and type III ceramide were labelled by DG kinase to determine if they can be phosphorylated. The assay was carried out using cardiolipin/octyl glucoside micelles as described in the Materials and Methods. Panel B: Lipids extracted from cells (3×10^6 cells/lane) that had been treated with $10 \mu\text{M}$ of the different compounds indicated were labelled by the DG kinase method to estimate the ceramide content. Type III ceramide (100, 400 and 1500 pmoles, from left to right) was used as standard. Panels C and D: Ceramide bands were scraped from the silica gel plates and counted in a scintillation counter.

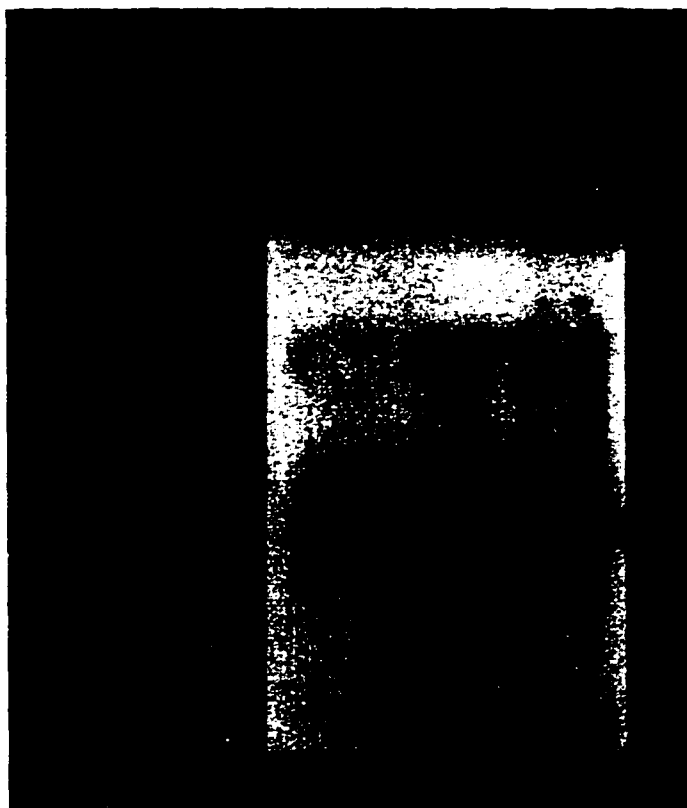


Fig. 7. In-gel renaturation of CAPK assay. An autoradiogram of a CAPK assay.

A 97 and 110 kDa proteins are autophosphorelated in the presence of either 10 μ M D-e-C₈-Cer or D-t-C₈-Cer. Each lane contains equal amounts of membrane proteins isolated from U937 cells. The control contains membrane proteins from untreated cells.



Fig. 8. Okadaic acid does not prevent DNA fragmentation induced by D-e-C₈-Cer.

Low molecular weight DNA isolated from U937 cells (5×10^6) treated with either 10 nM okadaic or 10 μ M D-e-C₈-Cer or with 1, 5 or 10 nM okadaic acid and 10 μ M D-e-C₈-Cer for 18 hr. Control cells were treated with ethanol (0.1% final volume) for 18 hr. MW, molecular weight markers (ϕ X174 DNA/Hae III)

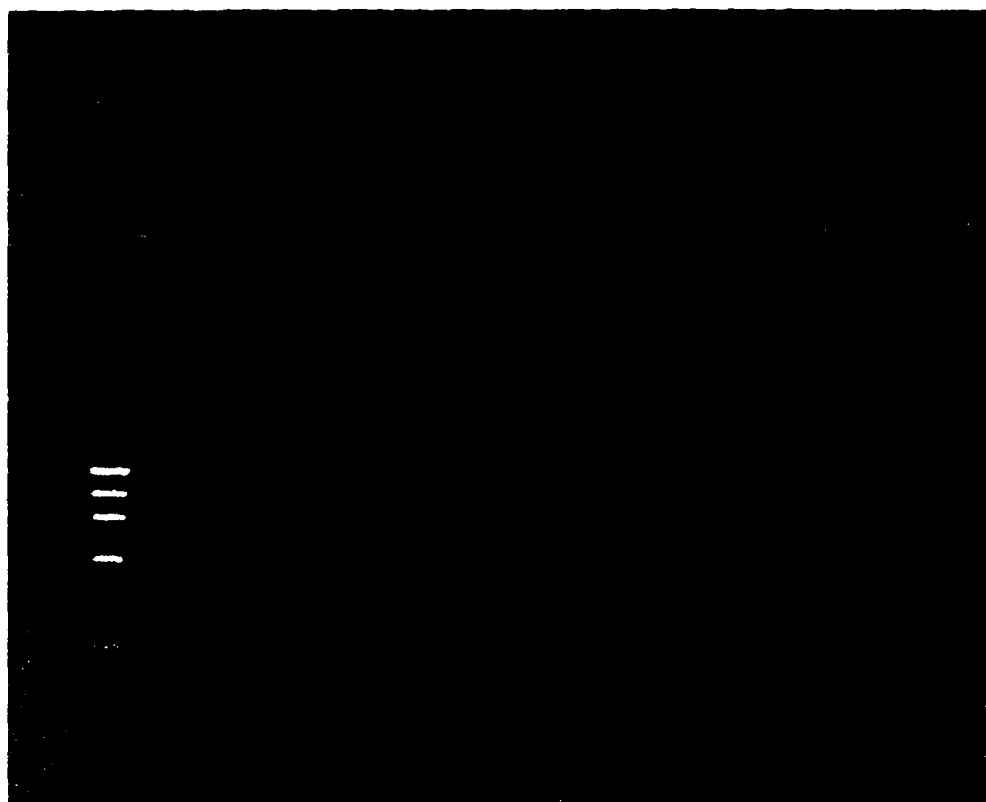


Fig. 9. Treatment of FL5.12 and FL5.12-bcl-2 cells with D-e-C₈-Cer.

FL5.12 and FL5.12-bcl2 cells (5×10^6) were treated with $10 \mu\text{M}$ D-e-C₈-Cer for 3, 6 and 12 hr. At the end of the incubation the low molecular weight DNA was isolated and electrophoresed in a 2% agarose gel. DNA isolated from cells treated with ethanol (0.1% final volume) were used as control. MW, molecular weight markers ($\phi\text{X174 DNA/Hae III}$)

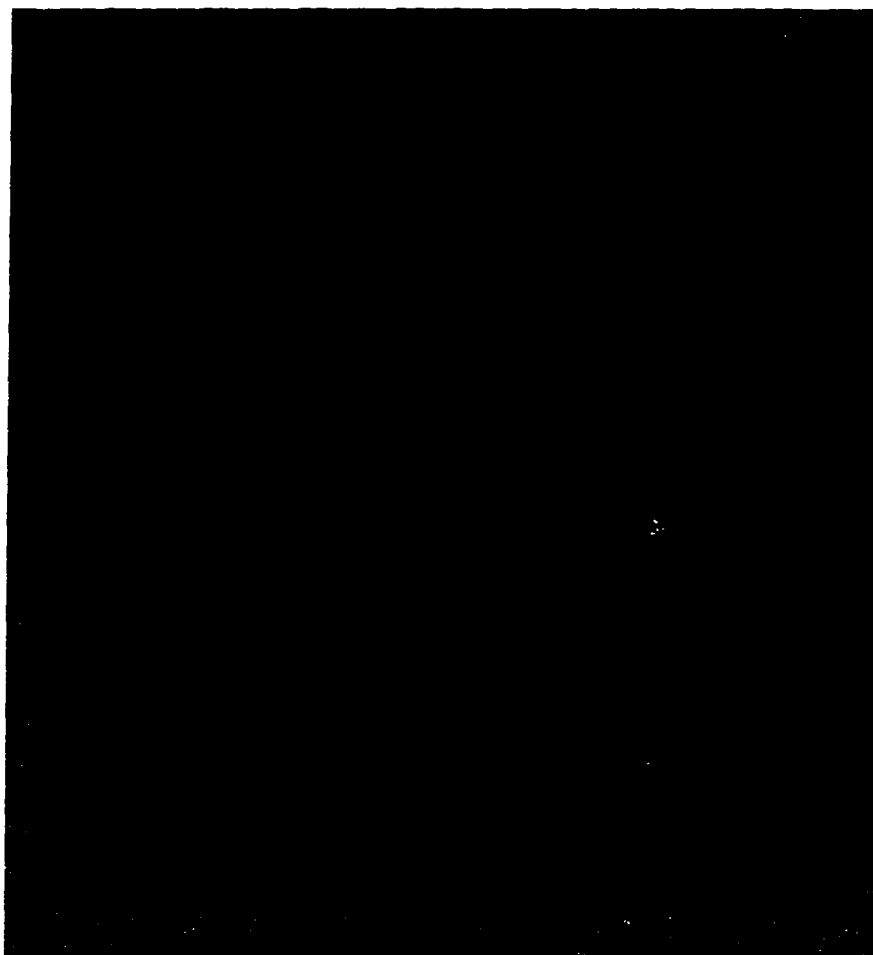


Fig. 10. FL5.12 and FL5.12-bcl2 cells were treated with the C₈-Cer stereoisomers or other analogs.

