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THE ROLE OF SPHINGOLIPIDS IN THE CONTROL OF APOPTOSIS IN HUMAN PLACENTAL PRIMARY TROPHOBLASTS

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY**

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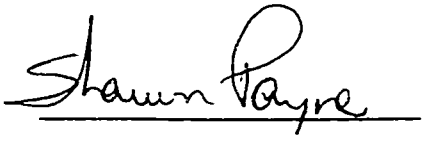

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **The Role of Sphingolipids in the Control of Apoptosis in Human Placental Primary Trophoblasts** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**.

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Abstract

Apoptosis in placental trophoblasts appears to be a normal, regulated event that can be induced by TNF/IFNγ (Tumour necrosis factor/interferon-gamma) and inhibited by EGF (epidermal growth factor). A higher than normal incidence of apoptosis in the placenta has been linked to some complications during pregnancy. In this study I investigated the role of sphingolipids in determining the fate of trophoblasts in response to TNF/IFNγ and EGF. In addition, I investigated whether trophoblasts express the death receptor Fas.

Cultured primary human cytotrophoblasts express Fas antigen on the cell surface. Western blot and RT-PCR analysis indicate that the Fas protein is full length. However, cross-linking anti-Fas antibody and Fas ligand expressing cells did not induce apoptosis. Pretreatment with IFNγ and treatment with protein synthesis inhibitors did not sensitize trophoblasts to Fas-mediated apoptosis. The role of Fas in trophoblast biology is still unclear.

Sphingolipids have been shown to play a role in both cytokine-induced cell death and survival. Activation of sphingomyelinase (SMase), and the resulting increase in cellular ceramide has been implicated in TNFα-induction of apoptosis while the activation of sphingosine kinase, and the increase in sphingosine 1-phosphate (SPP), have has been shown to be anti-apoptotic. Exogenous ceramide and acid SMase induce apoptosis in trophoblasts, which can be completely abrogated by cotreatment with EGF. EGF lowered endogenous basal ceramide levels and reduced the level of acid SMase-induced ceramide formation. An acidic ceramidase inhibitor increased cellular ceramide levels and induced apoptosis that could not be blocked by cotreatment with EGF.

Exogenous bacterial (neutral) SMase increased ceramide levels but did not induce apoptosis. In addition, an inhibitor of alkaline ceramidase increased ceramide levels but did not induce apoptosis nor inhibit EGF protection from apoptosis. These data would suggest a role for acid SMase and acid ceramidase in the control of apoptosis in trophoblasts. The addition of SPP to trophoblast cultures inhibited TNF/IFNγ and ceramide induced apoptosis. Inhibitors of sphingosine kinase induced apoptosis, which could not be blocked by EGF. Moreover, EGF activated sphingosine kinase activity, suggesting a role for sphingosine kinase activity, and therefore SPP, in EGF induced protection from apoptosis as well as presents a novel, EGF-regulated mechanism for the activation of sphingosine kinase.

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ABBREVIATIONS

aSMase	Acid sphingomyelinase
bp	base pair
BSA	Bovine serum albumin
bSMase	Bacterial sphingomyelinase
CAPK	Ceramide activated protein kinase
CAPP	Ceramide activated protein phosphatase
CHX	Cycloheximide
CT	Cytotrophoblasts
DDW	Double distilled water
DED	Death effector domain
DISC	Death-inducing signalling complex
D-MAPP	D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol.
Edg	Endothelial differentiation gene
EGF	Epidermal Growth Factor
FADD	Fas associated death domain
FCS	Fetal calf serum
FLICE	FADD-like ICE (Caspase 8)
FLIP	FLICE inhibitory protein
IFNγ	Interferon-γ
IMDM	Iscove's Modified Dulbecco's medium
IUGR	Intrauterine growth retardation
JNK	Jun N-terminal Kinase
kbp	kilobase pair
kDa	Kilodaltons
MAPK/ERK	Mitogen activated protein kinase/extracellular response kinase
MDC	Monodansylcadaverine
MHC	Major histocompatibility complex

MTT	3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide
NAME	N-nitro-L-arginine methyl ester
NMMA	N-monomethyl-L-arginine monoacetate salt
NO	Nitric Oxide
NOS	Nitric Oxide synthase
nSMase	Neutral sphingomyelinase
OE	*N*-oleoylethanolamine
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCD	Programmed Cell Death
PC-PLC	Phosphocholine specific-phospholipase C
PDGF	Platelet Derived Growth Factor
PMA	Phorbol myristate acetate
PS	Phosphatidylserine
RIP	Receptor-interacting protein
RT	Room temperature
SAPK	Stress activated protein kinase
SD	Standard Deviation
SODD	Silencer of death domain
SPP	Sphingosine 1-phosphate
ST	Syncytiotrophoblasts
TdT	Terminal deoxynucleotidyl transferase
TNF	Tumour necrosis factor α
TNF-RI	TNF Receptor Type 1 (p55)
TNF-RII	TNF Receptor Type 2 (p75)
TRADD	TNF-receptor associated death domain
TUNEL	TdT-mediated dUTP-biotin nick end labelling

Chapter 1 : Introduction

Programmed cell death (PCD) is a regulated, physiological cell death in which a cell plays an active role in its own demise and was first recognized as part of a natural process to remove excess cells during vertebrate embryonic development (Glucksman, 1951; Saunders, 1966). It is now evident that PCD plays a fundamental role in a variety of physiologic and pathologic situations in post-developmental cells. In a multicellular organism the number of cells in any given organ or tissue must be controlled to maintain a constant size. Cells that could interfere with organ function, such as senescent or damaged cells, must also be removed. PCD also plays a critical role in immune system functions. Autoreactive and non-functioning lymphocytes are eliminated by PCD, and the cytotoxic effects of cytotoxic immune cells and cytokines in the elimination of viral infected cells and transformed cells are due to PCD. PCD is therefore a tightly controlled and regulated event in the maintenance of tissue homeostasis

1.1 Programmed Cell Death/Apoptosis

In describing cell death, apoptosis is often used synonymously with PCD, and all non-necrotic forms of cell death have been classified as apoptosis. However, apoptosis describes a particular form of programmed cell death characterized by particular morphological changes that occur during death (Kerr *et al.*, 1972). The chromatin condenses and compacts against the nuclear membrane, the cell shrinks while the cell membrane remains intact, cytoplasmic proteins are extensively cross-linked by the action of transglutaminase, and the cell detaches from the adjacent cells (reviewed in Allen *et al.*, 1997). The cell forms cytoplasmic buds, or "blebs", which eventually detach from the cell to form membrane bound fragments known as apoptotic bodies. Sometimes the entire cell will condense into a single apoptotic body. The integrity of apoptotic bodies and containment of the cytoplasmic contents is probably maintained by the action of transglutaminase and the resultant cross-linking of the proteins (Piacentini *et al.*, 1991; Fesus *et al.*, 1991). Apoptotic bodies are rapidly phagocytosed by macrophages and surrounding epithelial cells. The containment of cellular contents within membrane-

bound bodies and their rapid phagocytosis prevent their spillage into the surrounding tissues, therefore prevent an ensuing inflammatory response that could damage the neighbouring cells and tissues.

One of the hallmark biochemical events of apoptosis has been the internucleosomal cleavage of double stranded DNA into 180-200 bp fragments by Ca^{2+}/Mg^{2+}-dependent endonucleases. Large DNA fragments (50-300 kbp) appear first, followed by the smaller fragments that may be derived from the larger fragments (Wyllie, 1980b; Sun & Cohen, 1994). DNA cleavage does not appear to be the lethal event in apoptosis but may serve as a mechanism to break the DNA into smaller pieces for packaging and disposal. However, internucleosomal DNA fragmentation does not always occur in cells displaying characteristic apoptotic morphology, such as nuclear condensation and blebbing.

Very early in apoptosis the cell undergoes plasma membrane changes, namely the appearance of phosphatidylserine (PS) on the outer leaflet of the cell membrane (Fadok *et al.*, 1992). In healthy cells, PS is normally found on the inner leaflet of the plasma membrane. During apoptosis there is a redistribution of lipids and PS rapidly appears in the outer leaflet. This event is common to many cells types undergoing apoptosis (Martin *et al.*, 1995a). The mechanism of PS redistribution is not clearly understood, but appears to require the activation of a non-specific lipid "scramblase" (Verhoven *et al.*, 1995; Bratton *et al.*, 1997), and the loss of aminophospholipid translocase activity (Bratton *et al.*, 1997), which normally maintains the asymmetrical distribution of PS. The appearance of PS on the cell surface is believed to serve as the recognition signal on apoptotic cells for phagocytosis by macrophages and epithelial cells (Fadok *et al.*, 1992a, b).

1.2 Non-apoptotic Cell Death

1.2.1 Necrosis

Necrosis is a physiologically relevant form of cell death that is not programmed cell death, and does not occur in a developmental context. It is generally induced by physical trauma to a cell, such as a noxious compound or physical insult that disrupts

osmotic balance (Wyllie *et al.*, 1980a; Farber, 1990). The result is cellular swelling, including organelle damage, until the cell membrane ruptures and cellular contents are spilled into the surrounding tissue which can lead to an inflammatory response and second tissue damage. It is generally considered a "passive" cell death in which the cell does not play a role in its own demise. Although necrosis does not appear to be under any physiological control, it has physiological implications due to the tissue damage from the ensuing inflammatory response.

1.2.2 Apoptosis vs Necrosis

As previously stated, apoptosis is not synonymous with PCD, and therefore cell death cannot be classified into the simple dichotomy of apoptosis and necrosis. As early as 1973 (Schweichel & Merker, 1973), and more recently (Clarke, 1990; Zakeri *et al.*, 1995), it was recognized that not all PCD in developing tissues fits neatly into the apoptosis category but display a "necrotic-like" morphology. These include the appearance of large cytoplasmic vacuoles, lack of internucleosomal DNA fragmentation, and little or no pyknosis (reviewed in Kitanaka & Kuchino, 1999). Death receptor (Fas and TNF receptor)-induced death *in vitro* can sometimes be "necrotic-like", depending on the cell type, death receptor ligation, and signalling pathways activated (Schulze-Osthoff *et al.*, 1994; Vercammen *et al.*, 1998a,b). It is therefore important to recognize that there can be different forms of PCD that may be under the control of different cellular mechanisms, yet morphologically look more like necrosis.

1.3 Death Receptors

1.3.1 TNF Receptors

There are two TNF receptors, TNF-RI (p55) and TNF-RII (p75), which are expressed on most cell types (Tartaglia & Goedel, 1992). Binding of TNF to one or both of these receptors initiates a plethora of cell effects, such as cell death, cell proliferation, inhibition of cell proliferation, differentiation, expression of adhesion molecules, and secretion of cytokines, depending on the type of cell and the presence of other cytokines or growth factors. Most of the biological effects of TNF have been attributed to

signalling through TNF-RI, including the induction of apoptosis, and TNF-RI is sometimes referred to as a death receptor. However, there are multi-signalling events initiated by TNF-RI, including both apoptotic and anti-apoptotic, and combined with the potential of multiple cellular outcomes, relating a signalling event to a biological outcome has been problematic and the focus of intense research.

TNF receptors are the prototype receptors of the TNF receptor superfamily (Smith *et al.*, 1994). The family is characterized by a variable number of copies of a cysteine-rich motif in the extracellular domain but, as a family, their cytoplasmic domains share very little homology. Within the family however, the death receptors, such as TNF-R1 and Fas, contain a region of 68 amino acids in the cytoplasmic domain called the "death domain" (Ioth *et al.*, 1993; Tartaglia *et al.*, 1993), so called because mutational studies have shown that this region is required for the induction of death by the death receptors. The death domain is also a region of protein-protein interactions that promotes complexing with death domains of other proteins.

When trimeric TNF binds TNF-R1, the receptors initially trimerize resulting in the aggregation of their death domains, which results in the recruitment and of other cytosolic adaptor proteins containing death domains to form a large receptor-adaptor protein complex called the "death-inducing signalling complex", or DISC (Kischkel *et al.*, 1995). The first event appears to be the removal of a negative regulator called SODD (silencer of death domains: Jiang *et al.*, 1999), which prevents the binding of the adaptor molecules. SODD may be a mechanism of silencing the receptor until TNF binds, and may modulate the duration of the signal because it re-associates with the receptor within minutes of disassociation. The first of the adaptor proteins to make up the DISC is TRADD (TNF-receptor-associated death domain: Hsu *et al.*, 1995; Hsu *et al.*, 1996) (Figure 1.1). Like TNF-R1, it contains a death domain and associates with the receptor via the death domains. FADD (Fas associated death domain protein), another death domain-containing protein associates with TRADD via death domain interactions (Hsu *et al.*, 1996; Chinnaiyan *et al.*, 1996). In addition to a death domain, FADD contains a region called the "death effector domain" (DED), which binds to the DED of pro-caspase8 (FLICE), thereby recruiting pro-caspase 8 into the DISC and facilitating that

activation of caspase 8 (Boldwin *et al.*, 1995; Boldwin *et al.*, 1996; Muzio *et al.*, 1996). The activation and role of the caspase cascade is discussed in the following sections.

RIP (Receptor-interacting protein) is another protein recruited to the receptor complex via death domain interactions. In addition to a death domain it also contains a kinase domain capable of autophosphorylation and an intermediate region (Hsu *et al.*, 1996). It can associate with the receptor itself (Stanger *et al.*, 1995), albeit weakly, much stronger with TRADD (Hsu *et al.*, 1996) as well as with FADD and TRAF2 (TNF receptor-associated factor 2: reviewed in Darnay & Aggarwal, 1997). TRAF2-RIP interactions are not via death domains but may involve the kinase and intermediate domains. Overexpression of RIP or RIP lacking the kinase domain can induce apoptosis (Stanger *et al.*, 1995), and there is good evidence supporting a role of RIP in TNR-R1-induced NF-κB translocation and SAPK/JNK activation (see below). RIP may therefore play a role in the multi-signals generated by TNR-R1.

In addition to the formation of the DISC and the induction of apoptosis, TNF-R1 also stimulates NF-κB, which is independent of the induction of apoptosis and moreover, has been shown to be anti-apoptotic (Beg & Baltimore, 1996; Liu *et al.*, 1996; Wang *et al.*, 1996; Van Antwerp *et al.*, 1996; Qin *et al.*, 1999). NF-κB is activated when the inhibitory molecule, IκB, is phosphorylated and degraded, releasing it from NF-κB thereby permitting NFκB to translocate to the nucleus. IκB is phosphorylated by a multisubunit kinase containing the catalytic subunits IκB kinase α and β (IKKα and IKKβ) and a non-catalytic subunit IKKγ (NF-κB Essential Modulator; NEMO) (Mercurio *et al.*, 1997; Regnier *et al.*, 1997; Zandi *et al.*, 1997; Yamoaka *et al.*, 1998). This large complex, called a "signalosome" (Mercurio *et al.*, 1997) is recruited to TNF-R1. The adaptor molecules RIP and TRAF2 are involved in the activation of NF-κB (Hsu *et al.*, 1996; Ting *et al.*, 1996; Yeh *et al.*, 1997; Kelliher *et al.*, 1998), but defining their precise roles has been problematic because of the multiple interactions of RIP and TRAF2 with other receptor-complex components. More recently it was shown that TRAF2 is required for "signalosome" recruitment to the receptor and RIP is required for its activation (Devin *et al.*, 2000).

Another major signalling pathway induced by TNF-R1 is the sphingolipid

pathway, which will be thoroughly discussed in a subsequent section. TNF activates several neutral sphingomyelinases and an acid sphingomyelinase, leading to an increase in ceramide levels, which induces apoptosis (Obeid *et al.*, 1993; Jarvis *et al.*, 1994). The link between the receptor and the activation of these enzymes is not well understood. A region of the receptor corresponding to the death domain (Wiegmann *et al.*, 1994) and the recruitment of FADD to the receptor are required for the TNF-induced activation of acid SMase (Wiegmann *et al.*, 1999). A small region membrane-proximal to the death domain, designated neutral SMase activating domain (NSD) is necessary for neutral SMase activation (Adam *et al.*, 1996). FAN (Factor Associated with neutral SMase activation) associates with NSD and appears to be required for the activation of neutral SMase (Adam-Klages *et al.*, 1996).

TNF also activates the SAPK/JNK pathway (Westwick *et al.*, 1995; Natoli *et al.*, 1997; Allan-Yorke *et al.*, 1998). However, little is known about the receptor proximal events leading to its activation. A requirement for TRAF2 and RIP (Liu *et al.*, 1996; Lee *et al.*, 1997; Natoli *et al.*, 1997; Reinhard *et al.*, 1997) recruitment has been implicated in some studies while another using *rip*$^{-/-}$ mice could not find evidence for the requirement of RIP (Kelliher *et al.*, 1998). Signalling events between TRAF2 and/or RIP and SAPK/JNK are poorly understood. The role of SAPK/JNK in TNF-induced apoptosis is also not well understood. SAPK/JNK is generally considered to be involved in the induction of apoptosis (Xia *et al.*, 1995), and variety of other stimuli, such as Fas (Goillot *et al.*, 1997) and ionizing radiation (Haimovitz-Friedman *et al.*, 1994), that induce apoptosis also stimulate SAPK/JNK. The requirement for TRAF2 in TNR-R1-induced SAPK/JNK activation might suggest that SAPK/JNK plays an anti-apoptotic role. In fact, it has been shown that TNF-induced apoptosis and activation of SAPK/JNK may occur by two separate pathways in different cell types, and may play both apoptotic and anti-apoptotic roles in TNF-R1 signalling (Liu *et al.*, 1996; Natoli *et al.*, 1997). Moreover, early activation of SAPK/JNK by TNF may enhance cell survival in some cell types (Roulston *et* al., 1998). Given the complexity of TNF receptor signalling, it is conceivable that SAPK/JNK may play different roles in TNF-R1 signalling in different cell types.

1.3.2 Fas/CD95

Fas, also known as CD95, is a 40-45-kDa cell surface molecule of the TNF receptor family. Like all members of this family, it contains a region of cysteine-rich repeats in the extracellular domain (Smith *et al.*, 1994). Fas also contains the death domain in its cytoplasmic tail, a characteristic it shares with TNF-R1 (Ioth *et al.*, 1993; Tartaglia *et al.*, 1993). Thus, like TNF-R1, Fas is a death receptor that initiates apoptosis (Itoh *et al.*, 1991; Suda *et al.*, 1993). Fas and TNF-R1 also share some of the components of the receptor signalling complex, or the DISC, as described in the previous section. Fas does not recruit TRADD however, and FADD interacts directly with the death domain of Fas. Concomitantly, caspase-8 (FLICE/MACH) interacts with FADD via their death effector domains, resulting in the activation of caspase-8. Fas is ubiquitously expressed in a variety of tissues with high levels of expression in the thymus, liver, heart and kidney (Watanabe-Fukunaga *et al.*, 1992; reviewed in Nagata, 1997).

The ligand for Fas is Fas ligand (FasL). It is a 40-kDa-membrane protein and is a member of the TNF superfamily (Suda *et al.*, 1993; Beutler & van Huffel, 1994). There is also a 26-kDa functional soluble form of FasL (sFasL) that is produced by activated T cells and can be found in the sera of patients with granular lymphocytic leukemia and natural killer cell lymphoma (Tanaka *et al.*, 1996). FasL is expressed as an inducible protein on activated T cells. Non-activated T cells express FasL at low levels but upon activation, either through TCR activation signals or pharmacological agents such as ionomycin and PMA, FasL expression is up-regulated (Rouvier *et al.*, 1993; Yang *et al.*, 1995; Glass *et al.*, 1996). Activated B cells have also been shown to express FasL (Hahne *et al.* 1996). Although FasL was originally described in the context of T cell-mediated cytotoxicity, other cell types, such as epithelial cells of the eye (Griffith *et al.*, 1995), microglia (Spanaus *et al.*, 1998), Sertoli cells in the testis (Bellgrau *et al.*, 1995) and placental trophoblasts (Runic *et al.*, 1996; Hunt *et al.*, 1997), also express FasL.

The function of Fas-FasL mediated-apoptosis was first described as a T cell mediated event in the context of immune clearance and selection. Cytotoxic T cells, when activated, express FasL and can use Fas-FasL to trigger apoptosis in the cytotoxic

T cell response (Kagi *et al.*, 1994). Fas-FasL interactions also play a role in the down-regulation of the immune response and is involved in activation-induced apoptosis of peripheral T cells, a process that removes activated T cells once they have accomplished their task (Brunner *et al.*, 1995; Mogil *et al.*, 1995). Mice with defective Fas (*lpr*) or FasL (*gld*) expression suffer from lymphoproliferative disease where this is an accumulation of lymphocytes in the lymph nodes and spleen. In *lpr* mice, however, the lymphoproliferative phenotype can be prevented by expression of Fas as a transgene in lymphocytes (Wu *et al.*, 1994), suggesting a role of Fas in the prevention of accumulation of T cells. In addition to an immune function and T cell deletion, Fas-FasL induced cytotoxicity also plays a role in establishing and maintaining sites of immune privilege (Bellgrau *et al.*, 1995; Griffith *et al.*, 1995). These are sites where the cellular destruction from a cytotoxic T cell response would not be beneficial, such as the eye, testes and placenta. Constitutive expression of FasL in the sites induces apoptosis in activated T cells, thereby eliminating the potentially dangerous T cells as they enter or come in contact with these tissues.

1.4 Death Receptor-Induced Apoptosis Signalling Pathways

1.4.1 Caspases

The caspases (cysteine-aspartases) are a family of cytosolic proteases that are considered to be the executioners of apoptosis. The grouping into a family is based on a conserved cysteine residue in the catalytic region of the enzymes and they all have a requirement for an aspartate at the cleavage site of the substrate. The discovery that caspases play a role in apoptosis came from the observation that interleukin-1 β-converting enzyme (ICE) was homologous to the *C. elegans* death protein Ced-3 (Yuan *et al.*, 1993). ICE (caspases 1) became the first and prototype of the ICE family of proteases, later to be called caspases. Although ICE does not appear to play a significant role in apoptosis, its homology to Ced-3 initiated the search for other mammalian cysteine proteases and quickly led to the identification of the mammalian apoptotic ced-3 homolog, apopain/CPP32, or caspase 3 (Nicholson *et al.*, 1995). Since then, at least 14 mammalian caspases have been identified and have been grouped into two subfamilies:

1) those that play a role predominantly in the regulation and execution of apoptosis and 2) those that play a role predominantly in inflammation and cytokine processing (reviewed in Budihardjo *et al.*, 1999). All known caspases are synthesized as proenzymes, or zymogens, and require catalytic cleavage of the pro-domain to become active proteases. The catalytic enzyme responsible for the cleavage is another caspase, either one of the same type (autocatalyis) or a different caspase. The apoptosis caspases are again divided into two functional groups, the initiator caspases (e.g. caspase-8) and the executioner caspases (e.g. caspase-3 & -7).

In receptor-mediated apoptosis, the initiator caspase is caspase 8 (FLICE). As previously described, procaspase-8 is recruited to the DISC by associating with FADD (Boldwin *et al.*, 1995; Boldwin *et al.*, 1996; Muzio *et al.*, 1996). Procaspase-8 appears to have weak intrinsic proteolytic activity, and is converted to activate caspase 8 when they come into close proximity to, and cleave one another within the DISC (Martin *et* al., 1998; Muzio *et al.*, 1998; Yang *et al.*, 1998). Caspase-8 can then activate caspase-3 and -7 by direct cleavage (Muzio *et al.*, 1996; Srinivasula *et al.*, 1996). This direct activation of caspase-3 by caspase-8 may only be significant when there is a sufficiently high level of recruitment and activation of caspase-8 (Kuwana *et al.*, 1998; Scaffidi *et al.*, 1998). Cells capable of receptor-induced activation of caspase-3 in this manner are referred to as type I cells (Scaffidi *et al.*, 1998).

Alternatively, caspase 8 can cleave BID, a pro-apoptotic member of the bcl-2 family, which, when cleaved, translocates to the mitochondria and causes the release of cytochrome c (Li *et al.*, 1998; Luo *et al.*, 1998). The released cytochrome c initiates the oligomerization of Apaf-1 (Apoptotic protease-activating factor-1) and the recruitment of procaspase-9 to form the "apoptosome" (Green & Kroemer, 1998), and it is within the apoptosome that caspase-9 is activated (Li *et al.*, 1997). Cleavage of procaspase 9 may be by autocatalysis similar to the activation of procaspase 8 (Zou *et al.*, 1999). Activated caspase 9 is released from the complex and can directly activate caspase-3 and -7 (Slee *et al.*, 1999). Cells utilizing this death-receptor-induced pathway are referred to as type II cells.

Caspase-3 and -7 are referred to as executioner caspases because the cleavage of

their targets provides the basis for the morphological and biochemical changes observed during apoptosis. The list of caspase targets is extensive and includes nucleic acid repair enzymes (PARP; Lazebnik *et al.*, 1994; Germain *et al.*, 1999), nucleases (ICAD/DFF45; Liu *et al.*, 1997; Enari *et al.*, 1998; Tang *et al.*, 1998) that permit and participate in DNA degradation, structural proteins such as lamins (Lazebnik *et al.*, 1995) that function in the maintenance of nuclear integrity, the cytoskeletal component fodrin (Martin *et al.*, 1995b), as well as a variety of other structural and signalling proteins (reviewed in Miller, 1999). Feedback mechanisms also exist that can amplify the death signal. For example, caspase-3 can cleave caspase-6, which can cleave caspase-8, generating a potential amplification of the signal (Slee *et al.*, 1999). Anti-apoptotic molecules, such as NF-κB (Ravi *et al.*, 1998), bcl-2 (Cheng *et al.*, 1997) and TRAF1 (Irmler *et al.*, 2000) can also serve as caspase targets leading to their inactivation and amplifying the pro-apoptotic signal.

There is growing evidence that caspases may play a central role in the regulation of death receptor-induced apoptosis and the morphological characteristics of apoptosis but are not an absolute requirement for death-receptor-induced cell death. In some cell types, such as L-929 fibroblasts, the U937 monocytic cell line, and NIH 3T3 fibroblasts, the inhibition of caspases does not block death receptor-induced death but causes a switch from an apoptotic morphology to a necrotic-like morphology (Vercammen *et al.*, 1998a,b; Khwaja & Tatton, 1999). A similar phenomenon is seen in drug-induced cell death in B lymphocytes (Lemaire *et al.*, 1998). This has led to the idea that caspases, at least in some cell types, may not be the critical executioners of cell death, but instruct the cell to die by apoptosis when a death signal is received.

1.4.2 Sphingolipid Pathway

The sphingolipids are a large group of lipids which contain a sphingoid backbone. In 1884 J.L.W. Thudichum gave the name "sphingosine" ("Sphinx"-like) to the backbone of sphingolipids because of the many enigmas these lipids presented to the researcher (reviewed in Merrill *et al.*, 1997). In Greek mythology, the Sphinx posed a riddle to those it encountered and destroyed those who could not answer the riddle. For

nearly 100 years very little was known about the functional roles of sphingolipids other than a structural role in cell membranes. Interest in sphingolipids as something other than structural lipids was rekindled in 1980's when there was an increasing list of biological outcomes linked to both glyco- and phosphosphingolipids (Hannun & Bell, 1989). One of the early reports demonstrating that sphingomyelin and its derivatives may participate in signal transduction came with the observation that sphingosine inhibits PKC (Hannun *et al.*, 1986). Since then, sphingolipids have received much attention and are rapidly emerging as second messengers, and in some cases, first messengers, in a variety of signal transduction pathways.

Structurally, sphingolipids belong to 2 groups, sphingophospholipids and glycosphingolipids, and there are over 300 known sphingolipid metabolites (Bell *et al.*, 1993). The sphingophospholipid sphingomyelin and, in particular, its metabolite ceramide have received much attention since the initial observations that ceramide can induce DNA fragmentation (Obeid *et al.*, 1993; Jarvis *et al.*, 1994). In addition to apoptosis, ceramide may also play a role in signalling pathways activated by extracellular agents that induce cell proliferation (Hauser *et al.*, 1994), differentiation (Kim *et al.*, 1991) and adhesion molecule expression (Modhur *et al.*, 1996) in some cell types. But caution must be taken in assigning a role for ceramide in an agonist-induced biological event coinciding with an observed ceramide increase because the "signalling" lipid may be a ceramide metabolite (Figure 1.2). Other important metabolites shown to play a role in signal transduction are sphingosine, ceramide 1-phosphate and in particular, sphingosine 1-phosphate, for which there is a growing field of evidence supporting its role as a signalling messenger in cell survival (anti-apoptotic) and differentiation (see Section 1.5.1). The hydrolysis of sphingomyelin to generate ceramide by the activation of sphingomyelinases upon cell stimulation appears to be the primary source of "signalling" ceramide, and is referred to the "sphingomyelin cycle" or "sphingomyelin pathway" (Fig. 1.3). However, there is increasing evidence that ceramide derived from *de novo* synthesis from an agonist-induced increase in activity of one or more enzymes in the *de novo* synthesis pathway can also be a source of "signalling" ceramide.

1.4.2A Sphingomyelin/Ceramide Synthesis and Degradation

Sphingomyelin is synthesized from ceramide by the transfer of a phosphocholine to ceramide. Ceramide synthesis can occur, for the purposes of this review, by two pathways. One is by the sphingomyelin pathway whereby the phosphocholine headgroup is removed by the action of sphingomyelinases (Fig. 1.3), a reversal of sphingomyelin synthesis. The second is by *de novo* synthesis (Fig. 1.4). Serine and palmitoyl-CoA are condensed by the action of serine palmitoyltransferase (3-ketosphinganine synthase) to generate 3-ketosphinganine, followed by a rapid reduction by a reductase to sphinganine, which is then fatty acylated by ceramide synthase (dihydroceramide synthase: sphinganine (or sphingosine) N-acyltransferase) to dihydroceramide, followed by the induction of a double bond in the sphinganine backbone to generate ceramide. A phosphocholine headgroup is added to ceramide by sphingomyelin synthase to generate sphingomyelin (Merrill and Jones, 1990).

Degradation of sphingomyelin occurs by the action of sphingomyelinases (see next section) that remove the phosphocholine headgroup to yield ceramide. Ceramide can then be converted to glycosphingolipids, phosphorylated to ceramide 1-phosphate, or deacylated by the action of ceramidases to generate sphingosine (Fig. 1.2). Sphingosine can be reacylated by ceramide synthase to re-generate ceramide, or phosphorylated by sphingosine kinase to form sphingosine 1-phosphate. The role of sphingosine 1-phosphate as a bioactive lipid is reviewed in section 1.5.1.

1.4.2B Ceramide, Sphingomyelinases and Apoptosis

Abundant reports have demonstrated that cell-permeable short chain ceramides (C_2-, C_6-, C_8-ceramide; $C_{\#}$ denotes length of acyl side chain) (Obeid *et al.*, 1992; Jarvis *et al.*, 1994; Kaipa *et al.*, 1996; Cai *et al.*, 1997; Hartfield *et al.*, 1997; Veldman *et al.*, 1998), natural long chain ceramide (C_{16}-ceramide; Ji *et al.*, 1995) and exogenous bacterial (neutral) sphingomyelinase (Obeid *et al.*, 1993; Jarvis *et al.*, 1994a,b) to generate ceramide from sphingomyelin in the outer leaflet of the cell membrane, can induce apoptosis in many diverse cell types. Moreover, ceramide levels increase in cells

treated with apoptosis-inducing agents such as TNFα (Kim *et al.*, 1991; Modhur *et al.*, 1996), Fas (Tepper *et al.*, 1997), ionizing radiation (Haimovitz-Friedman *et al.*, 1994) and drugs such as daunorubicin (Bose *et al.*, 1995). Although the use of exogenous ceramides mimics the biological effects of apoptosis-inducing extracellular stimuli, it does not directly address the physiological relevance of ceramide in response to the stimulus, nor the mechanism by which ceramide is generated (sphingomyelin cycle vs *de novo* synthesis).

In most cases, the "signalling" ceramide appears to be from the hydrolysis of sphingomyelin by sphingomyelinases. There are at least 7 known sphingomyelinases that can be grouped into three groups based on pH optima: acidic, neutral and alkaline SMase. The lysosomal or acid SMase (aSMase) appears to be found in all tissues and is deficient in the lysosomal storage disorder Niemann-Pick disease. Niemann-Pick cells have proven to a valuable tool in helping decipher the role of acid SMase in signal transduction and apoptosis. There is also evidence to suggest that acid SMase is found in caveolae (Liu & Anderson, 1995). There is a neutral, Mg^{2+}-dependent, membrane-bound SMase (nSMase) as well as a number of other neutral SMase in varying tissues, such as the myelin sheath (Chakraborty *et al.*, 1997) or rat liver nuclei (Albi *et al.*, 1997), with varying Mg dependency, and an alkaline SMase in bile and the digestive tract. Both the aSMase and nSMase play roles in signal transduction.

Okazaki *et al* (1989) was one of the first to demonstrate that external cellular stimuli could activate neutral sphingomyelinase and sphingomyelin turnover in the vitamin D_3-induced differentiation of HL-60 cells. Since then, extensive studies have addressed the precise role of SMase in signal transduction and apoptosis. Early studies implicated nSMase in TNF-induced sphingomyelin turnover (Dressler *et al.*, 1992) but further studies have shown that TNF can activate both acid and neutral SMase. The current model of TNF-R1 signalling via sphingomyelinase is based on the work by Wiegmann *et al.* (1994). In this model, activation of aSMase leads to cell death and the activation of cell death associated pathways, such as JNK, whereas nSMase activation leads to cell survival or pro-inflammatory effects and the activation of MAPK/ERK pathway (Fig 1.1). TNF-R1 can activate both aSMase and nSMase via different domains

in the cytoplasmic tail of the receptor (Wiegmann *et al.*, 1994; Wiegmann *et al.*, 1999; Adams *et al.*, 1996: see section 1.3.1). The death domain, which is essential for TNF-R1-induced apoptosis, is also required for TNF-R1-induced activation of aSMase. Deletion of this domain abrogates TNF-induced apoptosis and aSMase activation but not nSMase activation and other TNF-R1 signalling events, such as ERK activation and NF-κB translocation. Further support is given with the findings that D609, an inhibitor of PC-PLC and the resultant aSMase activation, and other agents such as ammonium chloride, which alter the pH of acidic compartments, also inhibit TNF-induced apoptosis (Monney *et al.*, 1998), aSMase activation (Wiegmann *et al.*, 1994) and ceramide generation (Monney *et al.*, 1998). There is an extensive list of other reports demonstrating that TNF-R1 and Fas can activate aSMase in diverse cell types (Cifone *et al.*, 1993; Cai *et al.*, 1997; De Maria *et al.*, 1998).

Further support for a role of aSMase in apoptosis comes from the use of TNF-induced apoptosis resistant cell lines, murine aSMase knockout and natural aSMase deficient Niemann-Pick cells. A TNF-resistant U937 cell variant (Wright *et al.*, 1996) and a human breast carcinoma (Cai *et al.*, 1997) have impaired TNF-induced aSMase activation, yet other TNF-induced signalling appears to be intact and exogenous ceramide can still induce apoptosis. aSMase-deficient Niemann-Pick lymphoblasts fail to undergo radiation-induced apoptosis and ceramide-increase (Santana *et al.*, 1996). Restoration of aSMase by retroviral transfer of cDNA restored radiation-induced apoptosis and ceramide generation. Irradiated aSMase knockout mice also demonstrate reduced apoptosis and ceramide generation in lung (Santana *et al.*, 1996) and in central nervous system endothelial cells (Peña *et al.*, 2000).

