Institut für Molekularbiologie
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Regulation and function of acid sphingomyelinase (ASM) and ceramide in TRAIL-induced apoptosis

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ABBREVIATIONS

AIF	Apoptosis Inducing Factor
Akt/PKB	Protein Kinase B
AP-1	Activating Protein 1
Apaf-1	Apoptosis Protease Activating Factor 1
ASM	Acid Sphingomyelinse
ATP	Adenosine Trihosphate
BAD	Bcl-2 Antagonist of cell Death
Bak	Bcl-2 Antagonist/Killer
Bax	Bcl-2-Associated X protein
Bcl-2	B-cell CLL/lymphoma 2 protein
Bcl-X_L	Bcl-2 like protein
BH3	Bcl-2 Homology domain 3
Bid	BH3 Interacting Domain death agonist
Bim	Bcl-2 Interacting Mediator of cell death
CAPK	Ceramide-Activated Protein Kinase
CD95L	CD95 Ligand
cDNA	complementary Deoxyribonucleic Acid
CDP-choline	cytidine-5'-diphosphate choline
CEACAM	Carcinoembryonic Antigen-related Cell Adhesion Molecules
CFTR	Cystic Fibrosis Transmembrane conductance Regulator
CIA	Collagen Induced Arthritis
CRAC	Calcium Release Activated Channel
DAG	Diacylglycerol
DAP-3	Death-Associated Protein 3
DC	Dendritic cells
DcR1	Decoy Receptor 1
DcR2	Decoy Receptor 2
DD	Death Domain
DED	Death Effector Domain
DIABLO	Direct IAP Binding protein with Low pI
DISC	Death-Inducing Signaling Complex
DNA	Deoxyribonucleic Acid

DR4	Death Receptor 4
DR5	Death Receptor 5
EAE	Experimental Autoimmune Encephalomyelitis
ER	Endoplasmic reticulum
FADD	Fas-associated Death Domain
FcγRII	Fc gamma Receptor 2
FLICE	FADD-Like IL-1 Converting Enzyme
FLIP	FLICE Inhibitory Protein
GADD	Growth Arrest and DNA Damage
GC	Gas Chromatography
GSPT1/eRF3	G1 to S Phase Transition protein/polypeptide chain Release Factor 3
GTP	Guanosine 5'-Triphosphate
HPLC	High Performance Liquid Chromatography
HPTLC	High-Performance Thin Layer Chromatography
HtrA2/Omi	High Temperature Requirement protein
IAP	Inhibitor of Apoptosis Protein
IL-1	Interleukin 1
IL-2	Interleukin 2
InsP3	Inositol 1,4,5-trisphosphate Receptor
JNK	Jun N-terminal Kinase
KSR	Kinase Suppressor of Ras
LFA-1	Lymphocyte Function Associated antigen 1
LUV	Large Unilamellar Vesicles
MALDI	Matrix-Assisted Laser Desorption/Ionization
MAPK	Mitogen-Activated Protein Kinase
MEF	Mouse Embryonic Fibroblast
MOMP	Mitochondrial Outer Membrane Permeabilization
mRNA	messenger Ribonucleic Acid
MS	Mass Spectrometry
NF-κB	Nuclear Factor Kappa Beta

NGF	Nerve Growth Factor
NK cells	Natural Killer cells
Opa proteins	Opacity proteins
OPG	Osteoprotegerin
PAF	Platelet Activating Factor
PC	Phosphatidylcholine
PKC	Protein Kinase C
PLA_2	Phospholipase A2
PLAD	Pre-Ligand Assembly Domain
PPA_2	Protein Phosphatase A2
PPAR	Peroxisome Proliferator-Activated Receptor
PUMA	p53 Upregulated Modulator of Apoptosis
RANK	Receptor Activator of NF-κB
RANKL	Receptor Activator of NF-κB Ligand
RIP	Receptor Interacting Protein
RNAi	Ribonucleic Acid interference
ROS	Reactive Oxygen Species
RP-HPLC	Reversed Phase- High Performance Liquid Chromatography
SAPK	Stress-Activated Protein Kinase
SM	Sphingomyelin
Smac	Second Mitochondrial Activator of Caspase
TAK-1	Transforming growth factor beta-Activated Kinase 1
tBid	Truncated Bid
TNF	Tumour Necrosis Factor
TNFR1	Tumour Necrosis Factor Receptor 1
TRADD	TNF Receptor-Associated Death Domain
TRAIL	TNF-Related Apoptosis Inducing Ligand
UPR	Unfolded Protein Response
UV	Ultraviolet
XIAP	X-linked Inhibitor of Apoptosis Protein
zVAD-fmk	benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone

1. INTRODUCTION

1.1. Apoptosis

1.1.1. Overview of apoptosis

Programmed cell death (apoptosis) plays critical roles in development and maintenance of tissue homeostasis. Apoptosis is a highly conserved mechanism that has evolved to maintain cell numbers and cellular positioning within tissues comprised of different cell compartments. De-regulation of apoptosis leads to pathological disorders such as developmental defects, autoimmune diseases, neurodegeneration or cancer (Thompson, C.B., 1995).

Apoptosis is defined by its morphologic features. The characteristics of the apoptotic cell include chromatin condensation and nuclear fragmentation (pyknosis), plasma membrane blebbing, and cell shrinkage. Eventually, the cell breaks into small membrane-surrounded fragments (apoptotic bodies), which are cleared by phagocytosis, without inciting an inflammatory response (Savill and Fadok, 2000).

Intensive effort has been made to explore the molecular mechanisms of the apoptotic signalling pathways including the initiation, mediation, execution and regulation of apoptosis. In mammals, a wide array of external signals may trigger two major apoptotic pathways, namely the ***extrinsic (death receptor-initiated)*** or the ***intrinsic (mitochondria-initiated)*** pathway (Jin and El-Deiry, 2005). The two pathways converge on activation of caspase-3 and subsequently on other proteases and nucleases that drive the terminal events of apoptosis. Cellular apoptosis is tightly controlled by a complex regulatory network. Every step in the apoptotic cascade is monitored and controlled by certain pro-survival signals provided by families of anti-apoptotic molecules such as NF-κB, Akt/PKB, Bcl-2 and IAP (Vogelstein and Kinzler, 2004).

Because de-regulated apoptosis is the cause of many diseases including cancer (Thompson, C.B., 1995), knowledge of the molecular mechanisms of apoptosis can provide new approaches for therapeutic intervention.

1.1.2. Intrinsic (mitochondria-initiated) apoptotic pathways

Intrinsic apoptotic pathways are initiated inside cells. The main cellular compartment involved is the mitochondrion, although endoplasmic reticulum (ER) has also been shown to participate in intrinsic apoptosis. A pivotal event in this type of apoptosis consists of mitochondrial outer membrane permeabilization (MOMP), which is mainly mediated and controlled by members of the Bcl-2 family. Bcl-2 family proteins are critical death regulators that reside upstream of mitochondria and may be pro-apoptotic or anti-apoptotic (Tsujimoto Y., 2003). They can be divided into three main subclasses, defined in part by the homology shared within four conserved regions known as the Bcl-2 homology BH1–4 domains, corresponding to the α-helices that describe the structure and function. The first subclass contains anti-apoptotic members that conserve all four BH1-4 domains; members of this subclass include Bcl-2 (Chan and Yu, 2004), Bcl-X_L (Boise *et al.*, 1993), Mcl-1 (Zhou *et al.*, 1997), A1 (Chuang *et al.*, 1998), and Bcl-W (Gibson *et al.*, 1996). In the second subclass, the structure of the BH1-3 domains in Bcl-X_L creates a hydrophobic pocket that can accommodate the BH3 domain of a pro-apoptotic member. The third subclass contains multidomain pro-apoptotic members such as Bax (Oltvai *et al.*, 1993) and Bak (Chittenden *et al.*, 1995) that display sequence conservation in BH1-3 domains and the BH3-only proteins Bid (Li *et al.*, 1998; Luo *et al.*, 1998), Bad (Yang *et al.*, 1995), Bim (O'Connor *et al.*, 1998), Noxa (Oda *et al.*, 2000), and Puma (Yu *et al.*, 2001) that display sequence conservation only in the amphipathic α-helical BH3 region and require Bax and Bak to mediate cell death. Apoptotic changes in mitochondria seem to be predominantly mediated by Bax and Bak and cells deficient for both Bax and Bak demonstrated resistance to all tested intrinsic death pathway stimuli (Wei *et al.*, 2001). Bax migrates to mitochondria and integrates into the outer membrane following various death-inducing stimuli (Hsu *et al.*, 1997; Wolter *et al.*, 1997), while Bak in its inactive form is already constitutively present in mitochondrial membranes. BH3-only proteins, such as Bid or Bad, seem to regulate the function of Bax and Bak and a current model suggests that these proteins compete with binding partners of Bax or Bak in the cytosol or in mitochondria to free Bax or Bak and enable them to trigger apoptosis (Cory *et al.*, 2003). Free Bax and Bak may oligomerize and form pores and/or may interact with the PTP, p53 and/or other mitochondrial proteins to trigger apoptotic changes including cytochrome *c* release and mitochondrial depolarization (Cory *et al.*, 2003; Leu *et al.*, 2004).

MOMP results in cell death via two mechanisms: release of soluble mitochondrial intramembrane proteins and disruption of essential mitochondrial functions (Green and

Kroemer, 2004). Among the released molecules, the most important are cytochrome *c* and IAP antagonists such as Smac/DIABLO, HtrA2/Omi and GSPT1/eRF3. Cytochrome *c* binds to Apaf-1 and forms a complex called ***apoptosome***, which, in turn, recruits procaspase-9 leading to apoptosis via activation of executioner caspases-3, -6 and -7 (Zou *et al.*, 1997; Li *et al.*, 1997) (Figure 1.1). *In vivo* studies showed that the Apaf-1, caspase-9 or caspase-3 knock-out MEFs are resistant to various apoptotic stimuli (Hakem *et al.*, 1998; Kuida *et al.*, 1996; Cecconi *et al.*, 1998; Yoshid *et al.*, 1998). Smac/DIABLO, HtrA2/Omi and GSPT1/eRF3 bind IAPs in a manner similar to caspases, therefore functioning as competitive inhibitors (Du *et al.*, 2000; Suzuki *et al.*, 2001; Hegde *et al.*, 2003). The caspase co-activator Smac/DIABLO is released along with cytochrome *c* during apoptosis to neutralize the inhibitory activity of IAPs and promote cytochrome *c*-dependent caspase activation.

Processed caspase-9, -3 and -2 are also released together with cytochrome c from mitochondria *in vitro* by disruption of $\Delta\psi$ with an uncoupler or in cell death caused by staurosporine. These findings imply that the mitochondrial procaspase molecules participate in apoptosis induction (Katoh *et al.*, 2004).

In addition to proteins that can trigger or enhance caspase activation, mitochondria also releases pro-apoptotic proteins unrelated to caspase activation. AIF, once released to the cytosol, translocates to the nucleus in response to death stimuli including pneumococcus, p53, UV-B and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, H_2O_2, and *N*-methyl-aspartate. In the nucleus, AIF induces peripheral chromatin condensation and large-scale DNA fragmentation. AIF in association with a heat-labile cytosolic factor may also permeabilize mitochondrial membranes. None of these AIF effects can be prevented by addition of zVAD-fmk indicating that they are caspase-independent, but the cell death is characterized by various apoptotic features including cell shrinkage, cell surface exposure of phosphatidylserine, loss of mitochondrial membrane potential and large-scale DNA fragmentation (Susin *et al.*, 2000). Genetic evidence establishes an essential role for AIF during early mammalian development (Joza *et al.*, 2001). AIF was found to be critical for the first wave of programmed cell death required for embryonic morphogenesis and cavitation. In response to apoptotic stimuli, endonuclease G is also released from the mitochondria into the cytosol where it translocates to the nucleus and generates oligonucleosomal DNA fragmentation. It is essential for DNA fragmentation, particularly during caspase-independent apoptosis (Li *et al.*, 2001). Unlike caspase inhibitors, Bcl-2 effectively protects cells from both caspase-dependent and -independent apoptosis. This is more likely through its ability to prevent mitochondrial

membrane permeabilization and the resultant release of potent pro-apoptotic molecules like endonuclease G (Donovan and Cotter, 2004).

Besides the mitochondria, the endoplasmic reticulum (ER) is a second compartment participating in intrinsic apoptosis. In the ER, quality control mechanisms ensure that only properly folded proteins are passed along the secretory pathway. Stress to the ER including oxidative stress, chemical toxicity, treatment with Ca^{2+} ionophores or exposure to inhibitors of glycosylation can result in misfolded proteins and perturbed calcium homeostasis, which provokes the unfolded protein response (UPR) (Breckenridge *et al.*, 2003). Release of calcium from the ER into the cytosol is in many cases required for stress-induced apoptosis. The ER is the major intracellular store of Ca^{2+} ions. Efflux of Ca^{2+} ions from the ER is often associated with uptake into the mitochondria. In staurosporine and ceramide-induced apoptosis, calcium acts as a messenger that coordinates the amplification loop between the mitochondria and the ER. A small amount of cytochrome *c* released from the mitochondria diffuses to the adjacent endoplasmic reticulum (ER) and binds to InsP3 receptors, thereby enhancing calcium release from the endoplasmic reticulum. The released calcium causes a mass exodus of cytochrome *c* from all mitochondria. The positive feedback finally results in the dramatic activation of caspases. This process may be an important event in disorders such as myocardial infarction, Alzheimer's disease and stroke (Rao *et al.*, 2004). Either Bax or Bak is required for ER stress-induced apoptosis. Recent findings suggest that both of them localize and function at the ER. Bax and Bak have been implicated in maintaining homeostatic concentrations of Ca^{2+} in the ER. Release of Ca^{2+} from ER stores and its uptake into mitochondria is impaired in Bax and Bak double knockout mice, which results in reduced apoptosis in response to certain death stimuli (Nutt *et al.*, 2002). So far, only caspase-12 activation during ER-mediated apoptosis has been reported. Upon activation, caspase-12 translocates from the ER to the cytosol, where it directly cleaves procaspase-9 to activate caspase-3. Caspase-$12^{-/-}$ mice were also found to be resistant to Aß peptide-induced apoptosis in an Alzheimer's disease model. However, functional caspase-12 has only been cloned from the mouse and rat, and the existence of a human isoform of caspase-12 remains controversial (Szegezdi *et al.*, 2003).

1.1.3. Extrinsic (death receptor-initiated) apoptotic pathways

The extrinsic pathway is activated by ligand-bound death receptors, mainly including TNF-TNFR1, CD95L/CD95 and TRAIL/DR4 or DR5. The TNF receptor superfamily is characterized by the presence of cysteine-rich domains that mediate binding between ligands and these transmembrane receptors. Among them, the death receptors TNFR1, CD95, DR4 and DR5 are best characterized for induction of apoptosis (Ashkenazi and Dixit, 1998). Pre-assembly of death receptors through a distinct region of their extracellular domain called PLAD (pre-ligand assembly domain) seems to be critical for ligand binding (Chan *et al.*, 2000). Upon binding of the ligand to the death receptors, the death-inducing signalling complex (DISC) is formed by association of the receptor death domains (DD) to the DD of an adaptor protein, such as FADD, TRADD or RIP. The adaptor proteins, in turn, have a death effector domain (DED) which binds to the DED of procaspase-8. Following autoproteolytic cleavage of procaspase-8, caspase-8 is released from the DISC and activates mainly caspase-3, thus inducing apoptosis (Walczak and Krammer, 2000) (Figure 1.1).

The initial model of death-receptor-induced apoptosis suggested that the simple trimerization of the receptors upon binding to their respective ligands is sufficient to initiate the apoptotic signal. However, recent studies demonstrated that a more massive aggregation of the receptors, the so called ***receptor clustering,*** is required for successful induction of apoptosis (see Discussion).

1.1.4. Cross-talk between extrinsic and intrinsic pathways

Based on the requirement of the intrinsic pathway for apoptosis induced by death receptors, cells can be divided in ***type I*** and ***type II*** cells. In type I cells, the intrinsic pathway does not participate at all in induction of apoptosis via the death receptors. However, in type II cells, only a small amount of FADD and caspase-8 are recruited to DISC, thus insufficient activation of caspase-8 requires involvement of mitochondria to finally induce apoptosis (Barnhart *et al.*, 2003).

Death receptor-mediated apoptotic signalling can activate the mitochondrial pathway through the BH3-only protein, Bid. Caspase-8 activated in the DISC is sufficient to cleave cytosolic Bid. Cleaved Bid (tBid) then translocates to the mitochondria, which leads to mitochondrial dysfunction and apoptosome formation through activation of Bax/Bak (Figure 1.1) (Li *et al.*,

1998; Luo *et al.*, 1998). Although the apoptotic mitochondrial events occur in both types of cells, only the apoptosis in type II cells is abrogated if the mitochondrial pathway is blocked by overexpression of Bcl-2 (Danial and Korsmeyer, 2004). It was originally believed that the activation of caspase-9 and -3 in the apoptosome would function as the amplification loop which can compensate the weak DISC formation and caspase-8 activation (Stennicke and Salvesen, 2000). However, the evidence that hepatocytes derived from the Apaf-$1^{-/-}$ and caspase-$9^{-/-}$ mice exhibit the similar sensitivity to CD95-mediated apoptosis suggests that other factors are required for the amplification (Yoshida *et al.*, 1998; Zou *et al.*, 2003). This controversy is reconciled by the finding that the second group of molecules including Smac/DIABLO, are released from the mitochondria during death receptor-mediated apoptosis. This protein interacts with and sequesters XIAP to remove its inhibition from caspase-3 and caspase-9 (Deng *et al.*, 2002). Caspase-3 then can undergo a second proteolytic step to be fully activated. This portion of fully activated caspase-3 subsequently cleaves many proteins, such as XIAP, to facilitate the apoptotic feedback, functioning as amplification loop and finally resulting in irreversible apoptosis (Engels *et al.*, 2000).

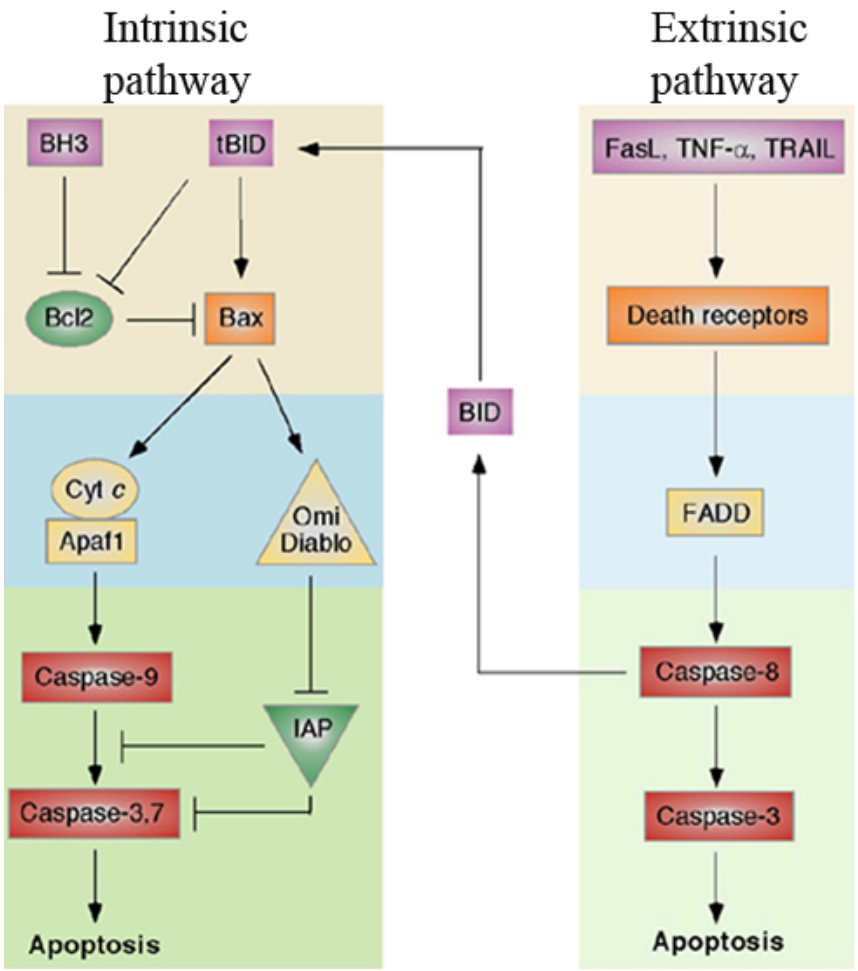


Figure 1.1. Schematic representation of extrinsic and intrinsic apoptosis pathways. The extrinsic pathway is initiated by activation of death receptors upon binding to their respective ligands. The intrinsic pathway employs release of various factors from the mitochondria. The two pathways can communicate via cleavage of Bid and its translocation to the mitochondria.

Not only does mitochondrial dysfunction influence caspase activation in death receptor-mediated apoptosis, but cross-talk exists between the upstream component of the extrinsic and the intrinsic pathways. The typical example is p53-mediated apoptosis. In response to DNA damage, p53 not only targets the intrinsic apoptotic molecules like Bax and Puma to activate the mitochondrial apoptotic pathway, but also up-regulates the extrinsic pathway genes like CD95L and KILLER/DR5 (Wu *et al.*, 1997; Kasibhatla *et al.*, 1998). It was shown that DNA damage-induced apoptosis can proceed through death receptor signalling (Hipfner and Cohen, 2004). These reports would argue for a contribution of death receptor signalling to the intrinsic apoptotic pathway.

1.2. Ceramide-enriched membrane platforms

1.2.1. Plasma membrane organization

The classical 'fluid mosaic model' of the plasma membrane suggested by Singer and Nicolson in 1972 has been modified by many studies in the last years (Singer and Nicolson, 1972).