Panel A: FL5.12 cells (5×10^6) were treated with 10 μ M C₈-Cer stereoisomers and analogues for 18 hr. At the end of the incubation the cells were lysed and the low molecular weight DNA was electrophoresed in a 2% agarose gel. Panel B: The same as panel A but use FL5.12-bcl2 cells instead. In control the cells were treated with ethanol (0.1% final volume) instead of ceramide. MW, molecular weight markers (ϕ X174 DNA/Hae III).

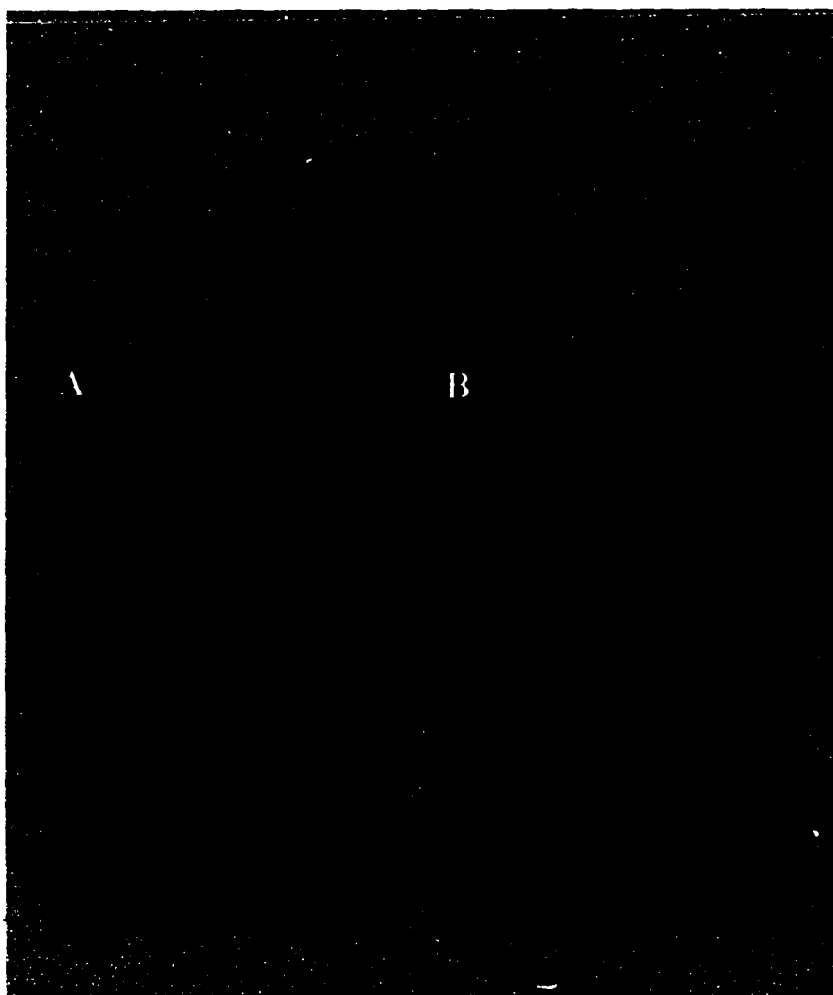


Fig. 11. Time course of DNA fragmentation induced by TNF- α and D-e-C₈-Cer.

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA extracted from U937 cells (5×10^6) treated with 10 ng/ml TNF- α for 30, 60, 90, 120, 180 and 360 min or with 10 μ M D-e-C₈-Cer for 3, 6, 12 and 18 hr. Panel A: DNA fragmentation ladders from U937 cells treated with TNF- α for 30, 60, 90, 120, 180 or 360 min. The last lane contains DNA from U937 cells treated with 10 μ M D-e-C₈-Cer for 360 min. Cells incubated with media containing 1% FCS for 360 min were used as control. Panel B: DNA fragmentation ladders from U937 cells treated with D-e-C₈-Cer for 3, 6, 12 and 18 hr. The last lane contains DNA from cells treated with TNF- α for 18 hr. Cells treated with ethanol (0.1% final volume) were used as control. MW, molecular weight markers (ϕ X174 DNA/Hae III).

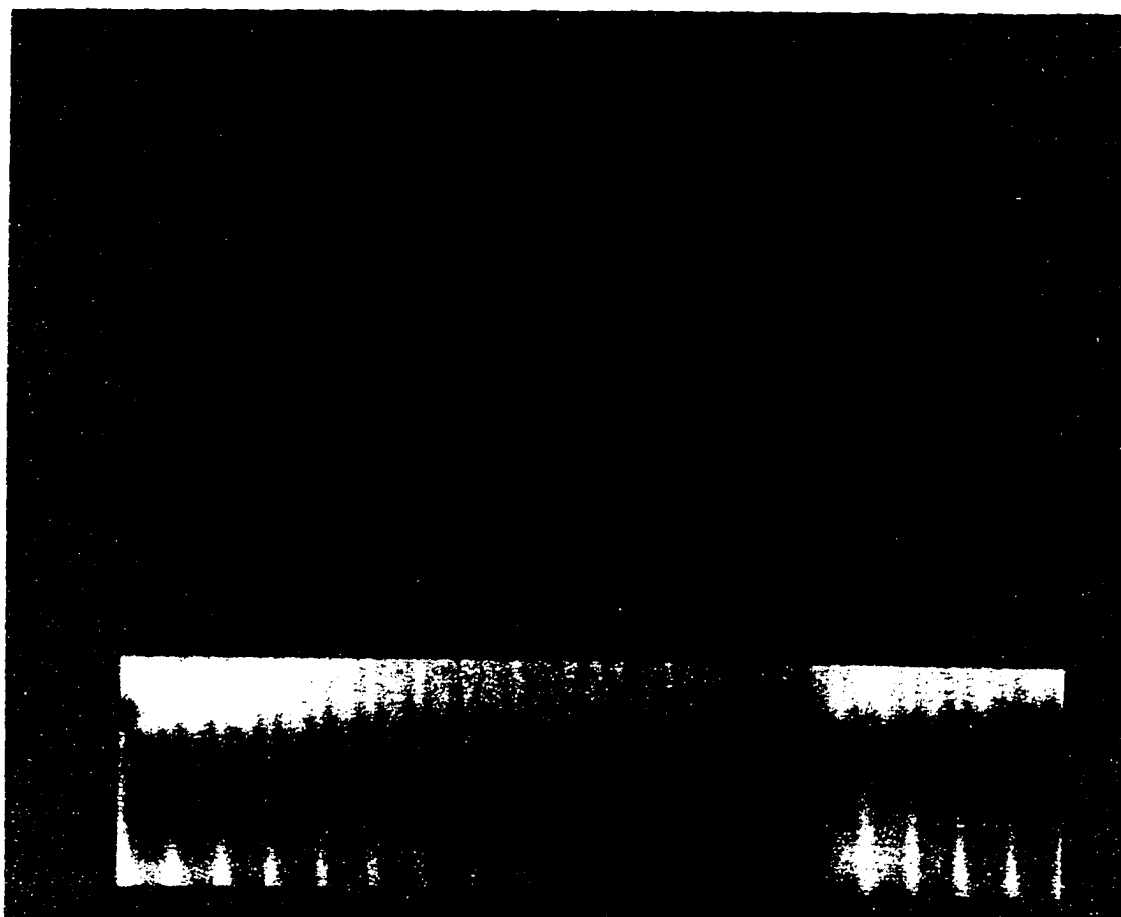


Fig. 12. Endogenous levels of ceramide in cells treated with TNF- α or D-e-C₈-Cer.