The link between TNF-R1 (and Fas) and the activation of SMase is not well understood. The death domain of TNF-R1 and Fas and the recruitment of FADD to the receptor are required for aSMase activation (Wiegmann *et al.*, 1999). TNF-R1-induced aSmase activation has been proposed to be dependent on PC-PLC activation and the subsequent generation of DAG (Schutze *et al.*, 1992; Wiegmann *et al.*, 1994). These events can be blocked by D609, an inhibitor of PC-PLC, suggesting that PC-PLC activation is upstream of aSMase activation. Other reports have also shown that D609

inhibits TNF-induced apoptosis (Machleidt *et al.*, 1996; Sortino *et al.*, 1996). However, data derived from D609 must be interpreted with caution because of the potential lack of specificity of the compound. In fact, D609 may also inhibit sphingomyelin synthase (Luberto & Hannun, 1998), whose activity also increases DAG levels. Receptor internalization is a potential mechanism by which the receptor and the associated signalling components can enter an acidic, aSMase compartment, and in fact, TNF-R1 internalization does occur (Tsujimoto *et al.*, 1995). Indeed, inhibition of TNF-R1 internalization by MDC, an inhibitor of endocytosis-mediated receptor internalization, does inhibit TNF-induced aSMase activation as well as events linked to aSMase activation, such as JNK activation and apoptosis (Schutze *et al.*, 1999). Cell membrane nSMase was not affected. TNF-induced nSMase activation is mediated thorough an adaptor molecule FAN, which associates with a region of the receptor called the nSMase-activation domain (NSD) (Adam *et al.*, 1996; Adam-Klages *et al.*, 1996), but little else is known.

This model of distinct SMases generating the same product, ceramide, in distinct cellular compartments (plasma membrane vs acid compartment), and the linking of different SMases to different cellular events, would suggest different roles for ceramide in signalling depending on the location of the ceramide. Ceramide in the plasma membrane would potentially interact with different cellular components than ceramide in an endosome or other acidic compartment. The sphingomyelin accessible to nSMase appears to be on the inner leaflet of the plasma membrane (Linardic & Hannun, 1994; Andrieu *et al.*, 1996; Zhang *et al.*, 1997), although the majority of sphingomyelin in the plasma membrane is on the outer leaflet (Merrill & Jones, 1990). Ceramide generated on the inner leaflet would be more accessible to cytosolic components than ceramide on the outer leaflet. Moreover, Zhang *et al.* (1997) demonstrated by transfecting cells with bSMase (neutral SMase) that the signalling ceramide on the inner leaflet of the plasma membrane is distinct from ceramide generated on the outer leaflet by exogenous bSMase. As described previously, the link between TNF-R1 and acid SMase, and how a signal reaches the acid compartment to generate signalling ceramide, is not well understood. One possibility is aSMase is activated directly in an acidic

endosomal/lysosomal compartment by yet unidentified signalling pathways to generate signalling ceramide. Another possibility is aSMase is found, and activated in caveolae, which are rich in sphingomyelin (Brown & Rose, 1992) and have been implicated as sites of ligand-induced receptor clustering and signal transduction for receptors such as the EGF (Mineo *et al.*, 1996) and PDGF receptors (Liu *et al.*, 1996). Il-1β and NGF–induced aSMase activation and ceramide generation have been localized to caveolae, or caveloae-like structures (Liu & Anderson, 1995; Bilderback *et al.*, 1997). Caveolae could provide an acidic compartment (Kamen *et al.*, 1988; Prasad *et al.*, 1994) for the enzyme, and their internalization cycle (Anderson, 1993) could provide a mechanism of delivering the signalling ceramide to intracellular compartments. In fact, TNF-mediated apoptosis has been shown to be initiated in caveolae-like domains (Ko *et al.*, 1999) and TNF-R1 internalization is required for TNF-mediated apoptosis (Schutze *et al.*, 1999). Exactly how ceramide found on the lumenal side of an acidic endosomal/lysosomal compartment interacts with, or transmits a signal to its non-endosomal targets is not understood. However, fluorescent, short chain ceramide analogues have been shown to readily flip-flop across membranes (Pagano & Martin, 1988; Bai & Pagano, 1997), suggesting that natural ceramide may also be able to flip-flop, although there is no direct evidence for natural ceramide flip-flop.

The kinetics and magnitude of ceramide changes in response to an extracellular stimulus varies depending on the type of cell and stimulus. TNF-induced ceramide increases have peaked at 20%-60% above basal levels within a few minutes and rapidly returned to basal levels (Schutz *et al* 1992; Obeid *et al.*,1993) or peaked at 1-2 hours (Kim *et al.*, 1991; Modhur *et al.*, 1996). Others have shown rapid ceramide increases that have been maintained for up to one hour (Rayes *et al.*, 1996; Verheij *et al.*, 1996). More recently it has been shown that TNF can induce waves of ceramide, one at 2-3 minutes, another at 40 minutes and a third at 2 hours, which appear to result from the sequential activation of nSMase, ceramide synthase and aSMase respectively (Bourteele *et al.*, 1998). Fas-induced ceramide changes have been rapid and as high as 2000% between 10 and 30 minutes in HuT78 T lymphoma cells (Cifone *et al.*, 1993; Cifone *et al.*, 1995) or a more modest 100% in 10 minutes in freshly isolated T lymphocytes (De Maria *et al.*,

1996). Tepper *et al.* (1997) however, reported a gradual 6-fold increase over 8 hours in Jurkat cells while Gamard *et al.* (1997) reported a gradual increase starting at 4 hours and reaching a 6-7-fold increase by 20 hours. Cuvillier *et al.* (2000) demonstrated a Fas-induced biphasic response in Jurkats, with an initial increase at 30 minutes, a return to basal levels followed by a gradual 1.5-2-fold increase by 6 hours. Serum deprivation, which causes cell cycle arrest and apoptosis, also induced late and sustained ceramide increases, from 3-fold in 24 hours to 15-fold by 96 hours (Jayadev *et al.*, 1994; Dbaibo *et al.*, 1995). Assigning specific signalling roles to ceramide has been problematic due to the heterogeneity of cellular responses. It has been proposed that the signalling ceramide in apoptosis is not the very early, transient increase (2-5 minutes), but the later sustained increase (Hannun 1996).

Some of the effects attributed to ceramide may be mediated by a ceramide metabolite, such as sphingosine, which is produced by the deacylation of ceramide by ceramidase. Sphingosine is produced in response to TNF in human neutrophils (Ohta *et al.*, 1994) and cardiac myocytes (Oral *et al.*, 1997), in response to Fas ligation in Jurkat cells (Cuvillier *et al.*, 2000), or in response to PMA-induced differentiation and apoptosis in HL-60 cells (Ohta *et al.*, 1995). In the case of Jurkat cells, Fas ligation was also shown to activate ceramidase activity. Exogenous sphingosine can also induce apoptosis (Ohta *et al.*, 1995; Jarvis *et al.*, 1996; Cuvillier *et al.*, 2000), or potentiate ceramide-induced apoptosis (Jarvis *et al.*, 1996). As with ceramide, sphingosine can be metabolized to other sphingolipids, and the apoptotic effects may be mediated by its conversion back to ceramide by the action of ceramide synthase. To rule out this possibility, these studies used the mycotoxin fumonisin B1, a known inhibitor of ceramide synthase (Merrill *et al.*, 1993).

The roles of aSMase, nSMase, and ceramide in TNF-R1 and Fas signalling, as well as apoptosis, are not without controversy. The different kinetics of ceramide generation in different studies has been a major source of confusion in assigning roles of ceramide in signal transduction and apoptosis, leading to the suggestion that ceramide may be a consequence of, and not an active mediator in the induction of apoptosis. The induction of apoptosis by exogenous ceramide or exogenous SMase would argue in

favour of an active role for ceramide in apoptosis. The use of synthetic short chain cell-permeable ceramide to induce apoptosis has been criticized as being non-physiological (Betts *et al.*, 1994; Ji *et al.*, 1995). However, long chain ceramides have also been used to induce apoptosis with similar results as the short chain (Ji *et al.*, 1995). Some authors have reported that TNF does not induce an increase in ceramide levels (Betts *et al.*,1994). In a cell comparison study, Bettaieb *et al.* (1996) showed that TNF-induced ceramide increases in U937 cells but not in another leukemic cells line KG1a. In another comparison study, exogenous ceramide induced apoptosis in U937 and HL-60 cells, but not in Raji cells, where it induced cell cycle arrest (Hirokawa *et al.*, 1996) These results demonstrate that under the same conditions, ceramide responses can be quite different in different cell types. Also, given the variable kinetics of ceramide generation with cell type and stimulus, it is possible to miss a transient increase, or the long-term ceramide increase if only a single point or early time points are investigated. Ceramide may also be rapidly metabolized to another sphingolipid metabolite, such as sphingosine, which has been shown to mediate apoptosis (Cuvillier *et al.*, 2000). Modur *et al.* (1996) demonstrated a TNF-induced ceramide increase and sphingomyelin decrease in HUVEC's, but the ceramide increase was not to the same magnitude as the sphingomyelin decrease, suggesting that ceramide can be rapidly metabolized. Given these possibilities it is conceivable that the role of ceramide can be underestimated in some cases.

Some of these arguments are being put to rest with findings that overexpression of anti-apoptotic molecules, such as bcl-2, can block apoptosis and block exogenous ceramide-induced apoptosis, but not the ceramide increase (Dbaibo *et al.*, 1997). Tetrapepetide inhibitors of caspase-3 have blocked Fas and ceramide-induced apoptosis (Tepper *et al.*,1997; Mizushima *et al.*, 1996; Suzuki *et al.*, 1997) but not Fas-induced ceramide increases (Tepper *et al.*, 1997). More recently it was reported that Fas-induced ceramide is dependent on FADD and initiator caspase 8 activation, and can be blocked by the caspase 8 inhibitor crm A (Grullich *et al.*, 2000). Others have shown that TNF-induced apoptosis and ceramide generation are blocked by crm A, but crm A does not block ceramide-induced apoptosis (Dbaibo *et al.*, 1997). These studies would suggest

that ceramide generation lies between the initiator and effector stages of death receptor-induced apoptosis. Others have suggested that ceramide has no role in early Fas signalling, or no role in Fas-induced apoptosis. The caspase inhibitor zVAD has been used to inhibit caspase-3, Fas-induced apoptosis and ceramide generation, drawing the conclusion that ceramide is after effector caspase activation, therefore not involved in signalling (Sillence & Allan, 1997). However, zVAD is a broad caspase inhibitor, and can inhibit both caspase-3 and –8. In another study fumonisin B1 was shown to block TNF-induced apoptosis but not Fas-induced apoptosis (Laouar *et al.*, 1999). The conclusion was ceramide is involved in TNF, but not Fas-induced apoptosis. Fumonisin B1 however, inhibits *de novo* ceramide synthesis, not ceramide generated from the sphingomyelin cycle and therefore did not address the sphingomyelin cycle in Fas signalling.

The use of murine acid SMase$^{-/-}$ and human naturally deficient Niemann-Pick cells have helped define the role of ceramide and aSMase in apoptosis, particularly for radiation-induced apoptosis. aSMase-deficient murine lung (Santana *et al.*, 1996), CNS endothelial cells (Pena *et al.*, 2000), and human lymphoblasts (Santana *et al.*, 1996) do not undergo radiation-induced apoptosis. Niemann-Pick lymphoblasts also show impaired Fas-induced apoptosis (De Maria *et al.*, 1998) and aSMase knockout mice are more resistant to anti-Fas-induced death (Lin *et al.*, 2000). These studies also revealed tissue and cell-specific difference in requirements for aSMase. Thymic tissue was not as resistant in aSMase$^{-/-}$ to radiation as lung tissue, and anti-Fas treatment failed to induce hepatocyte apoptosis in aSMase$^{-/-}$ animals whereas lymphocyte apoptosis was not affected. These comparison studies support the idea that aSMase and ceramide is indispensable in some tissues and cells but not in others. There are studies that report conflicting data. Bosen-de Cock *et al.* (1998) reported a similar reduction in Fas-induced apoptosis in Niemann-Pick lymphoblasts as De Maria *et al.* (1998), but could not restore apoptosis to normal with retroviral restoration of aSMase. Retroviral restoration of aSMase in Niemann-Pick lymphoblasts did restore radiation-induced apoptosis (Santana *et al.*, 1996).

The Krönke group model (Wiegmann *et al.*, 1994) makes a clear delineation in

the roles of aSMase and nSMase in apoptosis and cell signalling. TNF-induced aSMase ceramide is apoptotic while nSMase ceramide in the plasma membrane is not apoptotic but involved in other TNF-induced events. The aSMase-deficient cell data described above would support this dichotomy since nSMase remains functional. However, it may not be that simple. Exogenous bacterial (neutral) SMase generates ceramide in the plasma membrane and induces apoptosis in some cells (Obeid *et al.*, 1993; Jarvis *et al.*, 1994). This ceramide would be on the outer leaflet of the plasma membrane and there is evidence to suggest that plasma membrane signalling ceramide derived from the action of endogenous nSMase is on the inner leaflet (Linardic & Hannun, 1994; Andrieu *et al.*, 1996; Zhang *et al.*, 1997), questioning the validity of exogenous bacterial SMase in generating signalling ceramide. Zhang *et al.* (1997) did report, however, that ceramide on the inner leaflet could induce apoptosis and there are reports that TNF induces apoptosis in mammary carcinoma MCF7 cells by the activation of nSMase (Liu *et al.*, 1998) and serum withdrawl activates nSMase in Molt-4cells (Jayadev *et al.*, 1995). On the other hand, there are cases in which exogenous ceramide does but bacterial SMase does not induced apoptosis (Escargueil-Blanc *et al.*, 1998; Veldman *et al.*, 1998), suggesting that the signalling frunction of aSMase and nSMase-derived ceramide may depend on the cell type.

1.4.2C *de novo* Ceramide and Apoptosis

Ceramide as a signalling molecule has largely been studied in the context of the sphingomyelin cycle and the activation of SMases. There is increasing evidence demonstrating that ceramide derived from *de novo* synthesis may participate in cell signalling and specific enzymes in the *de novo* synthesis pathway can be regulated by cell stimuli. The anti-cancer drug daunorubicin-induced apoptosis is mediated by ceramide generated from increased activity of ceramide synthase and not sphingomyelinase (Bose *et al.*, 1995). Fumonisin B1, a natural specific inhibitor of ceramide synthase, blocked daunorubicin-induced apoptosis and ceramide elevation. In hen granulosa cells, fumonisin B1 blocked daunorubicin but not UV-induced apoptosis (Witty *et al.*, 1996), suggesting that the same cell can potentially use different pathways

to generate ceramide in response to different stimuli. Serine palmitoyltransferase, the initial and rate limiting enzyme in *de novo* synthesis, has been shown to be up-regulated by the chemotherapy drug etoposide (Perry *et al.*, 2000). TNF induces apoptosis and ceramide elevation without a concomitant decrease in sphingomyelin in bovine cerebral endothelial cells (Xu *et al.*, 1998). Fumonisin B1 blocked both the TNF-induced ceramide increase and apoptosis, demonstrating that *de novo* ceramide synthesis can play a role in TNF signalling. Moreover, TNF-induced activation of the sphingomyelin cycle and *de novo* synthesis may not be mutually exclusive in the same cell. Bourteele *et al.* (1998) demonstrated waves of TNF-induced ceramide increases, the first from nSMase and the second from the activation of ceramide synthase. The angiotensin type 2 receptor also mediates ceramide increases and apoptosis without a concomitant decrease in sphingomyelin or increase in SMase activity (Lehtonen *et al.*, 1999). These studies identify the *de novo* ceramide synthesis pathway as an inducible and regulated pathway in ceramide signalling and may operate instead of, or in addition to the sphingomyelin cycle.

1.4.2D Ceramide Targets

Cellular targets for ceramide include a ceramide-activated protein phosphatase (CAPP) and a ceramide-activated protein kinase (CAPK). CAPP arrests cell cycle in yeast (Nickels & Broach, 1996), and both c-myc (Wolff *et al.*, 1994) and c-Jun (Reyes *et al.*, 1996) act as targets for CAPP in leukemic cells. More recently, CAPP was found to inhibit the pro-survival molecules bcl-2 (Ruvolo *et al.*, 1999) and PKB/Akt (Schubert *et al.*, 2000) by dephosphorylation. It had previously been shown that ceramide could inhibit Akt (Zhou *et al.*, 1998). CAPK was first described as a kinase that could phosphorylate the EGF receptor (Goldkorn *et al.*, 1991; Mathias *et al.*, 1991). CAPK and KSR (kinase suppressor of Ras) have since been identified as the same molecule (Zhang *et al.*, 1997). CAPK/KSR links ceramide to Ras, Raf and MEK1, a pathway which also links ceramide to the dephosphorylation and activation of BAD (Basu *et al.*, 1998), a pro-apoptotic bcl-2 family member.

The SAPK/JNK pathway is also a reported target for ceramide (Westwick *et al.*,

1995; Cuvillier *et al.*, 1996; Verheij *et al.*, 1996; Huang *et al.*, 1997). This pathway is generally associated with cell death (Xia *et al.*, 1995), although this may not be the case for all cell types. SAPK/JNK does not appear to be a ceramide target *per se*, but the pathway is activated by ceramide-mediated activation of upstream signalling intermediates, MEK4 (Verheij *et al.*, 1996), Rac1 (Brenner *et al.*, 1997), and TAK1 (Shirakabe *et al.*, 1997), although there is no evidence to suggest that ceramide interacts directly with these intermediates. In HL-60 cells, ceramide increased both c-jun and c-fos expression, while antisense c-jun inhibited ceramide-induced apoptosis (Kondo *et al.*, 2000), strongly supporting a role of the JNK pathway in ceramide-induced apoptosis. In the case of UV-induced JNK activation, results from Niemann-Pick lymphoblasts suggest that aSMase derived ceramide may be signalling ceramide (Huang *et al.*,1997).

Different PKC isoforms are targets for, and may respond differently, to ceramide. Ceramide induced the translocation of PKC isoforms δ and ε from the membrane to the cytosol (Sawai *et al.*, 1997) and inactivates PKC-α (Lee *et al.*, 1996). PKC activation generally antagonizes death apoptosis (Obeid *et al.*, 1993; Jarvis *et al.*, 1994b;) and inhibitors of PKC induce apoptosis (Jarvis *et al.*, 1994c). On the other hand, ceramide induces translocation (Galve-Roperh *et al.*, 1997) and activation (Muller *et al.*, 1995) of the atypical PKC-ζ.

Ceramide also inhibits phospholipase D activity (Gomez-Muñoz *et al.*, 1994), which may be mediated by ceramide inactivation of ARF, RhoA, PKC-α and -β (Abousalham *et al.*, 1997), signalling molecules which are know to stimulate PLD activity. PLD activity generates phosphatidate (PA), which can be converted to lysoPA, both of which are signalling molecules mediating a variety of biological effects, including mitogenesis (reviewed in Martin *et al.*, 1994).

There are a number of reports to suggest that ceramide activates NF-κB (Reddy *et al.*, 1994; Machleidt *et al.*, 1994; Boland & O'Neill, 1998), while others suggest that ceramide does not activate, but may inhibit phorbol ester-activated NF-κB (Gamard *et al.*, 1997). Although exogenous ceramide may influence NF-κB activity, it is unclear whether ceramide is involved in TNF-induced NF-κB activation because there are reports showing that in the absence of a ceramide increase or SMase activation, TNF can

still induce NF-κB activation (Betts *et al.*, 1994; Johns *et al.*, 1994; Zumbansen & Stoffel, 1997).

As previously described, the inhibition of ceramide-induced apoptosis by inhibitors of caspase-3 would place ceramide upstream of caspase-3 activation. Exogenous cell permeable ceramides can induce caspase-3 proteolytic cleavage and activation (Mizushima *et al.*, 1996; Tepper *et al.*, 1997; Ito *et al.*, 1999; Cuvillier *et al.*, 2000), although there is no evidence to suggest it is a direct activation. Instead, ceramide-induced activation of caspase-3 may be mediated by the release of cytochrome c from the mitochondria. Ceramide can directly inhibit the mitochondrial respiratory chain complex (Gudz *et al.*, 1997), induce cytochrome c release (Ghafourifar *et al.*, 1999; Ito *et al.*, 1999; Cuvillier *et al.*, 2000), and activate caspase 9 (Cuvillier *et al.*, 2000), events which lead to the activation of caspase-3.

1.5 Anti-Apoptotis Mechanisms

TNF does not induce apoptosis in all cell types, and in many tumour cell lines, TNF and Fas-induced apoptosis requires protein synthesis inhibitors, suggesting that there are anti-apoptotic mechanisms that override the death signal. Some of these inhibitors can be receptor proximal, such as SODD (Jiang *et al.*, 1999), which prevents constitutive activation of TNF-R1 as well as silence the receptor after signalling, and FLIP/I-FLICE (Hu *et al.*, 1997; Irmler *et al.*,1997;), which binds to both Fas and TNF-R1 complexes to prevent the recruitment of caspase 8. Other anti-apoptotic proteins are distal to the receptor, such as members of the bcl-2 family. Bcl-2 itself may function at the level of the mitochondria by preventing the release of cytochrome c (reviewed in Wang *et al.*, 1999; Pellegrini & Strasser, 1999). Bcl-$_{xL}$ can dimerize with, and sequester Apaf-1, thus preventing formation of the apoptosome. NF-κB translocation is generally considered to be anti-apoptotic, and may do so by transcriptional upregulation of pro-survival bcl-2 family members (Tamatani *et al.*, 1999; Zong *et al.*, 1999). Moreover, bcl-2 can upregulate NF-κB activation (de Moissac *et al.*, 1998), generating a potential pro-survival amplification loop. Activation of PKB/Akt can promote cell survival in several ways. It can phosphorylate BAD (Datta *et al.*, 1997), thereby preventing its

dimerization with, and inactivation of the pro-survival Bcl-$_{xL}$. Secondly, it can directly phosphorylate and inhibit caspase-9 (Cardone *et al.*, 1998). There is also a new family of apoptosis inhibitors called IAPs, which may inhibit apoptosis by direct inhibition of caspases (reviewed in Deveraux & Reed, 1999).

1.5.1 Sphingosine 1-Phosphate and Survival

Sphingosine 1-phosphate (SPP), a ceramide metabolite, is emerging as an important bioactive sphingolipid mediating effects opposing those of ceramide. SPP acts as a second messenger in PDGF and serum-induced mitogenesis in fibroblasts (Olivera & Spiegel, 1993), inhibits TNF (Cuvillier *et al.*, 1996), Fas ligation (Cuvillier *et al.*, 1998), 1α, 25-dihydroxyvitamin D_3 (Kleuser *et al.*, 1998), SMase (Kleuser *et al.*, 1998) and ceramide-induced apoptosis (Cuvillier *et al.*, 1996), as well as play a role in neuronal survival and differentiation (Edsall *et al.*, 1997; Rius *et al.*, 1997), and angiogenesis (Wang *et al.*, 1999). SPP is generated by the phosphorylation of sphingosine by sphingosine kinase. Two human and two murine sphingosine kinase isoforms have been identified and cloned, each with a wide tissue distribution (Kohama *et al.*, 1998; Liu *et al.*, 2000; Nava *et al.*, 2000). Growth factors, such as PDGF and NGF, which play roles in cell growth and differentiation, activate sphingosine kinase leading to increased cellular SPP levels (Olivera & Spiegel, 1993; Edsall *et al.*, 1997). In the case of NGF, sphingosine kinase activity can remain elevated for more than several days (Edsall *et al.*, 1997; Rius *et al.*, 1997). The anti-apoptotic effects of PKC may be in part mediated through the activation of sphingosine kinase since PKC inhibitors also inhibit sphingosine kinase activity, and activators of PKC, such as phorbol esters, also activate sphingosine kinase (Edsall *et al.*, 1998). Additional support for the anti-apoptotic role of SPP comes from the transient over-expression of sphingosine kinase, which promotes cell growth and protects against serum deprivation- and ceramide-induced apoptosis (Olivera *et al.*, 1999). In addition, *N,N*-dimethylsphingosine, a competitive inhibitors of sphingosine kinase (Edsall *et al.*, 1998), promotes cell death (Ohta *et al.*, 1994; Sweeney *et al.*, 1996), inhibits the effects of sphingosine kinase overexpression (Olivera *et al.*, 1999), and inhibits the effects of NGF and PDGF.

SPP is not only a cell signalling second messenger, but acts as a first messenger by binding cell surface G protein-coupled Edg receptors (Lee *et al.*, 1998; Okamoto *et al.*, 1998; Van Brocklyn *et al.*, 1998). SPP is found at relatively high concentrations in plasma (0.5 μM) and activated platelets may be the principle source. Edg-1 has been recognized as the high affinity SPP receptor (reviewed in Spiegel & Milstien, 2000). More recently, Edg-8 was identified as a SPP receptor with a higher affinity (Im *et al.*, 2000), although its expression appears to be restricted to brain and spleen. Edg-3. and -5 also bind and function as SPP receptors, albeit with lower affinities (An *et al.*, 2000).

Due to its first and second messenger properties, identifying cellular targets for SPP has been problematic. The mitogenic and anti-apoptotic properties have been attributed to the intracellular, or 2nd messenger, SPP (Van Brocklyn *et al.*, 1998; Olivera *et al.*, 1999). Transient overexpression of sphingosine kinase increased intracellular SPP and protected against apoptosis, but SPP could not be detected in the culture medium, suggesting an intracellular sight of action. Microinjection of SPP is also mitogenic. Moreover, pertussis toxin (PT), which uncouples G_i from Edg-1 and thereby inhibiting G_i-mediated signals from Edg-1, did not prevent exogenous SPP from protecting against serum removal and ceramide-induced apoptosis in NIH3T3 fibroblasts. However, other Edg receptors, Edg-3 and Edg-5, can couple to PT-insensitive G-proteins (Windh *et al.*, 2000). SPP protection from apoptosis in T cells may be mediated by Edg receptors (Goetzl *et al.*, 1999). Also, HTC4 hepatoma cells can be rescued from serum withdrawal-induced apoptosis when transfected with Edg-3 and Edg-5 (An *et al.*, 2000). Given the different tissue distribution of Edg receptors (An *et al.*, 2000; Spiegel & Milstien, 2000), it raises the possibility that SPP protection from apoptosis may be mediated by different mechanisms in different cells.

The first and second messenger properties notwithstanding, SPP activates pro-survival events and signalling pathways. In U937 cells, SPP activates the ERK signalling pathway and inhibits TNF- and ceramide-induced SAPK/JNK activity (Cuvillier *et al.*, 1996). In Jurkat cells, SPP inhibits Fas-induced activation of caspase-3, -6, and -9, as well as the cleavage of PARP and lamins (Cuvillier *et al.*, 1998). Overexpression of sphingosine kinase also inhibits Fas and ceramide-induced cleavage and activation of

caspase-3 (Olivera *et al.*, 1998). In T cells, SPP reduced the levels of Bax, a pro-apoptotic bcl-2 family member, but did not affect bcl-2 and bcl-x_L levels (Goetzl *et al.*, 1999).

1.5.2 Ceramide-SPP Rheostat

The Spiegel group (Cuvillier *et al.*, 1996) has proposed a ceramide-SPP rheostat, or "biostat", in which the relative balance between intracellular ceramide and SPP may determine the fate of the cell (Fig 1.5). Tipping the balance in favour of ceramide or SPP depends on the extracellular stimuli, such as PDGF and TNF. Ceramide and SPP can then potentially activate opposing signalling cascades or events, and depending on the timing and magnitude of the signal, determine the fate of the cell.

1.6 The Placenta

The placenta acts as both an anchor to anchor the fetus to the uterine wall and conduit between mother and fetus. It provides for the transfer of nutrients, water and gases as well as for the elimination of metabolic waste products from the fetus. It also acts as an immunological barrier against maternal allogeneic responses and prevents the transfer of pathogens in maternal circulation from entering the fetus.

The placenta contains two principle types of villi, anchoring villi and floating villi. Anchoring villi embed into the maternal endometrium and serve to attach the placenta to the uterine wall. The floating villi do not come into contact with the endometrium, and as the name implies, they "float" in maternal blood, and serve as the principle site of exchange and barrier functions. Floating villi are covered by a layer of specialized cells, the trophoblasts. The outer most layer forms a continuous syncytium, made up of a multi-nucleated syncytiotrophoblast. It is these cells that come into direct contact with maternal blood. Underlying the syncytiotrophoblast lie the mononucleated cytotrophoblasts that divide and fuse to form the syncytiotrophoblast. Within the floating villous stroma lie fetal capillaries and stromal cells, such as fibroblasts and macrophages.

1.6.1 Life and Death of the Trophoblast

Apoptosis *per se*, is not an aberrant phenomenon in the villous placenta. The syncytiotrophoblast appears to constantly renew by loss of aged nuclei and cytoplasm which are segregated into protruding knots that pinch off into maternal circulation (Jones & Fox, 1977), and replenished through fusion of underlying cytotrophoblasts (Boyd & Hamilton, 1966). Electron microscopy observations of nuclei within the syncytiotrophoblast from placental bed biopsies (Nelson, 1996) and the observation of trophoblast apoptosis throughout gestation (Smith *et al.*, 1997a) suggest that apoptosis may be a normal placental aging process. During this process cell loss must be matched by cell replacement, and thereby maintain the placental function. However, a higher than normal incidence of trophoblast apoptosis is found in placentas from IUGR (intrauterine growth retardation) fetuses (Smith *et al.*, 1997b), suggesting aberrant apoptosis.

TNFα induces apoptosis in cultured cytotrophoblasts and syncytiotrophlasts in the absence of protein synthesis inhibitors (Yui *et al.*, 1994a). Apoptosis can be enhanced by co-culture with the pro-inflammatory cytokine IFNγ, which alone, does not induce apoptosis. High levels of TNF mRNA expression in placental tissues correlate with higher rates of fetal resorption (Tangri & Ragupathy, 1993) and injections of TNF or IFNα induce placental damage and fetal demise in rats and mice (Silen *et al.*, 1989; Chaout *et al.*, 1990). IUGR fetuses have elevated TNF in their amniotic fluid (Heyborne *et al.*, 1992) and microbial infection-induced TNF production has been implicated in pre-term labour (Casey *et al.*, 1989; Hillier *et al.*, 1993). This would imply that TNF is generally detrimental to the placenta and fetal survival. However, TNF is found in normal, healthy, placental tissue, and is produced by trophoblasts (Li *et al.*, 1992; Yang *et al.*, 1993), suggesting a physiological role during pregnancy. It is possible that TNF plays a role in normal trophoblast turnover. The TNF-induced cytotoxicity of trophoblasts must be balanced by cell renewal or prevention of excessive loss, which may be controlled by positive regulators of trophoblast growth, such as EGF (epidermal growth factor). EGF inhibits TNF/IFNγ-induced apoptosis in cultured cytotrophoblasts and syncytiotrophoblasts (Garcia-Lloret *et al.*, 1996), as well as influences trophoblast migration (Bass *et al.*, 1994) and differentiation, such as cytotrophoblast fusion to form

syncytiotrophoblasts (Morrish *et al.*, 1987). Moreover, EGF levels increase in maternal plasma and urine during pregnancy, whereas levels in mothers carrying IUGR babies only reach half the normal levels (Hofmann *et al.*, 1988).

TNF and IFNγ up-regulate expression of the adhesion molecule ICAM-1 on cultured trophoblasts, which mediates the adhesion of LPS-activated monocytes to the trophoblasts (Xiao *et al.*, 1997). In areas of bound monocytes, there is increased apoptosis and disruption of the trophoblast layer (Garcia-Lloret *et al.*, 2000). Activated monocytes in maternal circulation could bind and damage the villous syncytiotrophoblasts, thereby compromising the interface.

Given that TNF/IFNγ induces trophoblast apoptosis, and EGF antagonizes that apoptosis, it is conceivable that the interplay between these cytokines and growth factors determines the fate of the trophoblast, and consequently the fetus. An imbalance of these factors, or a defect in cell signalling, could lead to excessive trophoblast loss and a compromise in the maternal-fetal barrier.

Although much is known about TNF signalling and apoptosis, as well as EGF signalling, in other cell types, little is known about the signalling mechanisms in trophoblasts. EGF activates MAP kinases, PLCγ, and PI-3 kinase (van der Geer & Hunts, 1994), as well as inhibits Fas-mediated apoptosis in epithelial cells by an Akt-dependant mechanism (Gibson *et al.*, 1999), but nothing is known about how EGF antagonizes TNF-induced apoptosis in trophoblasts. Reactive oxygen and nitrogen intermediates, which have been shown to be involved in TNF-induced cytotoxicity in some cell types, are not involved in TNF-induced cytotoxicity in trophoblasts (Smith *et al.*, 1999). TNF/IFNγ induces apoptosis in 40-60% of trophoblasts in the absence of protein synthesis inhibitors (Yui *et al.*, 1994), suggesting that there are no constitutively active anti-apoptotic mechanisms that suppress apoptosis in these cells. Protein synthesis inhibitors enhance TNF/IFNγ-induced apoptosis , and Ho *et al.*, (1999) demonstrated that low bcl-2 expression in trophoblasts correlates with a higher frequency of apoptosis. These data would suggest that there is heterogeneity of trophoblasts with respect to protein synthesis inhibitor-sensitive anti-apoptotic mechanisms and bcl-2 expression. However, TNF/IFNγ or EGF did not alter the expression levels of bcl-2, suggesting that

it is not a cytokine-inducible factor in the regulation of apoptosis in trophoblasts.