Biological membranes of eukaryotic cells contain, in addition to glycerophosholipids, large amounts of sphingolipids and cholesterol, which predominantly localize to the outer leaflet of the plasma membrane. The most prevalent sphingolipid in the membrane is ***sphingomyelin***, which is composed of a hydrophobic ceramide moiety and a hydrophilic phosphorylcholine headgroup. Sphingolipids associate with each other via hydrophilic interactions or with cholesterol, via hydrogen bonds and van der Waal interactions (Simons and Ikonen, 1997; Harder and Simons, 1997; Brown and London, 1998). This leads to a lateral separation of sphingolipids and cholesterol into distinct microdomains, termed ***membrane rafts***. Cholesterol fills the voids between the bulky sphingolipids, thus stabilizing the rafts. The necessity of cholesterol for membrane rafts is demonstrated by disintegration of rafts

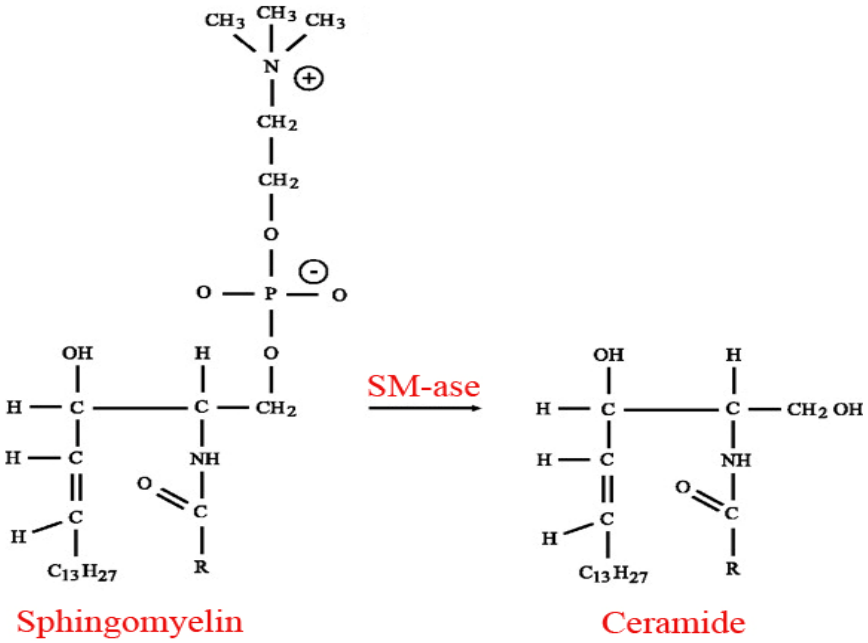
following pharmacological extraction of cholesterol from plasma membranes. The tight interaction between sphingolipids and cholesterol as well as their high local concentration result in a transition of these microdomains into a liquid-ordered or even gel-like phase, whereas the other domains of the plasma membrane exist in a more fluid, liquid-disordered phase (Simons and Ikonen, 1997; Brown and London, 1998; Kolesnick *et al.*, 2000).

1.2.2. Ceramide: biochemistry and synthesis

Ceramide is composed of D-*erythro*-sphingosine and a fatty acid containing 2-28 carbon atoms in the acyl chain. Most physiological ceramides have C_{16}-through C_{26} chains. Ceramide forms the backbone of complex sphingolipids such as cerebrosides, gangliosides and especially sphingomyelin.

Ceramide can be generated via two main pathways:
(a) *de novo* synthesis of ceramide from sphingosine or sphinganine by activation of ceramide synthase. This step occurs in the endoplasmic reticulum, after which ceramide is translocated to the Golgi apparatus, where it has a major function as a metabolic precursor of glycosphingolipids (Shimeno *et al.*, 1995; Bionda *et al.*, 2004).
(b) by hydrolysis of sphingomyelin following activation of sphingomyelinases, which can be acid, neutral or alkaline (Samet and Barenholz, 1999) (Figure 1.2).

In turn, sphingosine, sphingomyelin and glucosylceramide can be generated from ceramide by activation of enzymes such as sphingomyelin synthase, cerebrosidase or ceramide synthase. A schematic representation of reactions involving ceramide is depicted in Figure 1.3. Furthermore, the enzymes which mediate the key reactions of ceramide are listed in Table 1.



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Am J Physiol Regul Intergr Comp Physiol, 2006

Figure 1.2. Generation of ceramide from sphingomyelin. Upon activation of sphingomyelinases, sphingomyelin is hydrolysed with the subsequent release of ceramide and phosphorylcholine.

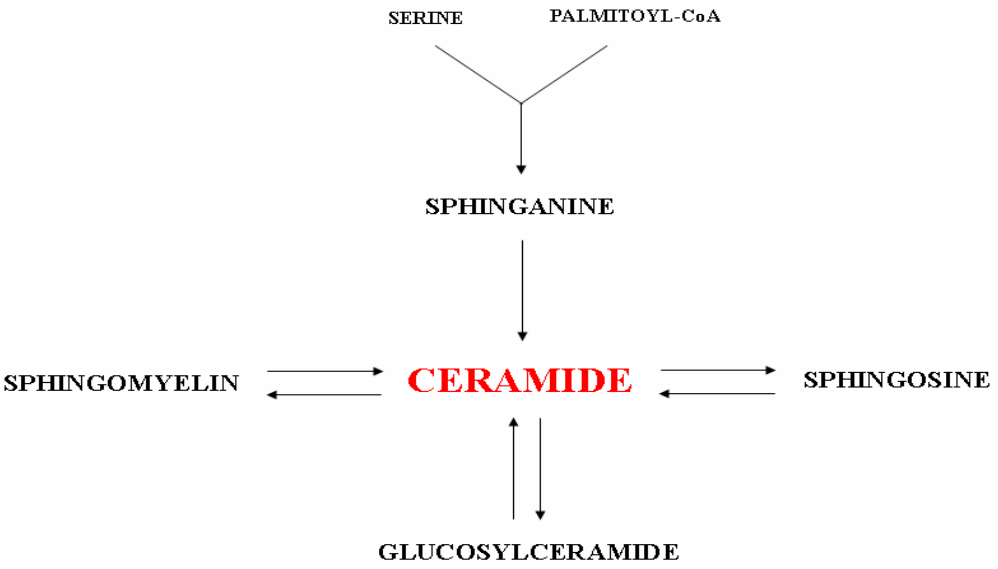

Figure 1.3. Schematic representation of biochemical reactions involving ceramide. Ceramide can be generated from sphinganine, sphingosine, sphingomyelin and glucosylceramide. The inverse reactions use ceramide to generate sphingosine, sphingomyelin and glucosylceramide.

STARTING POINT	PRODUCT	ENZYME
Sphinganine	Ceramide	Ceramide synthase
Sphingosine	Ceramide	Ceramide synthase
Sphingomyelin	Ceramide	Sphingomyelinase
Glucosylceramide	Ceramide	GlucosylCer synthase
Ceramide	Sphingomyelin	Sphingomyelin synthase
Ceramide	Sphingosine	Ceramidase
Ceramide	Glucosylceramide	Cerebrosidase

Table 1. Overview of ceramide key reactions and of the enzymes mediating these reactions

1.2.3. Ceramide: biophysical properties

The most commonly found ceramide (with acyl chains longer than 16 carbon atoms) are among the least polar, most hydrophobic lipids in membranes. Their solubility in water is negligible, thus free ceramides cannot exist in solution in biological fluids or in cytosol. In addition, long chain ceramides cannot give rise to micelles or other aggregates in aqueous suspension (Kolesnick *et al.*, 2000).

To explain the effects of ceramide observed *in vivo*, many studies investigated the behaviour of ceramides as they occur in artificial phospholipid bilayers. One main effect observed was that long-chain ceramides increase the order of the acyl chains in the bilayers, thus decreasing fluidity. Holopainen and co-workers (1997) demonstrated that a natural ceramide increases in a dose-dependant manner the acyl chain order in dimyristoyl-phosphatidylcholine bilayers in the fluid lamellar phase (Holopainen *et al.*, 1997). A similar effect was observed with a chemically defined C_{16}-Ceramide in bilayers composed of 1-palmitoyl-2-oleoylphosphatidylcholine (Holopainen *et al.*, 1998). Moreover, *in situ* generation of ceramide through the action of sphingomyelinase on bilayers containing phosphatidylcholine and sphingomyelin also led to a decreased fluidity (Holopainen *et al.*, 1998).

Another very important behaviour of ceramides was first observed by Huang and co-workers (1996). The authors showed that addition of ceramide induced lateral phase separation of fluid phospholipid bilayers into regions of gel and liquid crystalline (fluid) phases, with ceramide

partitioning largely into the gel phase (Huang *et al.*, 1996). Additional studies from Holopainen and colleagues (1997, 1998) detected ceramide-enriched microdomains in fluid phosphatidylcholine membranes (Holopainen *et al.*, 1997; Holopainen *et al.*, 1998), while Veiga *et al.* (1999) found lateral separation of ceramide-rich domains with as little as 5 mole% ceramide (Veiga *et al.*, 1999). Although the causes of domain formation by ceramides may be multiple, an important factor may be the high capacity of ceramides for hydrogen bonding. Both ceramide and sphingomyelin can act as acceptor and donor of hydrogen bonds through their hydroxyl and amide groups (Shah *et al.*, 1995a, b; Holopainen *et al.*, 1998) A recent study has provided evidence for hydrogen bonding between sphingomyelin and cholesterol as the basis for detergent insolubility of certain membrane fractions (Patra *et al.*, 1998).

1.2.4. Acid sphingomyelinase (ASM) and ceramide-enriched membrane platforms

Acid sphingomyelinase (ASM) is the first described and the best characterized sphingomyelinase (Samet and Barenholz, 1999). It catalyzes the degradation of sphingomyelin, a major lipid constituent of the extracellular side of eukaryotic plasma membranes, to ceramide and phosphorylcholine (Figure 1.2). Enzyme deficiency due to mutations in the ASM gene leads to Niemann–Pick disease, an autosomal recessive sphingolipidosis. The infantile type A of Niemann–Pick disease manifests itself in rapid neurodegeneration and patients die within three years, whereas Niemann–Pick disease type B patients suffer from a non-neurological visceral progression of this disorder (Schuchman and Miranda, 1997).

The enzyme was purified from urine (Quintern *et al.*, 1987) and the full-length cDNA encoding ASM was isolated (Quintern *et al.*, 1989; Schuchman *et al.*, 1991). The enzyme was shown to be a monomeric 72 kDa glycoprotein containing a protein core of 61 kDa. The full-length ASM-cDNA contains an open reading frame of 1890 bp encoding 629 amino acids. Biosynthesis studies in human fibroblasts revealed stepwise proteolytic processing of a 75-kDa ASM precursor to form the mature protein. Mature ASM possesses six potential N-glycosylation sites as was recently shown by N-terminal sequencing (Lansmann *et al.*, 1996) (Figure 1.4). Site-directed mutagenesis of the potential glycosylation sites and subsequent expression of mutated cDNA constructs indicated that at least five of them are used *in vivo* (Ferlinz *et al.*, 1997). ASM contans 17 cysteines and MALDI analysis revealed that 16 of the

cysteines are paired, forming eight disulfide bonds (Lansmann *et al.*, 2003) (Figure 4). Based on a process of elimination, the analysis shows that the C-terminal cysteine (Cys^{629}) is the unbridged free cysteine (Figure 1.4). Cys^{629} has been recently shown to be involved in the activation mechanism of the ASM via an oxidation reaction (Qiu *et al.*, 2003) (see Discussion).

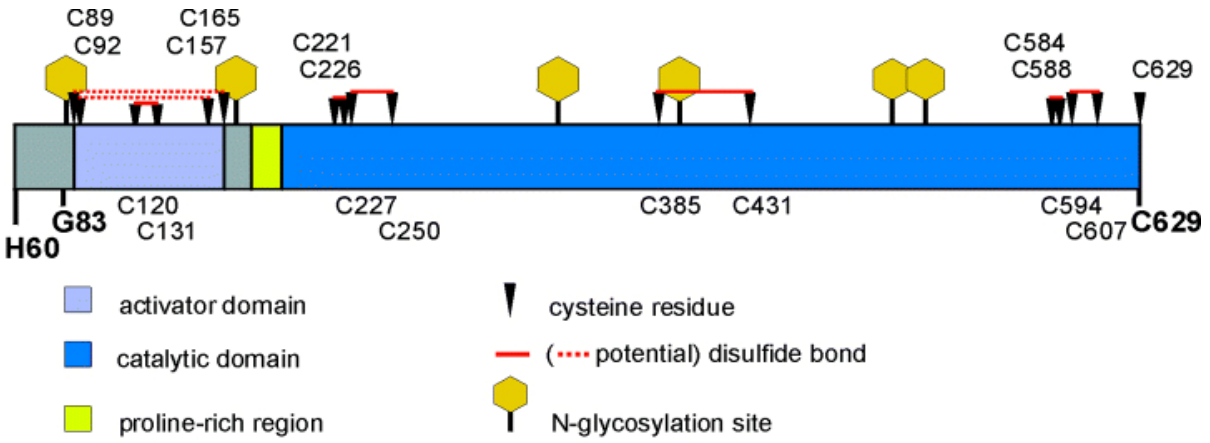

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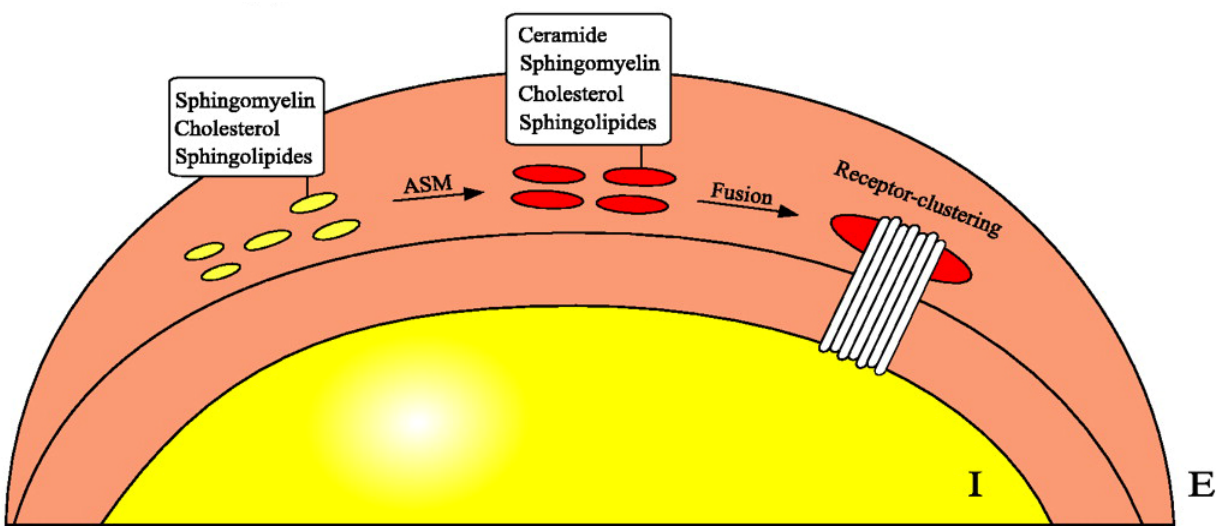
Figure 1.4. Schematic representation of ASM domain structure and disulfide bond pattern. The N-glycosylation sites are indicated. The disulfide bond pattern shows the presence of three bonds in the activator domain and five others in the catalytic domain. The terminal cysteine (Cys^{629}) is free.

In particular, generation of ceramide via activation of the ASM has been involved in stress responses and apoptosis. ASM induces hydrolysis of the sphingomyelin from membrane rafts and releases ceramide. Ceramide molecules dramatically change the biophysical properties of rafts because they self-associate (Nurminen *et al.*, 2002) and form small ceramide-enriched membrane domains (see chapter 1.2.3.), which spontaneously fuse and lead to formation of one or more large ***ceramide-enriched membrane platforms.***

Stimuli which were shown to induce the activation of the ASM and/or a release of ceramide and/or formation of ceramide-enriched membrane platforms include CD95 (Cifone *et al.*, 1994; Cremesti *et al.*, 2001; Grassme *et al.*, 2001a; Kirschnek *et al.*, 2000; Paris *et al.*, 2001b), CD40 (Grassme *et al.*, 2002), CD20 (Bezombes *et al.*, 2004), TNF (Garcia-Ruiz *et al.*, 2003; Schutze *et al.*, 1994; Zhang *et al.*, 2002), FcγRII (Abdel-Shakor *et al.*, 2004), CD5 (Simarro *et al.*, 1999), CD28 (Boucher *et al.*, 1995), IL-1 receptor (Mathias *et al.*, 1993), PAF

receptor (Goggel *et al.*, 2004), *P. aeruginosa* (Grassme *et al.*, 2003a), *S. aureus* (Esen *et al.*, 2001), *N. gonorrhoeae* (Grassme *et al.*, 1997), Sindbis virus (Jan *et al.*, 2000), Rhinovirus (Grassme *et al.*, 2005), γ-irradiation (Paris *et al.*, 2001a; Santana *et al.*, 1996), UV-light (Rotolo *et al.*, 2005; Charruyer *et al.*, 2005; Zhang, Y. *et al.*, 2001), or chemotherapeutic drugs (Morita *et al.*, 2000; Lacour *et al.*, 2004; Delmas *et al.*, 2004; Yabu *et al.*, 2005).

In general, ceramide-enriched membrane platforms seem to serve for re-organization and clustering of receptor molecules, such as CD95 (Grassme *et al.*, 2001 a, b), CD40 (Grassme *et al.*, 2002b), FcγRII (Abdel-Shakor *et al.*, 2004) and CD20 (Bezombes *et al.*, 2004). Receptor clustering leads to a high receptor density that seems to be required for effective transmission of the signal into cells (Figure 1.5). This is supported by the finding that ceramide-enriched membrane platforms amplify the signalling via CD95 approximately 100-fold (Grassme *et al.*, 2003b).



modified after Gulbins and Li,
Am J Physiol Regul Intergr Comp Physiol, 2006

Figure 1.5. Schematic representation of receptor clustering in ceramide-enriched membrane platforms. Upon stimulation of cells with the respective stimuli, ASM is activated and induces generation of ceramide from sphingomyelin. Ceramide accumulates in platforms on the cell surface and traps the receptors leading to a massive aggregation of these receptors within the platforms.

Ceramide may also 'flip' to the inner side of the cell membrane, although biophysical studies suggest that a spontaneous flip of ceramide through the membrane would occur slowly (Contreras *et al.*, 2005). However, intracellular ceramide might interact with phospholipase A2 (PLA_2) (Huwiler *et al.*, 2001), kinase suppressor of Ras (KSR) (Zhang *et al.*, 1997),

protein phosphatase A2 (PPA_2) (Dobrowsky *et al.*, 1993a, b; Dobrowsky and Hannun, 1993), protein kinase C (PKC) isoforms (Muller *et al.*, 1995), and/or c-Raf-1 (Yao *et al.*, 1995). Ceramide has been described to activate these proteins, resulting in regulation of cellular transcription, proliferation, and survival. Furthermore, the TNF receptor was shown to be rapidly internalized on activation, and most of the ceramide was released by the acid sphingomyelinase within the endosomes (Schneider-Brachert *et al.*, 2004). Endosomal ceramide binds to and activates cathepsin D (Heinrich *et al.*, 1999; Schneider-Brachert *et al.*, 2004), which is released from endosomes into the cytoplasm to trigger apoptosis.

Another target of ceramide and ceramide-enriched membrane platforms, respectively, has been recently identified as ion channels. In particular, it was demonstrated that ceramide inhibits the potassium channel Kv1.3 and calcium release activated calcium channels (CRAC) in lymphocytes (Gulbins *et al.*, 1997; Church *et al.*, 2005; Lepple-Wienhues *et al.*, 1999; Szabo *et al.*, 1996). Both channels are central for activation, differentiation, proliferation, and regulation of apoptosis; however, at present, the exact mechanism how ceramide blocks these channels is not known.

Finally, recent studies indicated an important function of ceramide in infectious biology. Bacteria, such as *N. gonorrhoeae,* and *P.aeruginosa* (Grassme *et al.*, 2003a; Grassme *et al.*, 1997) or viruses, such as Rhinovirus and Sindbis virus (Grassme *et al.*, 2005; Jan *et al.*, 2000), require formation of ceramide-enriched membrane platforms for infection of host cells. For *N. gonorrhoeae*, ceramide platforms seem to cluster receptors of the CEACAM-family that function as receptors for Opa proteins of the bacteria, suggesting that ceramide-enriched membrane platforms are required for the reorganization of *N. gonorrhoeae* receptors and intracellular signalling molecules that mediate internalization of the pathogens (Grassme *et al.*, 1997; Hauck *et al.*, 2000). Studies on *P. aeruginosa* showed that inhibition of the formation of ceramide-enriched membrane platforms prevented the induction of apoptosis of infected cells and internalization of the bacteria. Fluorescence microscopy studies revealed that ceramide-enriched membrane platforms serve to cluster at least two receptors that have been implied in infection with *P. aeruginosa,* namely CFTR and the CD95 receptor. The aggregation of CD95 in ceramide-enriched membrane platforms might be involved in the induction of apoptosis by *P. aeruginosa* (Grassme *et al.*, 2000). The function of CFTR clustering in ceramide-enriched membrane domains is less clear, but it might be involved in internalization of the bacteria and/or upregulation of CD95 on the cell surface of infected cells (Cannon *et al.*, 2003; Pier *et al.*, 1996). Rhinovirus infections resulted in rapid activation of

the ASM and formation of ceramide platforms which seem to be involved in the uptake of the viruses (Grassme *et al.*, 2005), while Sindbis virus- induced ASM and ceramide is required for induction of apoptosis in infected neurons (Jan *et al.*, 2000).

1.3. TNF-related apoptosis-inducing ligand (TRAIL)

1.3.1. Overview of TRAIL

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), also designated as Apo2L, is a type II transmembrane protein of about 33-35 kDa (Liabakk *et al.*, 2002), which was originally identified on the basis of sequence homology to CD95 ligand (CD95L) and TNF (Wiley *et al.*, 1995; Pitti *et al.*, 1996). Like other TNF family members, TRAIL forms a homotrimer, which cross-links three receptor molecules on the surface of target cells (Hymowitz *et al.*, 2000; Pan *et al.*, 1997).