Labeling and quantitation of the endogenous level of ceramide of U937 cells treated with 10 ng/ml TNF- α or 10 μ M D-e-C₈-Cer for various times. Ceramide bands were scraped from the silica gel plates and counted in a scintillation counter. Left upper panel: U937 cells treated with TNF- α for 60, 120, 180 and 360 min. Increase in endogenous ceramide is expressed as percent increase from control (untreated) cells. Top right panel: Endogenous ceramide levels from U937 cells treated with D-e-C₈-Cer for 2, 7.5, 15, 30, 60 and 180 min. Panel C: The autoradiogram of the TLC plate containing the ceramide bands that were scraped and counted in a scintillation counter shown in the right upper panel.

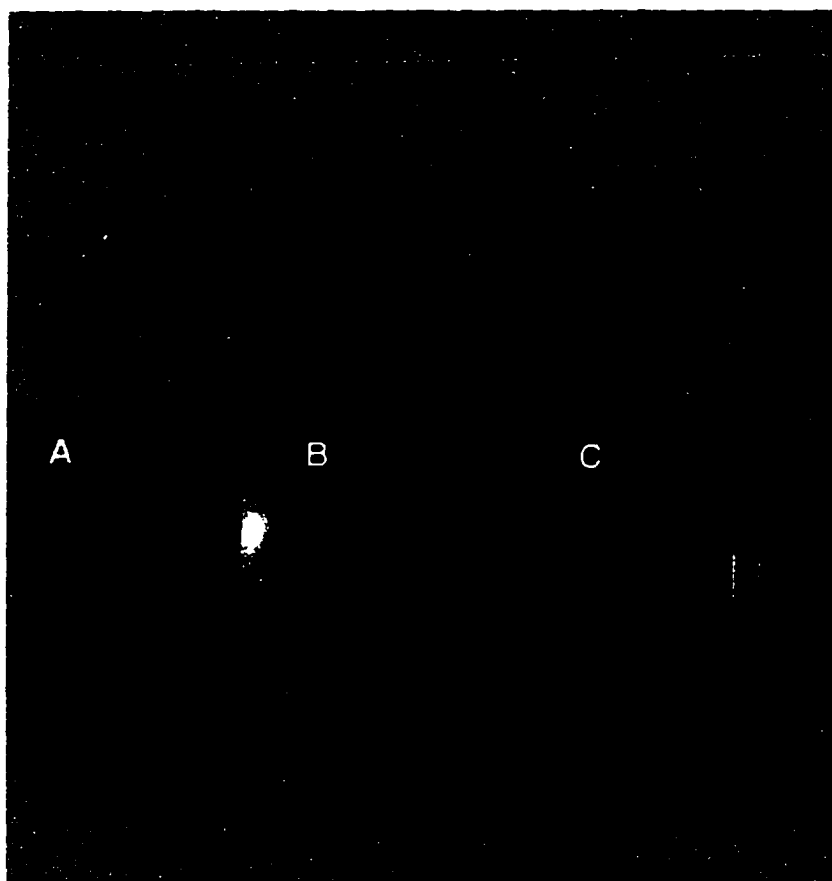


Fig. 13. Agarose gel electrophoresis of DNA isolated from U937 cells transiently exposed to TNF- α or D-e-C₈-Cer.

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA extracted from U937 cells (5×10^6) transiently exposed to 10ng/ml TNF- α for 1, 2.5, 30 or 60 or to 10 μ M D-e-C₈-Cer. Panel A: U937 cells were treated with D-e-C₈-Cer for 3 hr, and returned to media containing 1 or 10% FCS with or without 10 μ M D-e-C₈-Cer. The incubation was continued for 15 more hr before the isolation of DNA. Cells treated with 0.1% ethanol for 3 hr were returned to media containing 1% or 10% FCS and used as controls (first 2 lanes, respectively). Panel B: U937 cells treated with TNF- α for 1, 2.5, 30, and 60 min and returned to TNF- α -free media for a total of 180 min before the isolation of DNA. The last two lanes contain DNA from cells treated with 10 ng/ml TNF- α or 10 μ M D-e-C₈-Cer for 180 min, respectively. Cells incubated in media containing 1% FCS for 180 min were used as controls. MW, molecular weight markers (ϕ X174 DNA/Hae III). Panel C: DNA from U937 cells treated with TNF- α for 2 min and returned to TNF- α -free media containing 10 or 1% FCS media for a total of 120 min. The last two lanes contain DNA from cells treated with TNF- α or D-e-C₈-Cer for 120, respectively. The Control is the same as in panel B.

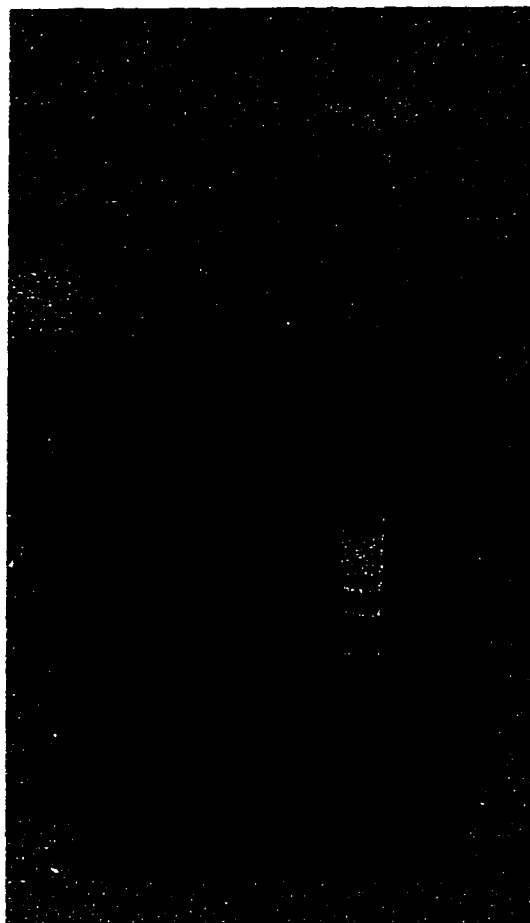
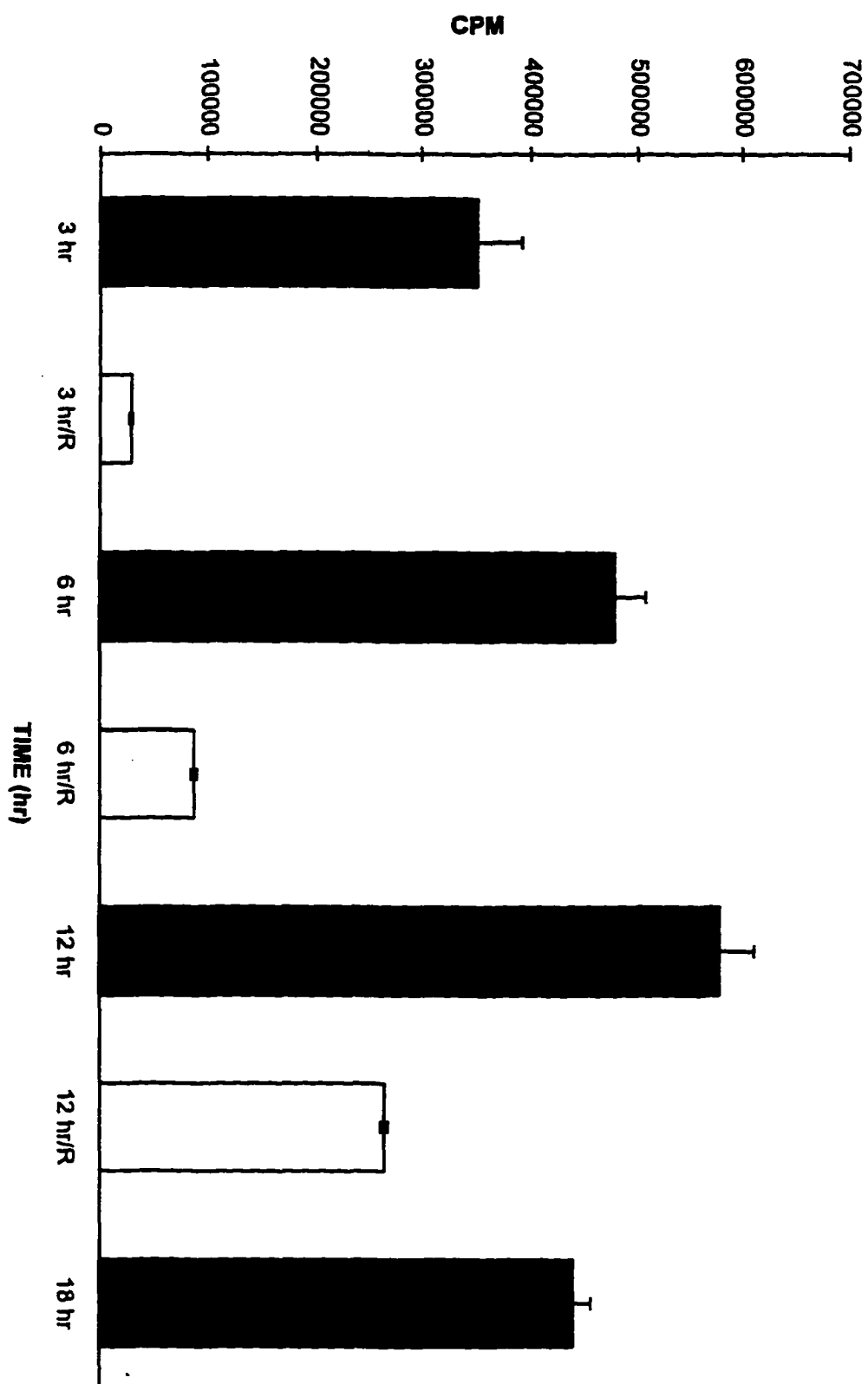