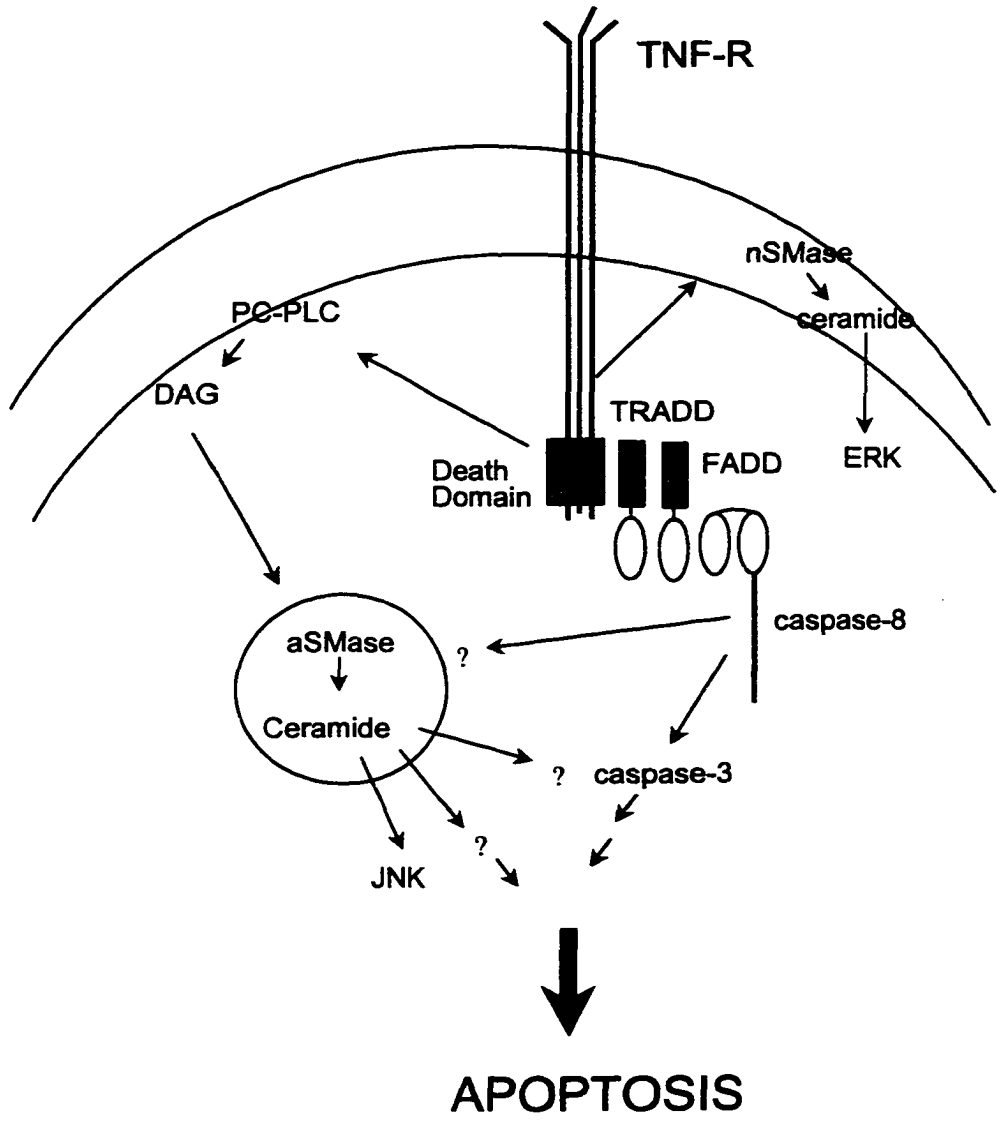


Figure 1.1 : Simplified schematic of TNF-R1-associated factors and signalling pathways leading to apoptosis. "?" indicate conflicting data or unknown intermediates.

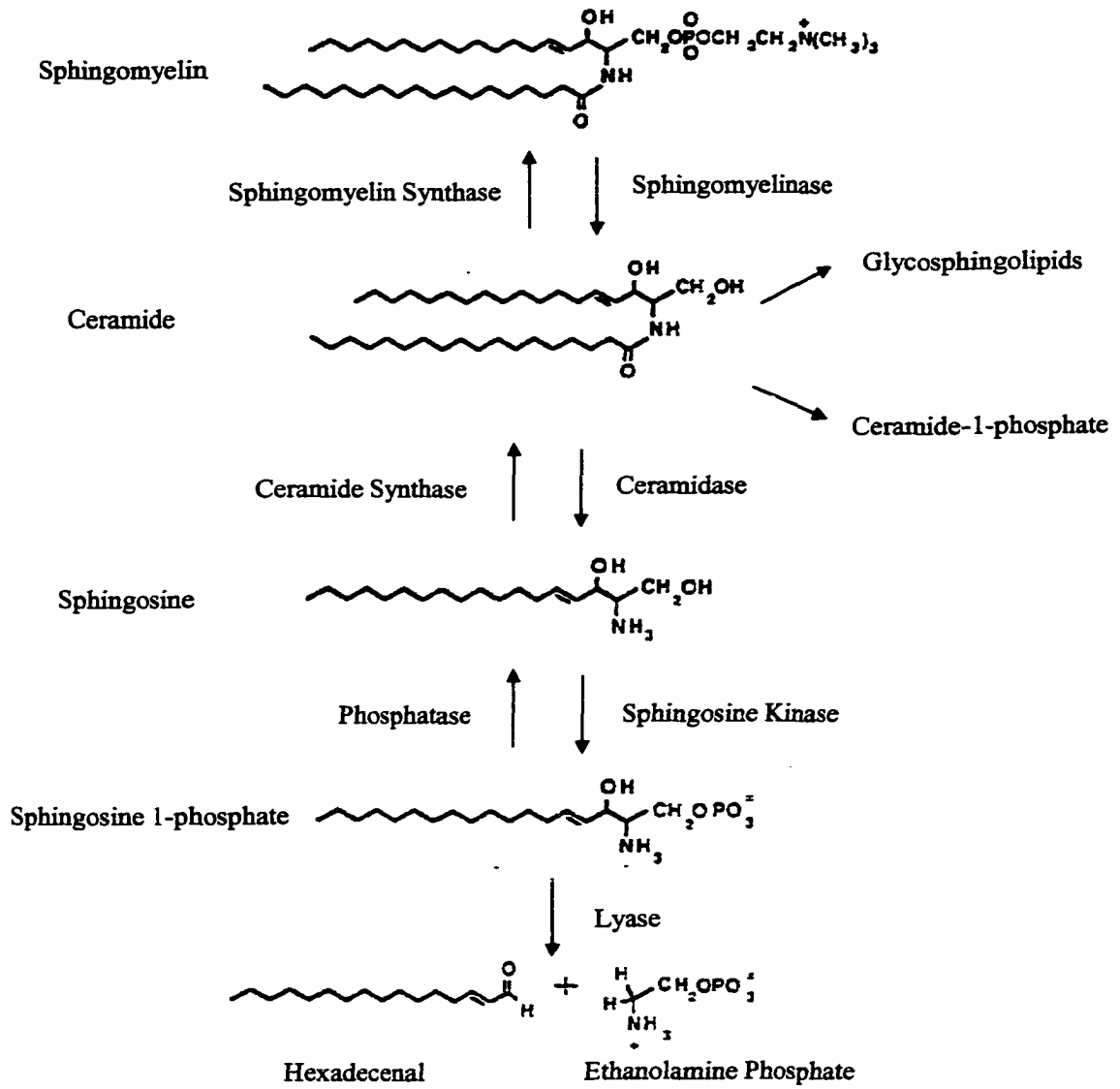


Figure 1.2 : Sphingomyelin Pathway and Metabolites. Simplified schematic biochemical pathways of sphingomyelin/ceramide metabolism and enzymes responsible.

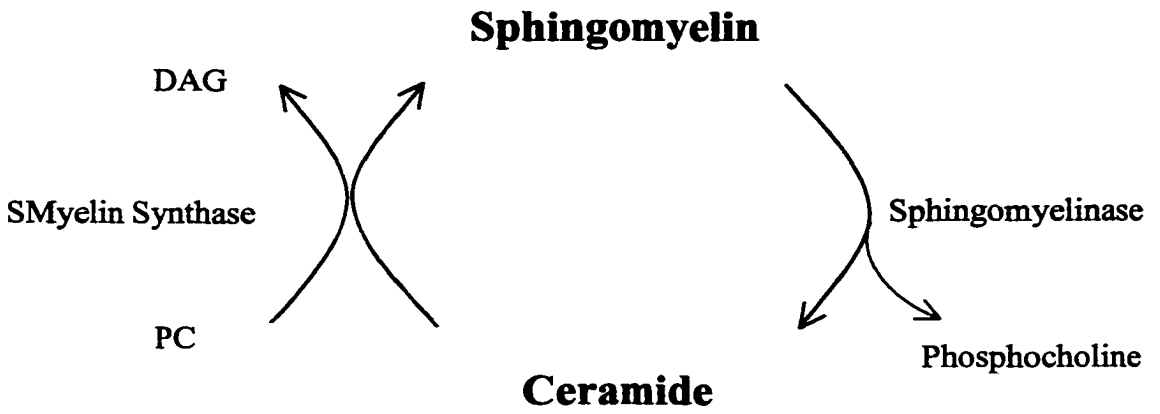


Figure 1.3 : Sphingomyelin Cycle. Sphingomyelinase converts sphingomyelin to ceramide by the removal of phosphocholine. Ceramide can be converted back to sphingomyelin by the transfer of phosphocholine from phosphatidylcholine to ceramide, generating sphingomyelin and DAG.

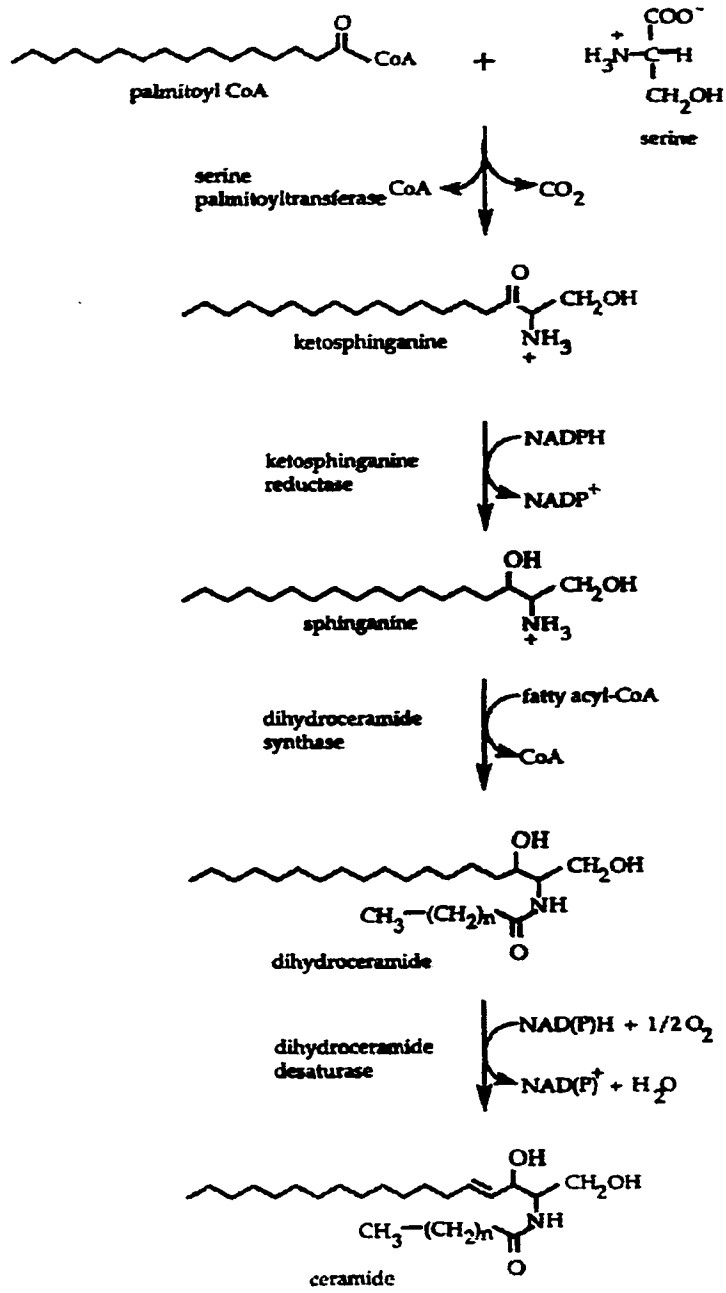


Figure 1.4 : *De Novo* Ceramide Synthesis Pathway.

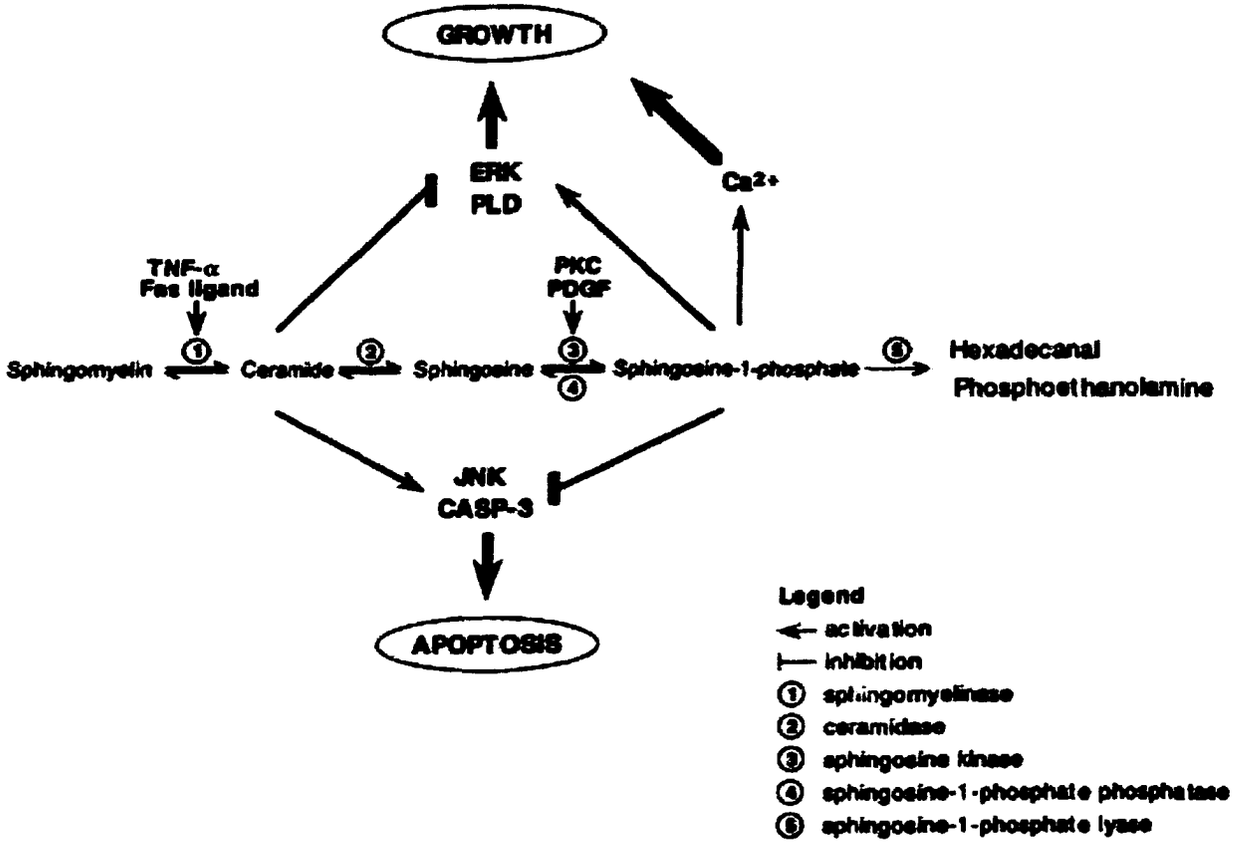


Figure 1.5 : Ceramide:SPP Biostat. Adapted from Spiegel (1999). TNF and Fas activate sphingomyelinase and thereby increase ceramide levels that induce apoptosis and inhibit pro-survival factors. In contrast, PDGF activates sphingosine kinase thereby elevating SPP levels that promote cell growth and inhibit pro-apoptotic factors.

Chapter 2 :Hypothesis and Rationale

2.1 Hypothesis

Apoptosis is a physiological cell death that is a normal part of cell turnover in many organs. However, cell loss must be controlled and balanced by cell renewal to maintain organ homoeostasis. There is an increasing knowledge of complex biochemical pathways that control the induction and inhibition of apoptosis. The complexity of these pathways, and the findings that different pathways may be active in different cell types, has led to some controversy and confusion. One such pathway is the sphingolipid pathway in the induction and inhibition of apoptosis. As described in the previous chapter, apoptosis in primary human placental trophoblasts is under the control of cytokines. TNF/IFNγ induces apoptosis whereas EGF inhibits cytokine-induced apoptosis. A high incidence of apoptosis in the placenta is linked to placental insufficiency. However, little is known about the role sphingolipids play in the control of apoptosis by these factors in trophoblasts. Moreover, although sphingolipids are involved in growth factor signalling such as PDGF and NGF, little is known about the role sphingolipids may play in EGF signalling in general. ***My general hypothesis is that sphingolipids act as signalling intermediates in death receptor-induced apoptosis and EGF inhibition of apoptosis in human primary trophoblast cells. More specifically, the pro-apoptotic sphingolipid ceramide is involved in trophoblast apoptosis, and the EGF inhibits apoptosis by influencing ceramide levels or upregulating the pro-survival sphingolipid, sphingosine 1-phosphate.***

2.2 Rationale

I decided to study apoptosis, and the role of sphingolipids in death receptor and EGF signalling in human placental primary trophoblasts for the following reasons:

1. Villous trophoblasts are the primary cells that make up the maternal-fetal interface. Multinucleated syncytiotrophoblasts (ST) form a single layer of cells that baths in maternal blood, and are formed by the fusion of mononucleated cytotrophoblasts (CT) that lie immediately beneath. The integrity of this layer is critical for proper placental function, which includes transfer of nutrients and removal of fetal waste products, as well as the prevention of transfer of infectious agents from mother to fetus.

2. Apoptosis of the trophoblast layer appears to be a normal event, with a constant loss of the syncytium and renewal by fusion of cytotrophoblasts. TNF/IFNγ induces apoptosis in cytotrophoblasts and EGF inhibits apoptosis. Given that these factors are produced by the placenta, and EGF is also important in the development of the placenta, they may play a role in the control of apoptosis in the placenta. However, excessive loss may compromise the barrier, leading to vertical transmission of disease, fetal loss or complications such as IUGR, which is correlated with increased TNF levels. A net loss could result from increased loss of ST or loss of CT, since these cells are the progenitors of ST. Understanding the mechanisms of TNF and EGF signalling in trophoblasts could lead to a better understanding of trophoblast turnover and therefore, better therapies to control it.

3. Both Fas and Fas ligand are expressed in the placenta. Fas Ligand is expressed on trophoblasts but it is unknown whether Fas is expressed on trophoblasts. Fas and TNF-R1 employ similar signalling mechanisms. If Fas is expressed on trophoblasts, it presents the potential for juxtacrine killing of trophoblasts, and therefore another mechanism for controlling trophoblast turnover.

4. Ceramide and acid SMase, and in some cases, nSMase, have been implicated in death receptor-induced signalling pathways in apoptosis in many cell types. Nothing is known about these signalling pathways in trophoblasts. The Spiegel model proposes that the balance of ceramide (death) to SPP (survival), could determine cell fate. Although some growth factors, such as PDGF, have been shown to increase SPP levels and

activate ceramidase in some cell types, it is unknown how EGF affects ceramide and SPP levels in trophoblasts. Does EGF signal by increasing SPP and/or decreasing ceramide levels? Moreover, EGF is an important factor in regulating trophoblast differentiation, and SPP induces differentiation in some cell types. Does EGF exert some of its effects on trophoblasts by increasing SPP levels?

5. This lab has developed a technique to obtain highly pure (>99.9%) human, primary cytotrophoblasts. These cells can be cultured for up to 4 weeks, and can be induced to form syncytium by co-culture with EGF. Cell line models for trophoblasts generally do not behave like villous trophoblasts because they proliferate and express HLA Class I molecules. Our primary trophoblast cultures therefore make an excellent model for studying trophoblast biology, as well as cell signalling in general in primary cell cultures.

2.3 Experimental Approach

For all my studies I used cultures of purified CT using the purification and culture system developed in this lab. I first determined if Fas (CD95) was expressed on trophoblasts by immunohistochemical staining and cellular ELISA. To determine if Fas induced cell death, trophoblasts were treated with cross-linking anti-Fas antibody and FasL-expressing cells. These studies are described in Chapter 4.

Having shown that Fas did not mediate cell death, I focused on the role of ceramide and in TNF-R1-induced death and EGF protection from death. Cell viability and apoptosis was determined in trophoblast cultures after treatment with exogenous ceramide, aSMase and nSMase as well as cotreatment with EGF. Ceramide levels and SMase activity were measured directly after treatment with TNF/IFNγ and EGF. Inhibitors of aSMase and ceramidase were used to determine the role of these enzymes in trophoblast apoptosis. These experiments, described in Chapter 5, lead to the finding that ceramide and aSMase mediate trophoblast apoptosis, and EGF may inhibit apoptosis by affecting ceramide levels. I therefore next investigated the effect of EGF on the pro-survival sphingolipid, sphingosine 1-phosphate. The effects of SPP on trophoblast survival were determined by co-culturing cells with exogenous SPP and TNF/IFNγ and

ceramide, as well as the use of inhibitors of sphingosine kinase to lower SPP levels. The effects of cytokines and growth factors on sphingosine kinase activity were also determined by measuring sphingosine kinase activity.

Chapter 3 : Methods and Materials

3.1 General Methods

3.1.1.Isolation and purification of human term placental trophoblasts

Placental trophoblasts were obtained from human term placentas from spontaneous vaginal deliveries and elective caesarean sections. Cells were prepared from chorionic villous tissue by using trypsin-DNase digestion as described by Morrish *et al.* (1987). The removal of red blood cells and the subsequent purification of trophoblasts with the use of Ig-conjugated glass bead columns to eliminate CD9-positive and MHC Class I and II positive cells has been described by Yui *et al.* (1994b) and Kilani *et al.* (1997). Purified cells were routinely cryopreserved in 10% (v/v) dimethylsulfoxide under liquid nitrogen.

3.1.2 Culturing of human term placental trophoblasts

After being thawed rapidly in a 37°C water bath, cells were washed with warm Iscove's Modified Dulbecco's Medium (IMDM; Gibco BRL, Burlington, ON) supplemented with 0.5% (v/v) 60°C heat-inactivated fetal calf serum (FCS; Gibco BRL) and 100 U/ml penicillin/100 μg/ml streptomycin (Gibco BRL). Following resuspension in 10% FCS/IMDM, cells were plated at $1x10^5$ cells per 100μl per well in Nunclon 96-well plates (NUNC; Life Technologies, Burlington, ON) or $2x10^6$ cells per well in Nunc 6-well plates. These cell concentrations were used throughout the experiments unless otherwise stated. After a minimum of 4 hour incubation at 37°C in 5% (v/v) CO_2 to allow adherence, non-adherent cells were removed by gently washing 2 times with warm 0.5% (v/v) FCS/IMDM. Adherent cells were maintained in 10% (v/v) FCS/IMDM or 0.5% (v/v) FCS/IMDM, depending on the experiment, in a 37°C/5% CO_2 incubator.

3.1.3 Detection of apoptotic cells using TUNEL

This assay detects DNA fragmentation and is based on the method described by Gavrieli *et al.* (1992). Trophoblasts in 96-well dishes were washed once with warm PBS, fixed for 10 minutes with cold acetone/methanol (1:1) at RT, and then

washed 3 times with PBS. The fixed cultures were incubated in TdT buffer (30 mM Tris base, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride) for 10 minutes at RT. After removal of the buffer, 30 µl of a mixture containing 8.3 µM bio-16-dUTP (Boehringer Mannheim, Laval, Québec), 16.5 µM dATP (Boehringer Mannheim), and 5 U/µl TdT in Tdt buffer was added an incubated at 37°C for 1 hour. The reaction was stopped by adding 100 µl 2X SSC (300 mM NaCl, 30 mM sodium citrate) for 10 minutes. After washing 3 times with DDW, endogenous peroxidase activity was quenched with 3% H_2O_2 for 10 minutes, washed 3 times, then blocked with 3% (w/v) skim milk powder, 0.5% Tween 20 in PBS for 30 minutes at RT. ExtrAvidin-HRP (Sigma) diluted 1:75 in the blocking buffer was added an incubated at RT for an additional 30 minutes. The cells were then washed 5 times, colour developed with aminoethyl carbizone (AEC) substrate/chromogen bulk kit (DAKO, Mississauga, ON) until a red colour developed (approximately 5-10 minutes), washed 3 times with DDW, then counterstained with hematoxylin. Positive controls were generated by fixed cells with 1 µg/ml Dnase I (Sigma) prior to labelling.

3.1.4 MTT Assay

This was based on the method of Mosmann (1993). After the specified treatment of trophoblasts cultured in 96-well dishes, media was aspirated from the wells, replaced with 100 µl 0.5%FCS-IMDM containing 0.5 mg/ml MTT, incubated at 37°C for 4 hours, then 150 µl acidic isopropanol added and incubated overnight in the dark to allow the crystals to dissolve. Optical density was measured at 570 nm with a reference wavelength of 650 nm.

3.2 Methods and Materials Specific for Chapter 4

3.2.1Cells

HeLa cells were cultured in 10% FCS-IMDM, detached from culture flasks with 0.05% trypsin, then plated in 96-well plates at 10^4 cells/well and incubated overnight to allow the cells to adhere and become confluent. A1.1 cells (from D. Green, La Jolla, CA)

were cultured in 10%FCS-RPMI 1640 supplemented with L-glutamine, 100 U/ml penicillin/100 μg/ml streptomycin (Gibco), and 10^{-5} M 2-mercaptoethanol.

3.2.2 Immunohistochemical Staining of Fas/CD95

Trophoblasts were cultured in 96-well plates as described. After washing twice with PBS, cells were fixed with 50 μl ice-cold methanol:acetone (95:5) or 2% paraformaldehyde (120 mM sodium phosphate monobasic; pH 7.2- 7.4) for 5 minutes, washed 3 times with PBS, then blocked for 1 hour with 1% BSA-1% FCS-PBS at room temperature. The cells were then incubated with 5 μg/ml monoclonal anti-Fas antibody (CH11) (Immunotech, Westbrook, ME) or mouse IgM (Southern Biotechnology, Inc., Birmingham, AL) in 1%BSA-1% FCS-PBS overnight at 4°C. After being washed 3 times with PBS, cells were incubated for 2 hours at room temperature with goat anti-mouse Ig-HRP at 1:200 in 1% BSA-1% FCS-PBS, and then washed 4 times with PBS. The colour was developed with 3-amino-9-ethylcarbozole (AEC Kit; Zymed, San Francisco, CA). Parallel cultures of HeLa cells were included as positive controls.

3.2.3 Anti-Fas/CD95 Cytotoxicity

Trophoblasts and HeLa cells were cultured in 96-well plates as described. Culture medium was aspirated a replaced with fresh medium containing 1 μg/ml cycloheximide (Sigma) and either anti-CD95 (CH11) or normal mouse IgM at the varying concentrations indicated, and incubated at 37°C for varying times. In experiments in which the cells were pre-incubated with INFγ, the medium was aspirated, replaced with fresh medium containing 100 U/ml IFNγ (Collaborative Biomedical Products, Bedford, MA) and incubated 24 hours at 37°C. The medium was then replaced with fresh medium containing IFNγ, cycloheximide and antibodies, and incubated at 37°C for various times. Cell viability was determined by MTT assay as described.

3.2.4 A1.1-Induced Cytotoxicity

A1.1 cells were induced to express FasL by incubating in 10% FCS-RPMI with 10 ng/ml PMA (Sigma) and 3 μg/ml ionomycin (Sigma) for 2 hours at 37°C. The

activated cells were pelleted, washed twice, and resuspended in 10% FCS-IMDM. Mock activated cells were incubated for the same time period in 10% FCS-IMDM without PMA and ionomycin. One hundred thousand activated and mock-activated cells were then added to confluent trophoblast and HeLa cells in 96-well plates and incubated at 37°C for the times indicated. After gentle washing to remove A1.1 cells, cell viability was then determined by MTT assay and DNA fragmentation.

3.2.5 DNA Fragmentation Assay

This method is based on that described by Rosemary *et al.* (1994). HeLa cells and trophoblasts were cultured in 96-well plates as described. After adhering, cells were labelled overnight with 1 μCi/well tritiated thymidine ([^{3}H]TdR; Dupont NEN, Boston, MA) in 10% FCS-IMDM, washed once with medium, and incubated for 1 hour at 37°C in fresh 10% FCS-IMDM. The medium was then aspirated, replaced with 50 μl 10% FCS-IMDM containing 2 μg/ml CHX and 200 U/ml IFNγ, followed by 50 μl A1.1 cells (10^5 total), 50 μl medium containing 20 ng/ml TNFα, or medium alone. Wells receiving medium alone served to determine spontaneous fragmentation and total labelling. Several wells also received medium containing, in addition to CHX and IFNγ, 20 μg/ml of antagonistic anti-Fas (SM1/23) or isotype control antibody $IgG2_b$ before adding A1.1 cells. The final culture concentrations were 1 μg/ml CHX, 100 U/ml IFNγ and 10 ng/ml TNFα. Trophoblast cultures were then incubated for 18-24 hours and HeLa cultures incubated for 3-4 hours at 37°C. The medium was aspirated, and fragmented DNA released by lysing the cells with 100 μl 1% Triton X-100 (Sigma) in PBS with 2 mM EDTA (Sigma). The lysates were transferred to microfuge tubes, centrifuged at 13 000 x g for 10 minutes, and 50 μl of the supernatant transferred to scintillation vials plus 6 ml scintillation fluid. For total DNA labelling, cells were lysed with 2% SDS (sodium dodecyl sulphate; Bio-Rad) in 0.1 N NaOH, and 50 μl transferred to scintillation vials for scintillation counting. Per cent DNA fragmentation was calculated by:

$$\frac{\text{sample cpm}}{\text{total cpm}} \times 100$$

3.2.6 Cellular Elisa

Surface expression of Fas on viable cells was determined by cellular ELISA. Trophoblast cultures in 96-well plates were blocked for 1 hour with 1% BSA-1% normal goat serum-PBS at 4°C, followed by incubation at 4°C for 2 hours with 5 μg/ml monoclonal mouse anti-human Fas (SM1/23) or $IgG2_b$ in blocking buffer. The cells were washed 3 times with cold PBS, then incubated at 4°C for 1 hour with goat anti-mouse-HRP conjugate (Southern Biotechnology, Inc., Birmingham, AL) diluted 1:2000 in blocking buffer. After 5 washes with cold PBS, colour was developed with ABTS (Sigma) solution (2 mM ABTS (2,2'-azino-*bis*[3-ethylbenzthiazoline-6-sulfonic acid]), 45 mM disodium phosphate, 30 mM citric acid, 0.003% H_2O_2), and optical density (OD) measured at 405 nm with a reference wavelength of 490 nm.

3.2.7 Western Blot

This work was carried out by Dr. Steve Smith at the Department of Obstetrics, University of Nottingham, UK.

Cells were harvested in 25 mM Tris-HCl pH 7.5, 0.5% Triton X-100 and then sonicated for 5 seconds. PAGE of 10 μl of lysate equivalent to 2 x 10^4 cells was performed on 12% gels (8.3 cm x 7.4 cm x 0.75 mm) in SDS with a pH 6.8 3.5 % stacking gel at rt for 1.5 hrs in 120 V. Electrophoresed standards (Bio-Rad, Mississauga, ON) and lysate proteins were transferred electophoretically to a nitrocellulose membrane (0.22 μm pore size; MSI Inc., Westborough, MA) at 15 volts for 2.5 hrs with an EC140 Miniblot module (EC Aparatus). The membrane was blocked overnight at 4°C in 5-7% skim milk solution in 0.1 M Tris-HCl, 0.5M NaCl (TBS). Gels were routinely stained with Coomassie blue dye to confirm protein transfer. The blocked membrane was incubated with mouse anti-human Fas monoclonal IgM (CH11; Transduction Laboratories, Lexington, KY) at a dilution of 1:1000 in 0.1% Tween in TBS (TBST), and then with HRP-conjuagted goat anti-mouse (Jackson Immunoresearch, Biocan Scientific, ON) at a dilution of 1:20000 in TBST. Ig-reacted bands were visualized with

an enhanced chemiluminescence kit (Amersham Canada, Ltd, Oakville, ON).

3.2.8 RT-PCR

This work was carried out by Dr. Steve Smith at the Department of Obstetrics, University of Nottingham, UK.

1st strand cDNA was synthesised from 10 ng total RNA by reverse transcription with 50 units Moloney Murine Leukaemia Virus Reverse Transcriptase in the presence of 50mM TrisHCl (pH 8.3), 75mM KCL, 3mM $MgCl_2$, 10mM DTT, 10 units recombinant RNAse Inhibitor, 400μM dNTP's and 6μg/ml oligo(dT_{12-18}) primer in a total reaction volume of 50μl. cDNA synthesis was performed at 37°C for 60 mins and the reverse transcriptase was inactivated by heating at 94°C for 10 mins. Resulting cDNA stocks were stored at -20°C until use.

Specific sequences were amplified from 1-4μl of the cDNA stocks by nested PCR in a PTC-200 DNA Engine (GRI Ltd, Essex, UK). The first round of PCR for Fas was performed for 20 cycles using primers designed using the Primer3 program at Massachusetts Institute of Technology (http://www.genome.wi.mit.edu/cgi-bin/primer) based on Fas published sequence (Oehm et al 1992). These primers were designated Fas 1 and Fas 2. Second round PCR was then carried out using 0.2 μl of the above reaction, which was amplified for a further 30 cycles using primers designated Fas II F and Fas II R (Cascino et al 1995). Fas primers were obtained from GibcoBRL Life Tchnologies (Paisley, Scotland). PCRs for GAPDH and β-Actin were carried out for 32 cycles, using primers from Stratagene Ltd (La Jolla, CA). Primer pairs spanned intron-exon junctions to control for genomic DNA contamination. Oligonucleotides Fas1 and Fas2 span the transmembrane domain region of the Fas mRNA, thus detecting both full-length Fas and the transcript for the soluble FasTMDel. Primer sequences were as follows :

Fas1 (5'-CTCTGGTTCTTACGTCTGTTGCTA),

Fas2 (5'GCTTTGGATTTCATTTCTGAAGTT),

Fas II F (5'-CATGGCTTAGAAGTGGAAAT),

Fas II R (5'-ATTTATTGCCACTGTTTCAGG),

GAPDH1 (5'-CCACCCATGGCAAATTCCATGGCA)

GAPDH2 (5'-TCTAGACGGCAGGTCAGGTCCACC).

Amplimer sizes were 338bp (Fas), 275bp (FasTMDel), 661bp (β-Actin) and 600bp (GAPDH). PCR reactions were carried out in the presence of 0.75 units Taq Polymerase, 20mM TrisHCl (pH 8.4), 50mM KCL, 1.5mM $MgCl_2$, 200μM dNTP's and 1mM of each oligonucleotide in a total reaction volume of 30μl. PCR was performed with temperatures of 94°C for 3 mins, 60°C for 3 mins followed by a varying number of cycles (see above) at 72°C for 90 secs, 94°C for 45 secs and 60°C for 45 secs. Final extension was for 10 mins at 72°C. PCR products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining and UV fluorescence. PCR products were confirmed by restriction digestion of amplimers extracted from low-melting point agarose gels (results not shown).

3.3. Methods and Materials Specific to Chapter 5

3.3.1 Treatment of trophoblasts with ceramide and sphingomyelinase

C_2-ceramide and C_{16}-ceramide (*N*-palmitoyl-sphingosine) were from Biomol (Plymouth Meeting, MA). Acid sphingomyelinase from human placenta, bacterial neutral sphingomyelinase (*Staphylococcus aureus)*, and dihydro-C_{16}-ceramide (*N*-palmitoyl-DL-dihydrosphingosine) were from Sigma. The ceramides were first dissolved at 37°C in ethanol:dodecane (98:2), then diluted to the appropriate concentrations in 0.5% FCS-IMDM. The sphingomyelinases were diluted directly into 0.5% FCS-IMDM. The carrier for the acid SMase was 50% glycerol, 25 mM potassium phosphate, pH 4.5, 0.1% Triton X-100, and 0.05 mM PMSF.