While TRAIL mRNA is constitutively expressed in a wide variety of normal tissues, the expression of functional TRAIL protein appears to be rather restricted to immune cells, including T cells, NK cells, monocytes, dendritic cells (DCs) and neutrophils (Cretney *et al.*, 2002; Kayagaki *et al.*, 1999). Although the physiological role of TRAIL is not yet fully understood, genetically engineered TRAIL-deficient mice do not show gross abnormality, except for impaired tumour immunosurveillance (Zerafa *et al.*, 2005) and higher sensitivity to experimental autoimmune diseases, such as collagen-induced arthritis (CIA) (Lamhamedi-Cherradi, 2003a), streptozotocin-induced diabetes (Lamhamedi-Cherradi, 2003a, b), and experimental autoimmune encephalomyelitis (EAE) (Cretney *et al.*, 2005), suggesting that the main physiological role of TRAIL is in the immune system.

1.3.2. TRAIL receptors

Five distinct TRAIL receptor have been identified thus far: DR4 (TRAIL-R1) (Pan *et al.*, 1997), KILLER/DR5 (TRAIL-R2, TRICK2) (Sheridan *et al.*, 1997; Walczak *et al.*, 1997), DcR1 (TRAIL-R3, TRID) (Sheridan *et al.*, 1997; Degli-Esposti *et al.*, 1997a), DcR2 (TRAIL-R4, TRUNDD) (Degli-Esposti *et al.*, 1997b) and osteoprotegerin (OPG) (Emery *et al.*, 1998)

(Figure 1.6). Although all receptors have high sequence homology in their extracellular domains, the intracellular domains differ. Based on that, TRAIL receptors are divided into two categories: ***death receptors*** and ***decoy receptors***.

Both DR4 and DR5 contain a C-terminal death domain that signals downstream caspase activation to mediate TRAIL-induced apoptosis. DR4 is a 468 amino acid type I trans-membrane protein that contains a 23 amino acid signal sequence, a 226 amino acid extra-cellular region, a 19 amino acid trans-membrane segment and a 220 amino acid cytoplasmic domain. There are two cysteine-rich domains (Chaudhary *et al.*, 1997; MacFarlane *et al.*, 1997). DR5 is also a type I trans-membrane protein of 411 amino acids, with a very large (51 amino acid) signal sequence, a 132 amino acid extra-cellular region, a 22 amino acid trans-membrane domain and a 206 amino acid cytoplasmic domain. Like DR4, DR5 also has two cysteine-rich domains (MacFarlane *et al.*, 1997). In contrast to humans, mice do not express a receptor homologue of DR4 and the mouse DR5-homologue seems to be the only death-inducing receptor of TRAIL (Wu *et al.*, 1999b).

TRAIL-R3 is a 299 amino acid protein with a 23 amino acid signal sequence, a 217 amino acid extra-cellular region and a 19 amino acid trans-membrane domain. Lacking a cytoplasmic segment, TRAIL-R3 is bound by a GPI linker and seems to act mainly as a 'decoy receptor' and compete with death receptors DR4 and DR5 for TRAIL binding. TRAIL-R4 is similar in function to TRAIL-R3. Although TRAIL-R4 has a trans-membrane domain, it contains a truncated death domain which renders it unable to induce apoptosis.

The fifth TRAIL receptor, osteoprotegerin (OPG) is a soluble member of the TNF receptor family for which the best described action is the inhibition of RANKL-stimulated osteoclast formation. OPG can bind to RANKL and prevent interaction with its cognate receptor RANK (receptor activator of NF-κB). However, OPG can also interact with TRAIL. A role for OPG as a decoy receptor for TRAIL under physiological conditions remains unclear, because the affinity of OPG for TRAIL is rather weak compared to the other TRAIL receptors (Truneh *et al.*, 2000).

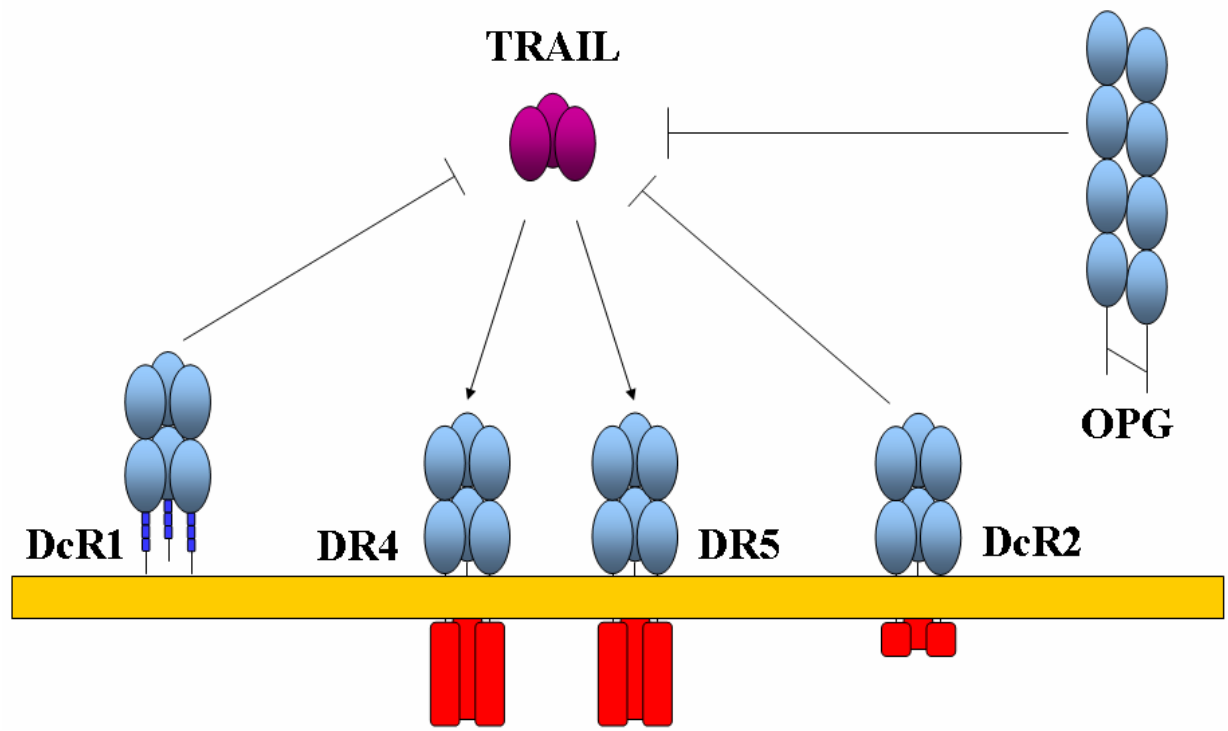


Figure 1.6. Schematic representation of TRAIL and its receptors TRAIL homotrimers cross-link one of the five receptors on the target cells: the two death receptors DR4 and DR5, two decoy receptors DcR1 and DcR2 or the soluble protein OPG.

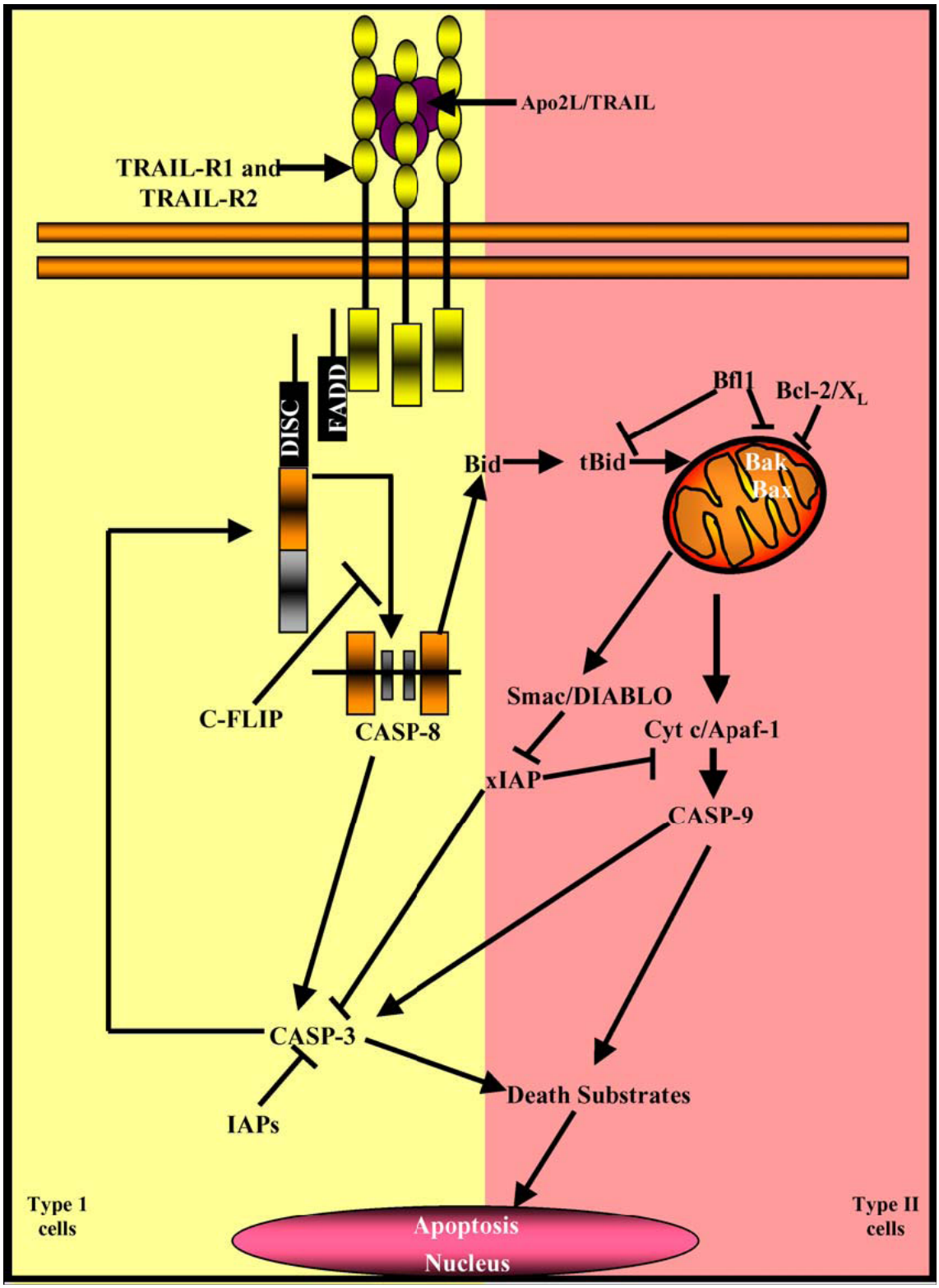
1.3.3. TRAIL-induced signalling pathways

The current model of TRAIL-induced apoptotic pathway suggests that binding of the ligand to DR4 or DR5 receptors results in the recruitment of the adaptor protein FADD, which is also crucially involved in triggering apoptotic signals initiated by stimulation of death receptors such as CD95 or TNF-R1. However, unlike stimulation via TNF-R1, which leads to the recruitment of other adaptor proteins, such as TRADD and RIP, FADD seems to be the main adaptor protein involved in signalling via TRAIL death receptors. FADD recruits proteins downstream in the pathway that actually effect apoptosis, such as procaspase-8 and forms what is known as the Apo2L/TRAIL-DISC (Figure 1.7). In this signalling complex, procaspase-8 undergoes auto-proteolytic activation. The formation of the DISC complex is pivotal in Apo2L/TRAIL-induced apoptosis. Mice or cell lines deficient in these molecules are completely protected from the apoptotic action of Apo2L/TRAIL (Kuang *et al.*, 2000). A recently identified adaptor molecule termed death-associated protein-3 (DAP-3) was found to directly bind the death domain of the Apo2L/TRAIL death receptors (Miyazaki and Reed, 2001). DAP-3 also associates with pro-caspase-8 and FADD, and functions to link FADD to TRAIL-R1 and TRAIL-R2 (Berger and Kretzler, 2002). However, the exact role of DAP-3

remains controversial, since it was demonstrated that DAP-3 is a ribosomal protein localised to the mitochondrial matrix and that it remains there during apoptosis (Green, D.R., 2000). In type I cells DR4 and DR5 stimulate caspase-8 processing to an extent, which is sufficient to activate the effector caspase, caspase-3. Alternatively, in cells where death receptor-induced, caspase-8-mediated activation of caspase-3 is limited (type II cells), small quantities of active caspase-8 are sufficient to trigger the mitochondria-mediated apoptotic signal.

Apoptosis initiated through the activation of the death receptors depends on cell-extrinsic signals. That is, engagement of death ligands that result in the formation and activation of DISC, which leads to the proteolytic activation of caspase-3 by caspase 8. On the other hand, cell intrinsic apoptosis is usually dependent on cues such as DNA damage or UV irradiation to induce apoptosis through the mitochondrial pathway. When this pathway is stimulated, the Bcl-2 family member Bax translocates to the mitochondria, the mitochondrial trans-membrane potential is reduced, and cytochrome *c* is released into the cytosol, activating caspase-9 and subsequently the effector caspases (Green D.R., 2000) (Figure 1.7).

Pro-apoptotic members of the Bcl-2 family such as Bax (or its homologue Bak) are counteracted by the anti-apoptotic members of the Bcl-2 family, such as Bcl- 2 or Bcl-X_L (Bouillet and Strasser, 2002). Proteins such as Bim, Bid, Puma and Noxa, contain only one of the four Bcl-2 homology domains (BH3) which are common to the rest of the Bcl-2 family and function only to augment the activity of the pro-apoptotic Bcl-2 family members (Bouillet and Strasser, 2002). When cleaved by caspase-8, Bid translocates to the mitochondria and activates Bax and Bak, providing a mechanism for crosstalk between the death receptors and the intrinsic pathway (Li *et al.*, 1998; Luo *et al.*, 1998) (Figure 1.7).



Bouralexis et al., Apoptosis, 2005

Figure 1.7. Schematic representation of TRAIL-induced apoptosis pathways. Upon binding of TRAIL to either DR4 or DR5, two types of apoptosis can occur: in type I cells the extrinsic apoptosis pathway is sufficient to kill the cells, while in type II cells, also the intrinsic pathway is employed for successful induction of apoptosis.

In addition to the proteolytic caspase cascade, caspase activity is further regulated by inhibitors of apoptosis (IAPs). The best characterized IAP is XIAP, which inhibits caspase-9 and caspase-3 by binding to their intermediate and fully cleaved forms (Deveraux and Reed, 1999). In cells that are undergoing apoptosis, caspases are liberated from IAP inhibition, by a process that is made possible by Smac/DIABLO (Du *et al.*, 2000; Verhagen *et al.*, 2000). Smac/DIABLO is released from the mitochondria of apoptotic cells and accelerates cell death activation by displacing XIAP from the caspases (Du *et al.*, 2000; Verhagen *et al.*, 2000) (Figure 1.7).

1.3.4. TRAIL and cancer therapy

Death ligands have been widely investigated in part due to their potential in cancer therapy. Unlike many conventional chemotherapeutic drugs, they stimulate cell death in tumour cells independently of the p53 tumour suppressor gene, which is often found inactivated in human cancers (Sigal and Rotter, 2000). However, clinical application of the prototypic death ligands CD95L and TNF has been hampered by toxicity to normal tissues. Intravenous TNF administration causes hypotension and a systemic inflammatory syndrome that resembles septic shock due to a strong activation of pro-inflammatory NF-κB. Injection of CD95L induces hepatocyte apoptosis and lethal liver failure in mice (Nagata S., 1997). Therefore, when TRAIL was shown to have relatively little toxicity towards many types of normal cells, it became a very promising cancer therapeutic agent (Ashkenazi *et al.*, 1999).

TRAIL appears to induce apoptosis predominantly in transformed cells (Wiley *et al.,* 1995; Pan *et al.,* 1997; Sheridan *et al.,* 1997), with the exception of some normal cells, such as stimulated splenocytes, memory- or dendritic cells (Hayakawa *et al.,* 2004; Janssen *et al.,* 2005; Ursini-Siegel *et al.,* 2002; Zhang, L. *et al.,* 2002). Initially, the high expression of decoy receptors in normal cells, but not tumour cells was proposed to be the major mechanism resulting in differential sensitivity to TRAIL (Ashkenazi and Dixit 1998). However, in most cancer cell lines, DR4 and/or DR5 are expressed, whereas decoy receptors expression is less frequent and does not correlate with resistance to TRAIL (Evdokiou *et al.*, 2002; Petak *et al.*, 2000; Nimmanapalli *et al.*, 2001). Thus, there are probably additional determinants of sensitivity besides decoy receptor expression.

Either DR4 or DR5 must be present on the cell surface to transduce the TRAIL signal. Deficiency or mutation of death receptors confers tumour cell resistance to TRAIL-induced apoptosis (Kim *et al.*, 2000; Lee *et al.*, 1999; McDonald *et al.*, 2001; Fisher *et al.*, 2001). Transport of TRAIL receptors to the cell membrane seems also very important to determine TRAIL sensitivity. Tumour cells may become resistant to TRAIL through regulation of the death receptor cell surface transport (Jin, Z. *et al.*, 2004).

Caspase expression and function appears to be frequently impaired by epigenetic mechanisms in cancer cells (Baylin and Bestor, 2000). Caspase-8 expression was found to be inactivated by hypermethylation of regulatory sequences of the caspase-8 gene in a number of different tumour cells derived from neuroblastoma, malignant brain tumours, Ewing tumour and small lung cell carcinoma and also in primary tumour samples. Importantly, restoration of caspase-8 expression by gene transfer or by de-methylation treatment sensitized resistant tumour cells for death receptor- or drug-induced apoptosis (Philchenkov *et al.*, 2004).

The caspase activation inhibitor FLIP acts as important negative regulator of TRAIL-induced apoptosis. Overexpression of FLIP protects cells from TRAIL-induced apoptosis (Kim *et al.*, 2003), and suppression of FLIP by RNAi sensitizes cells to TRAIL-induced apoptosis (Ricci *et al.*, 2004). A genetic screen in human placental cDNA retroviral library isolated c-FLIP(S) as a suppressor of TRAIL signalling in TRAIL-resistant clones (Burns and El-Deiry, 2001). Correlations between FLIP levels and TRAIL resistance have also been observed (Griffith *et al.*, 1998). Degradation of FLIP following exposure to a PPAR selective ligand apparently sensitizes tumour cells to Apo2L/TRAIL-induced apoptosis (Kim *et al.*, 2002). However, other studies failed to find such a correlation (Zhang *et al.*, 1999). Moreover, recent work suggests that FLIP can actually promote caspase-8 activation in response to death ligand (Micheau *et al.*, 2002; Zhang *et al.*, 1999). Thus more work is needed to further analyze the role of FLIP in the TRAIL resistance.

IAPs can inhibit TRAIL-induced apoptosis by modulating caspase activity (Suliman *et al.*, 2001), and transfection of Smac/DIABLO, an inhibitor of IAPs, overcomes the resistance to TRAIL-induced apoptosis (Deng *et al.*, 2002). A comparison of Apo2L/TRAIL-sensitive and -resistant melanoma cell lines showed a strong correlation between binding of XIAP to cleaved caspase-3 and TRAIL sensitivity. More Smac/DIABLO is released to the cytosol in TRAIL-sensitive cell lines (Zhang, X.D. et al 2001). Smac/DIABLO agonists sensitize human acute leukaemia Jurkat T cells for apoptosis induction by Apo2L/TRAIL and induces

regression of malignant glioma *in vivo* (Fulda *et al.*, 2002). A small molecule mimic of Smac strongly sensitizes both TNF and TRAIL-induced caspase activation and apoptosis (Li *et al.*, 2004).

Although TRAIL can activate the mitochondrial pathway, the importance of the mitochondrial pathway in TRAIL-induced apoptosis is still controversial. Overexpression of Bcl-2 or Bcl-X_L does not block TRAIL-induced apoptosis in lymphoid cells (Keogh *et al.*, 2000). In contrast, overexpression of Bcl-2 or Bcl-X_L inhibits TRAIL-induced apoptosis in human lung or prostate cancer cells (Munshi *et al.*, 2001). Thus, involvement of the mitochondria in TRAIL-induced apoptosis may depend on cell type, like type I and type II cells in CD95-induced apoptosis (Ozoren *et al.*, 2002). In cells requiring mitochondrial involvement, blockade of the mitochondrial pathway may change TRAIL sensitivity. For example, Bax-null HCT-116 cells are completely resistant to TRAIL-induced apoptosis.

Other cell signalling pathways may also be involved in the regulation of TRAIL sensitivity, including the Akt/PBK, Myc, NF-κB or p53 pathways. Constitutive activation of Akt/PBK in several cancer cells has been shown to confer resistance to TRAIL (Shankar and Srivastava, 2004; Martelli *et al.*, 2003). In contrast, Myc seems to be a positive regulator of TRAIL sensitivity (Tewari and Vidal, 2003). Myc has been shown to up-regulate expression of TRAIL receptors in some tumour cells (Wang *et al.*, 2004) c-Myc can enhance the apoptotic activity of death receptor signalling proteins by engaging the mitochondrial apoptotic pathway (Klefstrom *et al.*, 2002). p53 is another important regulator of TRAIL sensitivity. KILLER/DR5 was originally discovered as DNA damage-inducible, p53-regulated gene (Wu *et al.*, 1997). p53 may also regulate the expression of DR4 in a limited number of tumours (Liu *et al.*, 2004). TRAIL can activate NF-κB weakly both *in vivo* and *in vitro*. Although most studies have shown that NF-κB acts as an inhibitor of TRAIL-induced apoptosis (Eid *et al.*, 2002; Luo *et al.*, 2004), its role as an activator has also been reported (Shetty *et al.*, 2002).

Selective cytotoxicity to tumour cells by TRAIL makes it a promising death ligand for clinical application. However, many cancer cells are resistant to TRAIL-induced apoptosis. Application of TRAIL alone may not be effective in these cancers. Intensive effort has been made to sensitize TRAIL-resistant tumour cells. Various means have been found to act synergistically with TRAIL: chemotherapeutic drugs, such as doxorubicin, irradiation, histone deacetylase inhibitors, stress inducers, proteasome inhibitors, interferon, small molecules like peptide mimicking Smac and Bax, or molecules like retinoid or cyclooxygenase-2 inhibitors

(Shankar and Srivastava, 2004). The mechanisms underlying these synergies vary between therapies but all appear to be related to the TRAIL signalling pathway or regulatory mechanisms of TRAIL signalling. Many of these treatments have been applied in clinical cancer therapy, but toxicity is still a problem. Sub-toxic doses of some agents listed above combined with TRAIL may provide a way to efficiently kill tumour cells with less toxicity to normal cells.