Fig. 14. Agarose gel electrophoresis of DNA isolated from cells transiently exposed to D-e-C₈-Cer or D-t-C₈-Cer. Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA extracted from U937 cells (5×10^6) treated with $10 \mu\text{M}$ D-e-C₈-Cer or D-t-C₈-Cer for 3 hr, returned to media containing 1% FCS and further incubated for 15 more hr before the extraction of DNA. DNA from cells treated with D-e-C₈-Cer or D-t-C₈-Cer for 18 hr is shown (lanes 3 and 5). In control, the cells were exposed to ethanol (0.1% final volume) for 18 hr. MW, molecular weight markers ($\phi\text{X174 DNA/Hae III}$).

Fig. 15. Internalization and degradation of ceramide in U937 cells treated with D-e-C₈-Cer.

Internalization and degradation of ceramide in U937 cells treated with 10 μ M D-e-C₈-Cer for 3, 6, 12 and 18 hr. Shaded bars, intracellular ceramide in cells treated with D-e-C₈-Cer for 3, 6, 12 and 18 hr. Open bars, intracellular ceramide in cells treated with 10 μ M D-e-C₈-Cer for 3, 6 and 12 and then returned to ceramide-free media containing 1% FCS for 15, 12 and 6 hr, respectively.

CERAMIDE UPTAKE AND DEGRATATION IN U937 CELLS

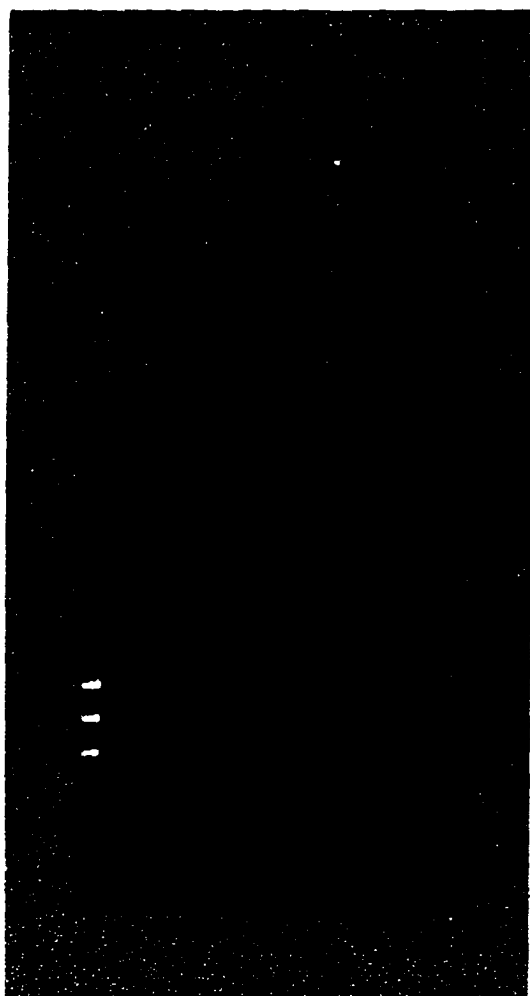


Fig. 16. Internalization of exogenous ceramide does not change the time required by TNF- α to kill cells.

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA isolated from U937 cells (5×10^6) treated with 10 ng/ml TNF- α or preexposed to 10 μ M D-e-C₈-Cer for 15 min before the addition of 10 ng/ml TNF- α . Lanes 4-7 contain DNA from cells treated with TNF- α for 30, 60, 90 and 120 min, respectively. Lanes 8-11 contain DNA from cells treated with D-e-C₈-Cer for 15 min and subsequently treated with 10 ng/ml TNF- α for 30, 60, 90 and 120 min. DNA from untreated cells was used as a control (lane 2). Lane 3 contains DNA from cells incubated with 10 μ M D-e-C₈-Cer for 120 min. MW, DNA molecular weight standards (ϕ X174 DNA/Hae III).

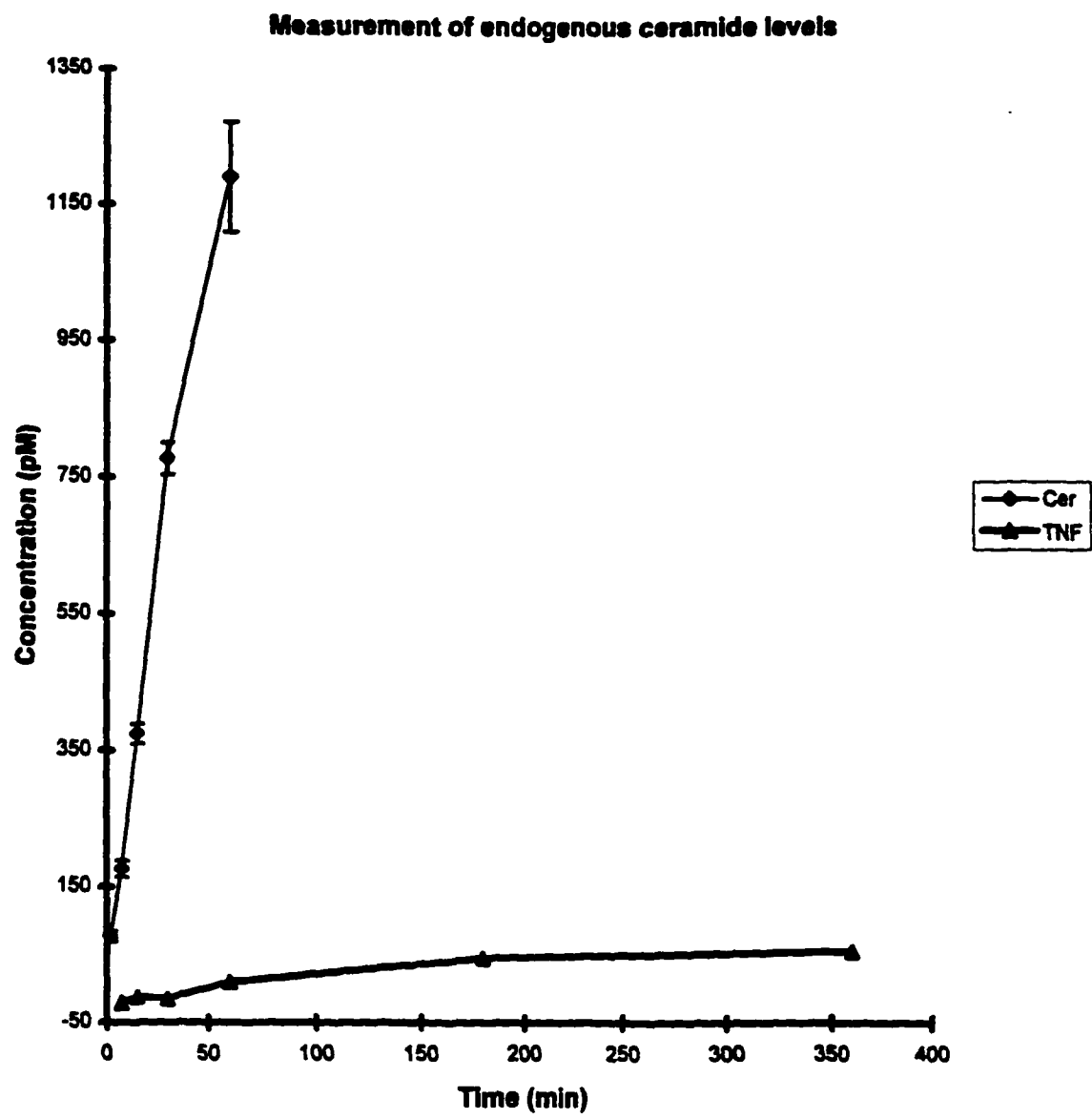


Fig. 17. Acid wash and trypsinization of U937 cells exposed to TNF- α .