Trophoblasts were cultured in 96-well plates. After adhering, the medium was aspirated and replaced with 100 μl 0.5% FCS-IMDM containing ceramide or sphingomyelinase with and without 10 ng/ml EGF and incubated at 37°C for specified times. Following incubation, cell viability and apoptosis were determined by MTT and TUNEL, respectively.

3.3.2 Treatment of trophoblasts with cytokines and SMase for ceramide determinations

Trophoblasts were plated at 2-3 x 10^6 cells per well in 6-well dishes (Nunclon) as described. For cytokine stimulations, the medium was aspirated and replaced with 1 ml 10% FCS-IMDM containing 10 ng/ml TNFα + 100 U/ml IFNγ with and/or without 10 ng/ml EGF. For SMase treatments, the medium was replaced with 1 ml 0.5% FCS-IMDM containing acid or neutral SMase, or vehicle, and with and without 10 ng/ml EGF. Both were incubated at 37°C for specified times and ceramide levels determined as described.

3.3.3 Inhibition of Acid Sphingomyelinase

D609 was purchased from Biomol and diluted to working concentrations in 10% FCS-IMDM with 10 ng/ml TNFα and 100 U/ml IFNγ. After specified times cell viability and apoptosis was determined by MTT and TUNEL.

3.3.4 Inhibitors of Ceramidase

N-oleoylethanolamine (OE), an inhibitor of acid ceramidase, was from Sigma, and D-MAPP, an inhibitor of alkaline ceramidase, was from Biomol. Both were first dissolved in DMSO and stored at –20°C before dilution to working concentrations in 0.5% FCS-IMDM for cell culture experiments. The working concentrations were 50 and 100 μM for OE and 10 μM for D-MAPP.

3.3.5 N-[(^{3}H]Palmitoyl)-D-Sphingosine (C_{16}-Ceramide) Synthesis

[^{3}H]C_{16}-ceramide was synthesized as described by Futerman and Pagano (1992). An *N*-hydroxysuccinimidyl (NHS) ester of palmitic acid was synthesized by continuous stirring 50 μmol of palmitic acid plus 500 μCi [^{3}H]palmitic acid (Dupont NEN; from Dr. David Brindley) with equal molar equivalents of NHS and dicyclohexylcarbodiimide (DCC) in 500 μl *N,N*-dimethylformamide (DMF) at room temperature for 3 days in the dark. The dicyclohexylurea precipitate was pelleted by centrifugation and the supernatant containing the succinimidyl ester of [^{3}H]palmitic acid was transferred to a clean Pyrex

tube. Fifty μM sphingosine (Biomol, Plymouth Meeting, MA) dissolved in DMF and 25 μl diisopropylethylamine (DIPE) were then added to the palmitic acid ester, the volume adjusted to 1 ml with DMF, and then incubated at 40°C with continuous stirring for 24 hours. The reaction was stopped by acidification with 50 μl 3N HCl, and the solvent evaporated under a stream of nitrogen gas. The product was extracted by a Bligh and Dyer (1959) extraction and purified by TLC using chloroform:methanol:acetic acid (94:1:5) as solvent. In this solvent ceramide migrates with an $R_f = 0.25$ while fatty acid migrates at $R_f = 0.95$. [^{3}H]ceramide was detected by autoradiography and eluded from the silica in chloroform:methanol (9:1). The specific activity of the final product was 1-2 x 10^4 dpm/nmol.

3.3.6 Ceramide Quantification (2 Methods)

Method 1 (DAG Kinase Assay): Trophoblasts were plated in 6-well dishes as described. After specified treatments with cytokines, cells were washed once with PBS. Lipids were extracted by adding 1 ml hexane:propanol (3:2) to each well for 30 minutes, transferred to 5-ml polypropylene tubes and completely dried under a stream of nitrogen. Two millilitres of methanol:chloroform:water (2:1:0.8) was added, capped, vortexed vigorously, then 520 μl each of chloroform and water added. The tubes were again vortexed vigorously, and the phases broken by centrifuging at 2000 rpm for 5 minutes. The top aqueous layer was aspirated and the bottom organic layer containing lipids was completely dried under a stream of nitrogen or overnight under vacuum.

Ceramide and DAG were enzymatically converted to ceramide-1-phosphate and phosphatidic (PA) acid, respectively, with [γ^{32}P]ATP by the DAG kinase assay as described by Preiss *et al.* (1986) and modified by Abousalham *et al.* (1997). A reaction mixture was prepared in sufficient amount to permit 100 μL per sample and standard. Standards of ceramide (Type III, Sigma) and DAG (Avanti Polar Lipids, Alabaster, AL) were prepared together in the range of 50 to 1000 pmoles to generate a standard curve. The reaction mixture per 100 μl was prepared with 20 μl reaction buffer (5 mM DETAPEC, 62.5 mM $MgCl_2$, 250 mM NaCl, 5 mM EGTA, 250 mM Imidazole/HCl, ph 6.6), 20 μl cardiolipin/N-octyl-β-D-glucopyranoside (5 mM cardiolipin (Avanti Polar

Lipids, Alabaster, AL), 7.5% N-octyl-β-D-glucopyranoside (ICN, Costa Mesa, CA), 1 mM DETAPEC, pH 6.6), 10 µl 100 mM DTT, 9 µl 11 mM ATP, 36 µl water, and 5 µl DAG kinase (Calbiochem, La Jolla, CA), then incubated for 20 minutes at 37°C. The final assay conditions were 50 mM imidazole, 1 mM DETAPEC, 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 10 mM DTT, 1 mM ATP, 1.5% N-octyl-β-D-glucopyranoside, 1 mM cardiolipin, and 0.01U DAG kinase. One µCi/assay [γ^{32}P]ATP was added to the reaction mixture, and then 100 µl of the mixture added to each sample. The samples were then vortexed, incubated in a 37°C water bath for 5 minutes, then sonicated in a sonicating water bath for 10 minutes. The tubes were then incubated for another 5 minutes at 37°C, resonicated for 10 minutes, then incubated at 37°C for 20 minutes. The lipids were extracted by adding 300 µl HCl (10 mM), 500 µl chloroform and 500 µl methanol, vortexed vigorously, extracted for 10 minutes at room temperature, followed by addition of 500 µl each of 10 mM HCl and chloroform. The tubes were vortexed centrifuged at 2000 rpm for 5 minutes to break the phases. After centrifugation, the aqueous phase (top) was aspirated and the organic phase evaporated under vacuum overnight. The dried lipids were dissolved in chloroform and spotted onto glass backed Silica Gel 60 Whatman TLC plates (VWR). The plates were developed in chloroform:methanol:ammonium hydroxide (65:35:7.5), and then in chloroform:acetone:acetic acid:methanol:water (10:4:3:2:1). Radioactive spots corresponding to ceramide-1-phosphate and PA were detected by autoradiography, scraped into scintillation vials, and radioactivity determined by scintillation counting. The quantity of the lipids was then determined by reference to the standard curve.

Method 2 ([^{3}H]Palmitic Acid Labelled Cells): Trophoblasts were plated in 6-well dishes as described. The adherent cells were then labelled with 5 µCi/ml [^{3}H]-palmitic acid (NEN, 45 Ci/mmol) in 10% FCS-IMDM for 48 hrs. After washing 3 times, the cells were treated as specified, and the lipids extracted as described above. The dried lipids were dissolved in chloroform and ceramide separated by TLC on Whatman Silica Gel 60 TLC plates (VWR Scientific, Mississauga, ON) in chloroform:methanol:acetic acid:water (85:4.5:5:0.5). In this solvent ceramide migrated with an R_f of 0.55-0.6 while sphingomyelin and phosphatidylcholine remained near the origin. Radioactive spots

corresponding to ceramide were identified by spraying the plate with En^{3}Hance (NEN Dupont) followed by autoradiography, and by comparison to authentic C_{16}-ceramide (Biomol, Plymouth Meeting, MA) identified by staining with iodine vapours. Radioactivity was determined by scintillation counting.

3.3.7 Acid Sphingomyelinase Assay

Trophoblasts were plated at 2-3 x 10^6 cells/well in 6-well dishes as described. After treatment, cells were washed once with cold PBS on ice, and then lysed by adding 400 µl 0.2% Triton X-100 to each well, scraping, and transferring to 1.5 ml microfuge tubes. After a 10 minute incubation on ice, the tubes were centrifuged for 5 minutes at 13000 rpm in a microfuge and the cell lysates transferred to fresh tubes. Protein concentration was determined by micro BCA (Pierce). Acid sphingomyelinase in the cell lysates was determined by combining 2.25 µl [N-methyl-^{14}C]sphingomyelin (NEN; 40-60 mCi/mmol; final concentration 0.2µCi/ml), 50 µl cell lysate (30-50 µg protein) plus 150 µl assay buffer (250 mM sodium acetate, pH 5.0, 1 mM EDTA) and incubating for 2 hours in a shaking 37°C water bath. Purified acid sphingomyelinase from human placenta (Sigma) was used as a positive control. After incubation, 800 µl chloroform:methanol (2:1) was added, vortexed, followed by the addition of 100 µl distilled water and centrifuged for 5 minutes to break phases. Aliquots (100 µl) of the top aqueous layer containing the hydrolysed phosphocholine were transferred to scintillation vials and scintillation counted.

3.3.8 Ceramidase Assay (2 Methods)

Method1: This method was based on that by Bielawska *et al.* (1996). Trophoblasts were plated at 2-3 x 10^6/well in 6-well plates. After treatment, the cells were scraped into 0.25M sucrose, 1 mM EDTA, disrupted by a few short bursts of a Vibra Cell™ probe sonicator (Sonics & Materials, Inc., CT). The lysates were centrifuged at about 800 x g for 5 minutes to remove whole cells and nuclei, after which the supernatants were centrifuged 100 000 x g for 1 hour. The membrane pellet was resuspended in appropriate acid (0.1M citric acid, pH 4.5) or alkaline (0.1M CAPSO, pH

9.5) buffers. Substrate mixture was prepared by mixing approximately 10 nmoles [^{3}H]ceramide (see section 3.3.5 for synthesis) with 100 µl of 0.1% Triton X-100 in chloroform/methanol (2:1) and 100 µl 0.2% sodium cholate in chloroform/methanol (2:1). The solvent was then evaporated under nitrogen gas at 70°C. Fifty µl buffer, 20 µl 50 mM $MgCl_2$, plus 100 µl cell extract was added to the tubes and incubated for 1 hour at 60°C. The reaction was stopped and [^{3}H]palmitic acid product was separated by adding 2 ml alkaline Dole's (isopropyl alcohol : heptane : NaOH ; 100 : 25 : 2.5), 1.2 ml heptane, plus 1 ml water. After vortexing and centrifugation (5 minutes at 2000 rpm) to break phases, the upper phase was discarded, and the bottom phase washed twice with heptane, discarding the upper phase. One ml 1N H_2SO_4 and 2 ml heptane were added to the bottom phase, vortexed, centrifuged, and aliquots of the upper phase containing the [^{3}H]palmitic acid were transferred to scintillation vials for scintillation counting.

Method2: This method was based on that by Huwiler *et al.* (1999). Trophoblasts were plated at 2-3 x 10^6/well in 6-well plates. After treatment, the cells were scraped into cold PBS on ice and the cells from 3 wells were combined, centrifuged at 800 x g for 5 minutes to pellet cells, and then the pellet resuspended in buffer (50 mM sodium acetate, pH 4.5, 0.5% Triton X-100, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM D-galactonic-γ-lactone for acid ceramidase, and 50 mM Tris, pH 8.0, 0.5% Triton X-100, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM D-galactonic-γ-lactone for alkaline ceramidase), and sonicated in a sonicating bath for 5 minutes. The samples were then centrifuged at 14 000 x g for 10 minutes and ceramidase activity in the supernatant assayed by combining 100 µl (approximately 20 µg protein: protein concentration in the supernatant was determined by BCA) of supernatant with 20 nCi [^{3}H]ceramide and incubated for 20 hours in a 37°C water bath. The reaction was stopped by adding 200 µl distilled water followed by 2 ml of chloroform:methanol (2:1) to extract lipids. The lower organic phase was evaporated under vacuum or under nitrogen, spotted onto TLC plates and resolved with chloroform:methanol:acetic acid:water (85:4.5:5:0.5), sprayed with En^{3}Hance spray, and radioactive spots corresponding to ceramide were scraped and transferred to scintillation vials for scintillation counting.

3.4 Methods and Materials Specific for Chapter 6

3.4.1 Sphingosine-1-phosphate

SPP was purchased from Biomol (Plymouth Meeting, MA), dissolved in warm methanol, aliquoted at 125 nmoles/tube, the methanol evaporated with a stream of nitrogen gas and the aliquots stored at –20°C. The dried aliquots were dissolved in 1 ml 0.4% BSA-IMDM and incubated with frequent vortexing in a water bath at 37°C for 30 minutes, then diluted to working concentrations in 0.5% FCS-IMDM. In co-treatment experiments with TNFα/IFNγ or sphingosine kinase inhibitors (see next section), the cytokines and inhibitors where added at specified concentrations directly to the working dilution of SPP.

3.4.2 Inhibitors of Sphingosine Kinase

Dihydrosphingosine (DHS) and dimethylsphingosine (DMS) were from Biomol, resuspended in DMSO, and diluted to final working concentrations in 0.5% FCS-IMDM.

3.4.3 Sphingosine Kinase Assay

Sphingosine kinase activity was determined based on the method of Olivera *et al.* (1998). Trophoblasts were plated in 6 well dishes (Nunc) at 2-3 x 10^6/well. After specified treatment, cells were washed once with cold PBS on ice, scraped into 500 µl cold PBS and pelleted at 800 x g for 5 minutes in a microfuge. The pellet was resuspended in 250 µl kinase buffer (20 mM Tris, pH 7.4, 20% glycerol, 1 mM disodium EDTA, 40 mM β-glycerophosphate, 0.5 mM 4-deoxypyridoxine, 1:100 dilution each of protease and phosphatase inhibitor cocktails (Sigma)), then cells disrupted by freeze thawing 5-6 times. Nuclei and cell fragments were pelleted by centrifugation at 800 x g for 5 minutes. Protein content in the cell extracts was determined by BCA (Pierce). One hundred µl cell extract was added to 80 µl kinase buffer, 10 µl of 20 mM sphingosine in 5% Triton X-100 (final assay concentration equals 100 nM), 5 µCi [γ^{32}P]ATP delivered in 10 µl 100 mM $MgCl_2$, and incubated with constant shaking for 30 minutes at 37°C. The reaction was stopped by immersing the tubes in an ice water bath plus the addition of 20 µl 1N HCl. [^{32}P]SPP was extracted by adding 800 µl chloroform:methanol:HCl

(100:200:1) followed by 240 µl each of chloroform and 1N KCl. The aqueous layer was aspirated and the organic layer dried under stream of nitrogen or overnight under vacuum. The dried samples were dissolved in chloroform: methanol (2:1), spotted onto TLC plates, and developed in butanol:acetic acid:water (3:1:1). [^{32}P]SPP bands were detected by autoradiography and scraped into scintillation vials for scintillation counting. Activity was normalized to protein and expressed as pmol SPP/mg/hour.

3.4.4 SPP Measurements

Trophoblast SPP levels were measured using the method described by Yatomi *et al.* (1995). SPP was extracted either under alkaline conditions as described or directly into water-saturated butanol. The extracts were acetylated with [^{3}H]acetic anhydride to generate [^{3}H]C_2-ceramide-1-phosphate that was then isolated by TLC. The amount of SPP in the cell extracts was calculated by extrapolation from a SPP standard curve.

Chapter 4 : Fas/CD95 Is Expressed on Placental Cytotrophoblasts But Does Not Induce a Death Signal

(Most of the data in this chapter has been published as an article entitled "Death Receptor Fas/APO-1/CD95 Expressed by Human Placental Cytotrophoblasts Does Not Mediate Apoptosis" by Payne, S.G., Smith, S.C., Davidge, S.T., Baker, P.N., & Guilbert, L.J. in *Biology of Reproduction* (1999) 60 : 1144-1150)

4.1 Introduction

Fas (CD95) is a 45-kDa cell surface "death" receptor that belongs to the TNF receptor family (Itoh *et* al., 1991; reviewed in Nagata, 1997). Like the TNF receptor p55 (TNF-R1), Fas contains a "death" domain required for the transduction of apoptotic signals that typify these receptors. Fas and TNF-R1 have similar signalling pathways and share some of the signalling intermediates in the signalling pathways that lead to apoptosis.

The ligand for Fas is Fas ligand (FasL) and it belongs to the TNF family. It is an inducible protein expressed on the cell surface of activated T cells and natural killer cells. It is these immune cells that participate in the Fas-FasL mediated elimination of virally infected and transformed cells. FasL is also constitutively expressed on specialized epithelial and endothelial cells that are associated with T cell selection and peripheral elimination, such as the eye, testis, brain and placenta (Griffith *et al.*, 1995; French *et al.*, 1996; Xerri *et al.*, 1997). In these sites FasL is thought to trigger apoptosis in homing T cells, thereby enforcing immune privilege (Griffith *et al.*, 1996; Streilein, 1996).

Both Fas and FasL are expressed in the placenta. FasL is expressed in deciduas, trophoblasts and fetal stroma, and is thought to control the migration of fetal cells into maternal tissue and maternal cells into fetal tissue (Bianchi *et al.*, 1996; Hunt & Hutter, 1996; Runic *et al.*, 1996). Fas has been shown to be expressed in choronic trophoblasts and selectively in endothelial cells (Xerri *et al.*, 1997; Salafia *et al.*, 1996), but has not been shown to be expressed in villous trophoblasts. Villous syncytiotrophoblasts are in

direct contact with maternal blood and form the maternal-fetal barrier (reviewed in Benirschke & Kaufmann, 1995). TNF-R1 is expressed in villous trophoblasts and mediates TNFα-induced apoptosis (Yui *et al.* 1996). It is suggested that maternal TNFα could lead to excessive apoptosis of villous trophoblasts and thereby compromise the maternal-fetal barrier (Yui *et al.*, 1994). It is conceivable that, if Fas is expressed on the villous trophoblasts, then activated maternal T cells expressing FasL could also induce apoptosis in villous trophoblasts.

Apoptosis *per se*, is not an aberrant phenomenon in the villous placenta. The syncytiotrophoblast appears to constantly renew by loss of aged nuclei and cytoplasm (Jones & Fox, 1977) and replenished through fusion of underlying cytotrophoblasts (Boyd & Hamilton, 1966). Electron microscopy observations of nuclei within the syncytiotrophoblast (Nelson, 1996) and the observation of trophoblast apoptosis throughout gestation (Smith *et al.*, 1997a) suggest that apoptosis may be a normal placental aging process. However, a higher than normal incidence of trophoblast apoptosis may lead to a compromise in the maternal-fetal barrier. Given that trophoblasts express FasL, the possibility exists for juxtacrine killing via trophoblast-expressed Fas and FasL. The addition of apoptosis induced from maternal sources, FasL and TNFα, could lead to a higher than normal incidence of apoptosis.

The expression of Fas protein does not always imply that the outcome will be apoptosis. There are splice variants that lack the signalling death domain and this Fas molecule does not transmit a death signal (Cascino *et al.*, 1996). Soluble forms of Fas exist, which lack the transmembrane domain, which may have antagonistic functions (Cascino *et al.*, 1995). In addition, Fas signal transduction can be regulated by viral and cellular-derived inhibitors, such as FLIP, which block the death signalling pathway (Irmler *et al.*, 1997; Srinivasula *et al.*, 1997; Hu *et al.*, 1997; Mannick *et al.*, 1997). In this study I therefore asked whether cytotrophoblasts express cell surface Fas and if it mediates apoptosis.

4.2 Results

4.2.1 Cell surface expression of Fas/CD95.

Fas expression on cultured cytotrophoblasts was determined immunohistochemically on paraformaldehyde and methanol:acetone fixed cells. Figure 4.1b shows that all cells expressed Fas, albeit at different levels. Some cells showed intense staining, especially in the perinuclear area of the cell, while others stained less intensely and more uniform. However, all the cells stained more intense than the isotype control cells (Figure 4.1a). To further verify that Fas was expressed on the cell surface, a cellular ELISA was done on live cells in culture. As shown in Figure 4.2, there was 4-fold increase in OD in wells treated with anti-Fas antibody versus isotype control antibody. Since this assay was done with live cells, the increase in OD reflects binding of antibody to cell surface Fas antigen, and not intracellular.

4.2.2 Fas mRNA and protein expression in cytotrophoblasts.

(The data in this section was collected by Dr. Steve Smith (Department of Obstetrics, City Hospital, University of Nottingham, Nottingham, UK) as his contribution to the collaborative work. It is included here solely to eliminate what would otherwise be gaps in the data.)

Alternative splice variants of Fas have been shown to exist. There are at least three soluble forms which lack the membrane-anchoring domain (Cascino *et al.*, 1995) and another which lacks the cytoplasmic signalling death domain (Cascino *et al.*, 1996). For these reasons it was important to determine if the Fas expressed by cytotrophoblasts was full length or a truncated variant. Fas mRNA was detected by RT-PCR using primers specific for a region that spans the transmembrane domain and includes part of the extracellular and cytoplasmic domains. The major product detected in whole placental tissue (Figure 4.3a) and purified cytotrophoblasts (Figure 4.3b) was identical to that of Jurkats (Figure 4.3a), a T cell line known to express full length Fas (Oehm *et al.*, 1992) In addition, the major product from cytotrophoblasts was between 300 and 400 bp, and the predicted size of the amplicon for transmembrane Fas is 338 bp. Western blot analysis of purified cytotrophoblasts revealed a 45-kDa protein (Figure 4.4), which is the

reported size of the full length Fas protein (Itoh *et al.*, 1991).

4.2.3 Activation of Fas with anti-Fas antibody does not induce apoptosis.

To determine if cytotrophoblast Fas mediated a death signal, we treated the cells with an IgM monoclonal anti-Fas (CH11). This antibody has been shown to induce Fas-mediated cell death (Yonehara *et al.*, 1989). HeLa cells have previously been shown to undergo Fas-mediated death (Deiss *et al.*, 1996) and were used in this study as a positive control. Treatment of HeLa cells with CH11 at 100 ng/ml in the presence of 1 μg/ml cycloheximide for 24 hrs reduced cell viability by 40% as measured by MTT (Figure 4.5). Cytotrophoblasts showed no loss in cell viability under the same conditions. The antibody was tested over a concentration range of 25 ng/ml to 200 ng/ml and had no effect on cytotrophoblast viability at any concentration (Figure 4.6).

We know that cytotrophoblast cultures require between 48 and 96 hours to lose viability when treated with TNFα/IFNγ (Yui *et al.*, 1994), and will do so in the absence of protein synthesis inhibitors. Thus it is possible that they also require longer treatment with anti-Fas. However, a 72 hour treatment with 100 ng/ml anti-Fas + IFNγ had no effect on cell viability. Parallel cultures of the same cells with TNFα reduced cell viability by about 50% (Figure 4.7), demonstrating that under these conditions cytotrophoblasts are able to undergo agonist-induced cell death.

4.2.4 Fas Ligand expressing cells did not stimulate cytotrophoblast death.

The use of cross-linking anti-Fas antibodies to activate Fas only mimic the effects of the physiological ligand for Fas, FasL. To determine whether functional cell-surface FasL could induce cytotrophoblast death, we used the T cell line A1.1 that can be induced to upregulate FasL expression when treated with PMA and ionomycin (Brunner *et al.*, 1995). However, we could not use the TUNEL assay to determine DNA fragmentation, a characteristic of apoptotic cell death, in these experiments because the A1-1 cells in the cultures made it problematic to count the TUNEL positive trophoblasts, and it was sometimes difficult to distinguish between TUNEL positive trophoblasts and A1-1 cells. We therefore decided to use [^{3}H]-thymidine labelled cells and measure the

amount of fragmented DNA released into the cytoplasm based on the method described in Garner *et al.* (1994). Since cytotrophoblasts are primary, non-dividing cells, we first we had to establish that cytotrophoblasts could be labelled with [^{3}H]-thymidine and fragmented DNA could be detected. Cytotrophoblasts were labelled overnight with 1 μCi [^{3}H]-thymidine, treated with TNFα/IFNγ for 20 hours, known to induce DNA fragmentation in cytotrophoblasts (Yui *et* al., 1994), and fragmented DNA determined as described in Methods and Materials. As shown in Figure 4.8 cytotrophoblasts can be labelled with [^{3}H]-thymidine, and DNA fragmentation induced by TNFα/IFNγ can be detected.

To establish A1-1 cytotoxic conditions, confluent HeLa cell cultures in 96-well plates were incubated for 3.5 hours with cycloheximide and varying numbers of A1-1 cells. As shown in Figure 4.9, activated A1-1 cells induce DNA fragmentation in HeLa cells, and the amount of fragmentation is dependant on the number of A1-1 cells added to the cultures. In cultures with 10^5 activated A1-1 cells, 14% of HeLa cell DNA was fragmented, and 5% fragmented with 2.5 x 10^4 A1-1 cells. Control, or non-activated A1-1 cells, had no effect (<1% DNA fragmentation). To demonstrate that the A1-1-induced DNA fragmentation was mediated by Fas-FasL interactions and not other apoptosis mediating factors, HeLa cells were incubated with an IgG anti-Fas monoclonal antibody (SM1/23) to block FasL interactions. Twenty μg/ml anti-Fas antibody completely, or nearly completely, blocked A1-1-induced DNA fragmentation, depending on the number of A1-1 cells (Figure 4.9). With 10^5 A1-1 cells, anti-Fas treatment reduced HeLa cell DNA fragmentation from 14% to 4%, and to control levels in cultures with 2.5 x 10^4 A1-1 cells. An $IgG2_b$ isotype control had no effect. This data demonstrates that activated A1-1 cells induced apoptosis is Fas mediated.

Having established a cellular FasL-induced apoptosis assay, we asked whether A1-1 cells could induce DNA fragmentation in cytotrophoblasts. Activated A1-1 cells did not induce DNA fragmentation in cytotrophoblasts that were also treated with 1 ug/ml CHX and 100 U/ml IFNγ for 20 hours (Figure 4.10). TNFα/IFNγ, however, under the same conditions, induced DNA fragmentation (16 %) in cytotrophoblasts, demonstrating that the cells were able to undergo agonist-induced apoptosis under these

conditions.

To further investigate whether FasL expressing cells affected cytotrophoblasts, cell viability was determined by MTT after a 20 hour incubation with 10^5 A1-1 cells in the presence of 1 μg/ml CHX and 100 U/ml IFNγ. As shown in Figure 4.11, activated A1-1 cells did not affect cytotrophoblast viability. HeLa cell viability however, was reduced by 60% under the same conditions. Cytotrophoblast cultures incubated with TNFα also saw a reduction in cell viability by about 20%, again demonstrating that under these conditions cytotrophoblasts can undergo agonist-induced cell death.

RT-PCR analysis demonstrated that Fas mRNA is also expressed in first trimester placentas (Figure 4.3a). T lymphocytes are susceptible to Fas-induced apoptosis at different stages of development and activation, therefore it is conceivable that trophoblasts have varying susceptibilities to Fas-induced apoptosis at different stages of gestation. However, activated A1-1 cells did not induce DNA fragmentation in cultured first trimester cytotrophoblasts (Figure 4.12).

4.2.5 Fas ligand expressing cells did not induce DNA fragmentation in syncytiotrophoblasts.

As described in Chapter I, mononucleated cytotrophoblasts fuse to form multinucleated syncytium, or syncytiotrophoblasts. In vivo, syncytiotrophoblasts appear to undergo constant loss and renewal (Jones & Fox, 1977), and the loss may be due to apoptosis (Smith et al., 1997). Although cytotrophoblasts do not appear to undergo FasL-induced apoptosis, it is possible that Fas may play a role in syncytiotrophoblast apoptosis. Syncytium formation can occur normally in basal medium but is accelerated by treatment with EGF. As shown in Figure 4.13, activated A1-1 cells did not induce DNA fragmentation in EGF-induced syncytiotrophoblasts. Under the same conditions, TNFα induced a small, significant ($p<0.05$) increase in DNA fragmentation. Garcia-Lloret *et al.* (1996) also demonstrated that syncytiotrophoblasts undergo TNFα-induced apoptosis, albeit to a lesser degree than cytotrophoblasts.

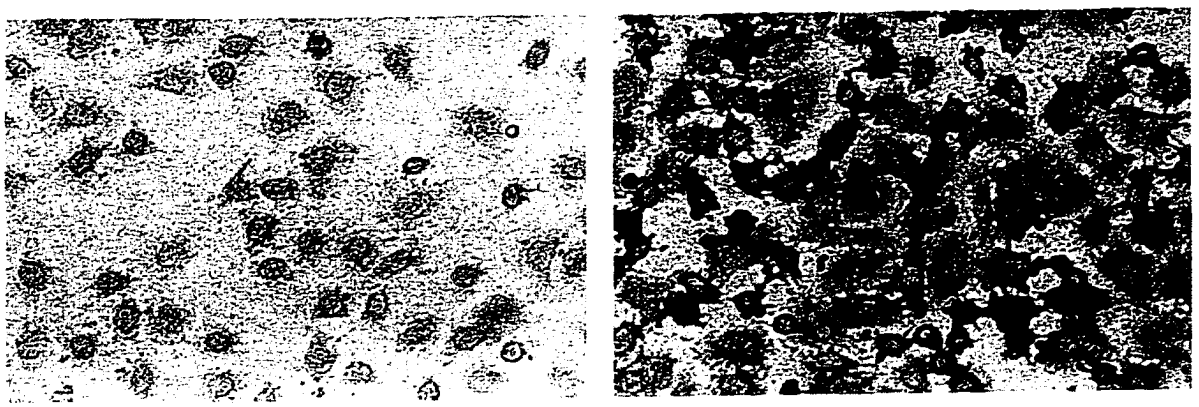
4.2.6 NOS inhibitors did not sensitize cytotrophoblasts to FasL-induced

apoptosis.

Cytotrophoblasts express the nitric oxide synthase (NOS) isozyme eNOS (Smith *et al.*, 1998) and produce nitric oxide (NO) (Guilbert *et al.*, unpublished data). Since NO has been shown to inhibit apoptosis in human B lymphocytes (Mannick *et al.*, 1994) and inhibits Fas-induced apoptosis in a variety of human cell lines such as T cell line Jurkat and promonocytic cell line U937 (Mannick *et al.*, 1997) without affecting Fas-expression levels, we asked whether NO blocked Fas-mediated apoptosis in cytotrophoblasts. To do this we used the competitive inhibitors of NOS, AG, L-NMMA and L-NAME (Southern & Szabo, 1996). However, these inhibitors at 0.5 mM did not increase A1.1 cell fragmentation of cytotrophoblast DNA (Figure 4.14). This data suggests that NO is not the inhibitor that is blocking Fas-mediated death in cytotrophoblasts.

4.3 Summary of Data

1. Cultured human cytotrophoblasts express Fas on the cell surface.
2. Cross-linking anti-Fas antibody does not induce cytotrophoblasts death
3. FasL-expressing cells do not induce death in cytotrophoblasts from first trimester and term placentas
4. FasL-expressing cells does not induce death in EGF-induced syncytiotrophoblasts
5. Inhibitors of NOS do not sensitize cytotrophoblasts to Fas-induced death.



A B

Figure 4.1 : Immunohistochemical staining of Fas on cultured human cytotrophoblasts. Trophoblasts were cultured overnight in 10%FCS-IMDM in 96-well dishes, fixed with methanol:acetone (or 2% paraformaldehyde) , and stained with 5 μg/ml mouse IgM isotype control (A) or anti-human Fas (B).

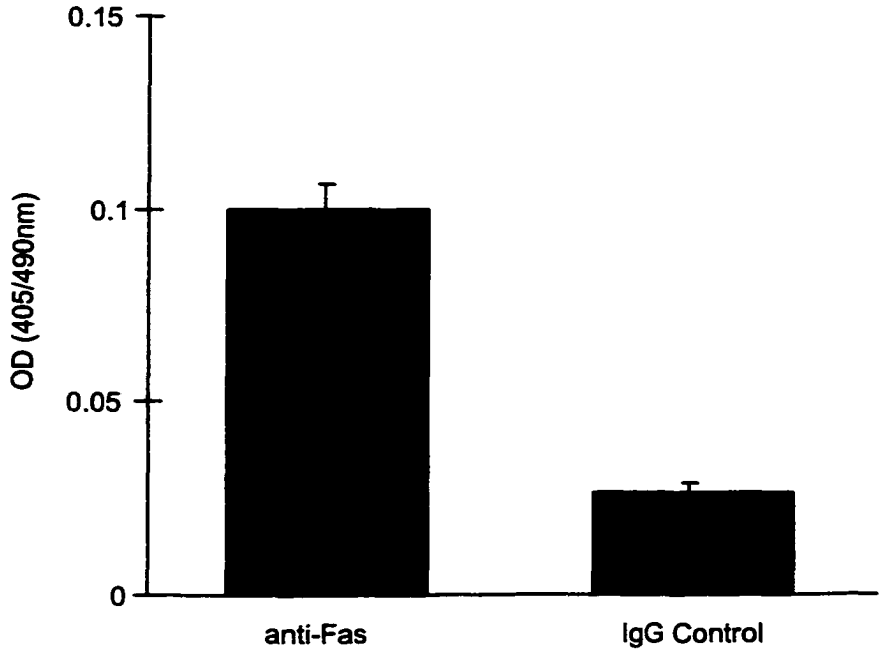


Figure 4.2 : Fas antigen expressed on the surface of cytotrophoblasts as shown by cellular ELISA. Cytotrophoblasts were plated at 1 x 10^5 in 96-well plates and incubated overnight at 37°C to allow the cells to adhere. After blocking on ice, the cells were incubated on ice with 5 μg/ml anti-Fas or $IgG2_b$ isotype control for 2 hours followed by a 1 hour incubation with goat anti-mouse Ig-HRP conjugate. Colour was developed with ABTS and optical density measured at 405 nm with a reference wavelength of 490 nm. The values represent an average OD of triplicate wells ± SD from one experiment, and is representative of three independent experiements. The two values are significantly different ($p<0.01$; Student's t-test).