1.4. Doxorubicin

1.4.1. Overview of doxorubicin

Doxorubicin (adriamycin or hydroxyldaunorubicin) belongs to the group of anthracyclines, which are a class of chemotherapeutic agents based upon daunosamine and tetra-hydro-naphthacene-dione (Figure 1.8). Anthracyclines, which include also daunorubicin, epirubicin and idarubicin, rank among the most effective anti-cancer drugs ever developed, and they are used to treat a wide range of cancers, including leukemias, lymphomas, breast, uterine, ovarian, and lung cancers (Weiss R.B., 1992). The major limitation in clinical use of anthracyclines consists in development of resistance in tumour cells as well as toxicity in healthy tissues (especially the heart). To avoid the latter, the maximum recommended cumulative doses of daunorubicin and doxorubicin were tentatively set at 500 and 450-600 mg/m^2, respectively.

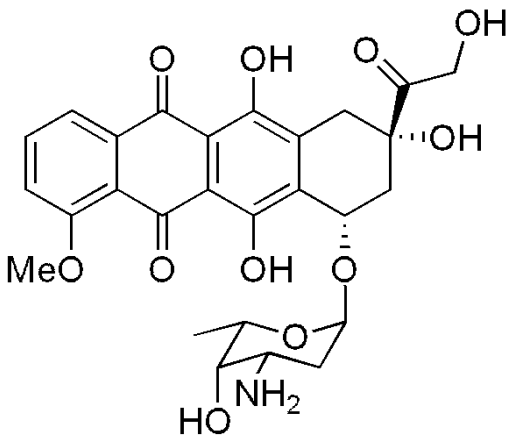


Figure 1.8. Structure of doxorubicin. The chemical structure of doxorubicin consists of a tetracyclic ring, with the sugar daunosamine attached by a glycosidic linkage. Structurally, doxorubicin is related to daunomycin (daunorubicin) and differs only in hydroxyl group substitution (instead of hydrogen) at the alkyl side chain at position '9' of the 'A' ring.

1.4.2. Signalling pathways activated by doxorubicin

Due to its wide use in cancer treatment, doxorubicin has been the object of intense basic research to identify the signalling pathways induced by this drug. The main mechanism of doxorubicin-induced apoptosis is DNA-damage. Binding of anthracyclines to DNA increases Tm of DNA melting point by 30°C as saturation of the potential binding sites is reached (Chaires *et al.*, 1982). Increase in Tm values of DNA is due to stabilization of DNA double helix resulting from the intercalation of the drug and can be used to explain DNA and RNA synthesis inhibition, as well as the effects of these drugs.

Although most of the interaction of the anthracycline antibiotics involves intercalation between the bases in DNA, these drugs can also alter the structure and function of this molecule (and hence chromatin) in many different ways. These structural changes may ultimately contribute to the apoptotic process induced by anthracyclines in cancer cells (Hale *et al.*, 1996). One such change involves the covalent modification of DNA (Purewal and Liehr, 1993). The formation of anthracycline-DNA adducts has been observed to occur both *in vitro* and *in vivo* (Purewal and Liehr, 1993) through a mechanism that involves the iron-complex of the drug (Parker *et al.*, 1999) The formaldehyde production resulting from the oxidative stress induced by the drug-metal complex produces a covalent attachment to the G-bases of DNA that functions as a virtual interstrand cross-linker (Parker *et al.*, 1999; Taatjes *et al.*, 1999). Such DNA cross-linking could participate in the chromatin aggregation process occurring during apoptosis.

The chelation of ions by anthracyclines can also result in the generation of reactive oxygen species (ROS), which is responsible for the free radical cytotoxicity of these drugs (Muller *et al.*, 1998). As it occurs with other quinines, these antibiotics can be enzymatically reduced to semiquinone radicals, which, in the presence of oxygen and iron, can result in the production of highly DNA-damaging oxygen radicals (Muller *et al.*, 1998).

The intercalation process itself results in a distortion of the DNA conformation that causes the inhibition of Topoisomerase II (Tewey *et al.*, 1984). The effects of anthracyclines on this enzyme have been extensively studied (Kellner *et al.*, 2002). In this instance, the mechanism of action involves the binding of the drug to the Topoisomerase II-DNA complexes forming a ternary complex (Binaschi *et al.*, 2001). Such binding interferes with the re-ligation step of the Topoisomerase resulting in double-stranded DNA breaks (Kellner *et al.*, 2002).

Recent studies suggest, however, that doxorubicin and anthracyclines in general can induce apoptosis regardless of DNA damage. Thus far, doxorubicin has been shown to induce apoptosis by direct release of cytochrome *c* from mitochondria (Clementi *et al.*, 2003), JNK activation (Yu *et al.*, 1996; Koyama *et al.*, 2006), p38 MAPK activation, which inhibited in turn the pro-survival factor Akt and Bad phosphorylation (Grethe *et al.*, 2006), PKC/JNK/Bak pathway stimulation (Panaretakis *et al.*, 2005) or induction/up-regulation of CD95L/CD95 system (Friesen *et al.*, 1999). Recently, it was shown that doxorubicin induces ceramide production; however, the pathway of ceramide generation remains controversial (Morita *et al.,* 2000; Andrieu-Abadie *et al.,* 1999; Gouaze *et al.,* 2001; Mercier *et al.,* 2003).

1.5. Aims of the study

Our group has demonstrated that activation of the ASM, the subsequent ceramide release and accumulation in platforms on the cell surface are crucial events for apoptosis induced by TNF-family member CD95. Generation of ceramide platforms served to trap and cluster the CD95 receptors, leading to the successful induction of apoptosis.

The present study will investigate whether stimulation of cells with TRAIL results in the activation of ASM, formation of ceramide-enriched membrane platforms and clustering of DR5 receptors at the plasma membrane. The importance of ASM and ceramide platforms for DR5 clustering and TRAIL-induced apoptosis will be tested by employing ASM-deficient cells.

Furthermore, the mechanism of ASM activation will be addressed. It will be investigated whether stimulation with TRAIL induces free radical oxygen production which might mediate activation of the ASM.

The last part of the study will focus on the mechanism of amplification of TRAIL-induced apoptosis by the chemostatic drug doxorubicin. It will be tested whether doxorubicin-induced ceramide release is able to sensitize cells to low doses of TRAIL. Moreover, the pathway of ceramide generation will be addressed.

2. MATERIALS

2.1. Chemicals

Acetic acid ($C_2H_4O_2$)	Merck, Darmstadt
Adenosine Tri-Phosphate (ATP)	Sigma-Aldrich Chemie GmbH, Steinheim
C_{16}-Ceramide	Biomol, PA, USA
Calcium chloride ($CaCl_2$)	Sigma-Aldrich Chemie GmbH, Steinheim
Cardiolipin	Sigma-Aldrich Chemie GmbH, Steinheim
Chloroform ($CHCl_3$)	Ridel-de Haen, Seelze
Cytohrome *c*	Sigma-Aldrich Chemie GmbH, Steinheim
DABCO (1,4-Diazabicyclo(2,2,2)octane)	Sigma-Aldrich Chemie GmbH, Steinheim
Deoxycholic acid ($C_{24}H_{40}O_4$)	Sigma-Aldrich Chemie GmbH, Steinheim
Diethylenetriaminepentaacetic Acid (DETAPAC)	Sigma-Aldrich Chemie GmbH, Steinheim
Dimethylsulfoxid (DMSO)	Sigma-Aldrich Chemie GmbH, Steinheim
Dithiothreitol (DTT)	Carl-Roth GmbH & Co, Karlsruhe
Doxorubicin hydrochloride (Adriblastin)	Pharmacia GmbH, Erlangen
Ethanol (C_2H_5OH)	Sigma-Aldrich Chemie GmbH, Steinheim
Ethylenediamine Tetraacetic Acid (EDTA)	Serva Electrophoresis GmbH, Heidelberg
FITC-Annexin Fluos	Roche Molecular Biochemicals, Penzberg

Glucose	Sigma-Aldrich Chemie GmbH, Steinheim
Glycerol	Fluka Chemie GmbH, Buchs
Hepes	Carl-Roth GmbH & Co, Karlsruhe
Histopaque (Ficoll)	Sigma-Aldrich Chemie GmbH, Steinheim
Hydrochloric acid (HCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Imidazole ($C_3H_4N_2$)	Sigma-Aldrich Chemie GmbH, Steinheim
Interleukin-2 (IL-2)	Roche Molecular Biochemicals, Penzberg
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich Chemie GmbH, Steinheim
Magnesium sulphate ($MgSO_4$)	Sigma-Aldrich Chemie GmbH, Steinheim
Methanol (CH_3OH)	Fluka Chemie GmbH, Buchs
Mineral oil	Sigma-Aldrich Chemie GmbH, Steinheim
Mowiol	Kuraray Specialities Europe GmbH, Frankfurt
N-acetylcysteine (NAC)	Sigma-Aldrich Chemie GmbH, Steinheim
N-octylglucopyranoside	Sigma-Aldrich Chemie GmbH, Steinheim
Paraformaldehide (PFA)	Sigma-Aldrich Chemie GmbH, Steinheim
Phytohemagglutinin (PHA)	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium chloride (KCl)	Sigma-Aldrich Chemie GmbH, Steinheim
Potassium dihydrogenphosphate (KH_2PO_4)	Sigma-Aldrich Chemie GmbH, Steinheim
Propidium Iodide (PI)	Sigma-Aldrich Chemie GmbH, Steinheim

Scintillation Cocktail	Beckman Coulter, Fullerton, CA, USA
Sodium acetate (CH_3COONa)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium chloride (NaCl)	Carl-Roth GmbH & Co, Karlsruhe
Sodium dodecyl sulphate (SDS)	Serva Electrophoresis GmbH, Heidelberg
Sodium fluoride (NaF)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium hydroxide (NaOH)	Sigma-Aldrich Chemie GmbH, Steinheim
Sodium phosphate (Na_2HPO_4)	Merck, Darmstadt
Sodium pyrophosphate ($Na_4P_2O_7$)	Sigma-Aldrich Chemie GmbH, Steinheim
Tiron	Fluka Chemie GmbH, Buchs
Tris	Carl-Roth GmbH & Co, Karlsruhe
Triton X-100	Sigma-Aldrich Chemie GmbH, Steinheim
Trypan Blue	Sigma-Aldrich Chemie GmbH, Steinheim

2.2. Tissue culture materials

RPMI-1640	Gibco/Invitrogen, Karlsruhe
MEM (EAGLE)	Gibco/Invitrogen, Karlsruhe
L-Glutamine	Gibco/Invitrogen, Karlsruhe
Sodium pyruvate	Gibco/Invitrogen, Karlsruhe
Peniccilin/Streptomycin	Gibco/Invitrogen, Karlsruhe
MEM non-essential aminoacids	Gibco/Invitrogen, Karlsruhe

Fetal Calf Serum (FCS)	Gibco/Invitrogen, Karlsruhe
Trypsin	Gibco/Invitrogen, Karlsruhe
Cell dissociation buffer enzyme-free	Gibco/Invitrogen, Karlsruhe
Tissue culture test plates	TPP, Trasadingen, Switzerland
Tissue culture flasks 75cm^2	TPP, Trasadingen, Switzerland

2.3. Antibodies and ligands

recombinant TRAIL/TNFSF10	R&D Systems
anti-DR5/TRAIL-R2 rabbit IgG	R&D Systems
anti-ceramide (clone 15B4) mouse IgM	Sigma
anti-CD3ε hamster IgG	BD-Pharmingen, San Diego, CA, USA
anti-6 x Histidine mouse IgG	Qiagen
FITC-goat anti-mouse IgM	Jackson Immunoresearch, West Grove, PA, USA
Cy3-goat anti-armenian hamster IgG	Jackson Immunoresearch, West Grove, PA, USA
Cy3-donkey anti-rabbit IgG	Jackson Immunoresearch, West Grove, PA, USA
Cy5-donkey anti-rabbit IgG	Jackson Immunoresearch, West Grove, PA, USA

2.4. PCR primers

ASM-PA 1-2 5’ –CGA GAC TGT TGC CAG ACA TC- 3’	Hermann GbR, Freiburg
ASM-PA 2-2 5’ –GGC TAC CCG TGA TAT TGC TG- 3’	Hermann GbR, Freiburg
ASM-PS-2 5’ –AGC CGT GTC CTC TTC CTT AC- 3’	Hermann GbR, Freiburg
Myco P1 5’ –GTG CCA GCA GCC GCG GTA ATA C- 3’	Hölle & Hüttner AG, Germany
Myco P4 5’ –TAC CTT GTT ACG ACT TCA CCC CA- 3’	Hölle & Hüttner AG, Germany

2.5. Cell lines

BJAB	established human Burkitt B-lymphoma cell line; gift from Dr. Verena Jendrossek (University of Tübingen)
A549	established human lung carcinoma cell line; American Type Culture Collection (ATCC), Manassas, USA
L929	established murine fibrosarccoma cell line; American Type Culture Collection (ATCC), Manassas, USA

All cell lines were tested monthly by PCR to exclude mycoplasma contamination.

2.6. Animals

ASM-deficient mice (on a C57Bl/6 background) were kindly provided by Dr. R. Kolesnick (Memorial Sloan-Kettering Cancer Center, NY, USA). Syngenic wild-type (WT) mice from the same heterozygous breeding were used as control.

ASM WT and ASM-deficient mice were propagated in the Animal Facility of the Uniklinikum Essen. Homozygocity of ASM WT and ASM-deficient mice was verified by PCR analysis.

Mice were housed in pathogen-free conditions under diurnal lighting alternated with a dark phase between 18.00 – 6.00, allowed daily food "Zuchthaltungsfutter Maus-Ratte 10 H 10" (Eggersmann) and water *ad libitum*.

All experiments have been approved by the state animal welfare board.

2.7. Radioactive substances

[^{32}P] gamma-ATP	Hartmann Analytic, Braunschweig
[^{14}C] Sphingomyelin	Perkin Elmer, Boston, MA, USA

2.8. Other materials

ADEFO X-Ray Developer Concentrate	Adefo Chemie GmbH, Dietzenbach
ADEFO X-Ray Fixer Concentrate	Adefo Chemie GmbH, Dietzenbach
anti-MHC Class II Microbeads	Miltenyi Biotec, Bergisch Gladbach
Cover slips 12 mm diameter	Carl-Roth GmbH & Co, Karlsruhe
Cryo 1C Freezing container	Nalgene, USA
Cryovials	Carl-Roth GmbH & Co, Karlsruhe

Cuvettes 10 x 4 x 45 mm	Sarstedt, Nümbrecht
FACS Polystyrene Round-Bottom Tubes	Beckton Dickinson Labware, Le Point de Claix, France
MACS LD Separation Columns	Miltenyi Biotec, Bergisch Gladbach
Microscopy glass slides 76 x 26 mm	Engelbrecht, Edermünde
Minisart syringe filters	Vivascience AG, Hannover
Neubauer chamber 0.1 mm	Marienfeld, Germany
Parafilm	Peckiney, Chicago, IL, USA
Polyethylene vials 20 ml	Packard, USA
PP-test tubes	Greine Bio-one GmbH, Frickenhausen
Silica G60 TLC plates	Merck, Darmstadt
***sn*-1,2-Diacylglycerol (DAG) Biotrak Assay Reagents System**	Amersham Biosciences, Freiburg
Steritop Vacuum-driven disposable top filters	Millipore, Billerica, MA, USA
Whatman filter paper	Whatman, Maidstone, UK
X-Ray films	Amersham Biosciences, Buckinghamshire, UK

2.9. Special laboratory equipment

Portable Datalogging Spectrophotometer	Bachofer, Reutlingen, Germany
BD FACSCalibur flow cytometer	BD Biosciences, San Jose, CA, USA
Leica DMIRE 2 microscope with TSP2 module	Leica Microsystems, Germany
TriCarb Liquid scintillation Analyzer	Perkin Elmer, USA
SpeedVac (Vacuum Concentrator)	Bachofer, Reutlingen, Germany
Sonorex bath sonicator	Bandelin electronic, Berlin, Germany

2.10. Buffers and Solutions

Annexin-binding buffer	10 mM Hepes pH 7.4 140 mM NaCl 5 mM $CaCl_2$
ASM lysis buffer	0.1% Triton X-100 50 mM sodium acetate pH 5.0
C_{16}-Ceramide stock solution	1 mM C_{16}-ceramide 10% N-octylglucopyranoside
Complete MEM (EAGLE)	500 ml MEM (EAGLE) 10% FCS 10 mM Hepes pH 7.4 2 mM L-Glutamine 1 mM sodium pyruvate 100 µM non-essential amino acids 100 Units/ml penicillin 100 µg/ml streptomycin
Complete RPMI-1640	500 ml RPMI-1640 10% FCS 10 mM Hepes pH 7.4 2 mM L-Glutamine 1 mM sodium pyruvate 100 µM non-essential amino acids 100 Units/ml penicillin 100 µg/ml streptomycin
DAG-assay Buffered Saline Solution	135 mM NaCl 1.5 mM $CaCl_2$ 0.5 mM $MgCl_2$ 5.6 mM Glucose 10 mM Hepes pH 7.2
DAG-assay detergent solution	7.5% N-octylglucopyranoside 5 mM cardiolipin 1mM DETAPAC
DAG-kinase diluent	1 mM DETAPAC (pH 6.6) 0.01 M imidazole/HCl
DAG-kinase reaction buffer	100 mM imidazole/HCl pH 6.6 100 mM NaCl 25 mM $MgCl_2$ 2 mM EDTA 2.8 mM DTT 5 µM ATP 10 µCi [^{32}P] gamma-ATP

Doxorubicin stock solution	3.4 mM Doxorubicin Ethanol
FACS buffer	Hepes/Saline pH 7.4 1% FCS
Hepes/Saline	132 mM NaCl 20 mM Hepes pH 7.4 5 mM KCl 1 mM $CaCl_2$ 0.7 mM $MgCl_2$ 0.8 mM $MgSO_4$
MACS buffer	PBS pH 7.2 0.5% BSA 2 mM EDTA
Moviol	6 g Glycerol 2.4 g Mowiol 6 ml H2O 12 ml 0.2 M Tris pH 8.5 0.1% DABCO
PFA stock solution	8% PFA PBS
Phosphate Buffered Saline (PBS)	4 mM $MgSO_4$ 7mM $CaCl_2$ 1.4 mM KH_2PO_4 137 mM NaCl 2.7 mM KCl 6.5 mM Na_2HPO_4
SDS-lysis buffer	0.1% SDS 0.5% deoxycholic acid 1% Triton X-100 10 mM EDTA 25 mM Hepes pH 7.3 10mM sodium pyrophosphate 10 mM NaF 125 mM NaCl
Trypsin	0.25% Trypsin 5 mM Glucose 1.3 mM EDTA

3. METHODS

3.1. Tissue culture techniques

3.1.1. Culture of established cell lines

3.1.1.1. Culture and passage of cells

BJAB lymphoma cells were maintained in complete RPMI-1640 medium; L929 and A549 cells were maintained in complete MEM (EAGLE) medium (see Materials) at 37°C in a 5% CO_2 atmosphere. For the adherent cells (L929 and A549) passage of cells was achieved by incubation with trypsin solution to dislodge the cells from the flask wall. Prior to that, the cultures were examined using a light microscope, to assess the degree of confluence. Media, PBS and trypsin were pre-warmed at 25°C. The cell monolayer was washed with PBS and trypsin was added. Detachment of cells was assessed by light microscopy. Cells were then re-suspended in medium, transferred to fresh flasks and kept incubated at 37°C.

3.1.1.2. Freezing and thawing of cells

The basic principle of successful cryo-preservation is a slow freeze and a quick thaw of cells. For the freezing step, DMSO was used to protect cells from ice crystal formation, which causes cell rupture. The freezing medium (see Materials) was prepared in advance and kept at 4°C. Cells were collected, counted with a Neubauer chamber and re-suspended at a concentration of 1 x 10^6 cells/ml in freezing medium. The cell suspension was transferred in cryo-protective vials which were placed at -80°C in a Cryo 1C Freezing Container overnight. For long-term storage, cells were placed in a liquid nitrogen storage vessel.

To thaw the cells, the vials from liquid nitrogen storage were transferred to a water bath at 37°C. After thawing, cells were washed with medium to dilute the DMSO, re-suspended in fresh medium, transferred to culture flasks and incubated at 37°C.

3.1.2. Culture of T splenocytes

3.1.2.1.Obtaining single-cell suspensions

For the studies employing murine splenic T cells, it was necessary to obtain initially a single cell suspension. To this purpose, spleens were collected under sterile conditions and mechanically disrupted between two sterile glass slides. Further passaging through 20 G needles ensured disruption of loose clumps. Finally, the cell suspension was left at rest for 5 minutes to allow settling of cell-clumps on the bottom of the plate. The supernatant was collected and used for further processing.

3.1.2.2. Separation of mononuclear cells

Separation of mononuclear cells (T and B splenocytes) was achieved by Histopaque (Ficoll) gradient centrifugation. The procedure was carried out in PP-tubes, by slowly pipeting the spleen-cells suspension over the pre-warmed (25°C) Histopaque (2:1 v/v). The samples were centrifuged at 800 x g for 20 min at room temperature, resulting in the accumulation of lymphocytes at the interface Histopaque/medium. After removing the upper layer, the lymphocyte layer was transferred in fresh PP-tubes and washed two times with RPMI-1640 at 450 x g, 5 min each.

3.1.2.3. Isolation of T cells by MACS

To obtain a pure T cell population, magnetic cell sorting (MACS) was used. To this purpose, splenic lymphocytes were counted with a Neubauer chamber, pelleted by centrifugation at 300 x g for 10 min, after which the pellet was re-suspended in 90 µl MACS buffer plus 10µl anti-MHC Class II microbeads per 10^7 cells and incubated for 15 min at 4°C. The anti-MHC Class II microbeads bind to MHC Class II-positive cells, therefore, they are appropriate for isolation of untouched T cells from a certain population (negative selection). After incubation with the microbeads, cells were washed with MACS buffer at 300 x g for 10 min and re-suspended in 500 µl MACS buffer (for up to 10^8 cells). Magnetic separation was performed by running the samples through LD depletion columns. The effluent representing the unlabeled T cells was collected.