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA isolated from cells treated with 0.57 pM/ml TNF- α for 2 min and washed in either 1 X PBS pH 7.4 or 1 X PBS pH 3.0 (acid wash) or treated with 100 μ g/ml trypsin. Cells were returned to media containing 1% FCS for a total of 2 hr before the extraction of the DNA.

Fig. 18. Comparison of the ceramide level in TNF- α and D-e-C₈-Cer treated cells.

U937 cells were treated with 10 ng/ml TNF- α for 7.5, 15, 30, 60, 180 and 360 min or with 10 μ M D-e-C₈-Cer for 2, 7.5, 15, 30 and 60 min. At the end of the incubation the cells were lysed and the intracellular ceramide was labeled as described in the Materials and Methods. Concentration (in pmoles) is expressed as the difference from control. $\blacktriangle$ = pmoles of ceramide from cells treated with TNF- α . $\blacklozenge$ = pmoles of ceramide from cells treated with D-e-C₈-Cer.



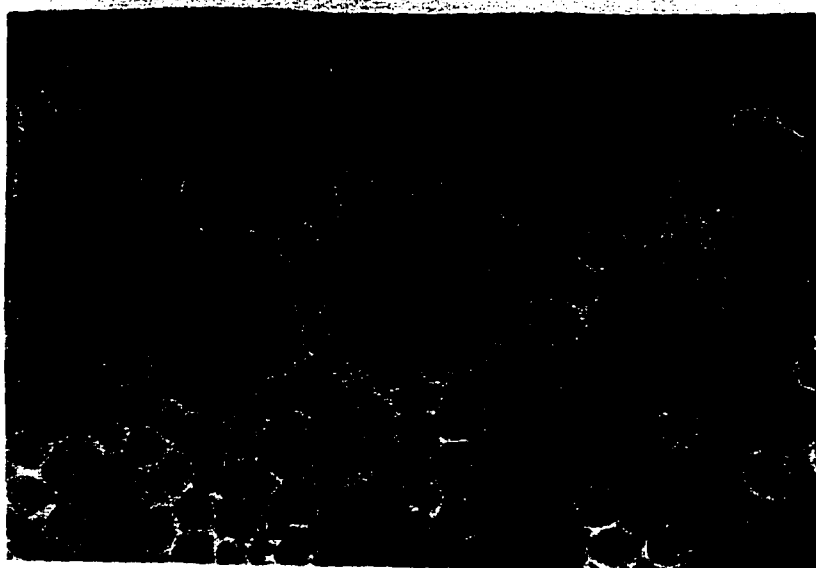
U937**UD-t-IN**

Fig. 19. Phase contrast microscopy of U937 and UDtIN cells. Untreated U937 and UDtIN cells were fixed in 3 % paraformaldehyde and photographed with a Leitz microscope (DMRB), magnification 400 X.

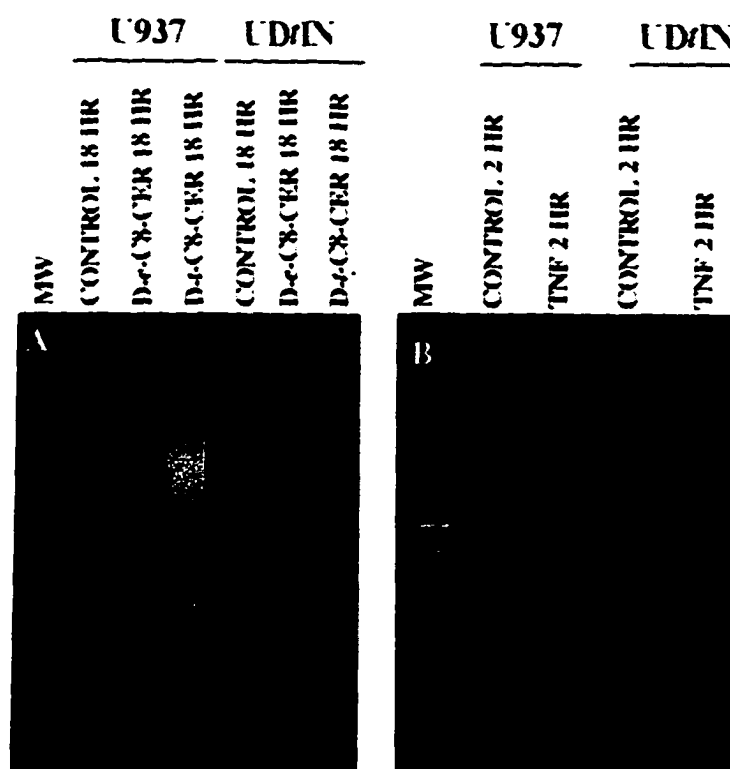


Fig. 20. Cell death induced by ceramide and TNF- α in U937 and UDtIN cells.

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA isolated from U937 and UDtIN cells (5×10^6) treated with $10 \mu\text{M}$ D-e-C₈-Cer or D-t-C₈-Cer or 10 ng/ml TNF- α . Panel A: U937 and UDtIN cells treated with $10 \mu\text{M}$ D-e-C₈-Cer or D-t-C₈-Cer for 18 hr. Panel B: U937 and UDtIN cells treated with or 10 ng/ml TNF- α for 2 hr. Cells treated with ethanol (0.1% final volume) were used as controls. MW, DNA molecular weight markers (ϕX174 DNA/Hae III).

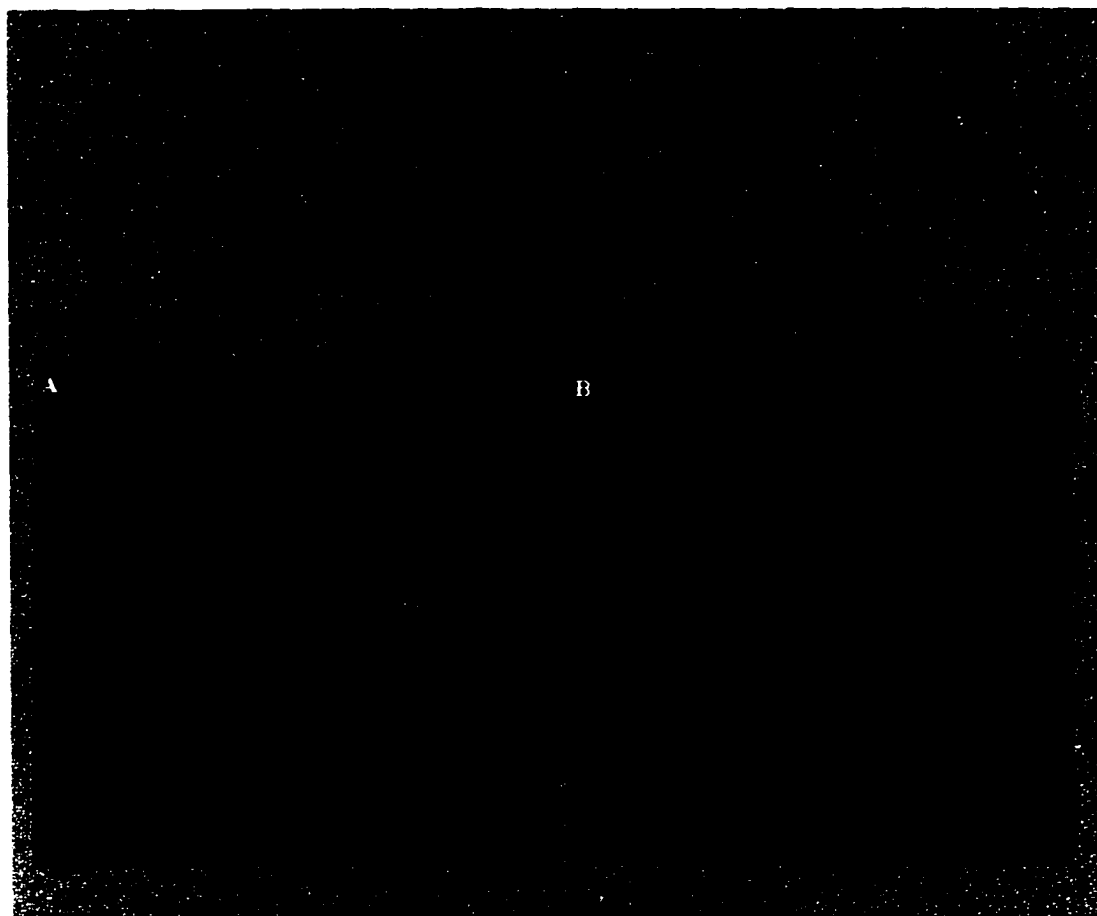


Fig. 21. Induction of cell death by C_8 -Cer stereoisomers and analogs in U937 and UDtIN cells.