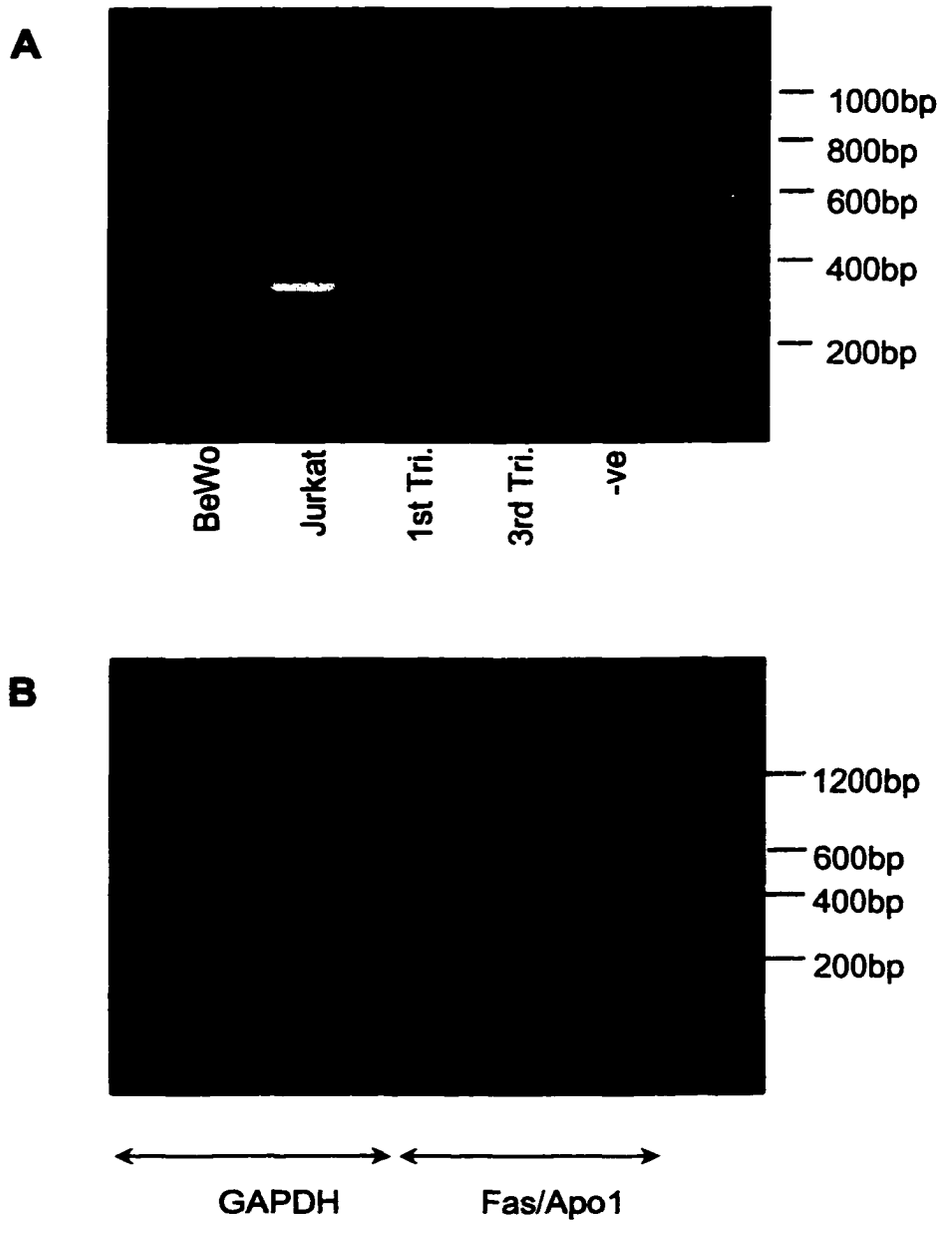


Figure 4.3 : Fas mRNA expression in first and third trimester placentas and cultured cytotrophoblasts. A. RT-PCR products from BeWo cells, Jurkat cells and first and third trimester placental tissue. B. Fas RT-PCR products from cultured cytotrophoblasts purified from term placentas. Shown are two pairs of amplifications for the house-keeping gene GAPDH and Fas from preparations from cytotrophoblasts from two different placentas. (Data collected by Dr. Steve Smith, University of Nottingham, UK)

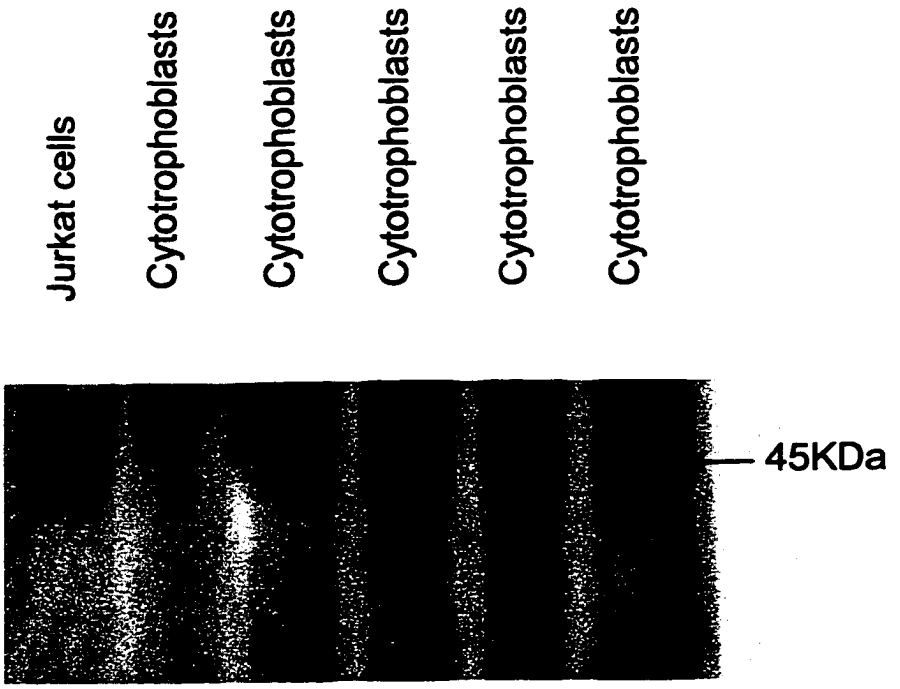


Figure 4.4 : Fas protein expressed in cytotrophoblasts purified from term placentas. Protein was extracted from Jurkat cells and 5 different preparations of purified cytotrophoblasts, separated by SDS-PAGE and Western Blotted using monoclonal anti-Fas (CH-11) as described in Methods and Materials. (Data collected by Dr. Steve Smith, University of Nottingham, UK)

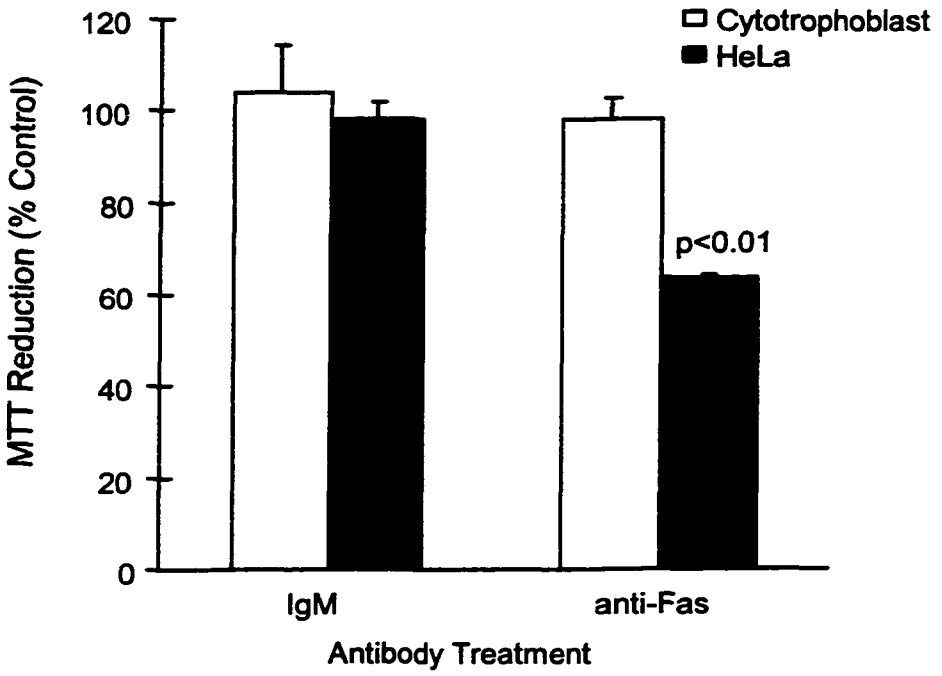


Figure 4.5 Anti-Fas antibody CH-11 does not affect cytotrophoblast cell viability. HeLa cells and cytotrophoblasts were cultured in 96-well plates, treated for 24 hours with 100 ng/ml anti-Fas (CH11) antibody or IgM in 10%FCS-IMDM with 1 μg/ml CHX, then cell viability determined by MTT assay. Per cent MTT reduction is calculated as a percentage of the OD's of cells cultured in basal culture (10% FCS-IMDM) medium with 1 μg/ml CHX.. Values shown are the average ± SD of triplicate determinations from a single experiment and are representative of 4 independent experiments with identical results. Statistical significance indicated is relative to IgM treated cells (Student's *t* test).

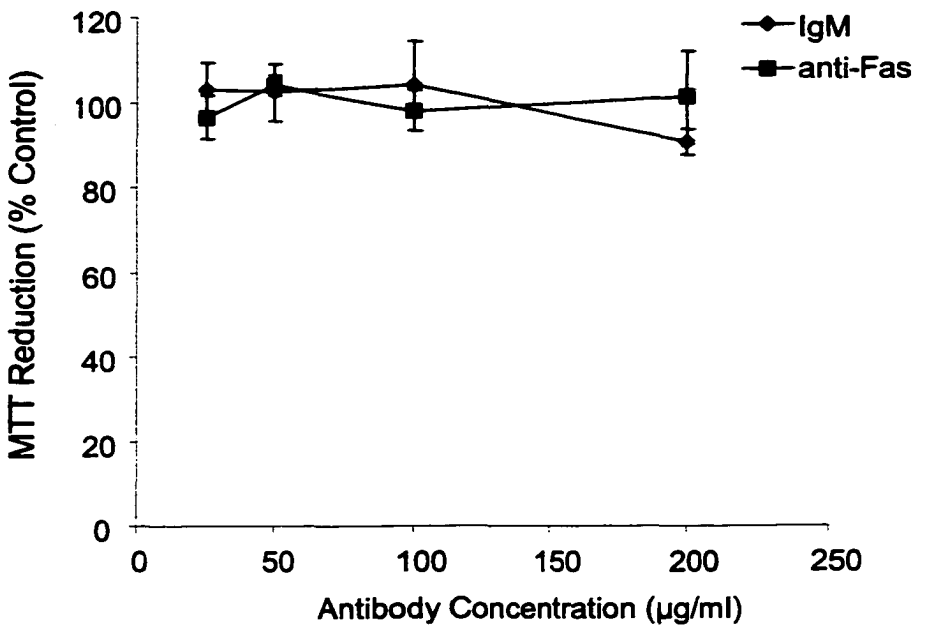


Figure 4.6 : Varying concentrations of anti-Fas (CH11) antibody did not affect cytotrophoblast cell viability. One x 10^5 cytotrophoblasts were cultured in 96-well plates, treated with varying concentrations of anti-Fas (CH11) antibody or IgM with 1 µg/ml CHX in 10%FCS-IMDM for 24 hours. MTT reduction is calculated as a percentage of the OD's of cells cultured in basal culture (10% FCS-IMDM) medium with 1 µg/ml CHX.. Values shown are the average ± SD of triplicate determinations from a single experiment and are representative of 2 independent experiments with identical results.

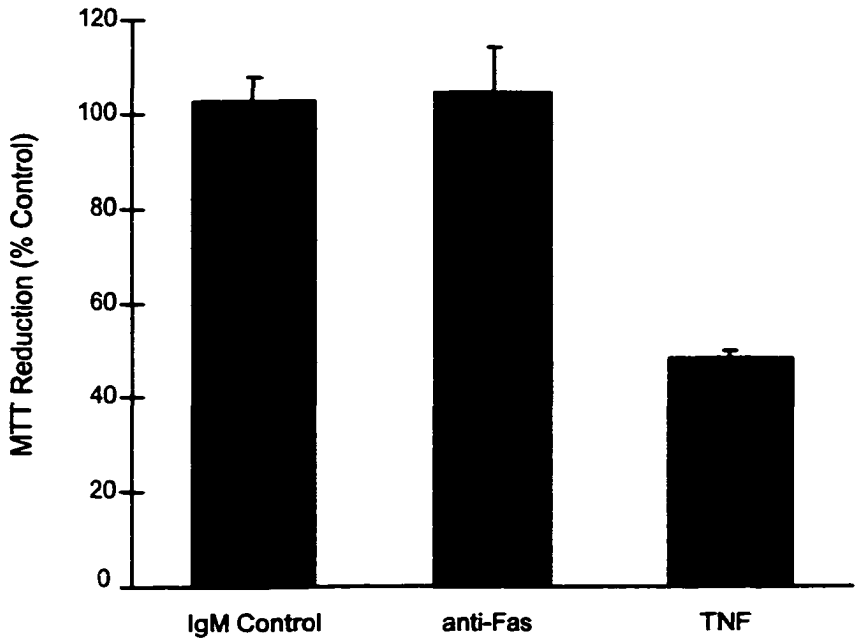


Figure 4.7 : Seventy two hour treatment of cytotrophoblast with anti-Fas (CH11) antibody did not affect cell viability. Cytotrophoblasts were plated at 1 x 10^5 per well, treated with 100 ng/ml anti-Fas (CH11) antibody, IgM, or 10 ng/ml TNFα with 100 U/ml IFNγ in 10% FCS-IMDM for 72 hours. MTT reduction is calculated as a percentage of the OD's of cells cultured in basal culture medium (10% FCS-IMDM). Values shown are the means ± SD of triplicate determinations from a single experiment and are representative of 2 independent experiments with identical results.

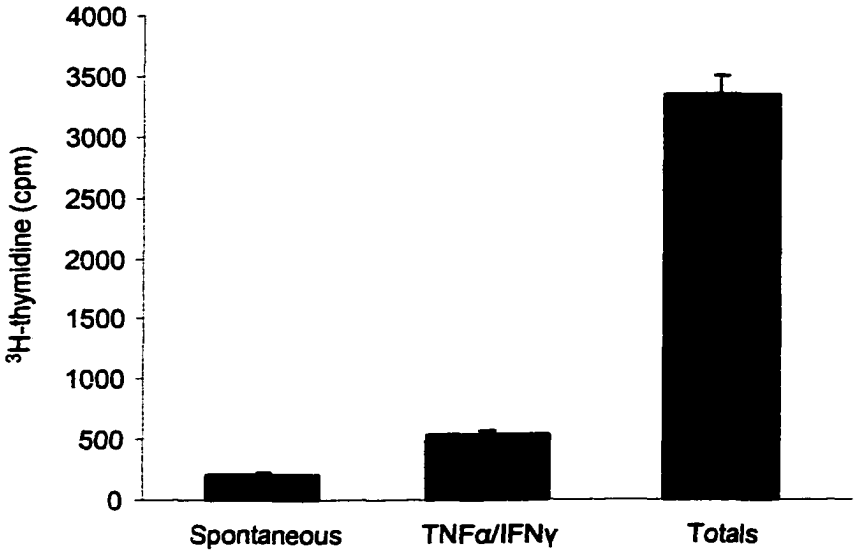


Figure 4.8 : Demonstration that non-dividing cytotrophoblasts can be labelled with [^{3}H]-thymidine and DNA fragmentation can be measured. Cytotrophoblasts were plated at 1 x 10^5 in 96 well plates and cultured for 4 hours to allow cells to adhere. The culture medium was replaced with fresh 10% FCS-IMDM containing 10 μCi/ml (1 μCi/well) and incubated overnight at 37°C. After labelling, cells were treated as indicated. Spontaneous, cells cultured in culture medium alone. TNFα/IFNγ, cells cultured with TNFα/IFNγ for 20 hours. Both sets were lysed with 1% Triton X-100, centrifuged, and nuclei-free supernatants taken for scintillation counting. Totals, cells were cultured with culture medium alone, lysed with 2% SDS, and aliquots of lysate were taken for scintillation counting. Values shown are the average ± SD of triplicate determinations from one experiment.

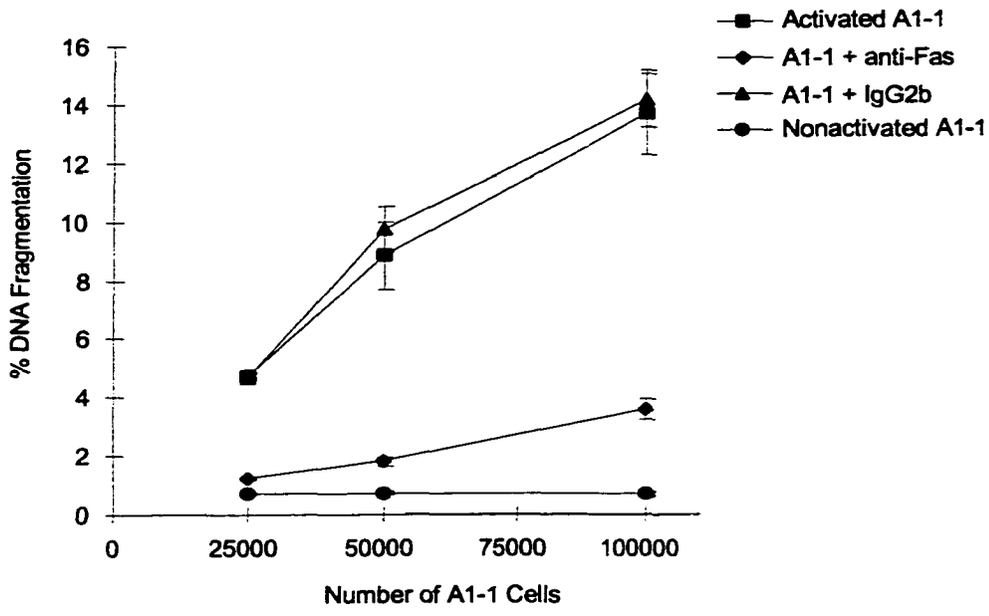


Figure 4.9 : Activated A1-1 cell induced DNA fragmentation in HeLa cells is dependant on Fas-FasL interactions. HeLa cells were plated at 1 x 10^4 cells per well in 96-well plates and incubated for 24 hours in 10% FCS-IMDM with 1 μCi [^{3}H]-thymidine. A1-1 cells were activated by incubating for 2 hours with 10 ng/ml PMA and 3 μg/ml ionomycin in 10% FCS-RPMI or mock activated by incubating in culture medium alone. A1-1 cell densities were adjusted and added to [^{3}H]-thymidine-labelled HeLa cells in a final concentration of 1 μg/ml CHX and incubated for 3.5 hours. Anti-Fas (SM1/23) or $IgG2_b$ was added just before the addition of A1-1 cells to a final concentration of 20 μg/ml. DNA fragmentation was measured as described in Methods and Materials. Results shown are means ± SD of triplicate determinations from one experiment and are representative of 2 independent experiments.

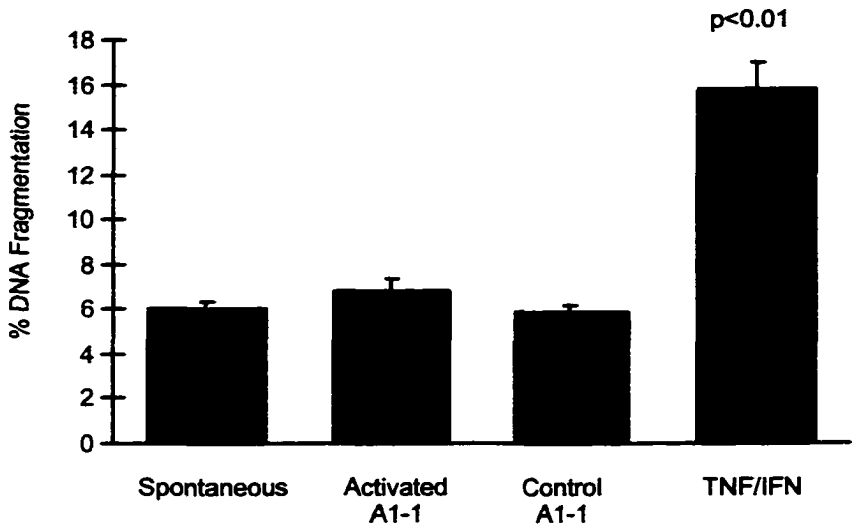


Figure 4.10 : Fas ligand expressing cells do not induce apoptosis in cytotrophoblasts. Cytotrophoblasts were plated at 1 x 10^5 cells per well in 96-well plates, [^{3}H]-thymidine labelled, then incubated with mock activated, activated A1-1 cells or TNFα in 10% FCS-IMDM with 1 μg/ml CHX and 100 U/ml IFNγ for 20 hours. DNA fragmentation was measured as described. Data are means ± SD from triplicate determinations and are representative of 3 independent experiments. Statistical significance shown is relative to cells in basal growth medium alone (spontaneous) (Student's *t* test).

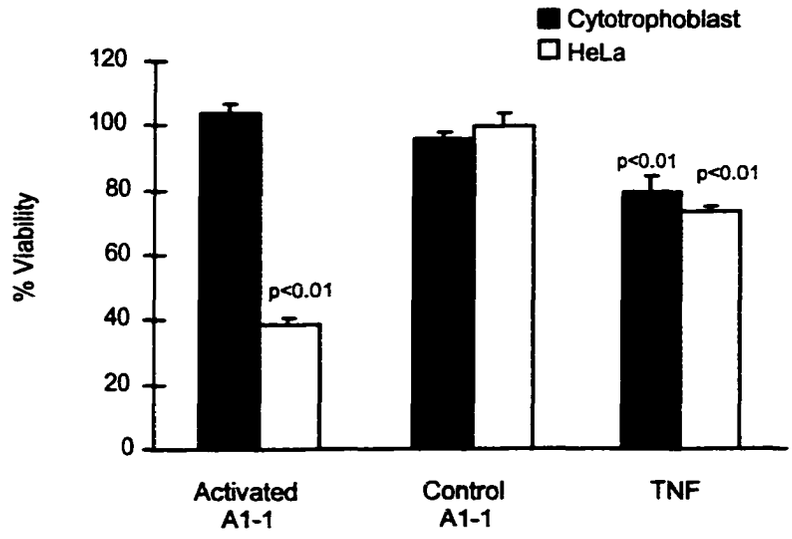


Figure 4.11 : Fas ligand expressing cells do not affect cytotrophoblast cell viability. Confluent HeLa cells and cytotrophoblasts plated at 1 x 10^5/well in 96-well plates were incubated with 1 x 10^5 mock activated, activated A1-1 cells or TNFα in 10% FCS-IMDM with 1 μg/ml CHX and 100 U/ml IFNγ for 24 hours. Cultures were washed with culture medium to remove A1-1 cells and cytotrophoblast cell viability was determined by MTT assay. Data are means ± SD of triplicate determinations and represent 1 of 3 independent experiments. Statistical significance shown is relative to untreated cells where viability equals 100% (Student's *t* test)

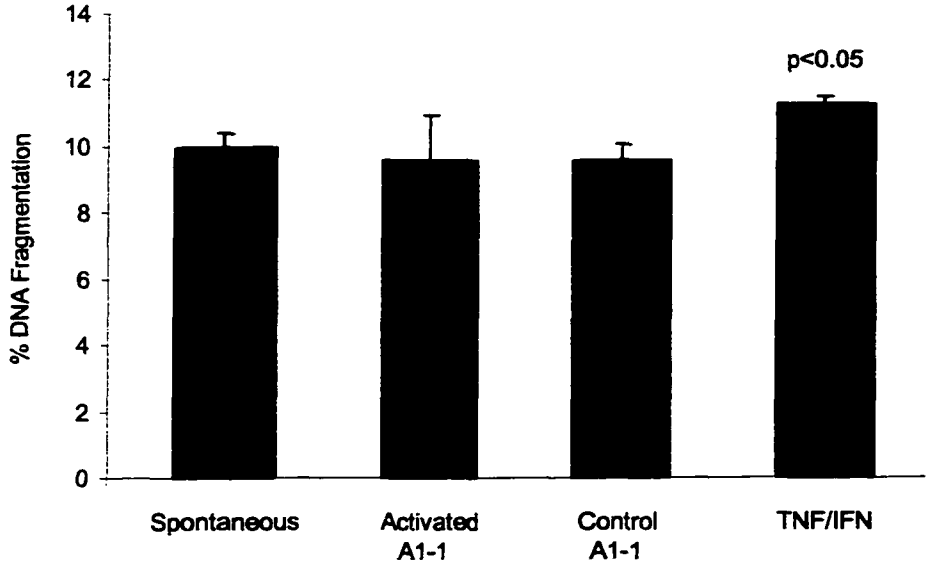


Figure 4.12 : Fas ligand expressing cells do not induce DNA fragmentation in cytotrophoblasts from first trimester placentas. Cytotrophoblasts purified from 1st trimester placentas were plated at 1 x 10^5/well in 96-well plates. After adherence (5 hours) in 10% FCS-IMDM, cells were gently washed with IMDM, then incubated overnight with 1 μCi [^{3}H]-thymidine in 10% FCS-IMDM. After washing, the cells were incubated for 24 hours with mock activated, ionomycin/PMA activated A1-1 cells or TNFα in 10% FCS-IMDM containing 1 μg/ml CHX and 100 U/ml IFNγ. DNA fragmentation was measured as described. Data are means ± SD of triplicate determinations. Statistical significance shown is relative to spontaneous DNA fragmentation ($p=0.043$; Student's *t* test).

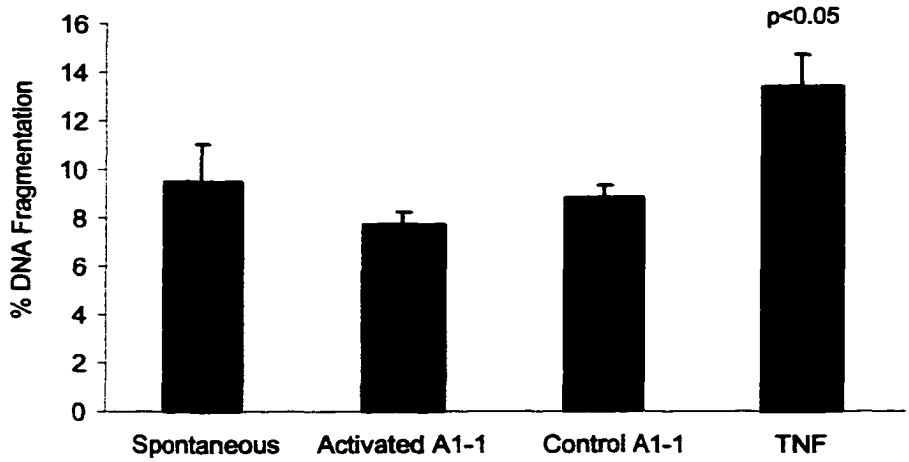


Figure 4.13 : Fas ligand expressing cells do not induce DNA fragmentation in syncytiotrophoblasts. Cytotrophoblasts were plated at 1 x 10^5/well in 96-well dishes, treated with 10 ng/ml EGF for 4 days to induce syncytium formation, and labelled with 1 μCi [^{3}H]-thymidine for 24 hours in 10% FCS-IMDM. After washing to remove EGF and [^{3}H]-thymidine, 1 x10^5 mock activated A1-1 cells, ionomycin/PMA-activated A1-1 cells or 10 ng/ml TNFα were added in 10% FCS-IMDM containing 100 U/ml and incubated for 18-20 hours. DNA fragmentation was measured as described. Data are mean ± SD of triplicate determinations. Statistical significance shown is relative to spontaneous DNA fragmentation (p=0.03; Student's *t* test).

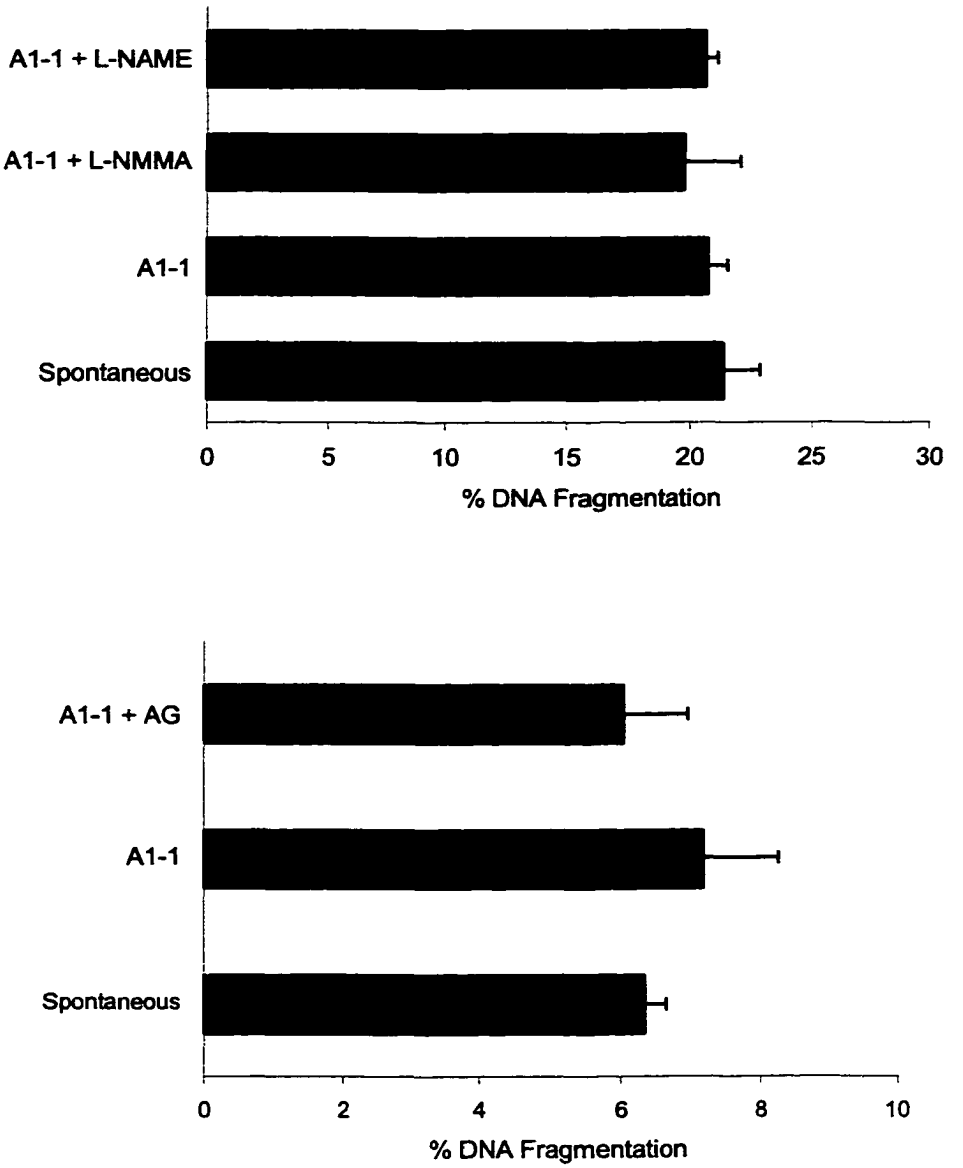


Figure 4.14 : Inhibitors of nitric oxide synthase do not up-regulate Fas ligand-induced DNA fragmentation in cytotrophoblasts. Cytotrophoblasts were plated at 1 x 10^5 in 96-well plates, labelled for 24 hours with [^{3}H]-thymidine, then incubated for 20 hours with ionomycin/PMA-activated A1-1 cells with 500 μM of the NOS inhibitors L-NAME, L-NMMA and AG in 10% FCS-IMDM. Data are mean ± SD of triplicate determinations. The L-NAME and L-NMMA experiments were done separate from the AG experiments with different batches of cytotrophoblasts cells and are therefore presented separately.

Chapter 5 : Ceramide and Acid Sphingomyelinase induce apoptosis in cytotrophoblasts, whereas EGF inhibits apoptosis and lowers ceramide

(Most of the data in this chapter has been published as an article entitled "Epidermal growth factor inhibits ceramide-induced apoptosis and lowers ceramide levels in primary placental trophoblasts" by Payne, SG., Brindley, DN., and Guilbert, LG. In *Journal of Cellular Physiology* (1999) 180 : 263-270)

5.1 Introduction

The sphingomyelin pathway, and the sphingolipid ceramide, is emerging as an important mediator of the effects of several extracellular stimuli, particularly stimuli which induce apoptosis, such as TNF and Fas. Signalling ceramide is derived from sphingomyelin by the action of at least two distinct sphingomyelinases, a cell-membrane neutral SMase (Chatterjee, 1993) and an acid SMase found in an endosomal compartment (Kolesnick *et al.*, 1991; Spence, 1993) and possibly caveolae (Liu & Anderson, 1995). Based on a model by Wiegmann *et al.* (1994), ceramide derived from acid SMase is thought to be the apoptosis-inducing ceramide whereas ceramide derived from neutral SMase is involved in other cellular responses. This may not always be the case since exogenous bacterial (neutral) SMase can induce apoptosis in some cell types (Kim *et al.*, 1991; Jarvis *et al.*, 1994).

TNF induces apoptosis in primary trophoblast cultures, and can be enhanced by co-culture with IFNγ, which alone has no cytotoxic effects (Yui *et al.*, 1994). Moreover, TNF/IFNγ-induced apoptosis can be blocked by EGF, which may provide a mechanism by which cell loss is controlled in the placenta to maintain placental function. Although the role of ceramide in TNF-induced apoptosis has been studied in a variety of tumour cell lines, nothing is known about the role of acid and neutral SMases, and ceramide in apoptosis in trophoblasts. As well, the effects of EGF on the sphingomyelin pathway are unknown. Another growth factor, PDGF, has been shown to activate ceramidase in rat mesangial cells (Coroneos *et al.*, 1995), which hydrolysis ceramide to free fatty acid and

sphingosine, thereby lowering ceramide levels. In this study I will therefore examine the role of acid and neutral SMase, and ceramide in trophoblast apoptosis, and how these may be affected by EGF.

5.2 Results

5.2.1 Ceramide and Acid SMase induce apoptosis in trophoblasts.

Trophoblast cultures were treated with 20 μM C_{16}-ceramide or 1 U/ml aSMase and apoptosis determined by TUNEL. C_{16}-ceramide induced apoptosis in trophoblasts, although the exact time at which DNA nicking occurred varied from experiment to experiment with different cell preparations but a similar pattern was seen in all experiments. In the experiment shown in Figure 5.1, 28% of ceramide treated trophoblasts have nicked DNA after 8 hours exposure whereas less than 5% of those exposed to dihydro-C_{16}-ceramide contained nicked DNA. Ceramide treatment also resulted in >75% loss of cells from culture as determined by MTT analysis after 24 hours (Fig 5.2). Acid SMase also induced DNA nicking. By 7 hours 15% of cells contained nicked DNA, and increased to 45% by 24 hours compared to only 12% of those exposed to carrier alone (Fig 5.3). Again, the timing and number of cells with nicked nuclei varied from experiment to experiment with different cell preparations, but a similar pattern was seen in all experiments.

5.2.2 EGF protects against ceramide and acid SMase-induced apoptosis.

Since EGF protects trophoblasts from TNF/IFNγ-induced apoptosis (Garcia-Lloret *et al.*, 1996), and the observation above that ceramide and aSMase induced apoptosis, we hypothesized that EGF would protect trophoblasts from ceramide-induced apoptosis. Treatment of cells with 10 ng/ml EGF completely inhibited ceramide-induced apoptosis (Fig 5.1). The number of cells with nicked DNA was reduced from 28% to <5% in the 8-hour experiment shown. In the longer term (24 hours) cultures however, where ceramide-induced >75% cell loss, EGF did not inhibit the ceramide-induced loss (Fig. 5.2). EGF also inhibited aSMase-induced apoptosis, reducing the number of cells with nicked DNA at 24 hours from 45% induced by aSMase alone to 13% at (Fig. 5.3).