3.1.2.4.Cultivation and stimulation of isolated T cells

After MACS separation, splenic T cells were washed two times with RPMI-1640 at 450 x g, 5 min each and cultured in complete RPMI-1640. To induce expression of DR5 receptors, T splenocytes were stimulated for 48-72 hrs with 10 µg/ml phytohemagglutinine (PHA) and 5 Units/ml murine interleukin-2 (IL-2).

3.1.3. Viability control by Trypan-blue exclusion

The Trypan Blue Dye exclusion is a screening test for cell mortality under *in vitro* conditions. Cells were taken in suspension, gently mixed with 0.4% Trypan Blue solution and counted after 3 min staining using a Neubauer chamber. The numbers of viable cells (seen as bright cells) and non-viable cells (stained blue) were evaluated using a light microscope with 20-fold magnification.

3.2. Cytometry techniques

3.2.1. Flow cytometry

Fluorescence Activated Cell Sorting (FACS) allows the analysis and sorting of cell populations based on their morphology and fluorescence after staining with different fluorochrome-conjugated antibodies.

3.2.1.1. Staining for surface molecules

To assess DR5 expression or release of ceramide on the plasma membrane, cells were treated as indicated, washed once in HEPES/Saline (see Materials) and fixed in 2% paraformaldehyde (PFA) in PBS (pH 7.4) for 15 min at room temperature. Cells were washed once in FACS buffer (see Materials) and incubated with the indicated primary antibodies for 45 min at room temperature. Anti-DR5 antibodies were diluted 1:100, anti-ceramide-antibodies were diluted 1:50 in FACS buffer. The samples were washed again and incubated with the respective fluorescent secondary antibodies for 30 min at room temperature, in the dark. Cy3-coupled antibodies were diluted 1:1000, while FITC-coupled antibodies were diluted 1:500 in FACS buffer. Cells were finally washed two times, re-suspended in 300 µl FACS buffer and transferred into FACS polystirene tubes.

When adherent cells (L929 and A549) were analysed for DR5 expression, particular care was taken when detaching cells from the flask/plate bottom. Since trypsin treatment can destroy surface molecules such as receptors, a non-enzymatic method (Cell Dissociation Buffer) was used to this purpose.

3.2.1.2. Detection of apoptotic cells

Detection of apoptotic cells was performed mainly by FITC-Annexin V staining. During early apoptotic cell death, phosphatidylserine is translocated to the outer leaflet of the plasma membrane and becomes available for Annexin, which is a calcium-dependant phospholipids-binding protein with high affinity for phosphatidylserine. After stimulation, 2 x 10^5 cells/sample were washed once in Annexin-binding buffer (see Materials) and incubated with FITC-Annexin V diluted 1:50 in Annexin-binding buffer for 15 min, at room temperature, in the dark. Cells were washed two more times, re-suspended in 300 µl Annexin-binding buffer and analysed by FACS.

To differentiate between apoptosis and necrosis, a FITC-Annexin V/Propidium iodide (PI) staining was performed. Apoptotic cells, with the exception of late apoptotic, become Annexin V-positive without becoming permeable to PI. PI is a nucleic acid dye, which emits red fluorescence when intercalated in nucleic acids. The dye enters the non-viable cells but does not penetrate the intact membrane of viable cells. This technique allows the differentiation of living cells (FITC-Annexin- PI-), early apoptotic (FITC-Annexin+ PI-), late apoptotic (FITC-Annexin+ PI+) or necrotic cells (FITC-Annexin- PI+). To this purpose, stimulated cells were incubated with FITC-Annexin and PI, both diluted 20 µl/ml in Annexin-binding buffer. After 15 min incubation at room temperature, samples were washed two times and analysed by FACS.

3.2.2. Immunofluorescence

To analyse DR5 and ceramide by fluorescence/confocal microscopy, cells were fixed and stained as described above (3.2.1.1.). In contrast to T splenocytes or BJAB cells, adherent cells (L929 and A549) were grown directly on cover-slips and the subsequent staining steps

were performed by placing the cover-slips face-down over drops of diluted antibodies on a piece of Parafilm. After staining, samples were embedded in Moviol (see Materials), and sealed with nail-polish 1 h later.

3.3. C_{16}-Ceramide reconstitution

ASM-deficient splenocytes and tumor cells were incubated with 1 or 2 μM C_{16}-ceramide (see Materials), respectively, for 10 min prior to TRAIL treatment. C_{16}-ceramide stock solution (1 mM) was prepared in 10% n-octylglucopyranoside and sonicated 10 min on ice prior to use. TRAIL was added at the indicated concentration and cell viability was determined by Trypan Blue exclusion and FACS analysis.

3.4. Acid sphingomyelinase (ASM) activity assay

The activity of the ASM was measured as the consumption of radioactive sphingomyelin to ceramide and phophorylcholine. To this purpose, activated splenic T-cells or tumor cells were incubated with 5 ng/ml and 100 ng/ml TRAIL, respectively, for the indicated times. Cells were centrifuged at 450 x g for 5 min at 4°C, lysed in 300 μl/sample ice cold ASM-lysis buffer (see Materials), followed by each three freeze/thawing and sonication cycles. The adherent cells (A549 and L929) were directly scraped into this buffer. Cell lysates were incubated with 0.05 μCi per sample [^{14}C]-labeled sphingomyelin (2 GBq/mmol) for 30 min at 37°C on a thermomixer. Since [^{14}C]-sphingomyelin is insoluble in water, it was first dried by SpeedVac centrifugation and solubilized into micelles in ASM-lysis buffer, using a bath sonicator for 10 min. Lipids were extracted by addition of 1 ml/sample chloroform:methanol

(2:1, v/v), followed by vigorous vortexing for 30 sec and centrifugation at 800 x g for 10 min. This results in separation of phosphorylcholine in the upper (aqueous) phase and ceramide in the lower (organic) phase. An aliquot (350 µl) of the upper phase was carefully collected and transferred in 20 ml polyethylene vials filled with 3.5 ml Scintillation Cocktail, followed by quantification by liquid scintillation counting.

3.5. Ceramide quantification by DAG-kinase assay

3.5.1. Lipid extraction and enzymatic reaction

The principle of DAG-kinase assay consists in conversion of ceramide to a quantifiable product (ceramide-1-phosphate) by the transfer of [^{32}P]phosphate from [^{32}P]gamma ATP to ceramide. To this purpose, stimulated cells were first extracted in $CHCl_3$:CH_3OH:1N HCl (100:100:1). The resulting biphasic mixture is composed of a lower lipid-containing organic phase, and an upper aqueous phase. The upper phase and any resulting precipitate at the interface were removed and the resulting lipids in the organic phase were concentrated by evaporation of the chloroform in a SpeedVac. Lipids were solubilized in 20 µl of a detergent solution (see Materials) and formation of micelles was achieved by sonication of the samples for 10 min in a bath sonicator. To start the reaction 60 µl of DAG-kinase reaction buffer was added to 10 µl of diluted enzyme (dilution 1:1 v/v in DAG-kinase diluent) and the samples were incubated for 30 min at room temperature on a thermomixer. The kinase reaction was stopped by extraction of the samples with 1 ml/sample $CHCl_3$:CH_3OH:1N HCl (100:100:1), 170 µl/sample Buffered Saline Solution (see Materials) and 30 µl of a 100 mM EDTA-solution, followed by vortexing. The resulting upper phase was removed, and the lower

organic phase was again concentrated by SpeedVac centrifugation. The dried lipids were dissolved in 20 µl/sample $CHCl_3$:CH_3OH (1:1 v/v).

3.5.2. Separation of lipids by Thin Layer Chromatography (TLC)

Lipids were separated on a Silica G60 TLC plate. A solvent system of $CHCl_3$:CH_3OH:CH_3COOH (65:15:5 v/v), was added to the TLC chamber, and was allowed to saturate the atmosphere for 1 h by using a sheet of Whatman filter paper. The silica plates were loaded with the solubilized lipids, placed into the TLC chamber and the solvent front was allowed to migrate to the top of the plate. The plate was then removed, air dried for 45 min and exposed to X-ray films for 24-72 hrs. Ceramide-spots were identified by co-migration with a C_{16}-ceramide 1-phosphate standard, scrapped from the plate into 20 ml polyethylene vials and quantified by liquid scintillation counting using the Cerenkov protocol.

3.6. Quantification of free radical oxygen (ROS) release

The reduction of ferricytochrome *c* to ferrocytochrome *c* has been used to measure rates of formation of oxygen radicals (ROS) by numerous enzymes. To this purpose, cells were stimulated with TRAIL or left untreated in the presence or absence of anti-oxidants. Cells were lysed with a SDS-containing buffer (see Materials) in which 1 mg/ml cytochrome *c* was dissolved. Samples were immediately transferred to 10 x 4 x 45 mm cuvettes, covered with mineral oil and absorbance at 550 nm was determined.

3.7. DNA techniques

3.7.1. DNA isolation

3.7.1.1. DNA isolation from mouse tails

For genotyping of ASM-WT and ASM-deficient mice, app. 1-2 mm of mouse tail was cut and placed into 80 µl Tissue Lysis Buffer (TLB) (see Materials). The samples were incubated at 56°C overnight and the volumes were raised to 800 µl with autoclaved ddH_2O.

3.7.1.2. DNA isolation from cell lines

To test cultured cell lines for the presence of Mycoplasma, 5 x 10^5 cells/sample were pelleted and re-suspended into 50 µl TLB. The samples were incubated at 56°C for 3 h followed by boiling at 95°C for 10 min. The volumes were then raised to 100 µl with autoclaved ddH_20.

3.7.2. Polymerase Chain Reaction (PCR)

3.7.2.1. ASM PCR

For the identification of ASM-WT, ASM heterozygotes or ASM-deficient mice by PCR, 1 µl of overnight tail digest (see 3.7.1.1.) was added to 1.2 µl 10 x PCR Buffer, 2.5. mM MgCl2, 1 µl dNTP mix 5 units/ml Taq Polymerase and 0.1 µl each of primers ASM-PA1-2, ASM-PA2-2 an ASM-PS-2 in 0.2 ml PCR tubes. The temperature of the lid of the PCR machine was raised to 104°C and the temperature of the PCR block was raised to 96°C for 17 min, after which the following cycle was carried out 35 times:

Denaturation: 95°C for 1 min

Annealing: 58°C for 1 min

Elongation: 72°C for 1 min 45 sec

After the last cycle, the PCR block remained at 72°C for 5 min, after which the samples were placed at 4°C.

3.7.2.2. Mycoplasma PCR

For the identification of mycoplasma by PCR, 1 µl of cell digest (see 3.7.1.2.) was added to 2.5 µl 10 x PCR Buffer, 4.1 mM $MgCl_2$, 0.5 µl dNTP mix 1.25 units/ml Taq Polymerase and 0.25 each of primers P1 and P4 in 0.2 ml PCR tubes. The temperature of the lid of the PCR machine was raised to 104°C and the temperature of the PCR block was raised to 96°C for 17 min, after which the following cycle was carried out 25 times:

Denaturation: 95°C for 1 min

Annealing: 60°C for 1 min

Elongation: 72°C for 1 min 30 sec

After the last cycle, the PCR block remained at 72°C for 7 min, after which the samples were placed at 4°C.

3.7.3. Agarose gel electrophoresis

To analyse the products of the PCR, a 1% agarose gel was poured in TBE buffer (see Materials) that contained 0.01 µg/ml ethidium bromide. The samples (15 µl) were loaded on the gel along with 0.1 µg/µl of a 100-bp-standard. The gel was run under 5 V/cm current. Visualization of the DNA fragments was performed under UV-light.

4. RESULTS

4.1. Role of Acid Sphingomyelinase (ASM) in TRAIL-induced apoptosis

4.1.1. ASM is necessary for TRAIL – induced apoptosis

To define the role of ASM for TRAIL-induced apoptosis ASM wild-type and ASM-deficient T splenocytes were employed in parallel. The splenocytes were activated by phytohemagglutinin (PHA) and IL-2 for 72 h to induce expression of DR5. Subsequent incubation with TRAIL resulted in a dose-dependant induction of apoptosis in ASM wild-type splenocytes, while deficiency of the ASM prevented TRAIL-induced apoptosis (Figure 4.1)

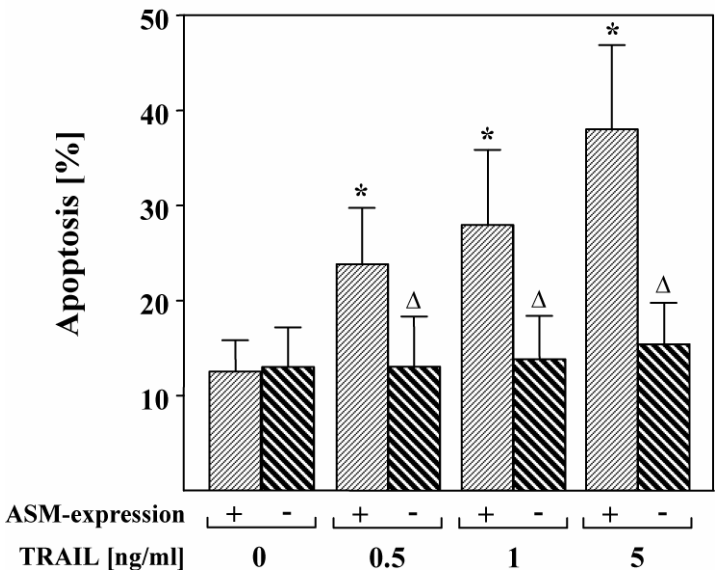
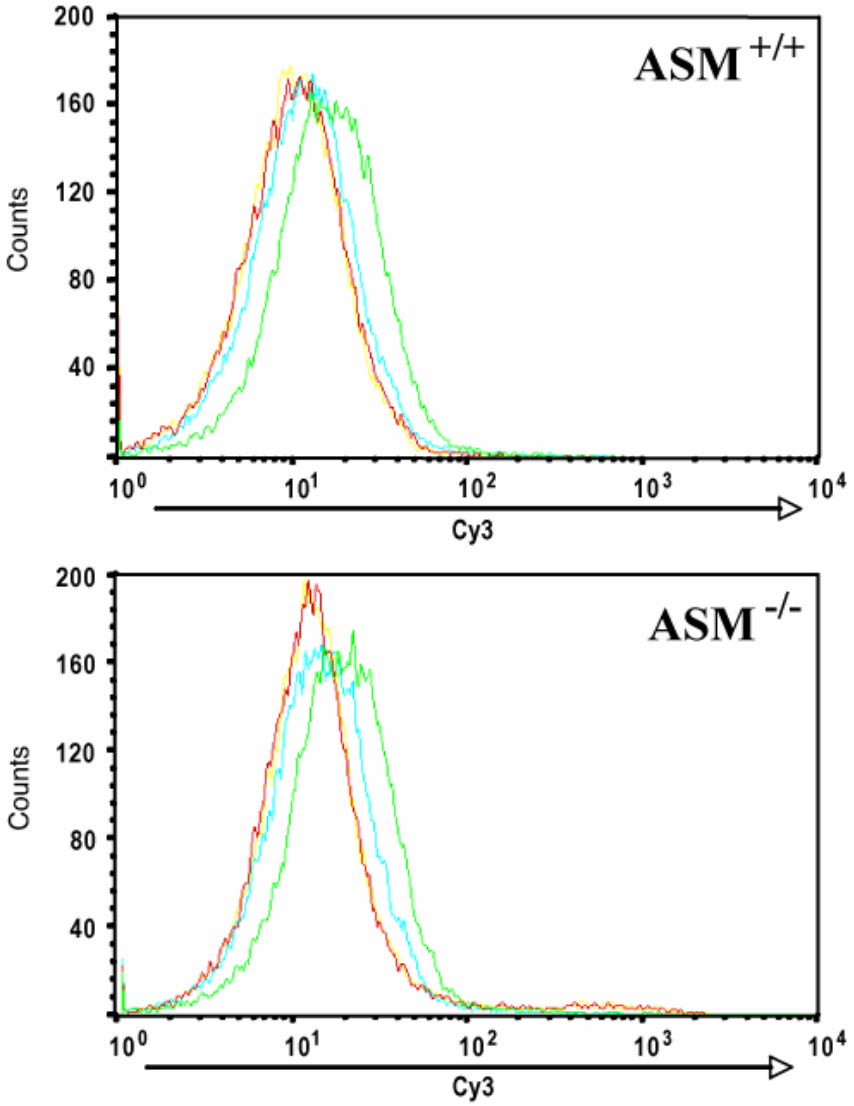


Figure 4.1. ASM is required for TRAIL – induced apoptosis.
Stimulation of wild-type splenocytes for 8 h with TRAIL results in a dose-dependent induction of apoptosis, while ASM-deficient cells are resistant to TRAIL-triggered apoptosis. Cellular apoptosis was determined by FACS analysis following FITC-Annexin V staining. The data show the mean ± S.D. of 3 independent experiments.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated samples or between ASM-positive and ASM-deficient cells are indicated by an asterisk* or a delta Δ, respectively.

Control experiments were performed to exclude the possibility that the difference in apoptosis is due to a different expression of DR5 between ASM wild-type and ASM-deficient cells. Expression of the receptor was confirmed by FACS analysis and did not significantly differ between the two cell types (Figure 4.2).



Yellow = unstimulated cells; Cy3-coupled secondary antibody only
Blue = unstimulated cells; anti-DR5 primary antibody and Cy3- coupled secondary antibody
Red = PHA / IL2 stimulated cells; Cy3-coupled secondary antibody only
Green = PHA / IL2 stimulated cells; anti-DR5 primary antibody and Cy3- coupled secondary antibody

Figure 4.2. DR5 expression is similar in ASM wild type and ASM- deficient T splenocytes.
T splenocytes from ASM wild-type (+/+) and ASM- deficient (-/-) mice were stimulated with 10 µg/ml PHA and 5 units/ml IL-2 to express DR5. Similar expression levels of DR5 in $ASM^{+/+}$ and $ASM^{-/-}$ cells compared to background (secondary antibody only) were confirmed by FACS analysis. DR5 staining was performed using a Cy3-coupled anti-DR5 antibody. The results are representative for 3 similar studies.

To confirm induction of apoptosis and exclude necrosis following TRAIL treatment, BJAB cells were incubated with 20 ng/ml or 100 ng/ml TRAIL for the indicated times, and a propidium iodide (PI)/FITC-Annexin V double staining was performed. The FACS analysis show a dose-dependent induction of apoptosis by TRAIL, while no significant necrosis was observed (Figure 4.3).

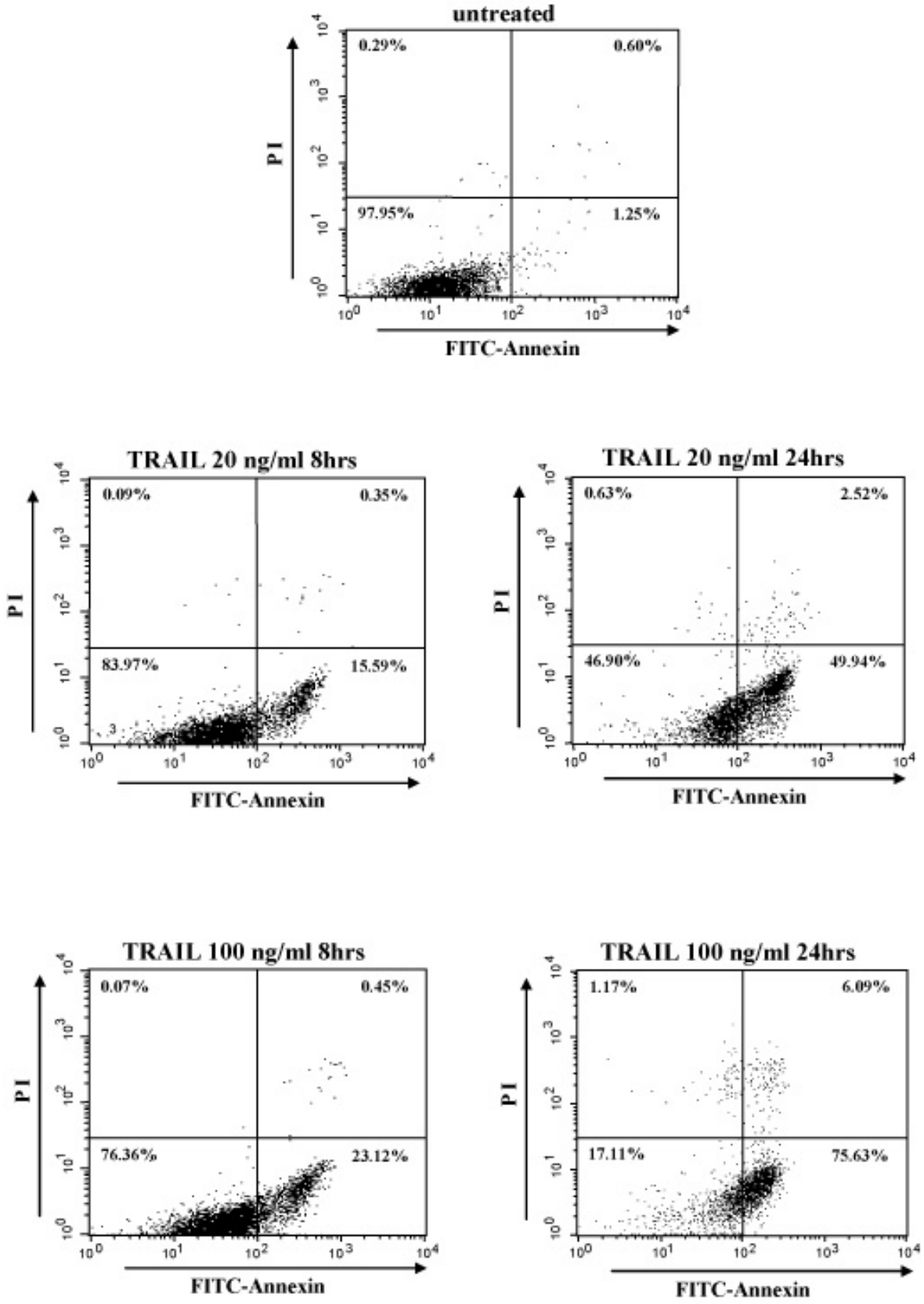


Figure 4.3. TRAIL induces apoptosis and not necrosis.
Induction of apoptosis in BJAB cells upon treatment with TRAIL was confirmed by staining with FITC-Annexin V and propidium iodide (PI). Viable cells appear in the lower left quadrant, apoptotic cells in the lower right quadrant and necrotic cells in the upper quadrants. FACS analysis was performed on 10.000 cells each; the percent distribution is given for each quadrant. The results are representative for 3 similar studies.