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA isolated from U937 and UDtIN cells (5×10^6) treated with D-e- C_8 -Cer, D-t- C_8 -Cer, D-e- C_8 -Ceramine, D-e- C_8 -cis- $\Delta 4$ -Cer and D-e- C_8 -4-yne-Cer for 3, 6 and 18 hr. Panel A: DNA fragmentation ladders from U937 cells treated with D-e- C_8 -Cer, D-t- C_8 -Cer, D-e- C_8 -Ceramine, D-e- C_8 -cis- $\Delta 4$ -Cer or D-e- C_8 -4-yne-Cer for 3, 6 and 18 hr. Panel B: Low molecular weight DNA from UDtIN cells treated with D-e- C_8 -Cer, D-t- C_8 -Cer, D-e- C_8 -Ceramine, D-e- C_8 -cis- $\Delta 4$ -Cer or D-e- C_8 -4-yne-Cer for 3, 6 and 18 hr. U937 and UDtIN cells treated with ethanol (0.1% final volume) for 6 or 18 hr were used as controls. MW, DNA molecular weight markers (ϕ X174 DNA/Hae III).

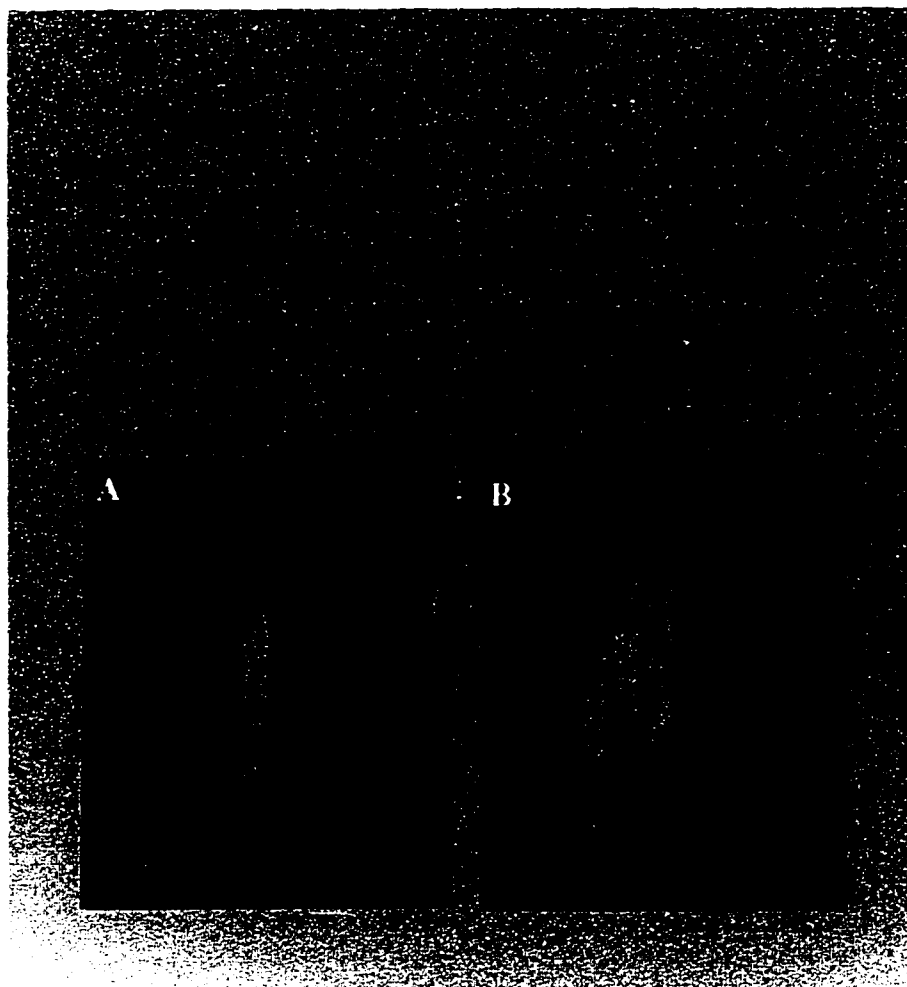


Fig. 22. Induction of cell death in U937 and UDtIN cells treated with ethanol and cycloheximide.

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA isolated from U937 and UDtIN cells (5×10^6) treated with $10 \mu\text{M}$ D-e- C_8 -Cer or D-t- C_8 -Cer or 2.5% ethanol or $50 \mu\text{M}$ cycloheximide for 3 or 18 hr. Panel A: DNA fragmentation ladders from U937 and UDtIN cells treated with D-e- C_8 -Cer, D-t- C_8 -Cer, ethanol or cycloheximide for 3 hr. Panel B: DNA fragmentation ladders from U937 and UDtIN cells treated with D-e- C_8 -Cer, D-t- C_8 -Cer, ethanol or cycloheximide for 18 hr. In control, cells were treated with ethanol (0.1% final volume). MW, DNA molecular weight markers ($\phi\text{X174 DNA/Hae III}$).

Fig. 23. Intracellular ceramide levels in UDtIN cells treated with D-e-C₈-Cer.

Endogenous ceramide labeling of UDtIN cells treated with 10 μ M D-e-C₈-Cer for 2, 7.5, 15, 30, 60, and 180 min. Endogenous ceramide was extracted and labeled as described in Materials and Methods. The radioactive ceramide bands were cut from the TLC plate and counted by scintillation counting. Known concentrations of ceramide type III were labeled in a similar manner and used as standards.