5.2.3 EGF lowers cytotrophoblast ceramide levels.

It was important to determine whether exogenous acid SMase added to the cultures could hydrolyse sphingomyelin to increase ceramide levels. Acid SMase has a pH optimum of ~5.0 (see Appendix I), and therefore would have to segregate into an acidic cellular compartment. As shown in Figure 5.4, acid SMase increased ceramide levels by 30% at 1.5 hours, and by 70% at 6 hours, indicating that the enzyme entered the appropriate acidic compartment.

Since EGF inhibited exogenous ceramide and acid SMase-induced apoptosis, we asked whether EGF affected acid SMase-induced and basal ceramide levels. EGF treatment alone lowered ceramide levels by 15% and 25% below control levels at 1.5 and 3 hours respectively (Fig. 5.4). EGF also lowered the acid SMase-induced ceramide increase by 20-30%, but not to levels observed in untreated cells.

Although these results tell us that EGF affects basal ceramide and exogenous acid SMase-induced ceramide increases, they do not tell us anything about the mechanism or whether EGF affects ceramide taken up from the culture medium in the experiments where EGF protected against exogenous ceramide-induced apoptosis. To help address these issues, trophoblasts were labelled with exogenous radiolabelled ceramide ([^{3}H]C_{16}-ceramide), treated with EGF and the amount of cellular tritiated ceramide measured. As shown in Figure 5.5, EGF lowered [^{3}H]C_{16}-ceramide by approximately 20% at 3 hours.

5.2.4 Exogenous bacterial (neutral) SMase does not induce apoptosis in trophoblasts.

Exogenous bacterial (neutral) SMase (bSMase) has been used in a number of studies to shown that ceramide can induce apoptosis (Obeid *et al.*, 1993; Jarvis *et al.*, 1994). We therefore asked whether neutral SMase, in addition to aSMase as shown above, could induce apoptosis in trophoblasts. As shown in Figures 5.6, bSMase increased ceramide levels. At 1 U/ml, equivalent to concentrations of aSMase used, ceramide levels increased to >600% above controls within 15 minutes, about 10-fold higher than that induced by aSMase. At 3 hours aSMase increased ceramide levels by

approximately 50% above controls (Figs. 5.4 & 5.6). However, 1 U/ml bSMase did not induce cell death by 24 hours (Fig. 5.8). By 48 hours there was still <10% cell loss from the cultures treated with bSMase whereas aSMase induced >80% cell loss as determined by MTT (Fig. 5.9).

The large ceramide increases were seen within minutes of adding bSMase (Fig. 5.6) to the cultures and persisted for 24 hours (Fig. 5.8), the last time point at which ceramide was measured. Since the ceramide levels were much greater than those induced by aSMase, bSMase was titered to generate ceramide levels (Fig. 5.8) comparable to those induced by aSmase (Figs. 5.4 & 5.6). At these lower concentrations, bSMase still did not induce cell death (Fig. 5.7). These data would support the idea that there are distinct signalling pools of ceramide.

5.2.5 TNF/IFN γ did not increase ceramide levels or activate aSMase.

Since TNF/IFNγ induced apoptosis in trophoblasts (Yui *et al.*, 1994), and the above observations that ceramide and aSMase induce apoptosis, we asked whether TNF/IFNγ increased aSMase activity and ceramide levels. Trophoblast cultures were treated with TNF/IFNγ for time points ranging from a few minutes to 24 hours. Ceramide levels were measured by the DAG kinase assay or from [^{3}H]palmitic acid labelled cells, and sphingomyelinase activity measured by the *in vitro* micellar assay. At all time points tested, TNF/IFNγ did not induce a measurable change in ceramide levels or aSMase activity.

To further investigate the role of the sphingomyelin cycle in TNF/IFNγ signalling, the effects of TNF/IFNγ on sphingomyelin levels were measured. It is possible that the sphingomyelin cycle is activated, resulting in a ceramide increase and a sphingomyelin decrease, but ceramide is rapidly metabolized. Sphingomyelin was measured in [^{3}H]choline-labelled cells by TLC and by the sphingomyelinase method as described. In several experiments there was an apparent ~20% drop in sphingomyelin levels between 3 and 4 hours in TNF/IFNγ-treated cells. However, this observation could not be repeated in other experiments using different preparations of cells

5.2.6 D609 inhibits TNF/IFN γ -induced apoptosis in trophoblasts.

Although TNF/IFNγ did not induce a measurable change in aSMase activity at the time points tested, we further investigated whether aSMase played a role in TNF/IFNγ-induced apoptotis. To do this we used D609, an inhibitor of PC-PLC, which is required for aSMase activation (Schutze *et al.*, 1992). D606 inhibited TNF/IFNγ-induced apoptosis (Fig 5.10). After 24 hours, 33% of trophoblasts treated with TNF/IFNγ contained nicked DNA, whereas cotreatment with 50 μg/ml D609 reduced the number of cells with nicked DNA to 10%, suggesting a role for aSMase in TNF/IFNγ-induced apoptosis in trophoblasts.

5.2.7 Inhibition of acid ceramidase, but not alkaline ceramidase, blocks EGF protection & induces apoptosis.

Since EGF protects trophoblasts from apoptosis and lowers ceramide levels, we hypothesized that ceramidase activity may be important for trophoblast survival and may be involved in the EGF-protection and ceramide decrease. If this were the case, then inhibitors of ceramidase should block the EGF effects. To address this possibility, two inhibitors of ceramidase were used: D-MAPP and *N*-oleoylethanolamine (OE). D-MAPP more potently inhibits alkaline ceramidase while OE is a more potent inhibitor of acid ceramidase (Bielawaska *et al.*, 1996).

We first determined the effects of the inhibitors on trophoblast viability and whether they blocked the protective effects of EGF on TNF/IFNγ-induced apoptosis. Since significant TNF/IFNγ-induced apoptosis is not induced until 12-18 hours, trophoblasts were incubated overnight with 100 μM OE and 10 μM D-MAPP. Greater than 80% of the cells were lost from cultures treated with OE for 24 hours (Fig 5.11), whereas D-MAPP had no effect of cell viability (Fig 5.12). Co-treatment with EGF did not block OE-induced cell loss. To determine if the OE-induced cell loss was by apoptosis, DNA nicking was determined by TUNEL. As shown in Figure 5.13, by 6 hours 50% of the cells treated with 50 μM and 100 μM OE contained nicked DNA, and co-treatment with EGF did not inhibit apoptosis. Concentrations of OE below 50 μM did not induce apoptosis nor affect cell viability. The effects of OE on EGF protection of

TNF/IFNγ-induced death could not be tested because OE induced cell death more rapidly than TNF/IFNγ. D-MAPP, however, did not affect EGF protection from TNF/IFNγ-induced trophoblast death (Fig. 5.12).

Since OE-induced apoptosis could not be blocked by EGF, and D-MAPP did not affect cell viability or EGF protection, we tested the effect of the inhibitors on cellular ceramide levels. Both OE and D-MAPP increased trophoblast ceramide levels. One hundred μM OE increased ceramide levels by 50% in 2 hours (Fig. 5.14) whereas 10 μM D-MAPP increased ceramide by 20% at 4 hours and 24 hours (Fig. 5.15). Moreover, EGF did not lower OE-induced ceramide increases (Fig. 5.14). Because the D-MAPP-induced ceramide increase was about half that of OE, its efficacy was tested with HL-60 cells, in which it induced a 40% ceramide increase at 4 hours (Fig. 5.15). In addition, we did not want to use concentrations higher than 10 μM because it can also inhibit acid ceramidase at higher concentrations (Bielawska *et al.*, 1996). We are at the highest range possible for alkaline ceramide inhibition and yet stay below the concentration which inhibits acid ceramidase.

This data would suggest a role for acid ceramidase, but not alkaline ceramidase, in EGF inhibition of apoptosis in trophoblasts, and that EGF-induced ceramide decrease is mediated, at least in part, by acid ceramidase. Moreover, the finding that D-MAPP increases ceramide but does not induce death, whereas OE increases ceramide and induces death, supports the idea that there are distinct apoptosis-inducing pools of ceramide.

To further investigate the role of a ceramidase in the anti-apoptotic effects of EGF, we attempted to measure ceramidase activity. However, ceramidase activity could not be detected using either of the two methods described in methods and materials. It is possible that in the amount of cellular protein recovered and used in the assays, ceramidase activity was below the detectable limits of the assay.

5.2.8 Sphingosine induces apoptosis in trophoblasts.

Sphingosine is a metabolite of ceramide that has been shown to be produced in response to TNF (Ohta *et al.*, 1994; Ohta *et al.*, 1997), and induces apoptosis (Ohta *et*

al., 1995; Cuvillier *et al.*, 2000). We therefore asked whether sphingosine induced apoptosis in cultured trophoblast and found that 30 μM sphingosine induced apoptosis in 20% of trophoblasts in 6 hours (Fig. 5.16). Since sphingosine can be converted to ceramide, and ceramide induced trophoblast apoptosis, ceramide levels were measured in cultures incubated with sphingosine, and were found to be increased by 60% above control by 3 hours (Fig. 5.17).

5.3 Summary of Results

1. Ceramide and acid sphingomyelinase induce apoptosis in cultured primary human trophoblasts.
2. EGF inhibits ceramide and acid sphingomyelinase-induced apoptosis
3. D609, a putative inhibitor of PC-PLC, blocked TNF/IFNγ-induced trophoblasts death
4. Bacterial (neutral) sphingomyelinase does not induce trophoblast apoptosis
5. TNF/IFNγ did not induce a measurable change in ceramide levels or aSMase activity in trophoblasts.
6. *N*-oleoylethanolamine, an inhibitor of acid ceramidase, increases ceramide levels and induces apoptosis that cannot be blocked by EGF
7. D-MAPP, an inhibitor of alkaline ceramidase, increased ceramide levels but does not induce death and does not block EGF.
8. Sphingosine induces apoptosis and increases ceramide levels in trophoblasts.

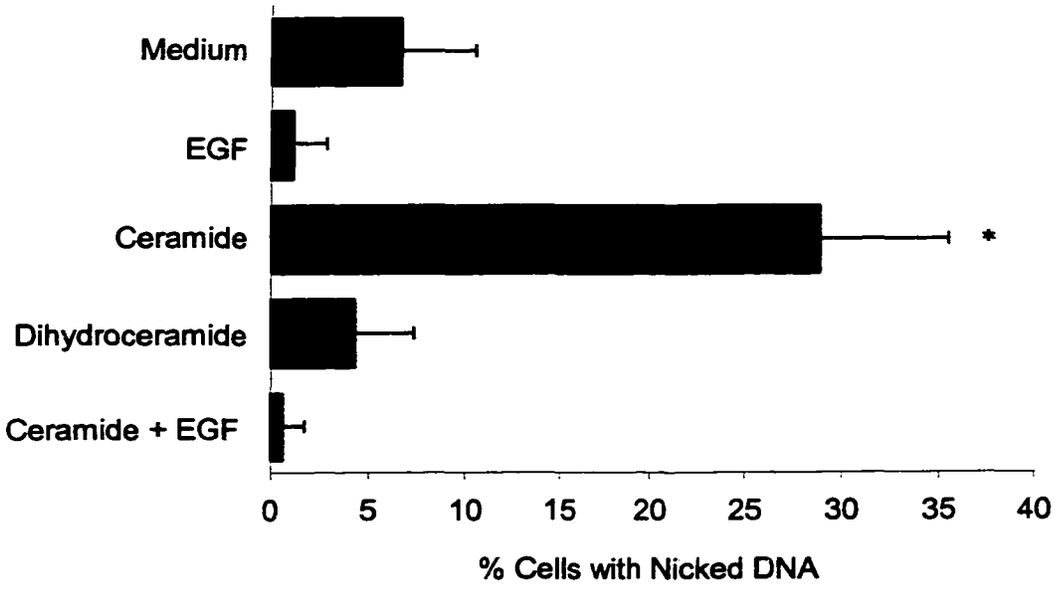


Figure 5.1 : Ceramide induces apoptosis in cytotrophoblasts and is inhibited by EGF. Trophoblasts were plated at 1 x 10^5 in 96-well plates and incubated with 20 μM C_{16}-ceramide, 20 μM dihydro-C_{16}-ceramide or 10 ng/ml EGF for 6-24 hours in 0.5%FCS-IMDM then processed for TUNEL. The data shown are from an 8-hour experiment. The exact time at which DNA occurred varied from experiment to experiment with different cell preparations but a similar pattern was seen in all experiments. Ceramide was first dissolved in ethanol:dodecane (98:2) before dilution in culture medium. The percentage of positive nuclei was determined by direct counting in 10 random fields, each containing approximately 100 nuclei. Values shown are mean ± SD from a single experiment and are representative of five separate experiments. * indicates statistical significance from cells in culture medium alone ($p < 0.05$; Student's *t*-test)

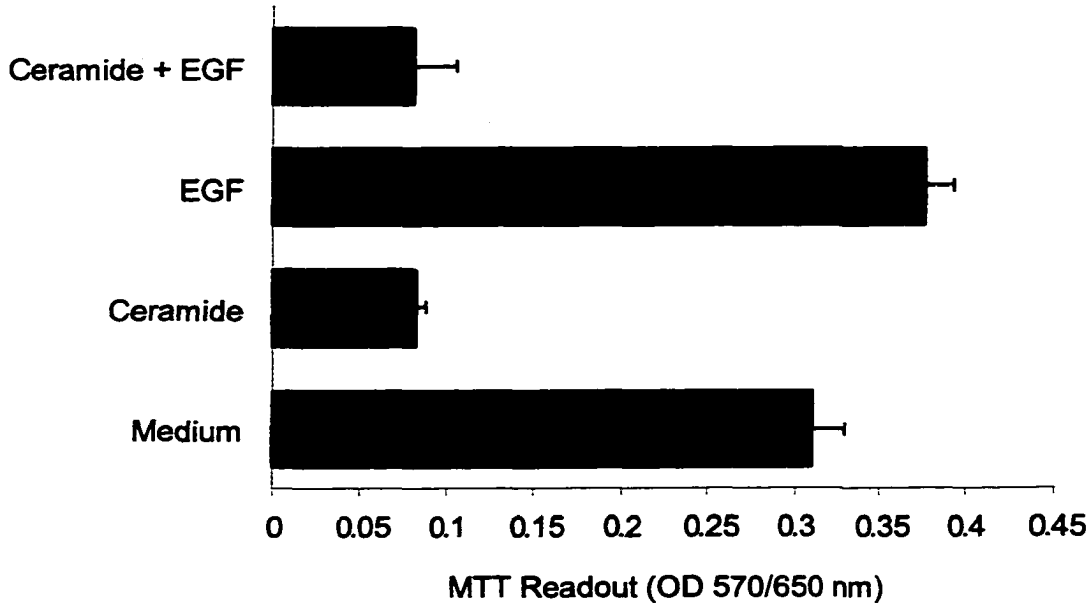


Fig 5.2 : Ceramide induces cell loss from cytotrophoblast cultures. Trophoblasts were plated at 1 x 10^5 in 96-well plates and incubated with 20 μM C_{16}-ceramide or 10 ng/ml EGF and viability determined by MTT. The data shown is from a 24-hour experiment, the earliest time point tested which showed >75% cell loss in ceramide-treated cultures. Data values shown are mean ± SD of triplicate determinations from a single experiment. The values are representative of 4 separate experiments, although the timing at which cell loss was observed varied from experiemtn to experiment with different cell preparations.

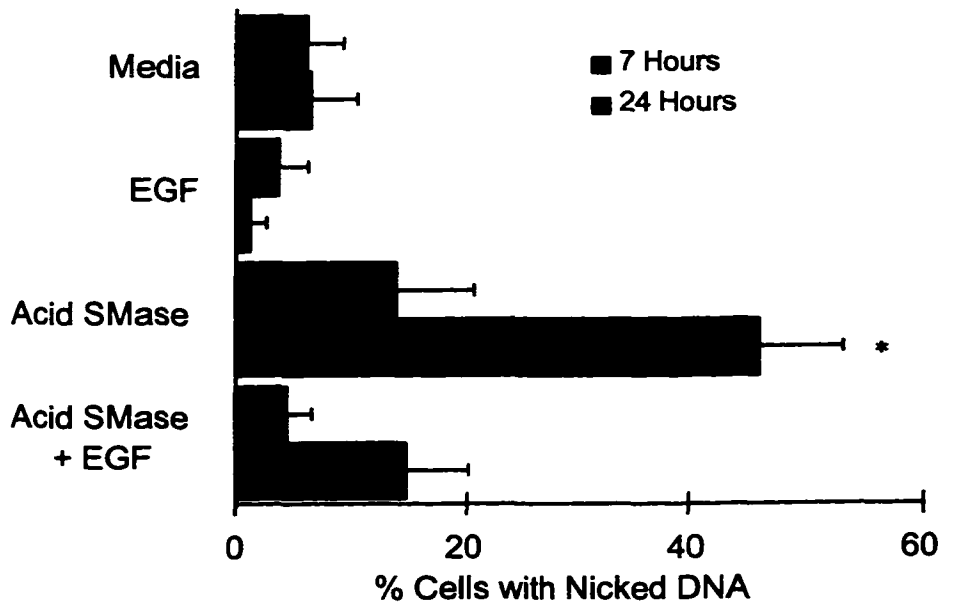


Fig 5.3 : Acid SMase induces apoptosis in cytotrophoblasts and is inhibited by EGF. Trophoblasts were plated at 1 x 10^5 in 96-well plates and incubated with 1 U/ml acid SMase with and without 10 ng/ml EGF in 0.5% FCS-IMDM. The percentage of positive nuclei was determined by direct counting in 10 random fields, each containing approximately 100 nuclei. Values shown are mean ± SD from a single experiment and are representative of three separate experiments. * indicates statistical significance from cells in culture medium alone ($p < 0.05$; Student's *t*-test)

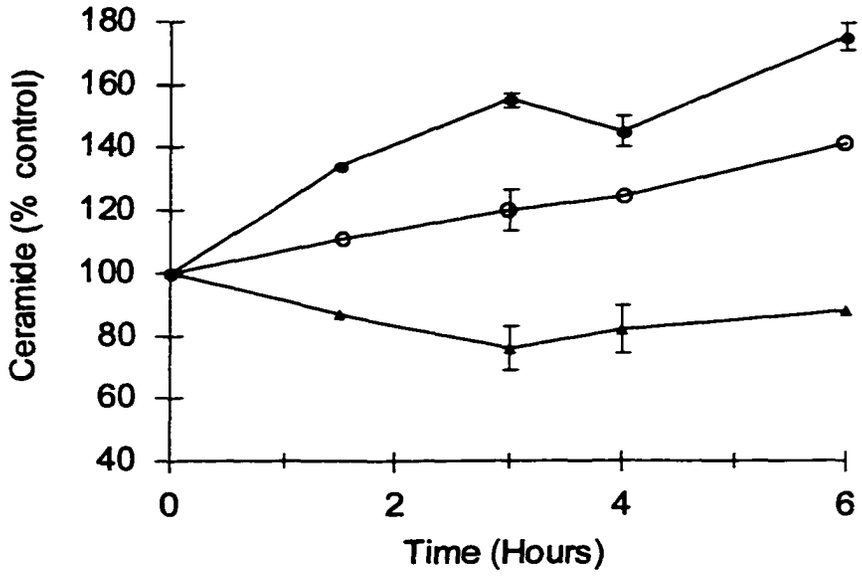


Figure 5.4 : Effect of acid SMase and EGF on ceramide levels in cultured cytotrophoblasts. Trophoblasts were plated at 2 x 10^6/well in 6-well dishes in 10% FCS-IMDM, then incubated with 1 U/ml aSMase (closed circles), aSMase + 10 ng/ml EGF (open circles), and 10 ng/ml EGF alone (closed triangles) from 0 to 6 hours. Lipids were then extracted and ceramide measured by DAG kinase assay. Data are expressed as a percent change of untreated controls (set at 100%). Points shown with SEM represent the mean of three to four separate determinations using different preparations of trophoblasts. Points without SEM represent single point determinations.

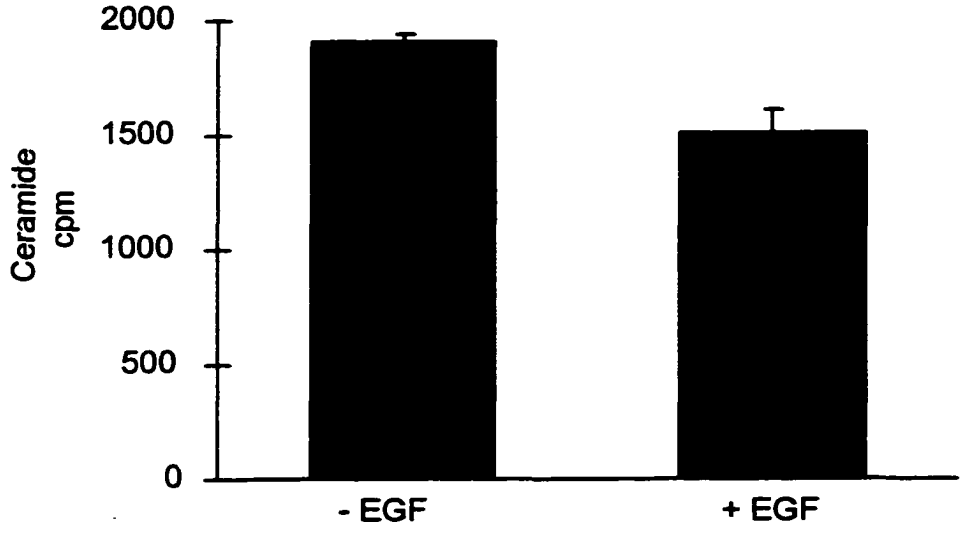


Figure 5.5 : EGF lowers ceramide levels in cultured trophoblasts. Trophoblasts were plated at 2 x 10^6/well in 6-well dishes in 10% FCS-IMDM, cultured with 15 μM [^{3}H]C_{16}-ceramide for 1.5 hours in 0.5% FCS-IMDM, then treated with 10 ng/ml EGF for 3 hours. Lipids were extracted and analysed by TLC. Data are expressed as average cpm ± SD of ceramide spots scraped from TLC plates. Shown is a representative experiment with triplicate determinations.

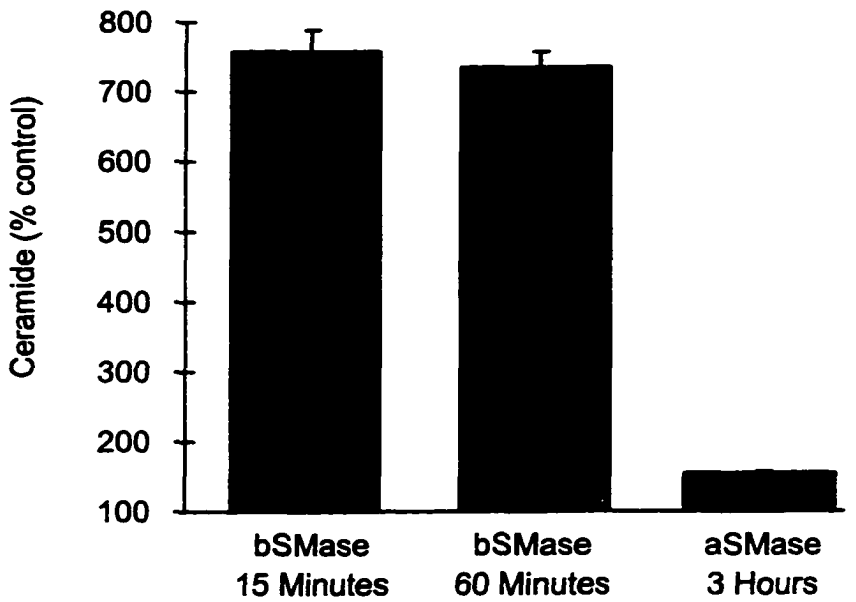


Figure 5.6 : Comparison of exogenous bSMase and aSMase-induced ceramide levels in trophoblasts. Trophoblasts were cultured in 0.5%FCS-IMDM with bSMase for 15 and 60 minutes or aSMase for 3 hours. Lipids were extracted and ceramide measured. Data are expressed as a percent of untreated controls (set at 100%). Each bar represents the mean ± range of two independent determinations.

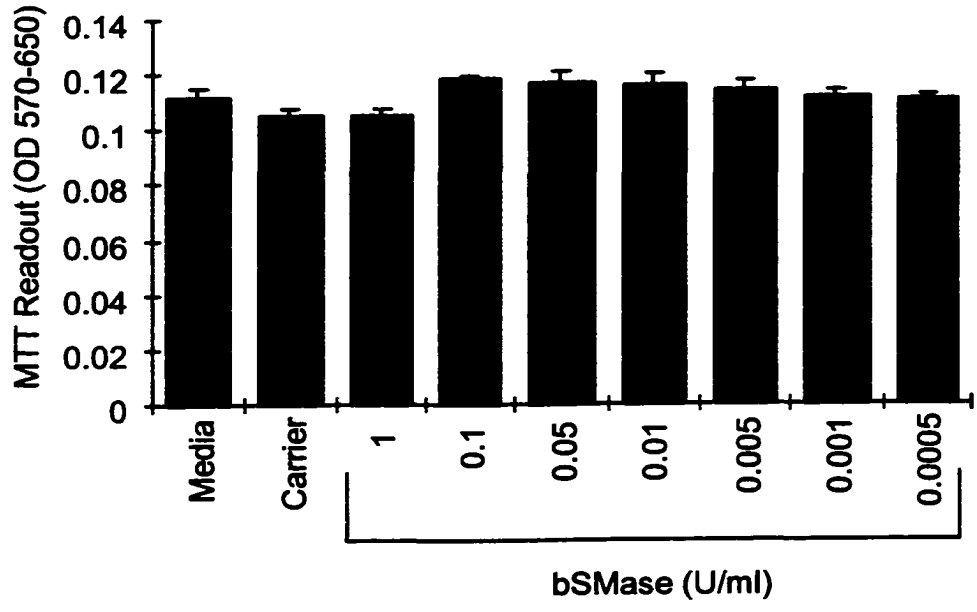


Figure 5.7 : Effect of bSMase on the viability of cultured trophoblasts. Trophoblasts were plated in 96-well dishes in 10%FCS-IMDM, then incubated with bSMase in 0.5%FCS-IMDM for 24 hours and cell viability determined by MTT. Data shown is representative of two separate experiments with different preparations of trophoblasts, and each bar represents mean ± SD of triplicate determinations.

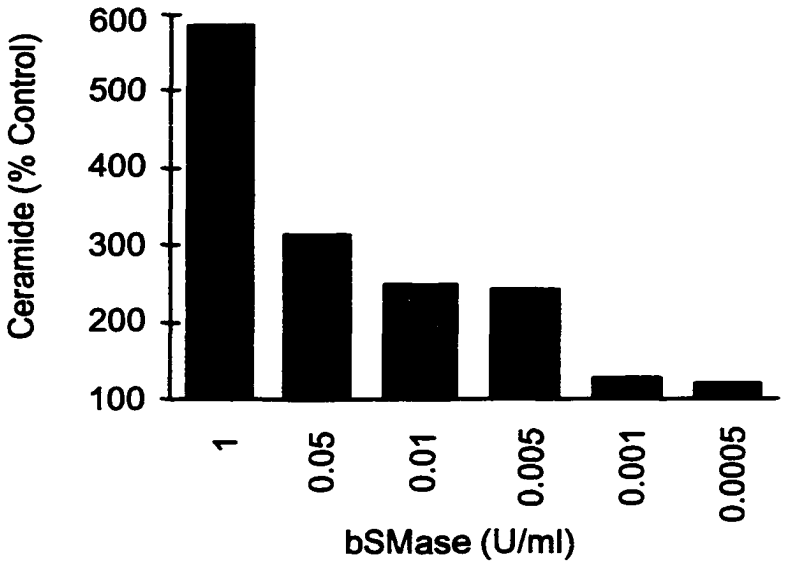


Figure 5.8 : Ceramide levels in trophoblasts treated with bSMase. Trophoblasts were plated at 2 x 10^6/well in 6-well plates, incubated with 5 μCi [^{3}H]palmitic acid for 48 hours, then incubated with bSMase for 24 hours. Lipids were extracted and analysed by TLC and scintillation counting. Data are expressed as percent change of untreated samples (set at 100%) calculated from cpm's. Shown is a representative of two independent experiments.

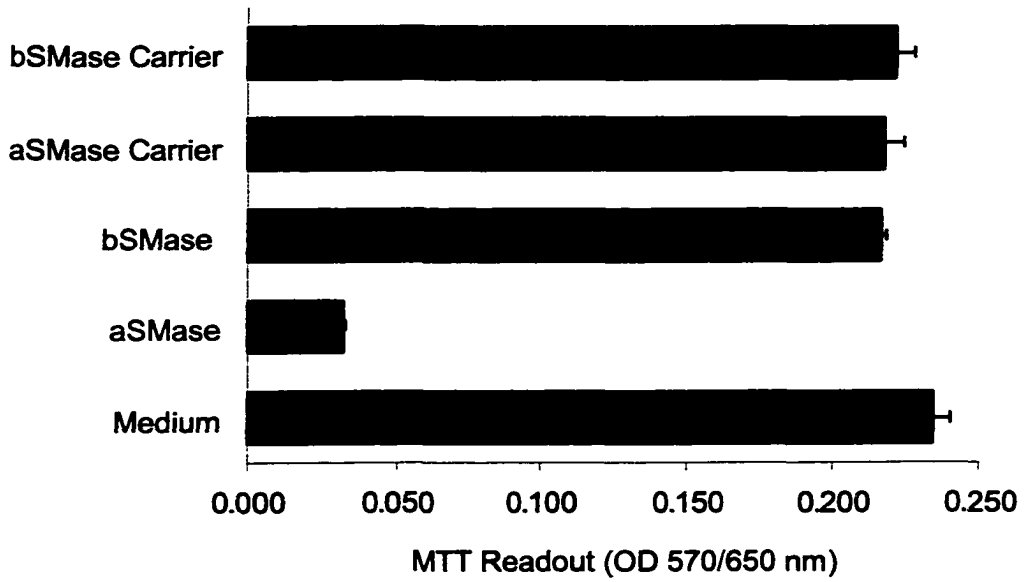


Figure 5.9 : Comparison of the effects aSMase and bSMase on trophoblast viability after 48 hours. Trophoblasts were plated in 96-well plates, treated with 1 U/ml bSMase and aSMase in 0.5%FCS-IMDM for 48 hours, and viability determined by MTT. Each bar represents mean ± SD of triplicate determinations from a single experiment, which was repeated twice with similar results.

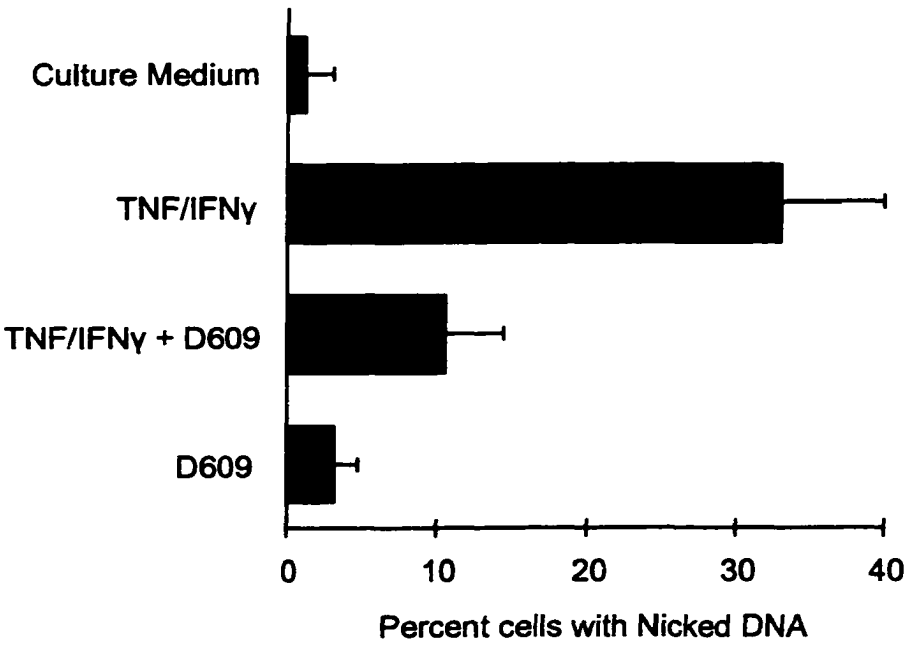


Figure 5.10 : D609 inhibits TNF/IFNγ-induced apoptosis. Trophoblasts were plated in 96-well plates, treated with 10 ng/ml TNF, 100 U/ml IFNγ, and 50 μg/ml D609 for 24 hours, then processed for TUNEL. Percent positive nuclei were determined as in Figure 5.1. Shown is a representative of two independent experiments.