4.1.2. TRAIL activates ASM

To test whether TRAIL activates ASM, wild-type T splenocytes were incubated with 5 ng/ml TRAIL for the indicated times. This resulted in a rapid activation of the ASM peaking approximately 10 min post treatment and declining thereafter (Figure 4.4a). Additionally, three other cell types (BJAB, L929 and A549 cells) were tested to determine whether TRAIL-induced ASM activation is a general phenomenon. Following incubation with 100 ng/ml TRAIL, the kinetics of ASM activation showed a similar pattern in these cells, with a peak at 10 min (for BJAB cells) (Figure 4.4b) and 20-30 min (for L929 and A549) (Figure 4.4c-d), after which it rapidly decreased.

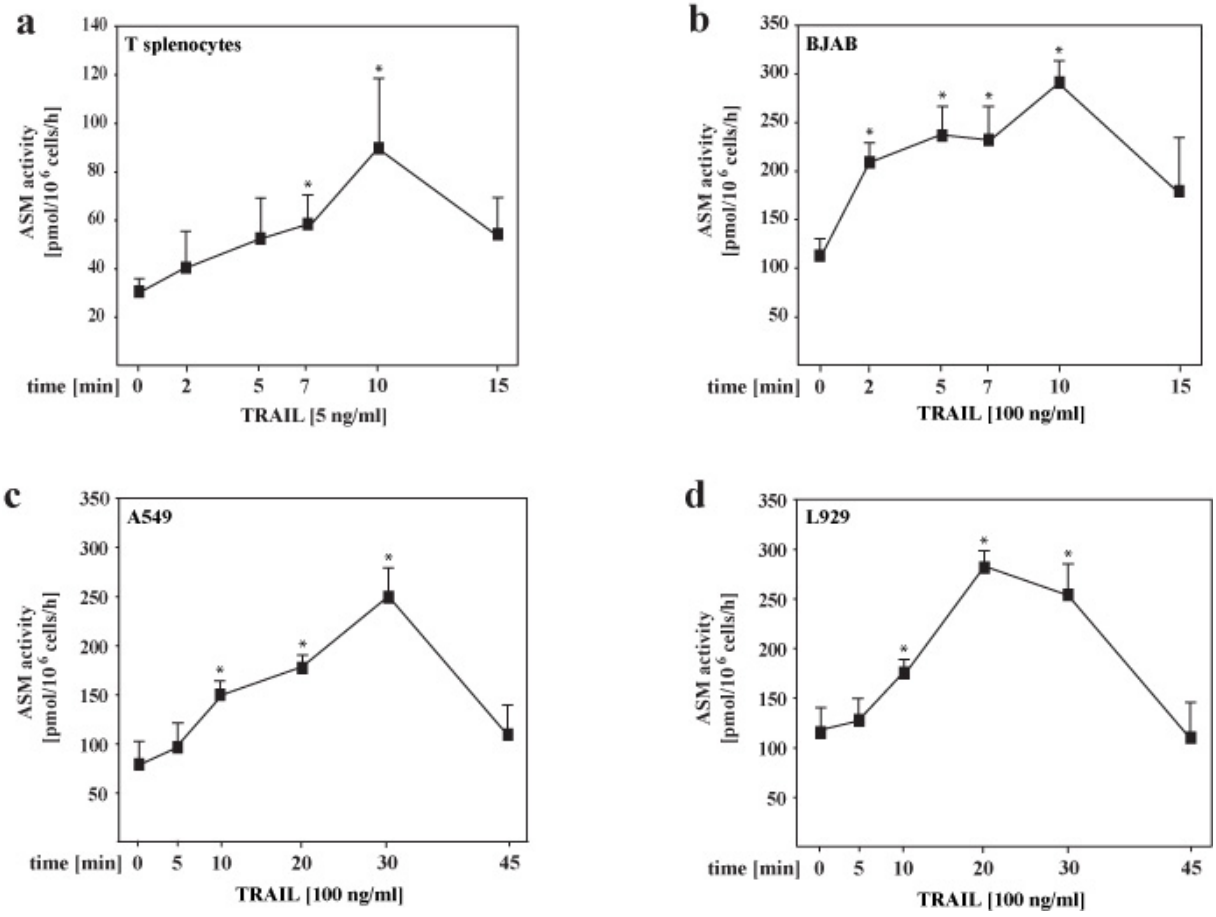
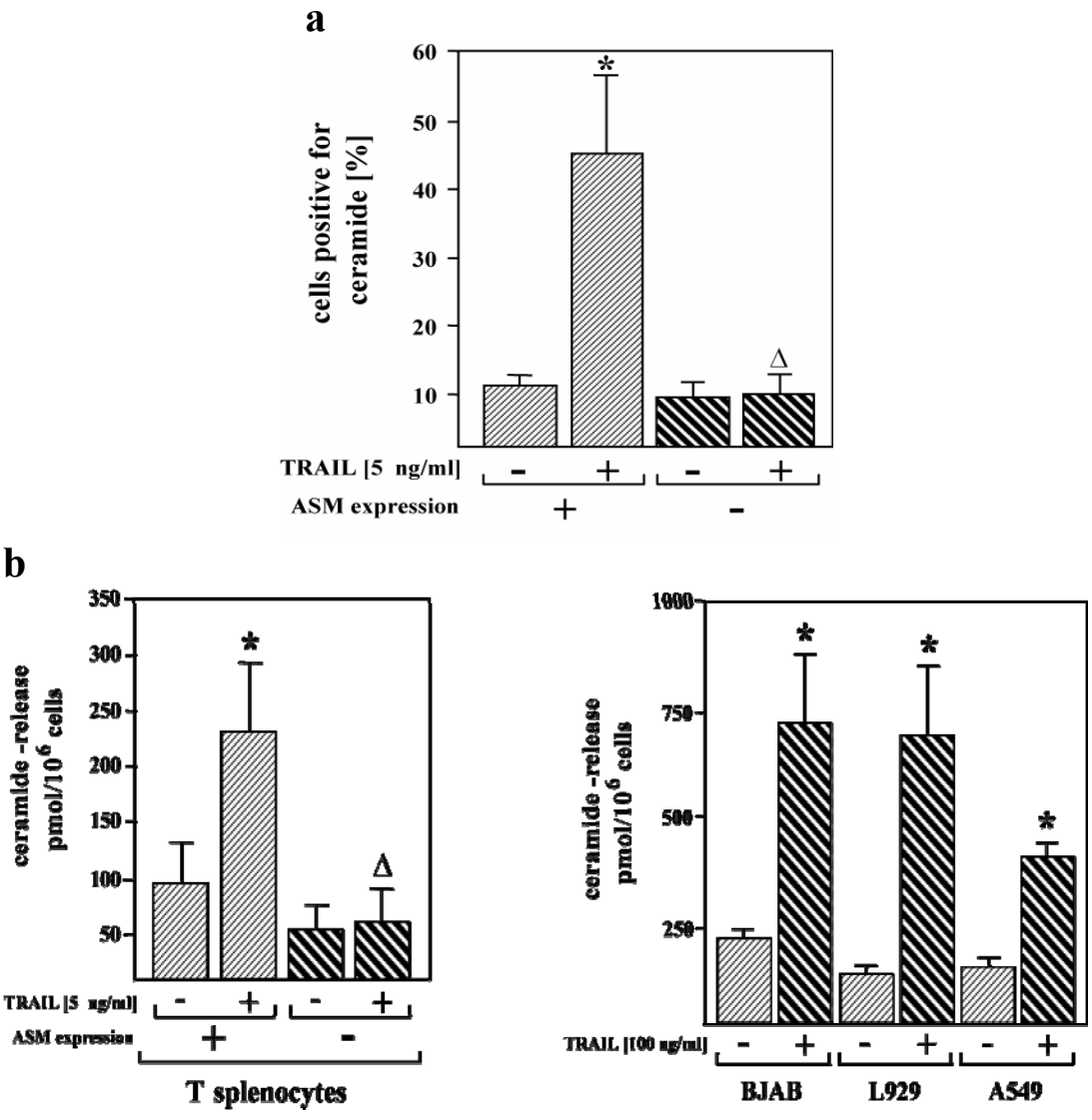


Figure 4.4. TRAIL activates ASM.
Stimulation of wild-type T splenocytes (**a**), BJAB (**b**), A549 (**c**) or L929 (**d**) cells with TRAIL results in rapid activation of the ASM. The activity of the ASM was determined by degradation of ^{14}C-labeled sphingomyelin to ^{14}C-labeled phosphorylcholine upon addition of the cell lysates from TRAIL -stimulated or -unstimulated cells. The data are the mean ± S.D. of 3 independent experiments.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated cells are indicated by an asterisk*.

4.1.3. TRAIL induces ceramide production/platforms via the ASM

Activation of ASM correlated with a release of ceramide in BJAB, A549, L929 cells and ASM wild-type T splenocytes, while ASM- deficient cells failed to respond to TRAIL with an increase of ceramide (Figure 4.5a-c). Splenocytes were stimulated for 10 min with 5 ng/ml TRAIL. BJAB cells were stimulated for 10 min with 100 ng/ml TRAIL, while L929 and A549 cells were stimulated for 20 min with 100 ng/ml TRAIL. Cellular ceramide was determined by FACS analysis (Figure 4.5a) employing monoclonal anti-ceramide antibodies to detect ceramide on the cell surface. Total cellular ceramide was assessed by DAG-kinase assay (Figure 4.5b). Finally, confocal microscopy studies confirmed the release of ceramide in ASM-positive, but not ASM-deficient T splenocytes following TRAIL treatment (Figure 4.5c).The confocal microscopy studies also revealed that ceramide accumulates in platforms onto the cell surface of the ASM wild type cells, while the ASM deficient splenocytes did not exhibit such platforms (Figure 4.5c).



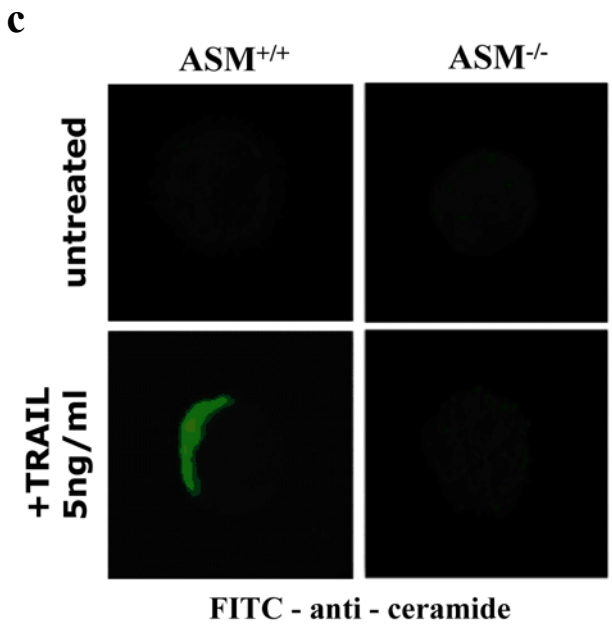


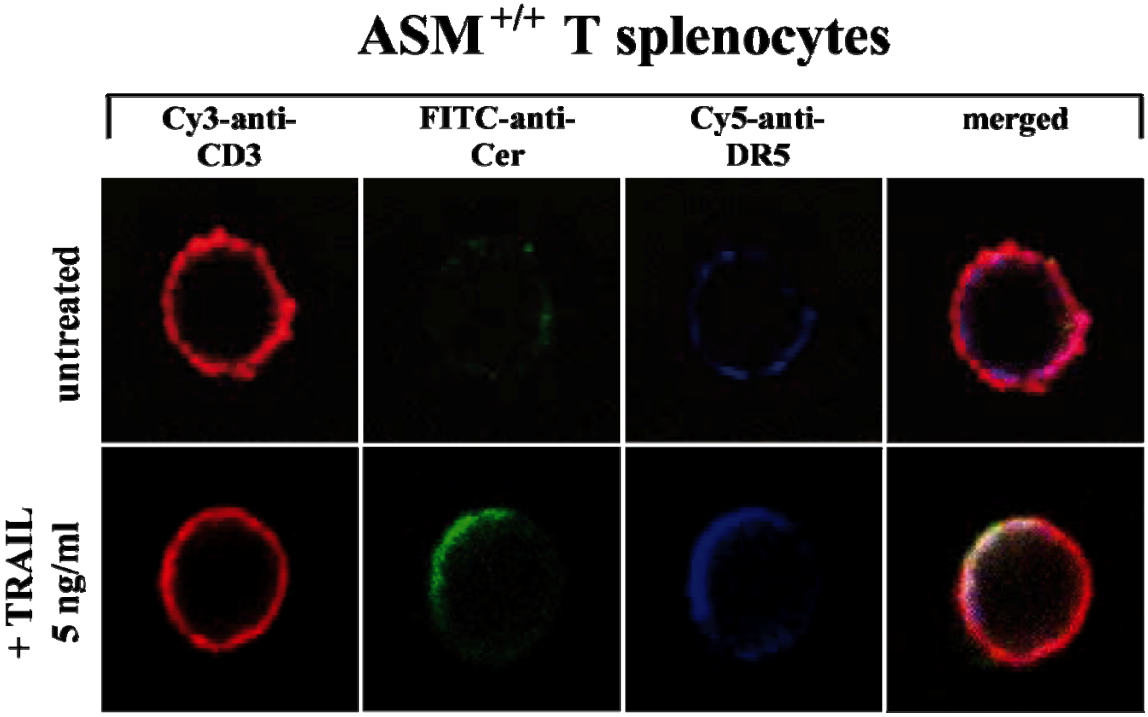
Figure 4.5. TRAIL induces ceramide release via the ASM.
(**a**) Stimulation of ASM wild type T splenocytes with TRAIL, induced a release of ceramide, while ASM-deficient cells failed to release ceramide upon TRAIL stimulation. Ceramide was determined by FACS analysis after staining with FITC-coupled anti-ceramide antibodies (clone 15B4). The data are the mean ± S.D. of 3 independent experiments.
(**b**) ASM-positive or -negative splenocytes, as well as BJAB, L929 and A549 cells were stimulated with TRAIL and cellular ceramide was determined by DAG-kinase assay (b). The DAG-kinase assay determines the cellular concentration of ceramide by phosphorylation of ceramide to ceramide-1-phosphate in the presence of [^{32}P]γATP. The data are the mean ± S.D. of 3 independent experiments.
(**c**) ASM-positive or -negative splenocytes were stimulated with TRAIL and surface ceramide was determined by confocal microscopy analysis after staining with FITC-coupled anti-ceramide antibodies (clone 15B4). The confocal microscopy studies display a typical result of 4 independent experiments with analysis of at least each 200 cells/sample.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated or ASM- positive and -negative samples are indicated by an asterisk * or a delta Δ, respectively.

4.1.4. TRAIL induces an ASM-dependant DR5 clustering within the ceramide platforms

Previous studies indicated that ceramide-enriched membrane platforms serve to reorganize activated receptor molecules in the plasma membrane (Grassme *et al.,* 2001a, b; Grassme *et al.,* 2002; Grassme *et al.,* 2003; Cremesti *et al.,* 2001; Abdel-Shakor *et al.,* 2004). To investigate whether DR5 clusters in ceramide platforms following TRAIL stimulation, ASM wild-type T splenocytes were incubated with 5 ng/ml TRAIL for 10 min, fixed in PFA and stained for ceramide and DR5 (Figure 4.6a). The results show a rapid formation of DR5 cluster that localized within the ceramide platforms, while no clustering was observed in untreated cells (Figure 4.6a). Very similar results were obtained for BJAB, A549 and L929

cells (Figure 4.6b-d) after stimulation with 100 ng/ml TRAIL. BJAB cells were stimulated for 10 min while L929 and A549 were incubated with TRAIL for 20 min.

a



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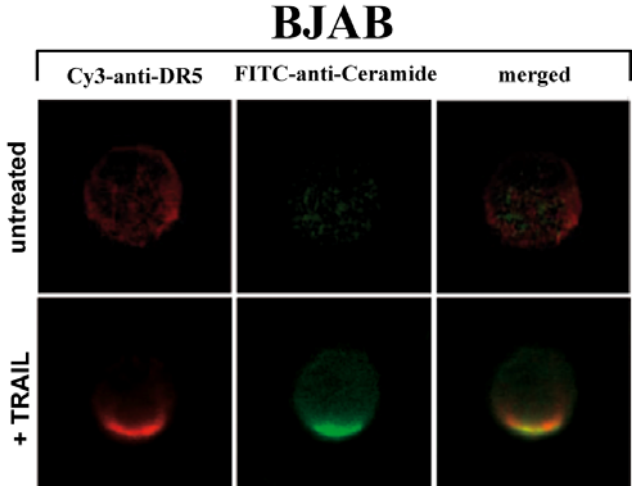


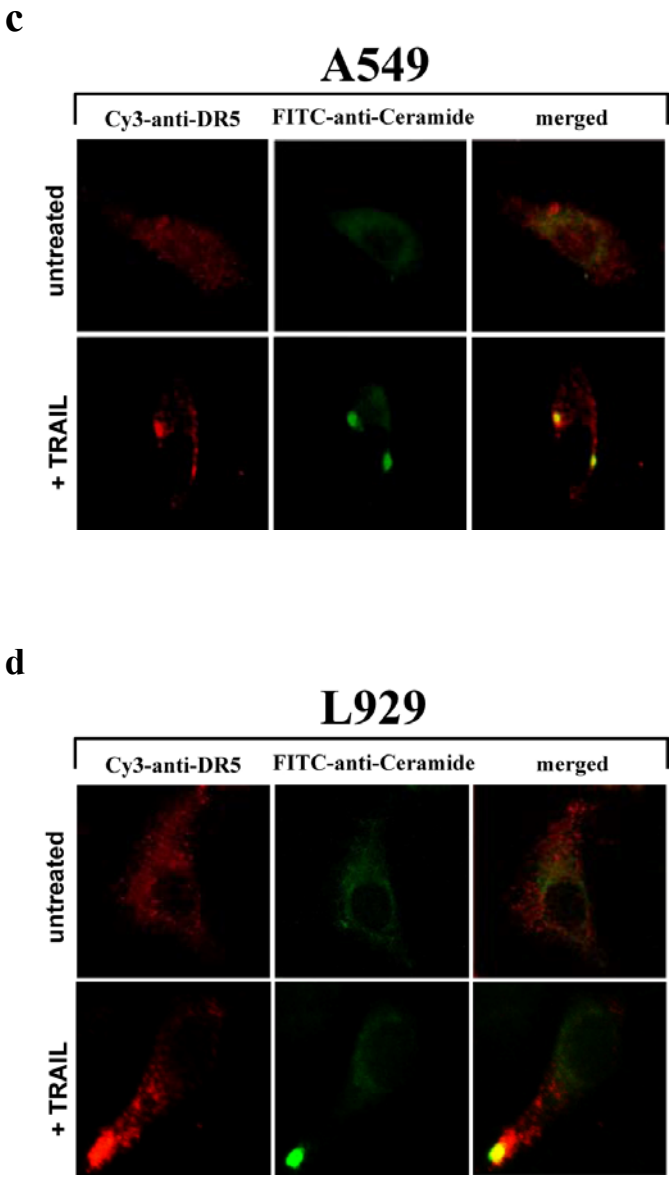


Figure 4.6. TRAIL induces DR5 clustering in ceramide-enriched membrane platforms.
(**a**) Stimulation of ASM wild-type T splenocytes with TRAIL results in clustering of DR5 within ceramide platforms. Cells were stained with FITC-coupled anti-ceramide antibodies (clone 15B4) and Cy5-coupled anti-DR5 antibodies. Staining of the cells with Cy3- labeled anti-CD3 antibodies served to exclude any non-T cells. The results are representative for 4 studies with analysis of each 200 cells/sample.
(**b-d**) Stimulation of BJAB, L929 and A549 cells with TRAIL results in clustering of DR5 within ceramide platforms. Cells were stained with FITC-coupled anti-ceramide antibodies (clone 15B4) and Cy3- coupled anti-DR5 antibodies. The results are representative for 3 studies with analysis of each 200 cells/sample.

To determine the importance of ASM for TRAIL-induced DR5 clustering, ASM wild-type and ASM-deficient T splenocytes were incubated with different concentrations of TRAIL ranging from 0.5 to 25 ng/ml for 10 min. Quantitative analysis of DR5 clustering was performed by confocal microscopy and showed a complete dependence of clustering on the expression of the ASM (Figure 4.7).

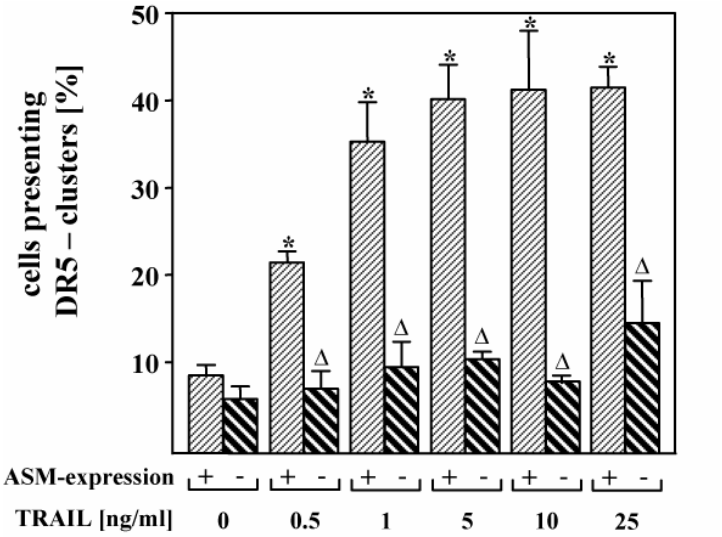


Figure 4.7. TRAIL-induced DR5 clustering is ASM- dependent.
Quantification of DR5 cluster in ASM wild-type and ASM- deficient T cells reveals that expression of ASM is required for DR5 clustering. Cells were stained with Cy3- coupled anti-DR5 antibodies and analysis was performed by confocal microscopy by counting DR5 cluster for at least 200 cells/sample. The data show the mean ± S.D. of 3 independent studies.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated or ASM- positive and -negative samples are indicated by an asterisk * or a delta Δ, respectively.