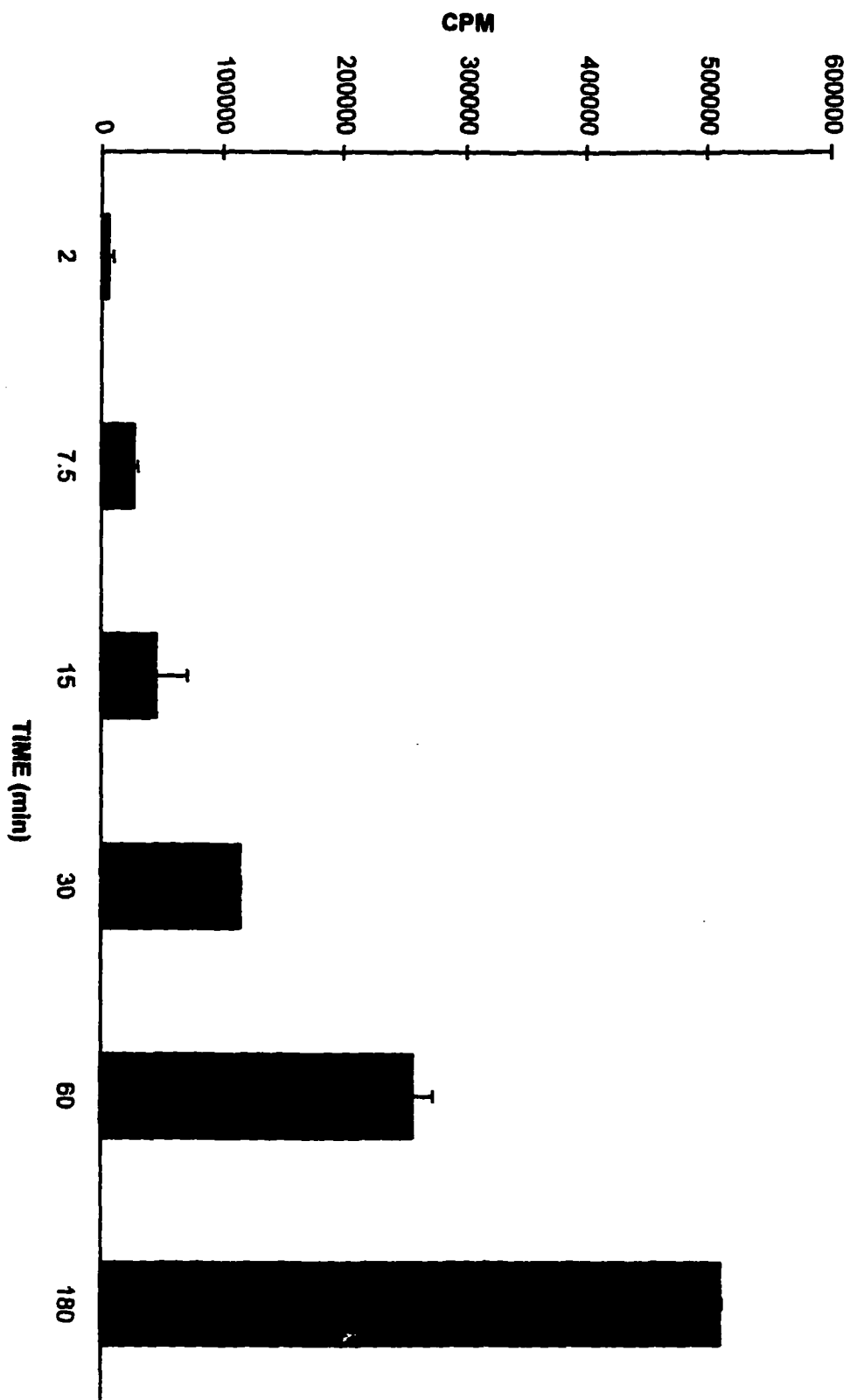
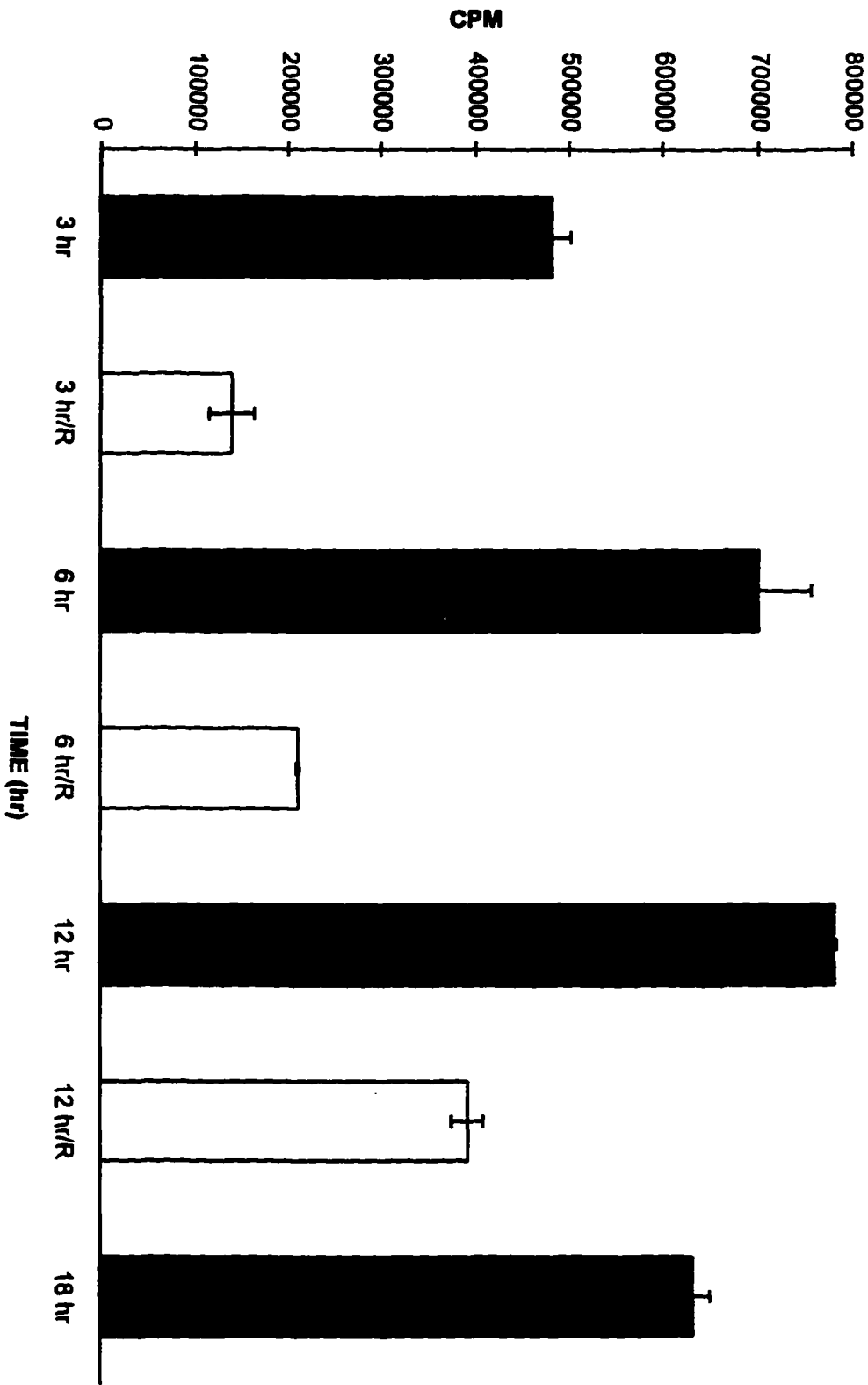
ENDOGENOUS CERAMIDE LEVELS IN CERAMIDE-TREATED UDIN CELLS

Fig. 24. Ceramide internalization and degradation in UDtIN cells.

Internalization and degradation of ceramide in UDtIN cells treated with 10 μM D-e-C₈-Cer for 3, 6, 12 and 18 hr. Ceramide was extracted and labeled as described in the Materials and Methods. Shaded bars, intracellular ceramide in cells treated with 10 μM D-e-C₈-Cer for 3, 6, 12 and 18 hr. Open bars, intracellular ceramide in cells treated with 10 μM D-e-C₈-Cer for 3, 6 and 12 and returned to ceramide-free media containing 1% FCS for 15, 12 and 6 hr, respectively.

CERAMIDE UPTAKE AND DEGRADATION IN U937 CELLS



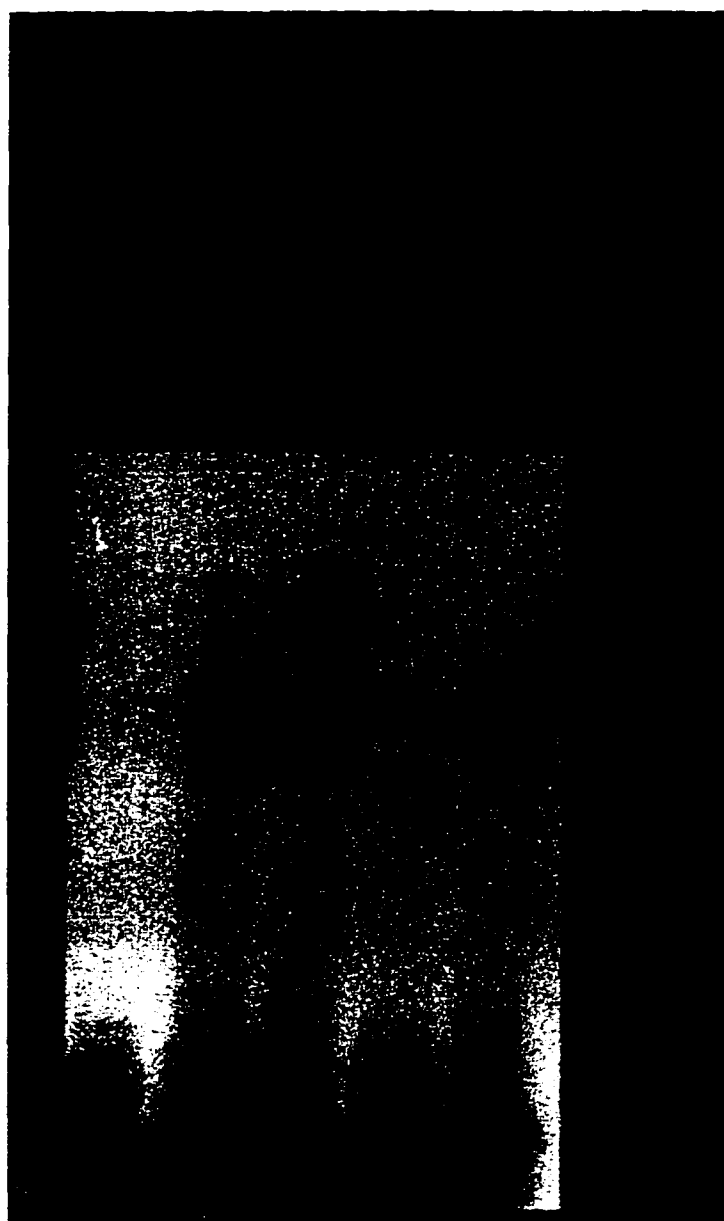


Fig. 25. Mobility shift assay of UDtIN cells treated with TNF- α or D-e-C₈-Cer.