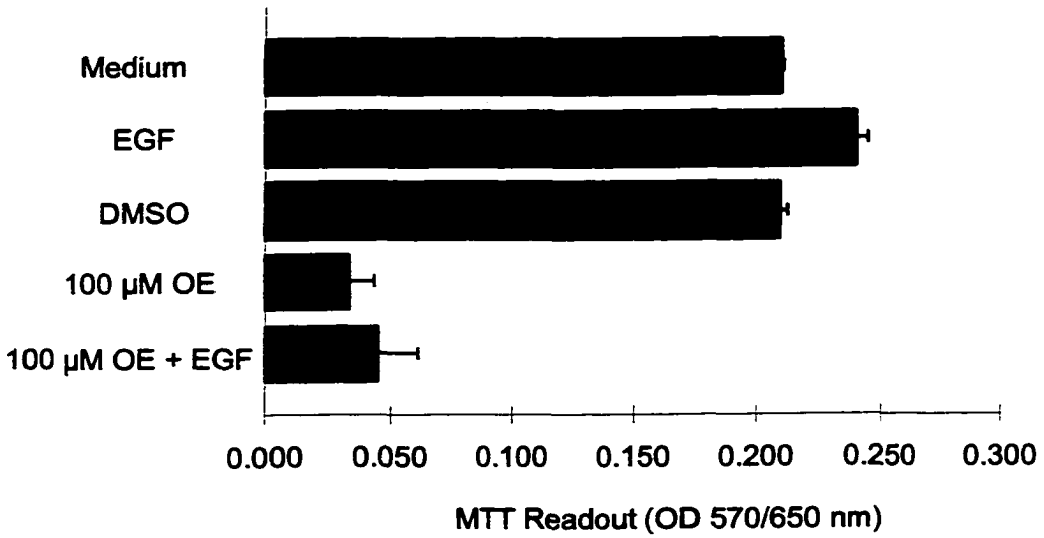


Figure 5.11 : Effect of *N*-oleoylethanolamine on the viability of cultured trophoblasts. Trophoblasts were plated in 96-well plates, treated with 100 µM OE, equivalent amount of DMSO (OE carrier), and 10 ng/ml EGF in 0.5%FCS-IMDM for 24 hours, then viability determined by MTT. Data values shown are mean ± SD of triplicate determinations from a single experiment, and are representative of 3 separate experiments

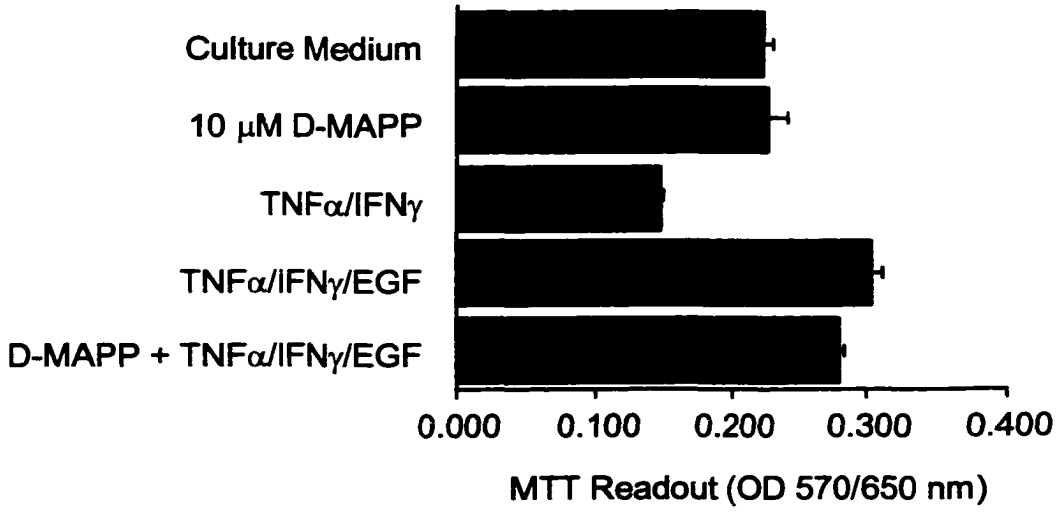


Figure 5.12 : Effect of D-MAPP on trophoblast viability and EGF protective effects. Trophoblasts were cultured in 96-well plates, incubated with 10 μM D-MAPP, 10 ng/ml TNF, 100 U/ml IFNγ, or 10 ng/ml EGF for 24 hours, and cell viability determined by MTT. Data values shown are mean ± SD of triplicate determinations from a single experiment, and are representative of 3 separate experiments

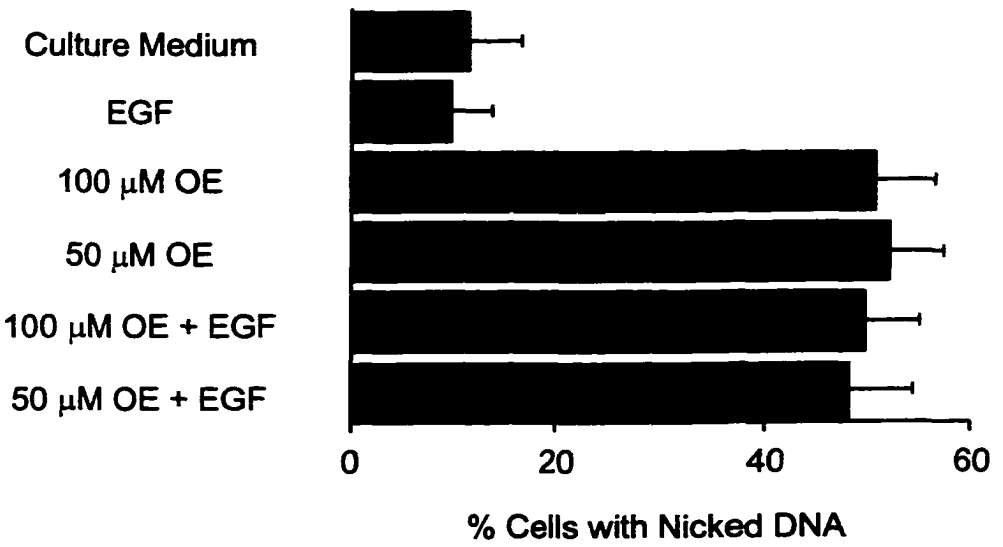


Figure 5.13 : Effect of OE on trophoblasts apoptosis. Trophoblasts were cultured in 96-well plates, incubated with 50 μM OE, 100 μM OE, and 10 ng/ml EGF in 0.5%FCS-IMDM for 6 hours, then processed for TUNEL. Percent positive nuclei were determined as in Figure 5.1.

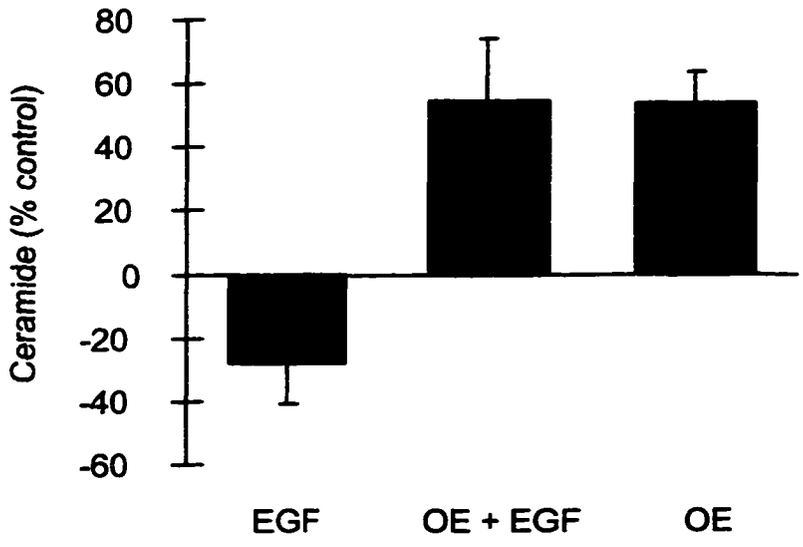


Figure 5.14 : Effect of OE on trophoblast ceramide levels. Trophoblasts were cultured in 6-well plates in 0.5%FCS-IMDM with 100 μM OE and 10 ng/ml EGF for 2 hours, then lipids extracted and ceramide measured. Data are expressed as a percent change from untreated samples. Data bars are mean ± range of duplicate determinations and are representative of 2 separate experiments.

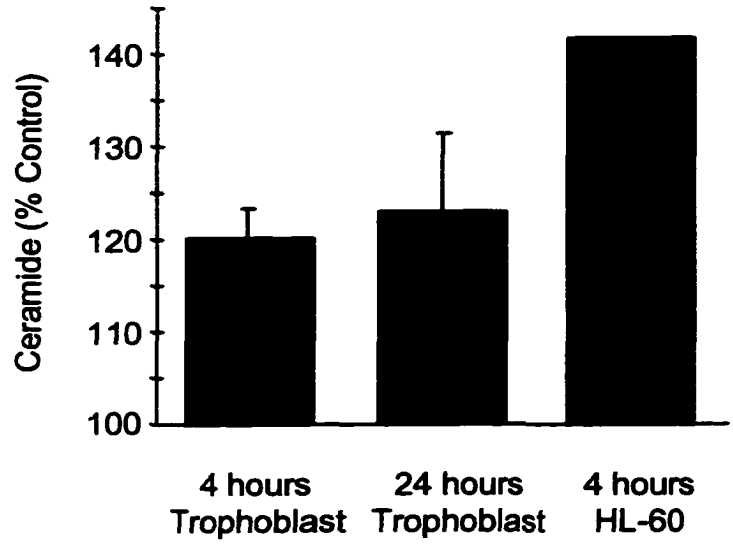


Figure 5.15 : Effect of D-MAPP on trophoblast ceramide levels. Trophoblasts were cultured at 2 x 10^6/well in 6-well dishes with 10 μM D-MAPP for 4 hours and 24 hours. HL-60 cells were treated with 10 μM D-MAPP for 4 hours. Lipids extracted and ceramide measured. Data are expressed as percent change from untreated controls (set at 100%) and are mean ± range of duplicate determinations. HL-60 data is from a single determination.

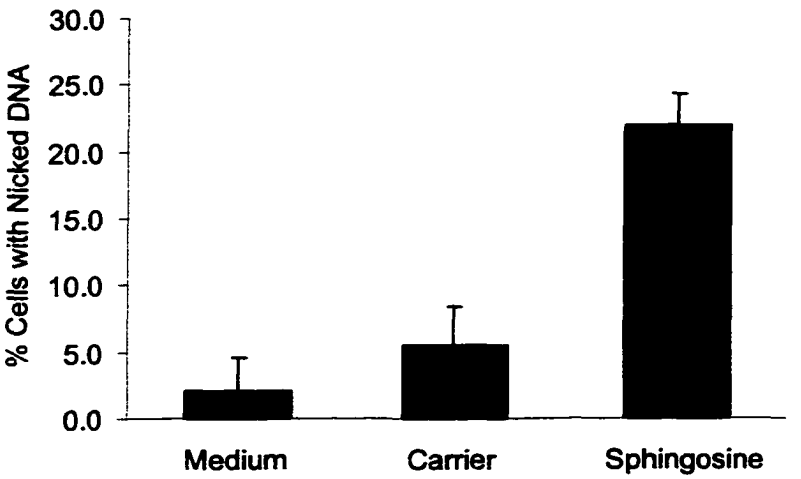


Figure 5.16 : Sphingosine induces apoptosis in cultured trophoblasts. Trophoblasts were cultured in 96-well plates in 0.5%FCS-IMDM with 30 μM sphingosine for 6 hours then processed for TUNEL. Sphingosine was first dissolved in DMSO (carrier) before dilution in culture medium. Percent positive nuclei were determined as in Figure 5.1.

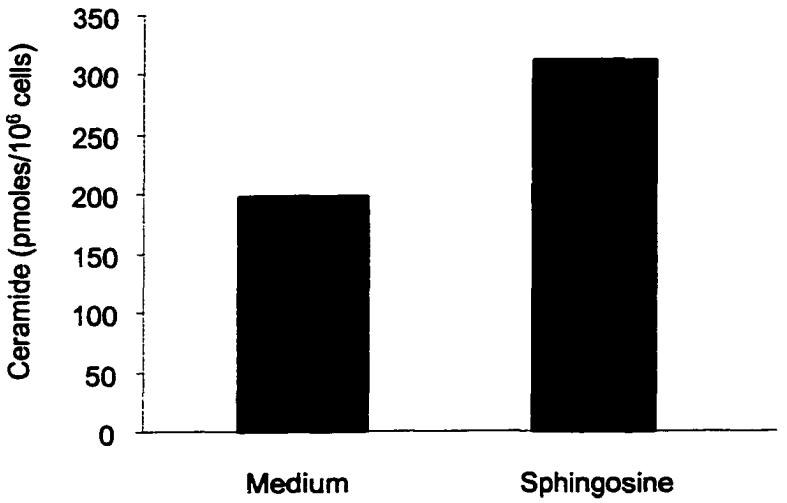


Figure 5.17 : Exogenous sphingosine induces an increase in ceramide levels in trophoblasts. Trophoblasts were cultured in 6-well plates and incubated with 30 μM sphingosine in 0.5%FCS-IMDM for 3 hours. Ceramide was measured by DAG Kinase assay as described. Data bars represent single point determinations from a single experiment.

Chapter 6 : Sphingosine 1-phosphate as a Mediator of the Effects of EGF

[The data in this chapter has been prepared as a manuscript for submission entitled "EGF activates sphingosine kinase and sphingosine 1-phosphate plays a role in the anti-apoptotic effects of EGF on primary human trophoblasts"]

6.1 Introduction

SPP is emerging as an important bioactive sphingolipid that promotes cell survival. It inhibits ceramide and TNF-induced apoptosis (Cuvillier *et al.*, 1996), plays a role in neuronal survival and differentiation (Edsall *et al.*, 1997), and acts as a second messenger in PDGF and serum-induced mitogenesis in fibroblasts (Olivera & Spiegel, 1993). The synthesis of SPP is mediated by sphingosine kinase, which is activated by PDGF, NGF and serum (Olivera & Spiegel, 1993; Edsall *et al.*, 1997), but not activated by EGF in fibroblasts (Olivera & Spiegel, 1993; Rani *et al.*, 1997). Inhibitors of sphingosine kinase reduce cellular SPP levels and induce apoptosis (Edsall *et al.*, 1998), suggesting that in some cell types sphingosine kinase activity and the resultant SPP may be required for cell survival.

The findings that ceramide induces apoptosis, and SPP inhibits ceramide-induced death and promotes cell survival, led the Speigel group to propose a ceramide:SPP biostat (Fig 1.5). This model proposes that the relative balance of ceramide and SPP determines the fate of the cell. Our data presented here has shown that ceramide induces apoptosis in trophoblasts, and EGF inhibits apoptosis. Moreover, EGF lowers ceramide levels, which could tip the balance of the "biostat" in favour of cell survival. The activation of sphingosine kinase by EGF, and thereby increase SPP levels, would further tip the balance in favour of cell survivor. We therefore asked whether SPP inhibits TNF/IFNγ- and ceramide-induced apoptosis, and whether EGF activates sphingosine kinase in human trophoblasts.

6.2 Results

6.2.1 SPP protects trophoblasts from TNF/IFNγ and ceramide induced apoptosis.

Trophoblasts cultures were treated with TNF/IFNγ with and without 10 μM SPP and incubated for 20 hours. TNF/IFNγ induced apoptosis in 17% of cells whereas cotreatment with SPP significantly reduced the number of cells with nicked DNA to 6% (Fig. 6.1). The number of cells with nicked DNA in cultures treated with SPP alone was about 2%, equivalent to those in medium alone. SPP concentrations less than 10 μM did not inhibit apoptosis.

SPP also reduced ceramide-induced apoptosis. In the experiment shown, C_{16}-ceramide induced DNA nicking in 13% of cells (Fig. 6.2). Cotreatment with 10 μM SPP reduced the number of apoptotic cells to 6%. Pretreatment with SPP before adding ceramide to the cultures further reduced the number of apoptotic cells to 4.5%, but was not significant from the non-pretreated cultures ($p>0.05$; Student's *t* test).

6.2.2 N, N-dimethylsphingosine (DMS) induces apoptosis in trophoblasts, which cannot be blocked by EGF.

The data described above demonstrated that the addition of exogenous SPP to trophoblast cultures inhibited TNF/IFNγ and ceramide-induced apoptosis, but does not answer whether SPP derived from endogenous sources inhibits apoptosis. If SPP is required for trophoblast survival we would expect that DMS, a competitive inhibitor of sphingosine kinase (Edsall *et al.*, 1998) would induce apoptosis. Twenty μM DMS rapidly induced apoptosis in trophoblasts (Fig. 6.3). At 3.5 hours, DMS induced apoptosis in 22% of trophoblasts. Cotreatement with SPP significantly reduced the number of cells with nicked DNA from 22 % to about 9% regardless of whether SPP was added 1 hour before or simultaneously with DMS.

In addition to inhibiting sphingosine kinase, DMS has also been shown to increase ceramide levels (Edsall *et al.*, 1998), and DMS-induced apoptosis could be in part due to the ceramide increase. We therefore measured ceramide levels in trophoblasts treated with DMS. By 2 hours there was a 40% increase in ceramide levels and a further 20% by 3 hours (Fig. 6.4). If DMS-induced apoptosis was mediated entirely by the

ceramide increase, we would expect EGF to inhibit DMS-induced apoptosis since EGF blocked ceramide-induced apoptosis. However, cotreatment with EGF did not significantly inhibit DMS-induced apoptosis ($p>0.05$) (Fig. 6.3). Pretreatment with EGF for 1 hour before adding DMS did reduce the level of DMS-induced apoptosis form 22% to 13%, and is significant at $p<0.05$ but not $p<0.01$ ($p=0.02$; Student's *t* test).

6.2.3 EGF activates sphingosine kinase.

Cultured trophoblasts were incubated with 10 ng/ml EGF and sphingosine kinase activity measured. As shown in Figure 6.5, there was a biphasic increase in sphingosine kinase activity. By 15 minutes there was a 40% increase over controls in sphingosine kinase activity and by 30 minutes activity returned to control levels (Fig. 6.5B). At approximately 3 hours sphingosine kinase activity begins to increase again and continues to increase to 3-fold above control levels by 24 hours, the last time point tested (Fig. 6.5A). Since PDGF has been shown to be a potent activator of sphingosine kinase whereas EGF was not in fibroblasts, we also determined the effects of PDGF on sphingosine kinase in trophoblasts. Like EGF, PDGF induced a moderate 1.5-fold increase by 3-4 hours, but unlike EGF, returned to control levels by 12 hours. In addition, PDGF, unlike EGF, had no effect on TNF/IFNγ-induced cytotoxicity of trophoblast cultures (Fig. 6.6).

We attempted to measure SPP levels using the [^{3}H]acetic anhydride acetylation method described in Methods and Materials. Although this method worked and was sensitive to 50 pmoles SPP, SPP levels from the trophoblasts lipid extracts were below the detectable levels of the method. We also employed a method developed in Dr. David Brindley's lab (personal communication). Cells were labelled with [^{32}P]orthophosphate to label lipid pools, and then [^{32}P]SPP in lipid extracts separated by a specific TLC protocol. Although this method proved useful in detecting SPP from trophoblast extracts, it was unsuitable for our needs due to concerns of culturing cells long term (12-24 hours) in phosphate-free culture medium as well as concerns of the equilibrium of labelled lipid pools.

6.2.4 Pertussis toxin does not inhibit the protective effects of SPP.

In addition to acting as second messenger signalling intermediate, SPP can act as a first messenger mediated through Edg receptors. Some of the effects are mediated by pertussis toxin (PT) sensitive G_i-coupled Edg receptors (e.g. Edg-1; Van Brocklyn *et al.*, 1998). To determine whether the SPP effects we observed were mediated by a G_i-coupled Edg receptor, we tested the effects of PT on the ability of SPP to inhibit TNF/IFNγ-induced apoptosis. As shown in Figure 6.7, 100 ng/ml PT did not inhibit SPP inhibition of TNF/IFNγ-induced apoptosis.

6.2.5 TNF/IFN γ activates sphingosine kinase.

Although TNF is generally associated with cell death, and SPP promotes cell survival and differentiation, TNF has been shown to activate sphingosine kinase in endothelial cells and mediate TNF-induced adhesion molecule expression (Xia *et al.*, 1998). We therefore asked whether TNF/IFNγ induced sphingosine kinase activity in trophoblasts. As shown in Figure 6.8, TNF/IFNγ activated sphingosine kinase in the two different preparations of trophoblasts tested, albeit at different levels. TNF/IFNγ induced kinase activity by 40% and 120% at 24 hours in the different trophoblast preparations. However, EGF-induced sphingosine kinase activity was greater than that of TNF/IFNγ in both cases, increased by approximately 300% and 180% respectively.

6.3 Summary of Results

1. SPP protects trophoblasts from TNF/IFNγ and ceramide-induced apoptosis.
2. *N,N*-Dimethylsphingosine, an inhibitor of sphingosine kinase, induces apoptosis and cannot be inhibited by co-treatment with EGF.
3. EGF activates sphingosine kinase activity in trophoblasts
4. SPP protection does not appear to be mediated a PT-sensitive G_i-coupled Edg receptor.
5. TNF/IFNγ activates sphingosine kinase, albeit at lower levels than EGF-induced activity.

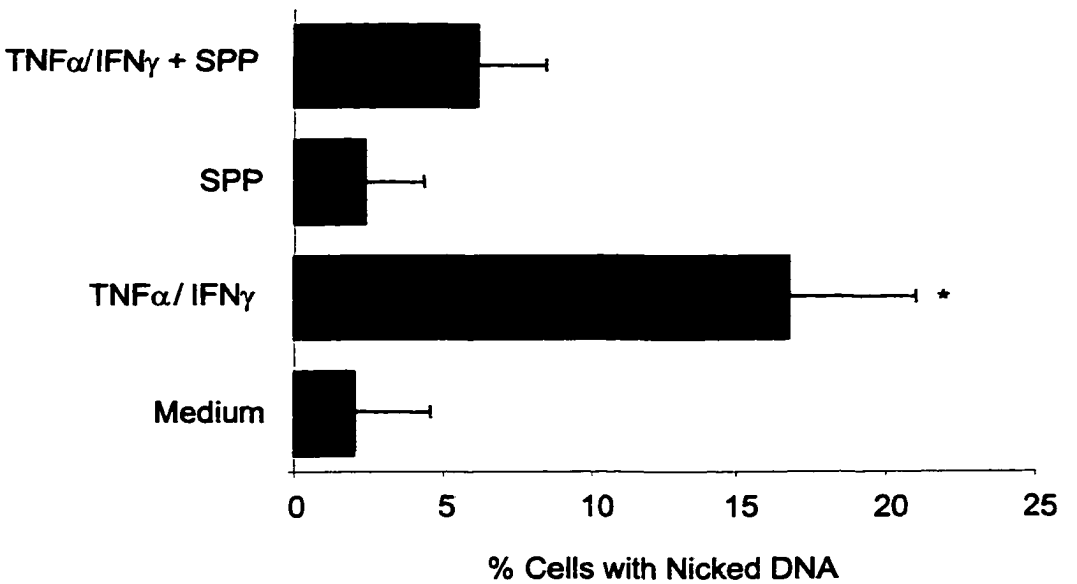


Figure 6.1 : SPP inhibits TNF/IFNγ-induced apoptosis. Trophoblasts were cultured in 96-well plates, treated with 10 ng/ml TNF and 100 U/ml IFNγ with and without 10 μM SPP for 20 hours, then processed for TUNEL. SPP was delivered to culture medium as a complex with BSA. The percentage of positive nuclei was determined by direct counting in 10 random fields, each containing approximately 100 nuclei. Values shown are mean ± SD from a single experiment and are representative of three separate experiments. * indicates statistical significance from cells in culture medium alone ($p < 0.05$; Student's *t*-test)

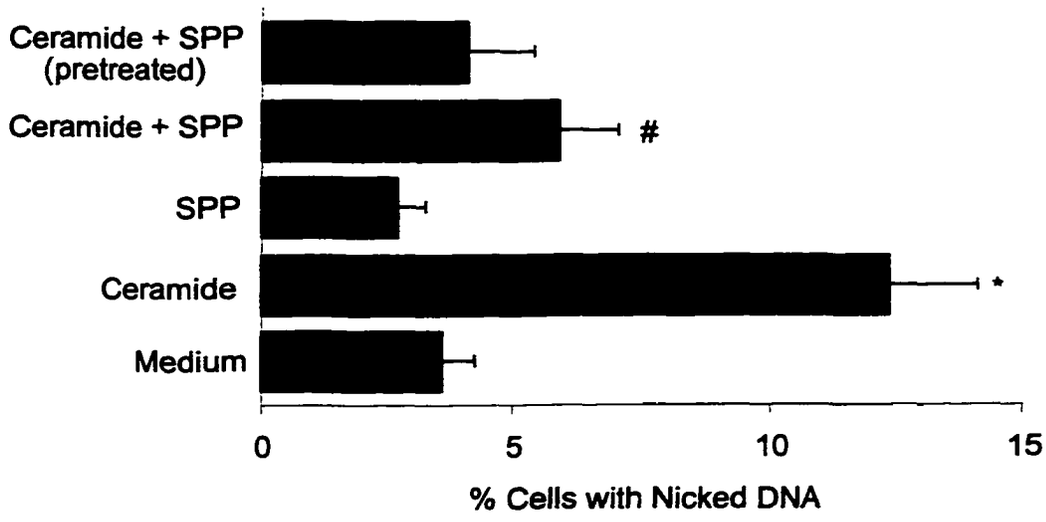


Figure 6.2 : SPP reduced ceramide-induced apoptosis in cultured trophoblasts. Trophoblasts were cultured in 96-well plates, incubated with 20 μM C_{16}-ceramide with and without 10 μM SPP for 20 hours, and processed for TUNEL. Pretreated cultures were pretreated with 10 μM SPP before adding ceramide. The exact time at which DNA occurred varied from experiment to experiment with different cell preparations but a similar pattern was seen in all experiments.

* $p<0.05$ compared to culture medium (Student's *t* test)

# $p<0.05$ compared to ceramide-treated cultures

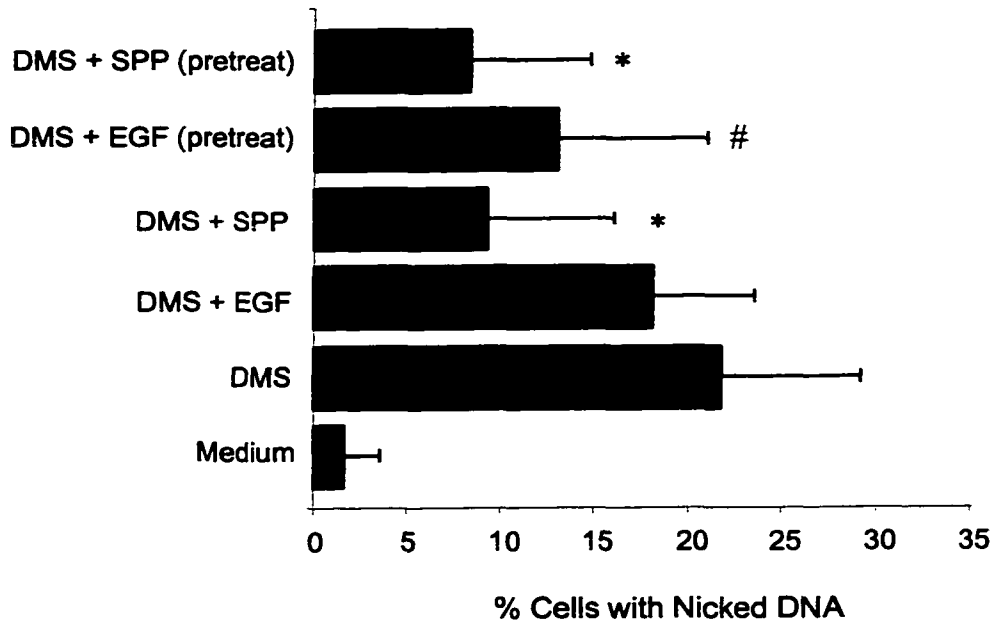


Figure 6.3 : EGF did not inhibit dimethylsphingosine (DMS)-induced apoptosis. Trophoblasts were cultured n 96-well plates in 0.5%FCS-IMDM with 20 μM DMS, 10 μM SPP and 10 ng/ml for 3.5 hours, then processed for TUNEL. Pretreated cultures were incubated with 10 μM SPP or 10 ng/ml EGF 1 hour before adding DMS. Values shown are mean ± SD from a single experiment and are representative of two separate experiments.

* $p<0.01$ compared to DMS alone (Student's *t* test)

# $p<0.05$ compared to DMS alone

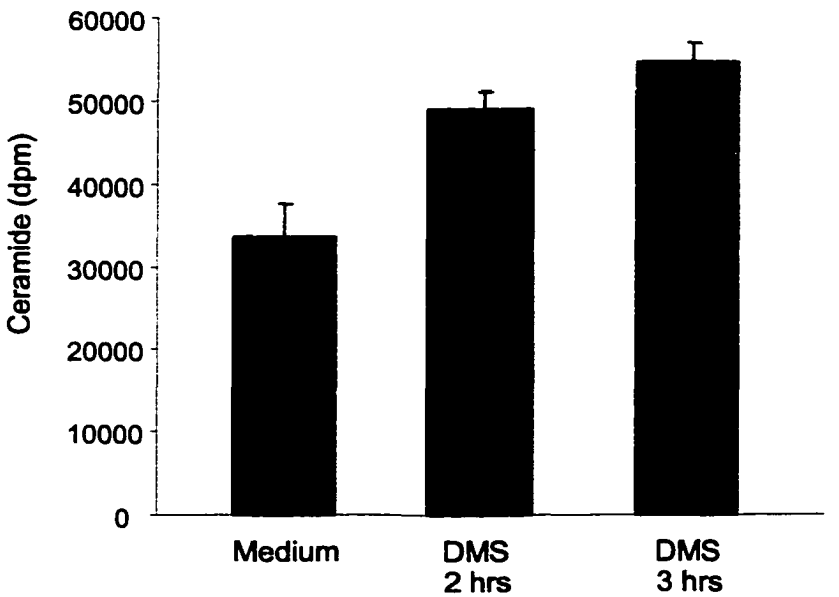
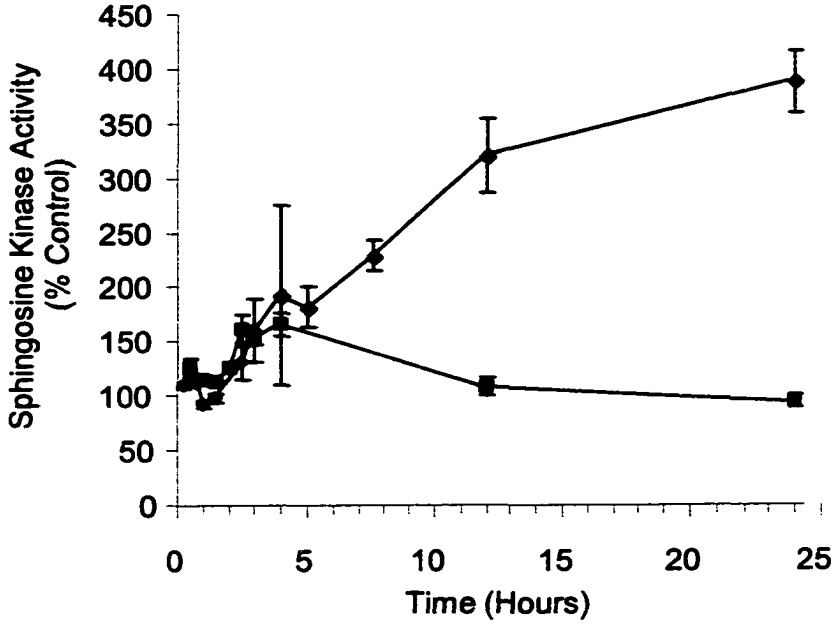


Figure 6.4 : DMS increased ceramide levels in cultured trophoblasts. Trophoblasts were cultured in 6-well dishes, labelled with 5 μCi [^{3}H]palmitic acid, and then incubated with 20 μM DMS in 0.5%FCS-IMDM for the time indicated. Ceramide levels were determined as described. Data are expressed as average dpm ± range of ceramide spots of duplicate samples scraped from TLC plates. The data is representative of 2 independent experiments.

A



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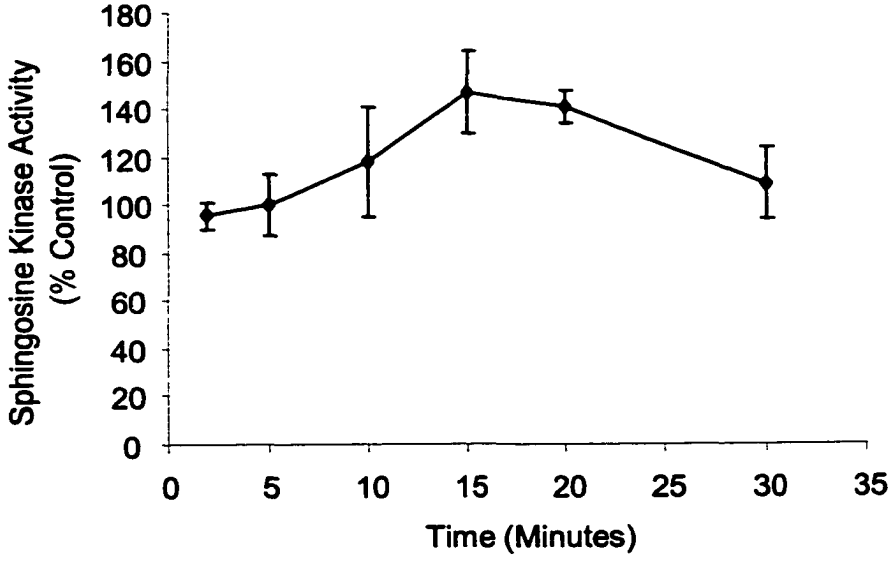

Figure 6.5 : EGF activated sphingosine kinase in cultured trophoblasts. Trophoblasts were cultured at 2-3 x 10^6/well in 6-well dishes, treated with 10 ng/ml EGF or PDGF and sphingosine kinase activity measured as described. Each data point is expressed as percent change of control (culture medium alone) set as 100%. A, EGF (*diamonds*) and PDGF (*squares*) stimulated sphingosine kinase activity. Data are expressed as mean ± SEM of 3 to 5 independent experiments. B, EGF stimulated sphingosine kinase activity between 2 and 30 minutes. Data are mean ± range of 2 independent experiments.

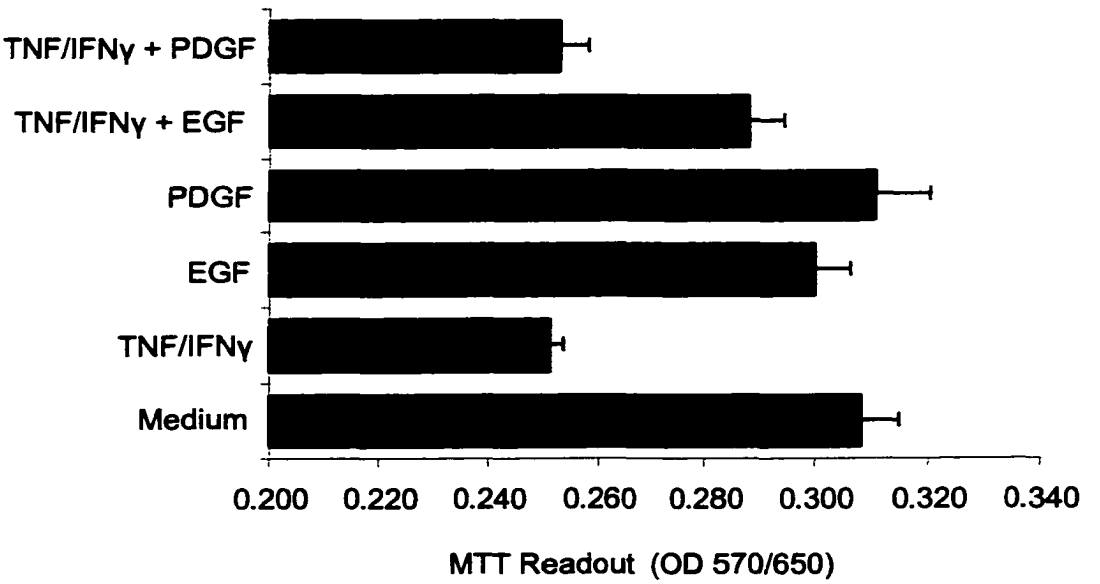

Figure 6.6 : PDGF does not inhibit TNF/IFNγ-induced cytotoxicity in trophoblasts. Trophoblasts were cultured in 96-well plates, treated with 10 ng/ml TNF + 100 U/ml IFNγ, 10 ng/ml EGF, or 10 ng/ml PDGF and incubated for 96 hours. Cell viability was determined by MTT. Data shown are mean of triplicate wells ± SD from one of two independent experiments using different preparations of trophoblasts.