Control experiments showed that TRAIL did not alter the total expression of DR5 as determined by FACS analysis (Figure 4.8), and the increase of the DR5 signal in the confocal microscopy studies is most likely caused by a massive reorganization and concentration of DR5 in the platform.

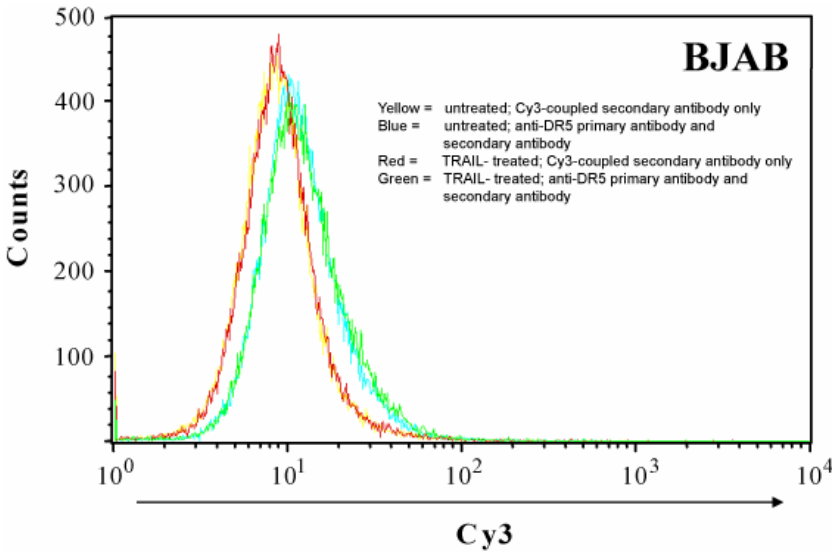


Figure 4.8. TRAIL does not alter the expression of DR5.
FACS analysis of 50.000 cells/sample indicated that TRAIL treatment does not increase DR5 expression on cell surface. Levels of DR5 were determined using Cy3-coupled anti-DR5 antibodies compared to background (secondary antibody only). The results are representative for 2 similar studies.

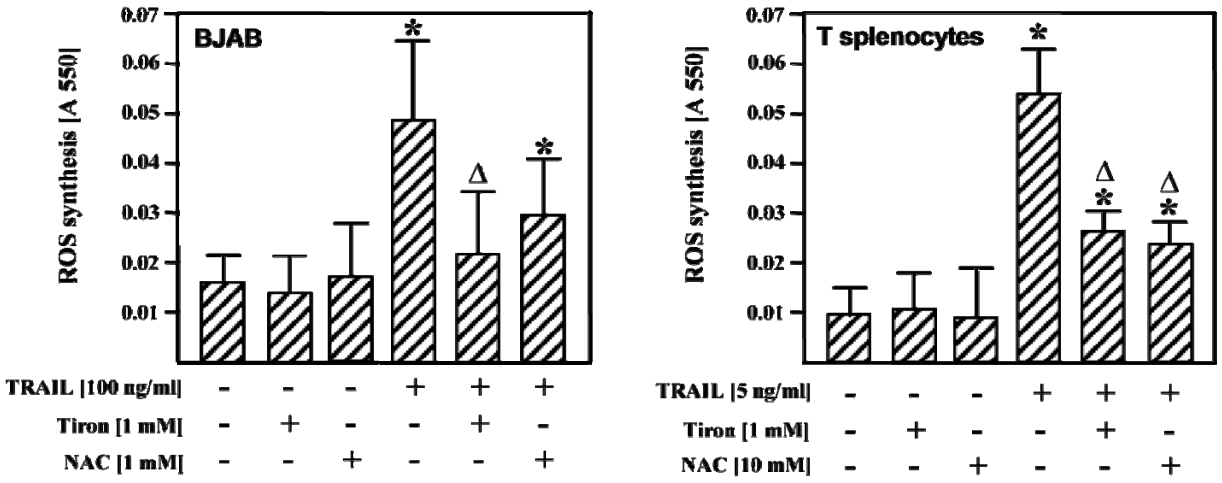
4.2. Mechanism of TRAIL- induced ASM activation

4.2.1. TRAIL induces Reactive Oxygen Species (ROS) production

Previous studies demonstrated a role of reactive oxygen species (ROS) in the induction of apoptosis by CD95 (Gulbins *et al.,* 1996; Liu *et al.,* 1996; Um *et al.,* 1996; Reinehr *et al.,* 2005). To test the hypothesis that the ASM is regulated by a redox mechanism, the release of reactive oxygen species (ROS) was determined upon stimulation of BJAB cells or ASM wild-type T splenocytes with TRAIL for 1 min. The results indicate a very rapid release of ROS upon TRAIL treatment, which was prevented by preincubation with the anti-oxidants Tiron and *N*-acetylcysteine (NAC) (Figure 4.9a).

Control experiments were performed to exclude the possibility that treatment with anti-oxidants prevents binding of TRAIL to the DR5 receptors, thus blocking TRAIL-induced signaling (Figure 4.9b). To this end, BJAB cells were stimulated for 10 min with TRAIL in the presence or absence of Tiron and NAC, respectively. The recombinant TRAIL used in our studies contains a 6 x Histidine domain, therefore, Cy3-coupled anti-6 x His antibodies were used to detect TRAIL bound to the BJAB cells. The FACS analysis shows no significant difference in TRAIL binding when cells were pre-treated with anti-oxidants (Figure 4.9b).

a



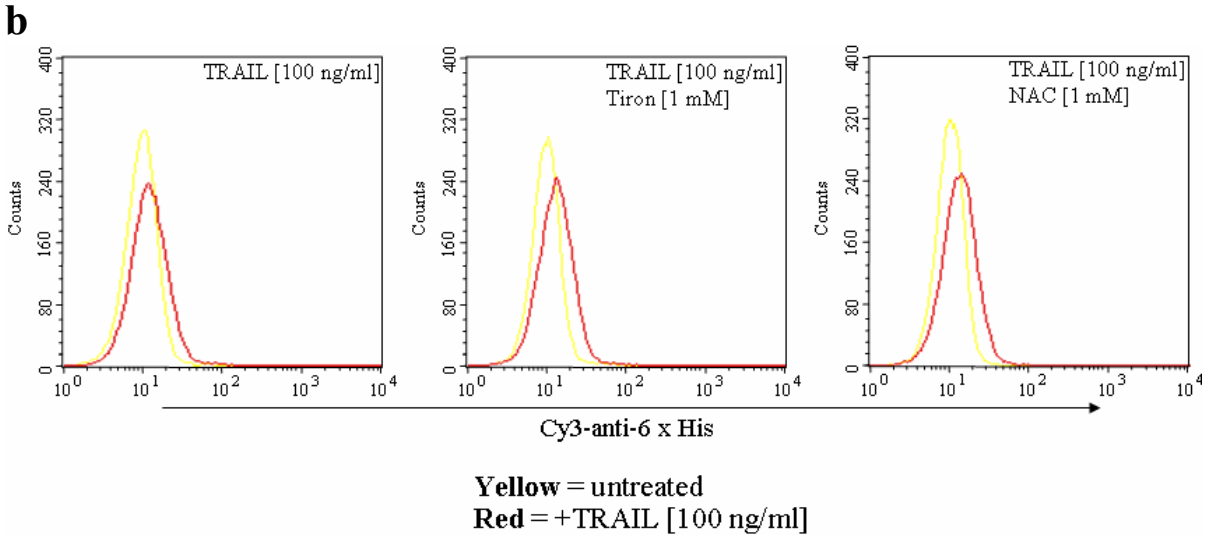


Figure 4.9. TRAIL induces Reactive Oxygen Species (ROS) production
(**a**) Stimulation of BJAB cells or T splenocytes with 100 ng/ml or 5 ng/ml TRAIL, respectively, for 1 min results in the release of ROS that are neutralized by pre-incubation with the anti-oxidants Tiron or N-acetylcysteine (NAC). Cells were lysed in a buffer containing cytochrome c and the change of absorption at 550 nm served as a measurement for the release of ROS. The data show the mean ± S.D. of 4 independent studies.
(**b**) BJAB cells were incubated or not with Tiron and NAC, respectively for 20 min. After stimulation with TRAIL for 10 min, cells were fixed, stained with Cy3-coupled anti-6 x His antibodies and analysed by FACS. The data are representative for 2 similar studies.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated or TRAIL-treated and anti-oxidants/TRAIL-treated samples are indicated by an asterisk * or a delta Δ, respectively.

4.2.2. TRAIL – induced ASM activation is ROS – dependent

To determine the relevance of TRAIL- induced ROS release for ASM activation, BJAB and ASM wild-type T splenocytes were incubated with Tiron and NAC prior to TRAIL treatment. This resulted in an inhibition of TRAIL-induced ASM activation, which demonstrates the importance of ROS regarding ASM activation mechanism (Figure 4.10).

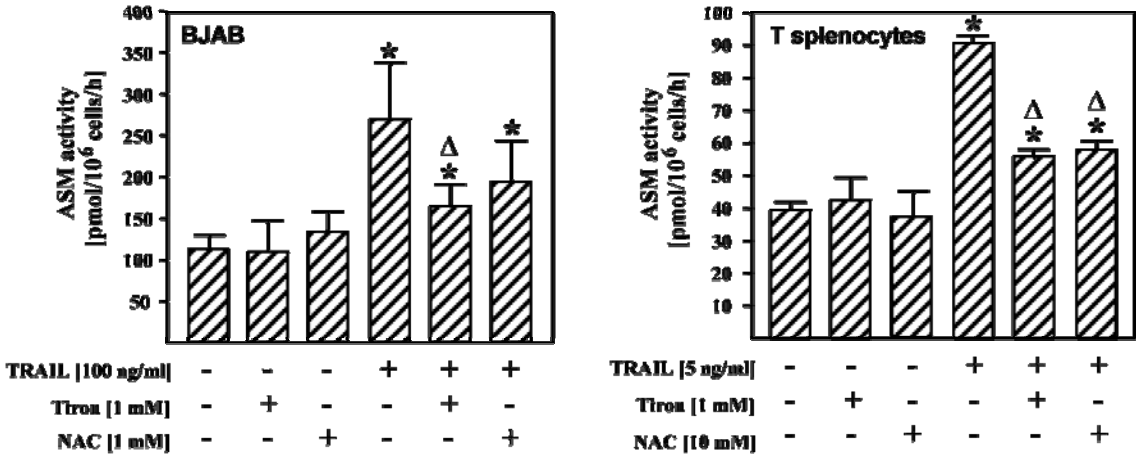


Figure 4.10. Anti-oxidants prevent TRAIL-induced ASM activation
BJAB cells and ASM wild-type T splenocytes were incubated with 100 ng/ml TRAIL and 5 ng/ml TRAIL, respectively, for 10 min in the presence or absence of anti-oxidants. ASM activity was determined by consumption of [^{14}C]sphingomyelin. The mean ± SD of 3 independent studies is displayed.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated or TRAIL-treated and anti-oxidants/TRAIL- treated samples are indicated by an asterisk * or a delta Δ, respectively.

4.2.3. ROS inhibition blocks ASM-mediated ceramide/DR5 clustering and apoptosis

Inhibition of ASM by Tiron and NAC treatment, respectively, correlated with an inhibition of TRAIL- induced ceramide platforms and DR5 cluster formation (Figure 4.11a) as well as prevention of apoptosis following TRAIL treatment (Figure 4.11b)

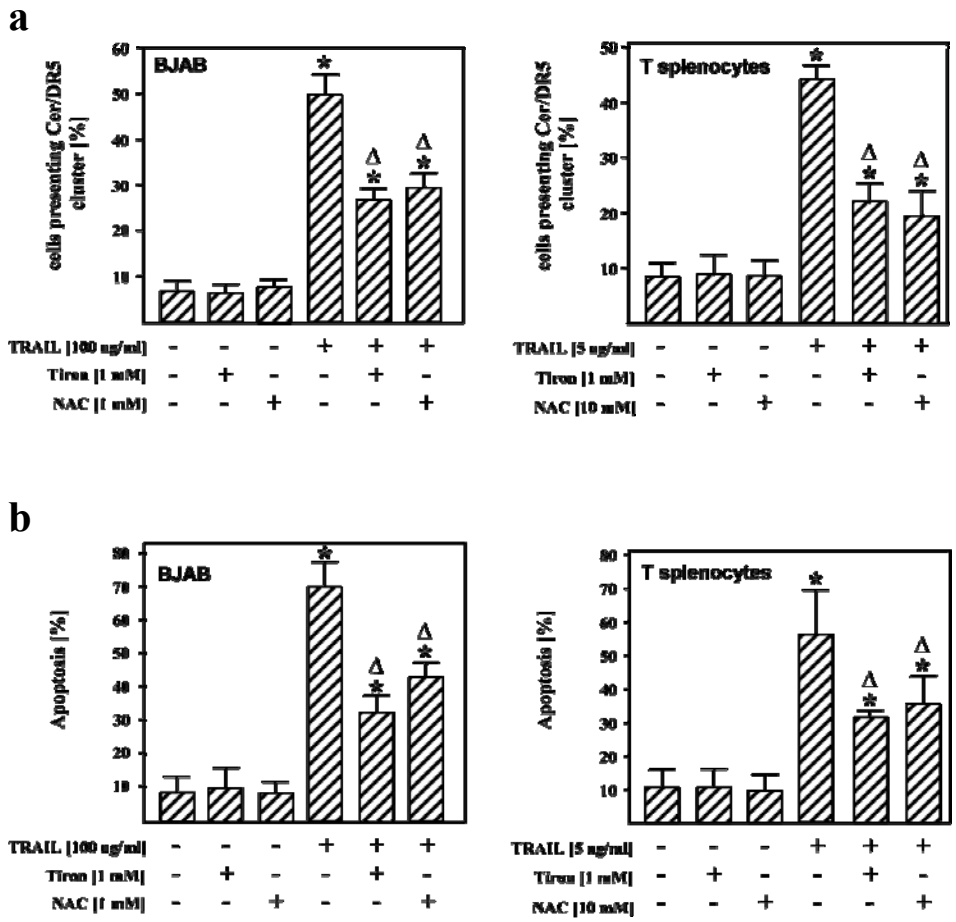
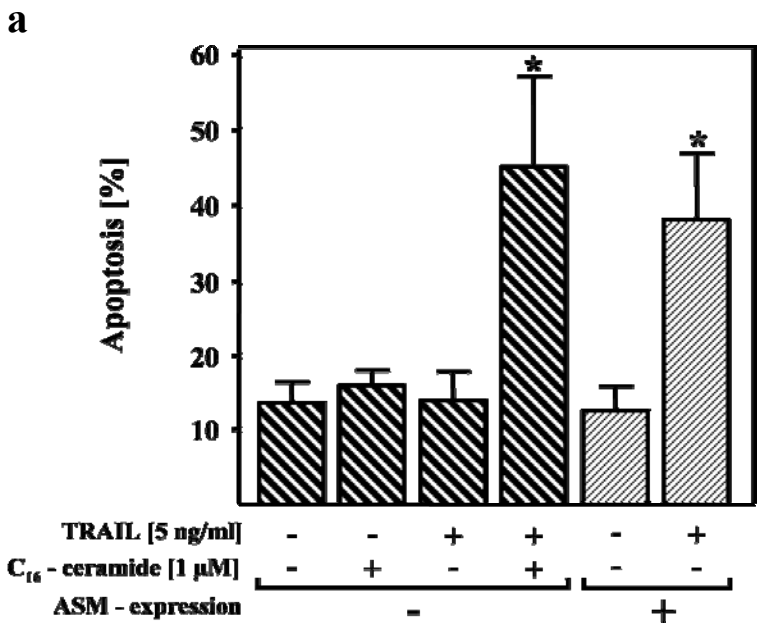


Figure 4.11. Anti-oxidants prevent TRAIL-induced ceramide/DR5 clustering and apoptosis
BJAB cells and ASM wild-type T splenocytes were incubated with NAC and Tiron 20 min prior to stimulation with 100 ng/ml TRAIL and 5 ng/ml TRAIL, respectively.
(**a**) Cells were fixed 10 min after TRAIL treatment and clustering of DR5 in ceramide-enriched membrane platforms was measured in at least 200 cells/sample by fluorescence microscopy analysis. The mean ± S.D. of 2 independent studies is displayed.
(**b**) Apoptosis was measured by FITC-Annexin V staining 24 h following TRAIL treatment. The mean ± S.D. of 3 independent studies is displayed.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated or TRAIL-treated and anti-oxidants/TRAIL- treated samples are indicated by an asterisk * or a delta Δ, respectively.

4.3. Role of ceramide and DR5 clustering for TRAIL – induced apoptosis

4.3.1. Ceramide reconstitution rescues apoptosis in ASM-deficient cells

To determine the significance of ceramide-enriched membrane platforms for TRAIL-induced apoptosis, it was tested if the addition of natural C_{16}-ceramide to ASM-deficient splenocytes that are unable to release ceramide in response to TRAIL treatment, converts the resistance of these cells to TRAIL-induced apoptosis. As demonstrated in Figure 4.12a, small amounts of natural C_{16}-ceramide were sufficient to restore sensitivity of ASM-deficient splenic T cells to TRAIL-induced apoptosis, although the cells lack ASM activity. Incubation with increasing doses of C_{16}-ceramide was performed to establish the dose of C_{16}-ceramide which is not lethal by itself, but is able to sensitize cells to TRAIL treatment (Figure 4.12b). The data suggest a dose response effect with 0.1 µM C_{16}-ceramide already able to restore some apoptosis triggered by TRAIL, while 0.5 µM C_{16}-ceramide are sufficient to fully restore TRAIL-triggered apoptosis in ASM-deficient cells (Figure 4.12b). Control experiments confirmed the formation of ceramide-enriched membrane platforms after addition of exogenous C_{16}-ceramide (Figure 4.12c)



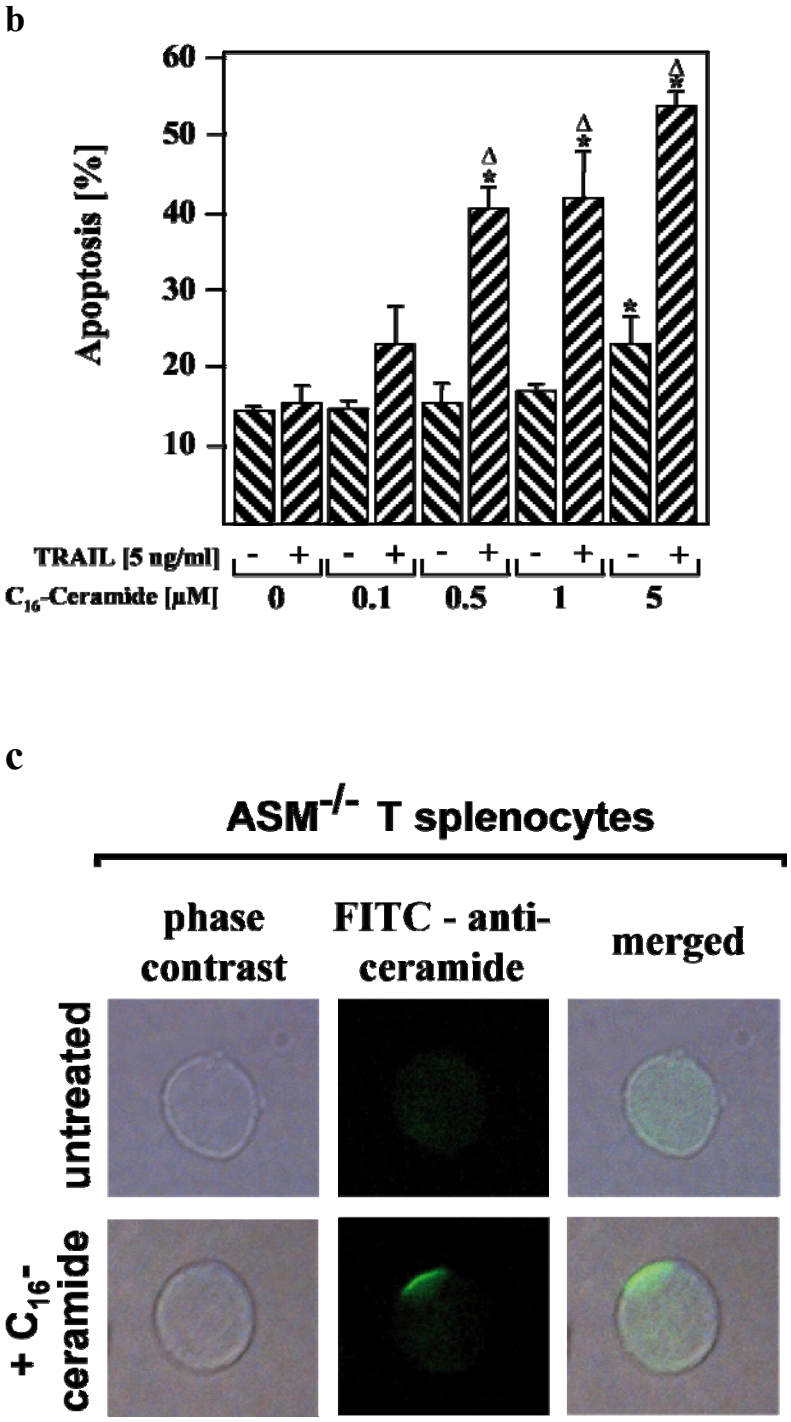


Figure 4.12. Natural C_{16}-ceramide restores TRAIL-induced apoptosis in ASM-deficient T splenocytes.
(**a**) Addition of 1 µM C_{16}-ceramide to ASM-deficient T cells restores TRAIL-induced apoptosis in these cells. The mean ± S.D. of 3 independent studies is displayed.
(**b**) ASM-deficient cells were incubated with increasing concentrations of C_{16}-ceramide, alone or in combination with TRAIL. Apoptosis was determined by FACS analysis 8 h after stimulation using FITC-Annexin V staining. The mean ± S.D. of 3 independent studies is displayed.
(**c**) Cells were incubated with 1 µM C_{16}-ceramide, fixed with PFA, stained with FITC-coupled anti-ceramide 15B4 antibodies and analysed by fluorescence microscopy. The results are representative for 2 studies with analysis of each 200 cells/sample.
Significant differences ($p \leq 0.05$, *t*-test) between untreated and C_{16}-ceramide- treated or C_{16}-ceramide/TRAIL-treated samples are labelled by an asterisk *, or a delta Δ, respectively.