UDtIN cells treated with 10 μ M D-e-C₈-Cer or 10 ng/ml TNF- α for 60 min and the nuclear extracts were isolated and electrophoresed in a non-denaturing acrylamide gel as described in Materials and Methods. The arrow indicates the complex formed by the translocated isoform of NF- κ B with the ³²P labeled oligonucleotide. The three lower bands represent non-translocated NF- κ B residing in the nucleus. The bands seen at the bottom of the gel in all samples represent free ³²P labelled probe.

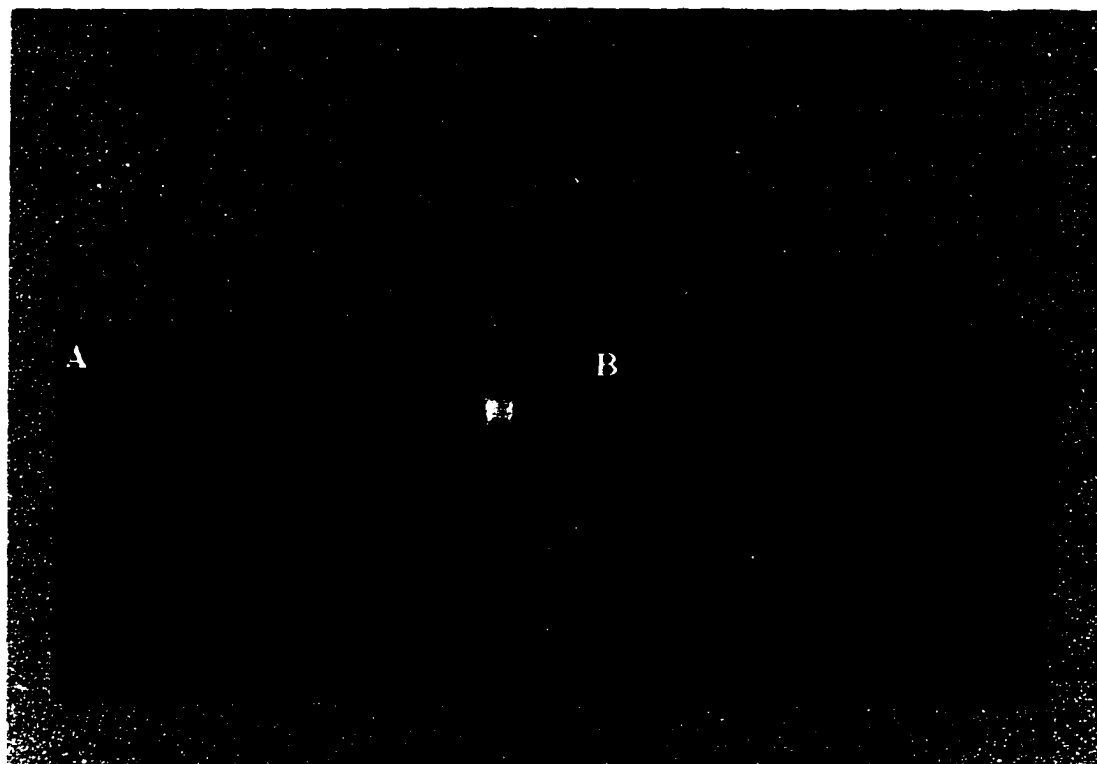


Fig. 26. The resistance of UDtIN cells depends on the activity of PP2A.

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA isolated from U937 or UDtIN cells (5×10^6) treated with $10 \mu\text{M}$ D-e- C_8 -Cer or 10 ng/ml $\text{TNF-}\alpha$ in the presence or absence of different concentrations of the PP2A phosphatase inhibitor okadaic acid. Panel A: UDtIN cells were treated with 0.1, 0.5, 1.0, 10.0 or 15 nM okadaic acid and $10 \mu\text{M}$ D-e- C_8 -Cer for 18 hr. Cells incubated in the presence of only D-e- C_8 -Cer or okadaic acid are also shown. Control cells were treated with 0.1% ethanol for 18 hr. Panel B: U937 and UDtIN cells were treated with both okadaic acid and $\text{TNF-}\alpha$ for 2 hr. Cells treated with only $\text{TNF-}\alpha$ or okadaic acid are also shown. In control, the cells were treated with ethanol (0.1% final volume) for 2 hr. MW, DNA molecular weight markers ($\phi\text{X174 DNA/Hae III}$).

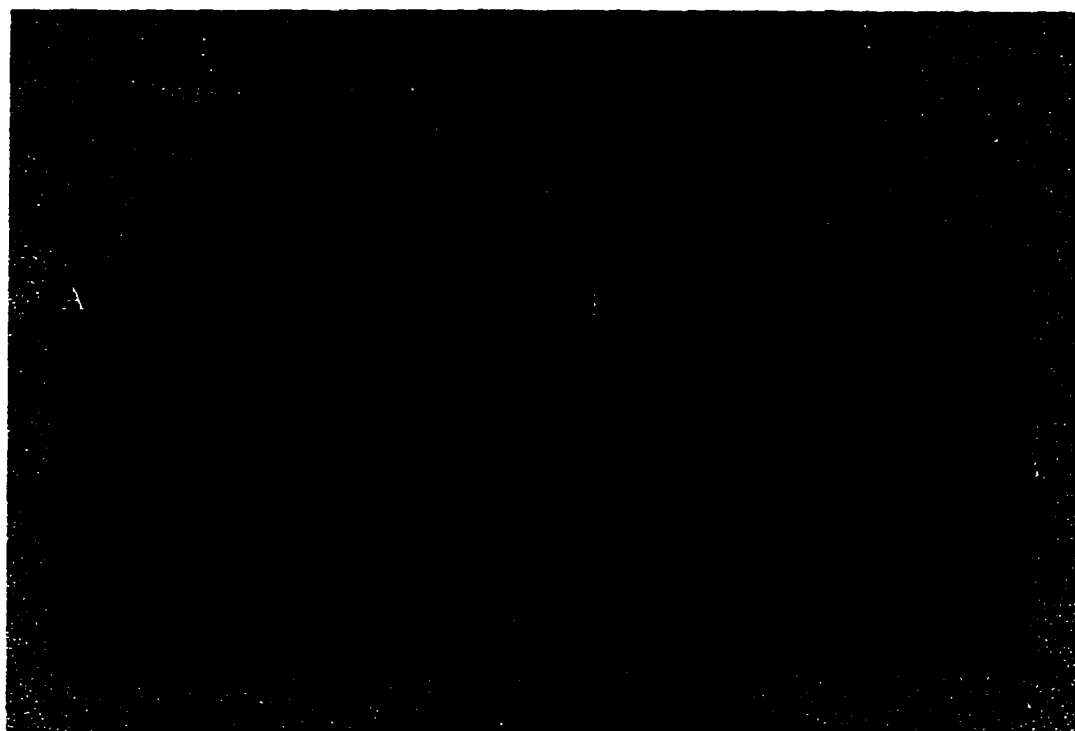


Fig. 27. Tautomycin and calyculin A do not sensitize UDtIN cells to killing by ceramide.

Agarose gel electrophoresis and ethidium bromide staining of low molecular weight DNA isolated from UDtIN cells (5×10^6) treated with different concentrations of the phosphatase inhibitors tautomycin or calyculin A and $10 \mu\text{M}$ D-e- C_8 -Cer for 18 hr. Panel A: UDtIN cells were treated with 160 nM tautomycin alone or with 40, 80 or 160 nM tautomycin and $10 \mu\text{M}$ D-e- C_8 -Cer for 18 hr. In control the cells treated with 0.1% ethanol for 18 hr. Panel B: UDtIN cells were treated for 18 hr with different concentrations of calyculin A alone or with both calyculin A and D-e- C_8 -Cer. In control, the cells were treated with ethanol (0.1% final volume) for 18 hr. MW, DNA molecular weight markers ($\phi\text{X174 DNA/Hae III}$).

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