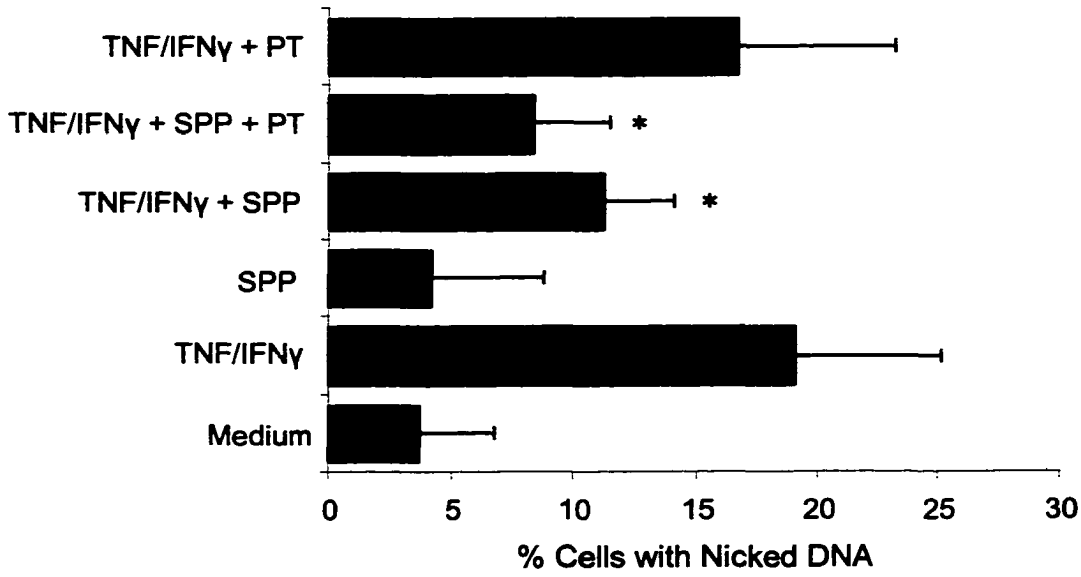

Figure 6.7 : Pertussis toxin does not inhibit SPP inhibition of apoptosis in trophoblasts. Trophoblasts were incubated with 10 ng/ml TNF + 100 U/ml IFNγ, 10 μM SPP and with and without 100 ng/ml pertussis toxin (PT) for 20 hours, then processed for TUNEL. The PT treated cells were pretreated with PT for 1 hour. Data are mean ± SD from a single experiment and are representative of two separate experiments
* $p<0.05$ compared to TNF/IFNγ alone (Student's *t* test)

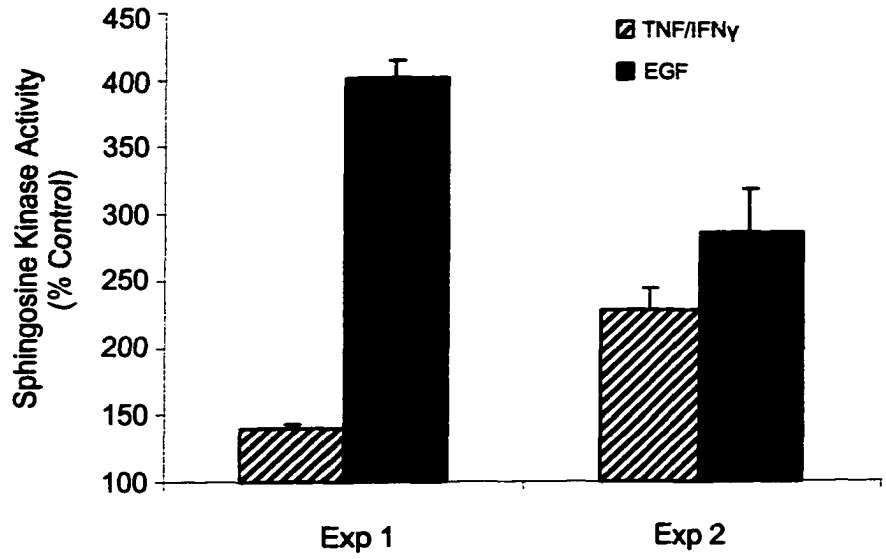

Figure 6.8 : TNF/IFNγ activates sphingosine kinase. Two different preparations of trophoblasts were cultured in 6-well plates, incubated with 10 ng/ml TNF + 100 U/ml IFNγ or 10 ng/ml EGF for 24 hours, and sphingosine kinase activity measured as described. Data are expressed as mean percent change from control (100%) ± range of duplicate determinations. Each experiment was done using a different preparation of trophoblasts.

Chapter 7 : Discussion

Despite a growing literature on death receptor-induced apoptosis in a variety of cell lines and tissues, little is known of the mechanisms of apoptosis in human primary trophoblasts. It is known that TNF/IFNγ induces apoptosis (Yui *et al.*, 1994) that is mediated by TNF-R1 (Yui *et al.*, 1996), and that EGF inhibits TNF/IFNγ-induced apoptosis (Garcia-Lloret *et al.*, 1996). The interplay of cytokines and growth factors could provide a regulatory mechanism for the control of normal trophoblast turnover in the placenta. If unregulated, it could lead to complications such as pre-eclampsia, IUGR, or vertical transmission of infectious agents, therefore making placental trophoblasts an important primary cell in which to study the mechanisms that control apoptosis. The pro-apoptotic signalling pathways and how EGF counters them in trophoblasts are largely unknown. Sphingolipids are known in other cell types to mediate life and death signals. I therefore investigated the role of sphingolipids in the control of life and death in primary trophoblasts using the culture system of purified trophoblasts developed in this lab.

One of the problems in the study of trophoblasts has been obtaining pure cell preparations. Cultured primary trophoblasts do not divide and contaminating fibroblasts could overgrow the cultures, skewing analysis. Yui *et al.* (1994b) developed a method that took advantage of the finding that trophoblasts do not express surface CD9 antigen (Morrish *et al.*, 1991), which is found on fibroblasts and other cells of the villous stroma. Using column-immunoelimination of trypsin-digested placental tissue, CD9 and MHC Class I expressing cells are removed, yielding primary cytotrophoblast cultures of more than 99.9% pure.

Recently, there has been discussion on the nature of the cells isolated using this method (Huppertz *et al.*, 1999). It was shown that many of the mononuclear cells isolated by this method are of multinuclear, syncytial origin and in fact, may already be programmed to die. These "mononuclear syncytial fragments" are relatively small and are formed when the fragile syncytium breaks up during the isolation procedure. Further worked revealed that varying stages of apoptotic morphology and characteristics (chromatin condensation, TUNEL positive nuclei) were observed in the cells of syncytial

origin but not cytotrophoblasts, leading to the conclusion that syncytiotrophoblasts are already programmed to die. At face value these findings may question the use of this isolation protocol to study the induction and inhibition of apoptosis in cells if the cells are already at varying stages of apoptosis. We do not dispute the finding that post-column cells may contain some syncytial fragments, and in fact, do find heterogeneity of cells in post-column cells. However, our experiments are not carried out on freshly purified cells but instead only on the population of cells that adheres to tissue culture plastic. This population of adherent cells ranges from 50%-80% of the post-column preparation and direct observations of the cultures have revealed that the relatively smaller "fragments" do not adhere and are washed away during the plating procedure. The adherent cells remain viable in culture for up to 4 weeks. Moreover, our adherent trophoblast cultures routinely contain less than 5% TUNEL positive nuclei, even after 24 - 48 hours in cultures, suggesting that these cells are not undergoing apoptosis before purification as defined by DNA nicking.

One important suggestion that came from the work of Huppertz *et al.* (1999) was that the initiation stage of apoptosis during trophoblast turnover might occur in the cytotrophoblasts. By some unknown mechanism apoptosis is then retarded until the cells fuse to form the syncytium and is then re-initiated at some stage. This was based on the observation that extracts from freshly isolated, unstimulated cytotrophoblasts contained relatively high caspase-8 activity but nearly undetectable capsase-3. On the other hand, syncytiotrophoblasts contained higher caspase-3 and -6 activity but low caspase-8. Their conclusion was that the initiator caspases are activated in cytotrophoblasts but subsequent activation of effector caspases is retarded in cytotrophoblasts. Although it is the syncytiotrophoblast that is exposed to maternal blood, and forms the crucial maternal-fetal barrier, the suggestion that apoptosis begins in the cytotrophoblast before fusion validates the use of cytotrophoblasts in experiments to explore the mechanisms that control apoptosis.

7.1 Fas/CD95 Expression on Trophoblasts

The principle aim of this study was to characterize Fas expression on

cytotrophoblast cultures. We know that TNF-R1 is expressed on cytotrophoblasts and mediates death (Yui *et al.*, 1996), therefore hypothesized that if Fas is expressed on trophoblasts, it could provide another mechanism for the induction of apoptosis in trophoblasts. We found that Fas is expressed on cultured first and third trimester cytotrophoblasts. Our observations that the protein could be detected by immunohistochemistry on all cytotrophoblasts in culture and by cellular ELISA on live cultured cells suggest that Fas is expressed on the cell surface. However, neither Fas antibody nor FasL-expressing cells induced cytotrophoblast apoptosis. Co-culture with IFNγ or protein synthesis inhibitors, conditions that enhance TNF-induced trophoblast apoptosis, did not sensitize trophoblasts to Fas-mediated death.

Down regulation of Fas signalling is not unique to trophoblasts. Activated T cells express FasL and transiently down-modulate Fas signalling, presumably to prevent auto-apoptosis within an immunologically functional time frame after antigen activation (reviewed in Nagata, 1997). The inhibitory mechanism(s) of Fas signalling in trophoblasts is unknown. Nitric oxide (NO) is produced by villous trophoblasts and has been shown to inhibit Fas responses in a variety of T and B cell lines (Mannick *et al.*, 1997). However our NO synthase inhibitor data would suggest that NO is not the mechanism in trophoblasts. Another mechanism first described in T cells is the protein FLIP, which prevents the binding of caspase 8 to FADD (Hu *et al.*, 1997; Irmler *et al.*, 1997; Srinivasula *et al.*, 1997), thereby preventing the assembly of the death-signalling complex. FLIP is transiently expressed in activated T cells and disappears when the cells become susceptible to Fas-mediated apoptosis. It is expressed in placental tissue (Irmler *et al.*, 1997), although no attempt was made to determine cell type. It is therefore a candidate for the observed Fas inhibition in cytotrophoblasts. However, FADD and caspase-8 are common to both Fas and TNR-R1, and FLIP inhibits both Fas and TNF-R1 mediated death (Hu *et al.*, 1997; Srinivasula *et al.*, 1997). TNF-R1-mediated death is not inhibited in cytotrophoblasts, suggesting that the inhibitor in trophoblasts is not common to both pathways. Further studies are currently in progress to determine the status of FLIP expression in trophoblasts.

Fas expression and signalling can be influenced by other cytokines. In murine

granulosa cells (Quirk *et al.*, 1998) and bone marrow stromal cells (Lepri *et* al., 1998), IFNγ upregulates Fas expression and Fas-mediated death. We observed Fas expression on trophoblasts in the absence of IFNγ treatment, and pre-treatment or cotreatment with IFNγ did not sensitize trophoblasts to Fas-mediated death. TNF has also been shown to upregulate Fas expression on endothelial cells (Sata *et al.*, 2000) and primary microglia cells (Lee *et al.*, 2000). TNF alone does not induce death in airway smooth muscle cells, but synergizes with Fas to enhance Fas-mediated death (Hamann *et al.*, 2000). These reports demonstrate that TNF and IFNγ can significantly affect Fas function. Given that TNF induced apoptosis in trophoblasts, it is conceivable that it may also affect Fas function in trophoblasts. The effect of TNF on Fas in trophoblasts was not tested and is worth investigating.

Although we investigated Fas in the context of cell death, we must not lose sight of the possibility that there may be other biological outcomes other than cell death as a result of Fas ligation. Human fetal astrocytes express Fas but do not undergo Fas-mediated death (Becher *et al.*, 1998). Recently it was shown that primary astrocytes do respond to Fas-ligation, but instead of apoptosis they upregulate expression of the chemokines MIP-1 and MIP-2 (Lee *et al.*, 2000). Does a similar phenomena occur in trophoblasts? It is conceivable that Fas ligation does not directly damage trophoblasts, but does so by stimulating chemokine expression and therefore mediating the recruitment of inflammatory cells, such as monocytes.

A control mechanism of Fas signalling in trophoblasts is understandable given that trophoblasts also express Fas ligand (Runic *et al.*, 1996). Similar to activated T cells, unregulated Fas signalling in trophoblasts could lead to auto-apoptosis. However, that does not mean there is no role for juxtacrine or auto-apoptosis of trophoblasts. The villous placenta grows and remodels during pregnancy, and the presence of Fas and FasL in the villous trophoblasts could help regulate trophoblast density. The distribution of apoptotic nuclei in trophoblasts of placentas from normal pregnancies would suggest a regulated tissue turnover by apoptosis. The higher than normal incidence of apoptosis in placentas from IUGR pregnancies (Smith *et al.*, 1997) and pre-eclampsia (Jones & Fox, 1980; Ghidini *et al.*, 1997) suggest that these complications may arise from an increased

trophoblast loss.

7.2 SMase/Ceramide and apoptosis

The data presented in Chapter 5 demonstrated that increasing ceramide levels in primary cytotrophoblasts, either directly by adding ceramide to the cultures or indirectly by adding aSMase, induced apoptosis. These findings are in agreement with other studies of other cell types where ceramide induced apoptosis (Obeid *et al.*, 1993; Jarvis *et al.*, 1994).

The role and relative contribution of aSMase in apoptosis is not clearly defined. Our data that exogenous aSMase induced apoptosis demonstrates that ceramide generated from the action of aSMase can induce apoptosis in trophoblasts, but does not address a physiological role. There are several lines of evidence supporting a role for aSMase in TNF (Higuchi *et al.*, 1996; Wiegmann *et al.*, 1994), Fas/CD95 (Cifone *et al.*, 1995) and radiation (Santana *et al.*, 1996) induced apoptosis. Some of this evidence comes from the use of Niemann-Pick lymphocytes and cells from aSMase$^{-/-}$ mice, whereas in the same systems others have suggested that aSMase does not play a significant role in apoptosis (Bosen-de Cock *et al.*, 1998; Lin *et al.*, 2000). Recent evidence may reconcile these discrepancies. Kirschnek *et al.* (2000) reported that aSMase may not be absolutely required for Fas/CD95-apoptosis, but depends on the amount and receptor aggregation status of the agonist. Under relatively low levels of stimulation, aSMase is required, but higher doses of cross-linking Fas-stimuli override the requirement for aSMase. This finding may explain why in some studies cross-linking anti-Fas antibody could induce apoptosis in Niemann-Pick lymphocytes. A similar study has not been reported for TNF-induced aSMase activation.

The role of nSMase in apoptosis is also unclear. The Kronke group model (Wiegmann *et al.*, 1994) proposes that nSMase is not involved in death receptor-induced apoptosis. The addition of exogenous bacterial (neutral) SMase to some cell types induces apoptosis (Kim *et al.*, 1991; Westwick *et al.*, 1995; Jarvis *et al.*, 1994) while in other cell types it does not (Zhang *et al.*, 1997; Veldman *et al.*, 1998). Our data is in agreement with the latter study. Bacterial SMase induced large increases in ceramide

levels in trophoblasts but did not induce apoptosis. There is evidence that ceramide generated by exogenous bSMase in the outer leaflet of the cell membrane is not part of the death signalling pathway in some cell types and does not reach the signalling pool on the inner leaflet (Linardic & Hannun, 1994; Zhang *et al.*, 1997). Ceramide generated on the inner leaflet by transfection of nSMase did induce apoptosis. These studies have led to the suggestion that there are unique pools of signalling ceramide that are functionally distinct.

Our finding that aSMase but not bSMase induces apoptosis argues in favour of the compartmentalized ceramide model in apoptosis. Our bSMase data clearly shows that a ceramide increase in the outer leaflet does not induce apoptosis in trophoblasts whereas ceramide derived from aSMase activity does. Exogenous aSMase (optimum pH 4.5-5.5 (Kolesnick, 1991)) must enter an acidic cellular compartment to be active. The nature of the compartment is unclear. Acid SMase is an endosomal/lysosomal enzyme (Spence 1993), and aSMase activity has been found in caveolae (Liu and Anderson, 1995). Trophoblasts are highly vacuolated and invaginated cells (Alpin, 1991). It is possible that exogenous aSMase added to the cultures enters the cell via caveolae, and is active in the caveolae or delivered to an intracellular compartment since caveolae can trap external molecules for intracellular delivery (Anderson, 1993). There is evidence to support the latter since secreted forms of aSMase, when added exogenously to macrophages, is endocytosed and targeted to lysosomes in an enzymatically active form (Schissel *et al.*, 1998).

Another question that remains is how exogenous ceramide enters the apoptotic pool and induces apoptosis whereas exogenous bSMase-derived ceramide in the outer leaflet does not. Exogenous ceramide would presumably be in contact with the outer leaflet. Zhang *et al.* (1997) has suggested that outer leaflet ceramide does not enter the apoptotic pool because of the slow rate of transfer (flipping) to the inner leaflet and may be metabolized before it can flip. Given that caveolae have been implicated in the apoptotic pool of ceramide, it is possible that exogenous ceramide may directly enter caveolae where it can enter the signalling pool directly, or be delivered to an internal pool.

We chose to investigate the effects of EGF on ceramide-induced apoptosis in trophoblasts for several reasons. EGF completely inhibits TNF/IFNγ-induced apoptosis in trophoblasts (Garcia-Lloret *et al.*, 1996). Secondly, other growth factors, such as PDGF, have been shown to activate ceramidase (Coroneos *et al.*, 1995), thereby lowering ceramide levels. Third, given that EGF protects primary trophoblasts from TNF/IFNγ-induced apoptosis *in vitro*, and is produced in the placental (Hofmann *et al.*, 1991) as well as plays a role in trophoblast differentiation (Morrish *et al.*, 1987), it is possible that EGF plays a critical role as a survival factor in the placenta. Here we have shown that EGF inhibits ceramide and aSMase-induced apoptosis and lowers trophoblast ceramide levels. Since EGF did not inhibit OE-induced apoptosis, or significantly reduce ceramide levels in OE-treated cells, it would appear that at least part of the mechanism in EGF protection is the activation of a ceramidase. We attempted to measure ceramidase activity but were unsuccessful. It is possible that with the amount of protein extracted from the trophoblast cultures ceramidase activity was below detectable limits of the assay. From our trophoblast cultures we could only obtain between 10% and 30% of the total protein levels used in other studies (Coroneos *et al.*, 1995; Huwiler *et al.*, 1999), and the problem was compounded by a limited number of primary cells, thereby limited the extent to which we could increase cell numbers in the experiments.

The data obtained from the ceramidase inhibitors also argues in favour of a compartmentalized ceramide model in apoptosis. OE preferentially inhibits acid ceramidase (Coroneos *et al.*, 1995) and D-MAPP inhibits alkaline ceramidase (Bielawaska *et al.*, 1996). Both inhibitors increased ceramide levels but only OE, the acid ceramidase inhibitor, induced apoptosis. Moreover, D-MAPP did not inhibit or reduce EGF protection of TNF/IFNγ-induced apoptosis, suggesting that alkaline ceramidase does not play a role in EGF protection from apoptosis.

Our data shows that EGF protects trophoblasts from exogenous ceramide-induced apoptosis in the shorter term cultures (8 hours), but does not inhibit ceramide-induced cell loss in the longer term (24 hours) cultures. We must bear in mind however, that we added relatively large concentrations of ceramide to the culture medium. It is conceivable that at the longer time points there is an overwhelming death signal from the

continual uptake and accumulation of ceramide from the culture medium. In addition, we cannot rule out the possibility that continues exposure to exogenous ceramide perturbs the cell membrane, potentially affecting cellular functions like adhesion.

Although ceramide and aSMase induced apoptosis, we could not detect a TNF/IFNγ-induced increase in ceramide levels or increased SMase activity. Since both aSMase and nSMase activity, as well as changes in ceramide levels, could be measured in trophoblasts, it would appear not to have been a problem with the methodologies, leading one to speculate that SMase and ceramide are not involved TNF/IFNγ signalling in trophoblasts. D609, a putative inhibitor of PC-PLC, which in turn inhibits aSMase, blocked TNF/IFNγ-induced apoptosis, supporting a role for aSMase. However, more recent evidence has demonstrated that D609 also inhibits sphingomyelin synthase, and some of the roles attributed to PC-PLC may be a result of the inhibition of sphingomyelin synthase (Luberto & Hannun, 1998). Therefore we must use caution in assigning a role for aSMase based on the data from D609. There are a number of possible reasons why we did not see TNF/IFNγ-induced changes in ceramide and aSMase. It is possible that ceramide is rapidly metabolized. Modur *et al.* (1996) demonstrated that TNF-induced ceramide increases in HUVECs were of a lesser magnitude than sphingomyelin breakdown, suggesting rapid metabolism. However, we could not find a consistent TNF/IFNγ-induced sphingomyelin decrease. Trophoblast cultures are not homogenous, and the amount TNF/IFNγ-induced apoptosis varies with different preparations of cells. TNFγ/IFNγ-induced cell loss can range from 40% to 60% (Yui *et al.*, 1994; and data presented here) in the same time period with different cell preparation. There are therefore a significant number of cells in the cultures that do not undergo TNF/IFNγ-induced apoptosis. It is possible that there is a TNF/IFNγ-induced ceramide increase but only in the cells that undergo apoptosis. Furthermore, if there is a very compartmentalized signalling pool of ceramide, it is possible that there can be relatively large ceramide increase in distinct cellular compartments. Ceramide levels are measured from total lipid extracts, therefore it is possible that a relatively large increase in a small compartment in only a percentage of the cells could go undetected with our methods. As stated above, these compartments could be caveolae or an acidic endosomal

compartment, and studies are currently underway to address these possibilities.

Very recently, another role for sphingolipids in apoptosis has been reported in Jurkat cells. Sphingomyelin loss during the execution phase may be required for some apoptotic cell morphologies, such as blebbing (Tepper *et al.*, 2000). During the loss of plasma membrane asymmetry, when phosphatidylserine flips to the outer leaflet, sphingomyelin moves to the inner leaflet where it is hydrolysed to ceramide. The loss of sphingomyelin causes a concomitant loss of cholesterol, and therefore changes the fluid properties of the membrane. This sphingomyelin loss, and ceramide generation, could account for the ceramide increase observed in some cell types at late stages of apoptosis. However, it does not address a signalling role for ceramide.

Our data does not address the contribution of ceramide from *de novo* synthesis. In some cell types, TNF (Xu *et al.*, 1998) as well as drug-induced ceramide increases (Perry *et al.*, 2000) result from activation of *de novo* synthesis and not from the sphingomyelin cycle. Another has shown that TNF can activate the sphingomyelin cycle and *de novo* synthesis in the same cell type, albeit with different kinetics (Bourteele *et al.*, 1998). Given that we could not measure an inducible change in SMase activity, it is an attractive hypothesis that TNF may activate the *de novo* synthesis pathway.

Exogenous sphingosine induced apoptosis in our trophoblast cultures, and recently it was reported that sphingosine mediates Fas-induced apoptosis in Jurkat cells (Cuvillier *et al.*, 2000). It is possible that sphingosine may contribute to apoptosis in trophoblasts, but we also observed an increase in ceramide levels in sphingosine-treated cultures. It is unclear if the resultant apoptosis was mediated by the ceramide, sphingosine, or both. This question could be addressed by the use of fumonisin B1, an inhibitor of ceramide synthase, which acylates sphingosine to ceramide. The role of sphingosine warrants further investigation.

The control of apoptosis by a growth factor, EGF, which modulates ceramide levels, fits the Spiegel Model (Olivera & Spiegel, 1993; Cuvillier *et al.*, 1996), or sphingolipid biostat. In this model, the relative balances of the sphingolipids ceramide and sphingosine 1-phosphate (SPP) controlled by TNF and PDGF, control the fate of the cell. Here we have shown that EGF lowers ceramide levels and promotes cell survival.

The effects of EGF on SPP levels are unknown. We therefore next investigated the effects of EGF on SPP.

7.3 SPP inhibits apoptosis and EGF activates Sphingosine Kinase

As previously described, SPP is a pro-survival sphingolipid (Olivera & Spiegel, 1993), opposing the effects of TNF-, ceramide- (Cuvillier *et al.*, 1996) and Fas-induced apoptosis (Cuvillier *et al.*, 1998). In Chapter 6 we have shown that exogenous SPP added to trophoblast cultures inhibited TNF/IFNγ and ceramide-induced apoptosis. The anti-apoptotic properties of SPP have been attributed to intracellular SPP (Van Brocklyn *et al.*, 1998). Given the high concentration of exogenous SPP required (10 μM), and the K_d (8 nM) of Edg-1, the so-called high affinity SPP receptor (Spiegel & Milstien, 2000), it is more likely that SPP is acting intracellularly as a second messenger, although we cannot rule out a first messenger function in trophoblasts. Pertussis toxin, which uncouples G_i from EDG-1, did not inhibit SPP effects, ruling out G_i-coupled receptor signalling. SPP does bind to other Edg receptors however, such as Edg-3 and -5, which can couple to PT-insensitive G-proteins (Windh *et al.*, 2000).

Olivera & Spiegel (1993) reported that PDGF, but not EGF, activated sphingosine kinase in fibroblasts, and that there is a divergence in PDGF and EGF signalling pathways (Rani *et al.*, 1997). We have shown here that EGF stimulates a sustained activation of sphingosine kinase whereas PDGF-induced sphingosine kinase activity is relatively modest and transient. Since the former studies were carried out, several different isoforms of sphingosine kinase have been identified (Kohama *et al.*, 1998; Liu *et al.*, 2000; Nava *et al.*, 2000). The assay for sphingosine kinase activity does not distinguish between the isoforms. It is conceivable that EGF and PDGF activate different isoforms in different cell types. Moreover, PDGF does not protect trophoblasts from apoptosis; therefore we would not expect PDGF activation of sphingosine kinase if SPP were an anti-apoptosis signalling molecule in trophoblasts.

Our data from experiments with the sphingosine kinase inhibitor DMS further support a role for sphingosine kinase in EGF signalling. EGF could not inhibit the effects of DMS. However, pre-treatment with EGF did reduce the level of DMS-induced

apoptosis. This is could be due to an EGF-induced increase in SPP levels during the pre-incubation period. DMS is a competitive inhibitor of sphingosine kinase and when added at the same time as EGF, it would prevent an EGF-induced SPP increase. However, a pre-treatment with EGF could increase SPP levels, and the addition of DMS would prevent any further increase. The pre-existing increase in SPP levels could exert its pro-survival effects for a period before it is metabolized, therefore decrease the amount of DMS-induced apoptosis. It must be noted however, that DMS can also inhibit PKC, and the pro-apoptotic properties of DMS may be mediated by inhibition of PKC. It is unknown if PKC inhibition induces apoptosis in trophoblasts. Therefore more specific inhibitors of PKC should be used to address this possibility.

TNF/IFNγ-induced activation of sphingosine kinase appears to be a contradiction. TNF/IFNγ induces apoptosis whereas sphingosine kinase activity, and the resultant increase in SPP, is anti-apoptotic (Cuvillier *et al.*, 1996). However, it is becoming more evident that TNF can activate multiple pathways, both apoptotic and anti-apoptotic. For example, TNF activates pro-apoptotic caspases (reviewed in Budihardjo *et al.*, 1999) and anti-apoptotic NF-κB (Beg & Baltimore, 1996; Qin *et al.*, 1999). TNF also activates sphingosine kinase in endothelial cells, which mediates TNF-induced adhesion molecule expression (Xia *et al.*, 1998). The biological processes which determine a cell's fate in response to these opposing signals are unknown. One determining factor could be the strength of the opposing signals, and how those signals are influenced by other extracellular signals.

EGF activation of sphingosine kinase in trophoblasts may have significance in other aspects of trophoblast biology. SPP, in addition to inhibiting apoptosis, promotes differentiation in neurons (Rius *et al.*, 1997) and cell migration in endothelial cells (HUVEC; Wang *et al.*, 1999). EGF is an important growth factor in the placenta, promoting trophoblast migration (Bass *et al.*, 1994) and differentiation (Morrish *et al.*, 1987; Garcia-Lloret *et al.*, 1996). Does SPP mediate some of these EGF-induced effects in trophoblasts?

7.4 Ceramide:SPP Biostat in Trophoblasts

Our data are consistent with the model that cell fate can be determined by the relative balance of ceramide to SPP. Increasing ceramide levels by adding exogenous ceramide or aSMase induces apoptosis. EGF inhibits apoptosis and was found to lower ceramide levels and increase sphingosine kinase activity, thereby increasing SPP levels. Inhibitors of sphingosine kinase, such as DMS, also induce apoptosis. Our data supports a role for distinct signalling pools of ceramide and SPP in the control of apoptosis in human trophoblasts, and moreover, presents a novel EGF-regulated mechanism for the activation of sphingosine kinase.

The finding that TNF also activates sphingosine kinase appears to be a contradiction to this model. If activation of sphingosine kinase promotes cell survival, how does TNF induce death if it also activates sphingosine kinase? However, TNF also increases ceramide levels, which does fit the model in that it is the balance of ceramide to SPP which determines the fate of the cell. Moreover, the balance is a function of time. That is, cell fate can be determined by when ceramide levels increase relative to when there is an increase in SPP levels.

7.5 Future Directions

It is still unknown why Fas does not induce a death signal in trophoblasts. Experiments are currently underway to determine if there is a receptor proximal inhibitor, such as FLIP, expressed in trophoblasts. FAP-1, a tyrosine phosphatase, is another factor that has been reported to suppress Fas signalling (Sato *et al.*, 1995) and studies are underway to determine if FAP-1 is expressed in trophoblasts. Recently it was shown that human thymocytes express a decoy Fas receptor (Jenkins *et al.*, 2000) that lacks the death domain. The decoy is expressed along with full length Fas and it may act as a dominant negative by mixing with wt Fas in the trimeric receptor complex. Although our data suggests that the major Fas transcript is full length Fas, it is possible that a similar transcript is produced at low copy number and was missed in our study. Further investigations to determine if trophoblasts express this, or a similar decoy receptor could help in understanding why Fas did not induce apoptosis. In addition, it is

possible that Fas is functional, but is stimulating the expression of chemokines or cytokines, and investigating this possibility could contribute to the understanding of novel Fas functions.

Although our data supports a role for ceramide and aSMase, we did not clearly show a link between aSMase, ceramide and TNF/IFNγ signalling. If, as we proposed, there are TNF/IFNγ-induced ceramide increases and aSMase activation in specific cellular compartments, then it may be possible to measure these changes in isolated acidic compartments, such as endosomes and caveolae. Inhibitors of aSMase (imipramine: Brenner *et al.*, 1998; Kirschnek *et al.*, 2000) are also now available with a greater specificity than D609, as well as inhibitors of nSMase, such as scyphostatin (Nara *et al.*, 1999). The use of these inhibitors may help clarify the role of aSMase and nSMase TNF-R1 signalling and apoptosis in trophoblasts. As well, the contribution of ceramide from *de novo* synthesis should be investigated. Specific inhibitors of enzymes in this pathway, as well as direct measurements of enzyme activity can help address this question.

Caspases play a central role in apoptosis in many cell types. In order to understand apoptosis, and how growth factors control apoptosis in trophoblasts, it is important to understand the role of caspases. Preliminary data from our lab indicate that TNF/IFNγ activates caspase-3 and -8, but how EGF, ceramide and/or SPP affects caspase activity is unknown. It would be interesting to speculate that ceramide would increase effecter caspase activity whereas SPP would inhibit it in trophoblasts.

Our experiments suggesting that SPP may be an anti-apoptotic molecule in trophoblasts is based the addition of SPP to the cultures and the observation that EGF activates sphingosine kinase. A more definitive answer into the role of SPP may be obtained by transfection of trophoblasts with sphingosine kinase and determine if it protects from TNF-induced apoptosis.

Since SPP induces differentiation in other cells types, the question arises: does it also promote differentiation, such as syncytialization, in trophoblasts? Fusion of cytotrophoblasts to form syncytiotrophoblasts is critical for normal placental function, and is promoted by EGF. In addition, if SPP promotes cytotrophoblast fusion, does it do

so by binding cell surface Edg receptors? Edg-3 is expressed in the placenta (Spiegel & Milstien, 2000), although there is no mention of cell type. Expression pattern of other Edg receptors on trophoblasts is unknown.

Although our studies in trophoblast survival focused on EGF effects on ceramide and SPP, EGF is known to activate other pro-survival signalling pathways. EGF stimulates MAPK/ERK activity (Van der Geer & Hunter, 1994), which is generally associated with cell survival and mitogenesis. Another pro-survival factor activated by EGF is Akt (Burgering & Coffer, 1995; Kulik *et al.*, 1998), and EGF inhibition of Fas-mediated death has been shown to require Akt activity (Gibson *et al.*, 1999). How these factors are influenced by ceramide and SPP are largely unknown, although ceramide (Zhou *et al.*, 1998), by acting through CAPP (Schubert *et al.*, 2000), inhibits Akt activity. The role of these factors in trophoblast survival warrants further investigation.

One of the principle aims of this study was to gain a better understanding of apoptosis in trophoblasts since increased apoptosis in the placental trophoblast layer may lead to complications, it is therefore important to carry out these studies in "diseased" or placentas from complicated pregnancies, such as IUGR and pre-eclampsia. Placentas from such pregnancies are available to our lab. Does TNF/IFNγ and ceramide induce higher levels apoptosis in these trophoblasts? Does SPP (and EGF) protect these trophoblasts from apoptosis to the same degree as it does in normal trophoblasts? Answering these questions may help lead to a better understanding as to why these complications exist, and consequently, potential therapies.

Chapter 8 : References

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Appendix I

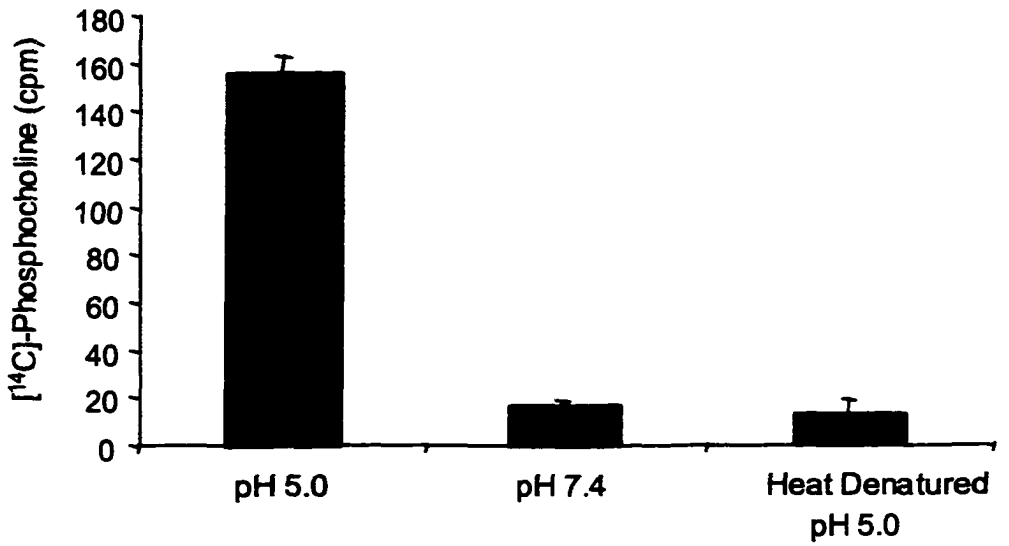

Figure A1 : Purified Acid SMase is Inactive at pH 7.4. SMase activity was measured as described in Methods and Materials. The activity of 100mU aSMase was measured at pH 5.0 and 7.4. Heat denatured (100°C for 5 minutes) enzyme served as a negative control. Data are expressed as mean cpm ± range of [^{14}C]-phosphocholine hydrolysed from [^{14}C]-sphingomyeline in separate experiements.