4.3.2. Forced DR5 clustering overcomes ASM deficiency

To determine whether clustering of DR5 alone is sufficient to induce apoptosis in ASM-deficient cells, ASM-deficient splenocytes were stimulated with low physiological and very high doses of aggregated TRAIL-ligand, respectively. While ASM-deficient splenic T cells were resistant to doses up to 100 ng/ml of TRAIL, they died by apoptosis after treatment with high doses of aggregated TRAIL (Figure 4.13a) indicating that forced clustering of DR5 in ASM-deficient cells is sufficient to overcome the genetic defect of the ASM. Fluorescence microscopy analysis of ASM-deficient splenocytes treated with high doses of TRAIL confirmed the formation of DR5 clusters in these cells (Figure 4.13b).

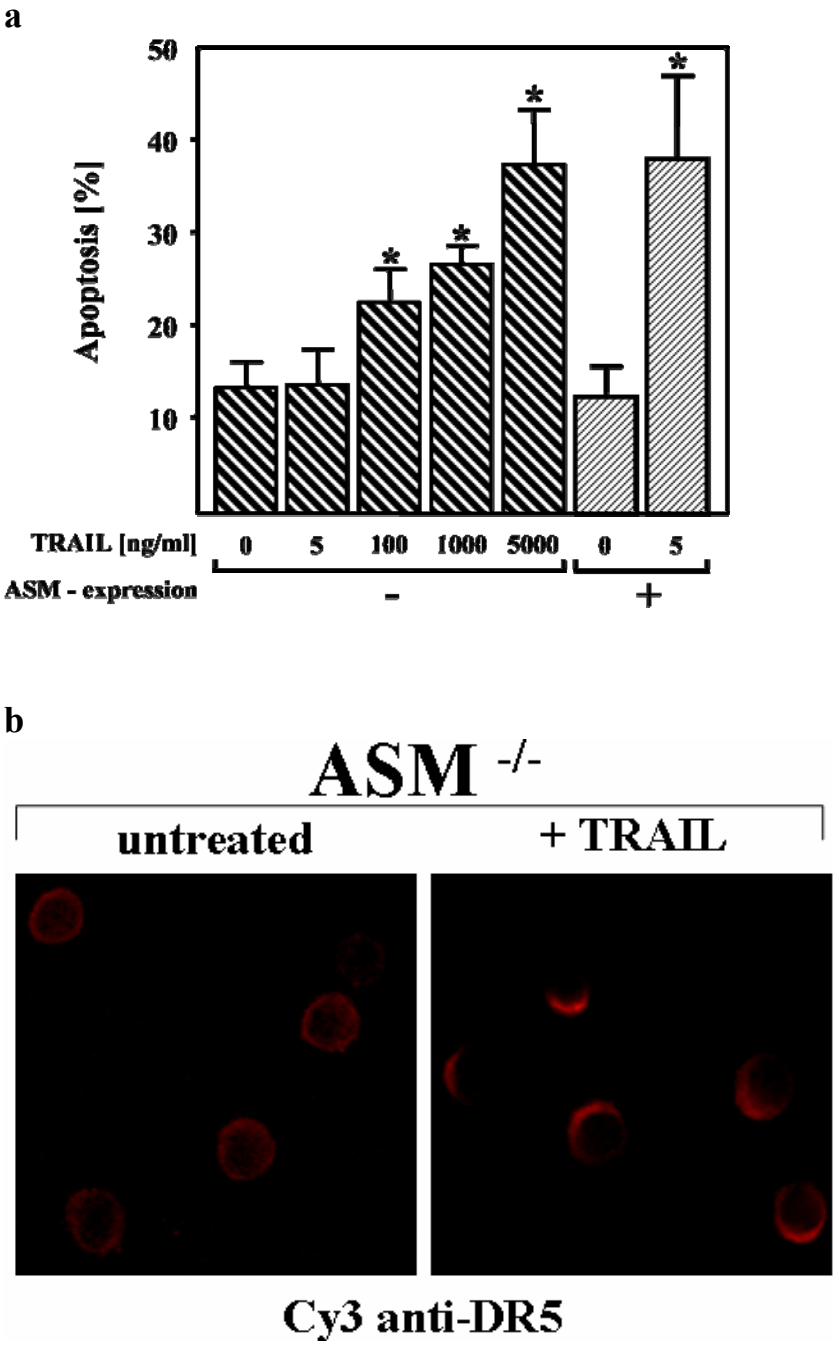


Figure 4.13. Forced clustering of DR5 restore TRAIL- induced apoptosis in ASM-deficient T cells.
(**a**) ASM-deficient splenocytes were treated with increasing doses of TRAIL. Apoptosis was determined by FITC- Annexin V staining 8 h after stimulation. The mean ± S.D. of 3 independent studies is displayed.
(**b**) ASM-deficient splenocytes were incubated with 100 ng/ml TRAIL for 10 min. Cells were fixed, stained with Cy3-labeled anti-DR5 antibodies and analysed by fluorescence microscopy. The results are representative for 3 similar studies.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated cells are indicated by an asterisk*.

4.3.3. Addition of ceramide amplifies the killing potential of low doses of TRAIL

The data displayed in Figures 4.12 and 4.13, showing that C_{16}-ceramide addition or forced clustering of DR5, respectively, restore TRAIL-induced apoptosis in ASM-deficient cells indicate that an interaction of TRAIL with DR5-trimers is not sufficient to trigger death. These data suggest that clustering of receptor molecules is critical to initiate apoptosis. However, very low doses of TRAIL are unable to induce activation of the ASM and formation of ceramide-enriched membrane platforms, although the ligand binds to DR5 and transforms the receptor into the active DR5-ligand complex. This suggests that below a certain threshold-dose TRAIL is unable to stimulate cells. We therefore hypothesized that a pharmacological induction of ceramide-enriched membrane platforms might be sufficient to amplify the effect of very low concentrations of TRAIL binding to DR5. To test this concept, a panel of tumour cells was treated with 10–100 ng/ml TRAIL to establish non-lethal doses of TRAIL for each cell line (Figure 4.14a). Next, cells were treated with 2 µM C_{16}-ceramide alone or in combination with the non-lethal does of TRAIL (Figure 4.14b). C_{16}-ceramide addition resulted in the formation of ceramide-enriched membrane platforms, but neither low doses (10–25 ng/ml) TRAIL nor 2 µM C_{16}-ceramide alone had any apoptotic effect on the tumour cells after 24 h incubation (Figure 4.14b). In contrast, the combination of 2 µM C_{16}-ceramide and low doses TRAIL killed the tumour cells as efficient as high doses of TRAIL (Figure 4.14b). This concept suggests that any drug or treatment inducing ceramide-enriched membrane platforms amplifies the effects of TRAIL on tumour cells and may permit the usage of a TRAIL dose *in vivo* that is without biological effect *per se*.

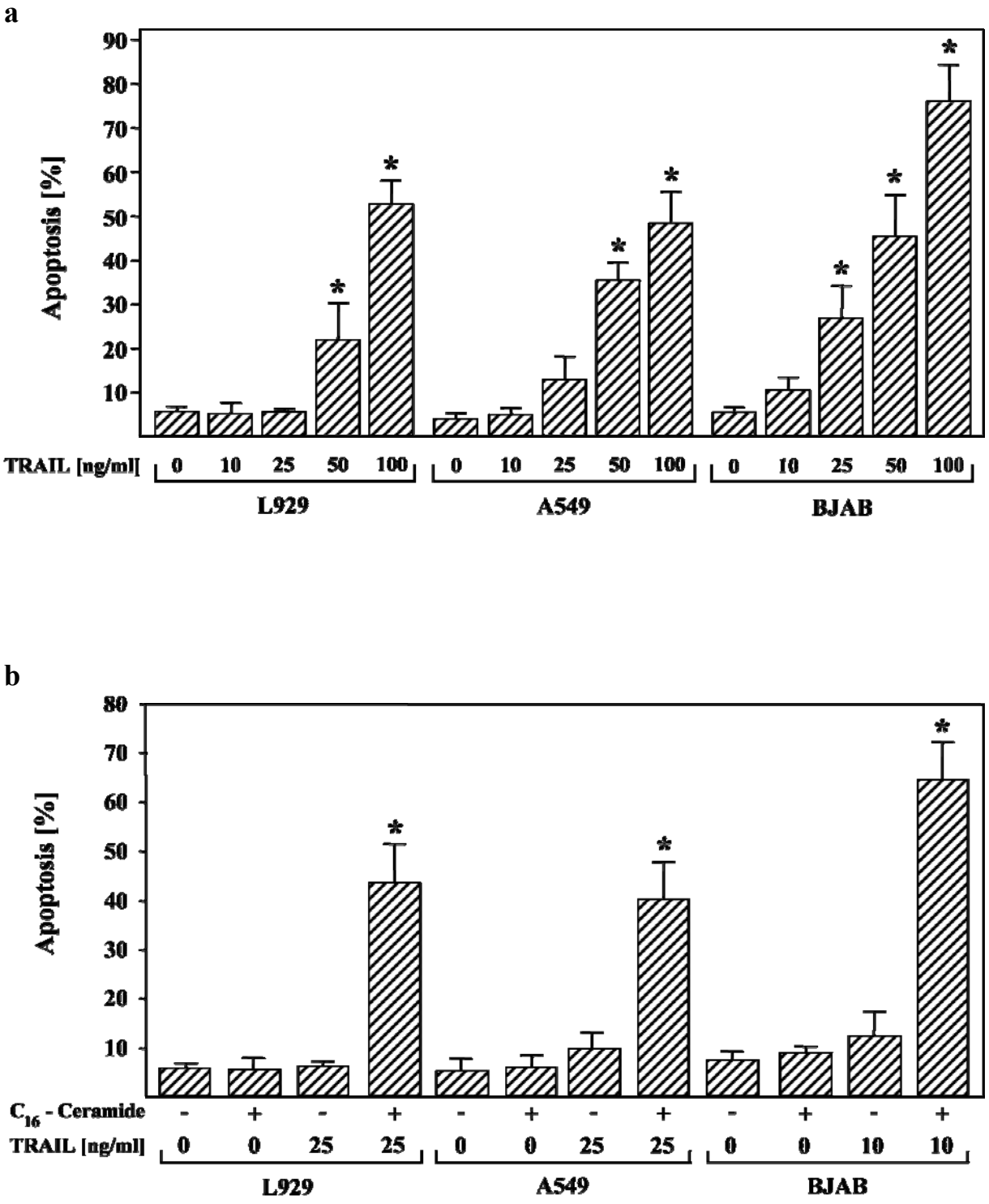


Figure 4.14. Amplification of TRAIL-induced apoptosis in tumour cells by ceramide-enriched membrane platforms

(**a**) Treatment of L929, BJAB and A549 tumour cells with increasing doses of TRAIL indicates that doses higher than 50 ng/ml are required to induce significant apoptosis in these cells. Apoptosis was determined by FACS analysis 24 h after stimulation. The mean ± S.D. of 3 independent studies is displayed.

(**b**) Addition of 2 µM M C_{16}-ceramide to the tumour cells greatly sensitizes the cells to TRAIL-induced apoptosis. The addition of C_{16}-ceramide facilitates high rates of apoptosis in the tumour cells at doses of TRAIL that are without significant effect if applied without C_{16}-ceramide. The mean ± S.D. of 3 independent studies is displayed.

Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated cells are indicated by an asterisk *.

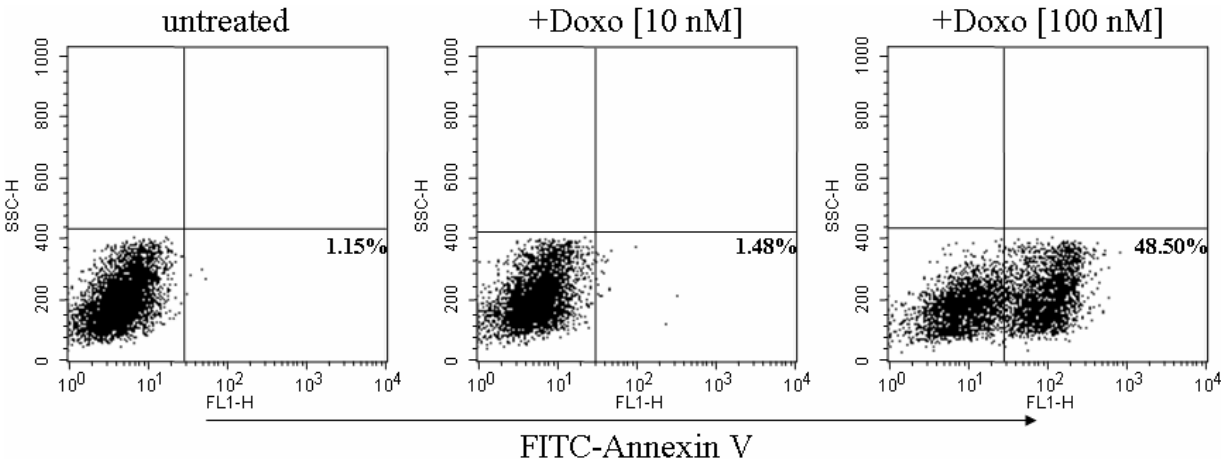
4.4. Doxorubicin-mediated amplification of TRAIL-induced apoptosis

The data described above demonstrate that ceramide plays a crucial role for TRAIL-induced apoptosis and that addition of C_{16}-ceramide sensitizes cells to apoptosis induced by very low doses of TRAIL. The next part of the study was designed to test this concept at a more physiological level using subtoxic doses of doxorubicin, a chemotherapeutic drug that has been previously shown to induce ceramide (Morita *et al.,* 2000; Andrieu-Abadie *et al.,* 1999; Gouaze *et al.,* 2001; Mercier *et al.,* 2003). To this end, it was investigated whether doxorubicin-induced ceramide release is sufficient to cluster DR5 and trigger apoptosis after stimulation with doses of TRAIL that are too low to activate the ASM, release ceramide, cluster DR5 and induce apoptosis by themselves.

4.4.1. Non-lethal doses of doxorubicin and TRAIL have a synergistic apoptotic effect

Preliminary experiments were performed on BJAB lymphocytes, which were treated with increasing concentrations of doxorubicin to establish the subtoxic dose of the drug. The FACS analysis performed 24 h later indicated that a dose of 10 nM doxorubicin has no effect on these cells (Figure 4.15a). Next BJAB lymphocytes were incubated with subtoxic doses of doxorubicin and TRAIL and apoptosis was determined after 24 h. The results demonstrate that separate treatment with neither drug at such low doses triggered apoptosis, while the combined treatment was able to induce significant apoptosis of these cells (Figure 4.15b).

a



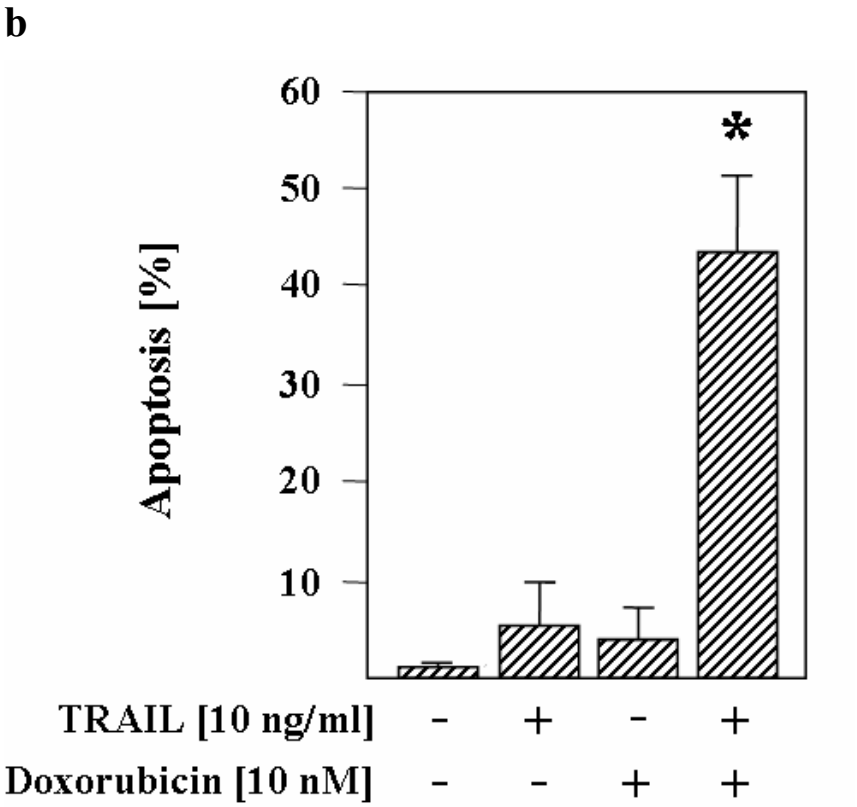


Figure 4.15. Doxorubicin enhances TRAIL-induced apoptosis
(**a**) BJAB cells were incubated with increasing doses of doxorubicin. After 24 h, cells were stained with FITC-Annexin V and analysed by FACS. The results are representative for 2 similar studies.
(**b**) BJAB cells were treated with either 10 ng/ml TRAIL, 10 nM doxorubicin, or both. Apoptosis was determined 24 h later by FACS after staining with FITC-Annexin V. Quantitative analysis indicates an increase of apoptosis in samples treated with both TRAIL and doxorubicin, while no apoptosis was observed for treatment with either drug alone. The data depict the mean ± S.D. of 5 independent experiments.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated cells are indicated by an asterisk*.

4.4.2. Doxorubicin treatment does not up-regulate DR5 expression

Previous studies indicated an increase of DR5 expression after treatment with cytostatic drugs, including doxorubicin (Sheikh *et al.,* 1998; Gibson *et al.,* 2000; Shankar *et al.,* 2005). To exclude that the synergistic effect of low doses of doxorubicin and TRAIL is due to doxorubicin-induced up-regulation of DR5 receptors expression, BJAB cells were incubated with 10 nM doxorubicin for 1 h and DR5 expression was analyzed by FACS (Figure 4.16). The results indicate that stimulation with such low doses of doxorubicin did not induce an up-regulation of the DR5 receptors (Figure 4.16).

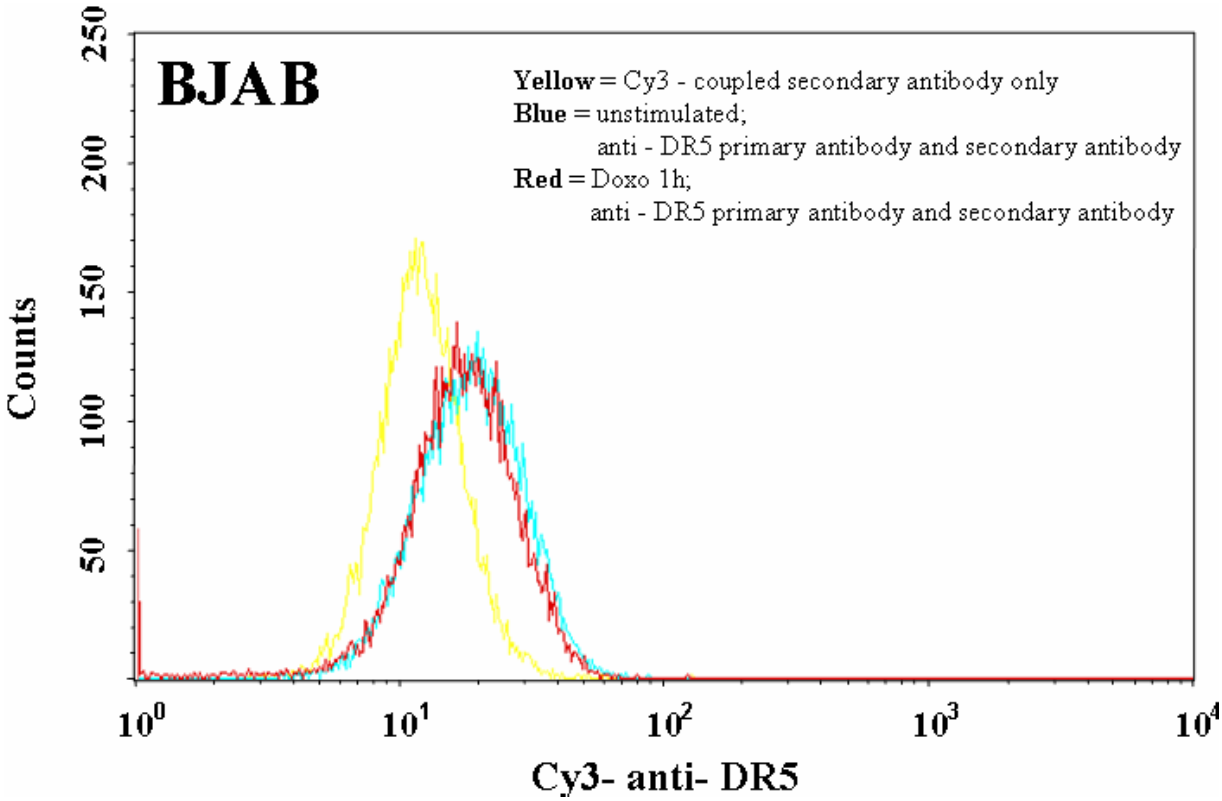


Figure 4.16. Low doses of doxorubicin do not increase the expression of DR5 receptors
BJAB cells were incubated with 10 nM doxorubicin for 1 h. After fixation and staining with Cy3-coupled anti-DR5 antibodies, cells were analysed by FACS. The results are representative for 3 similar studies.

4.4.3. Doxorubicin treatment induces ceramide release and platforms formation

To define the amplification mechanism mediating the synergy of doxorubicin and TRAIL to trigger apoptosis, it was tested whether doxorubicin at such low doses induces a release of ceramide. To this purpose, BJAB lymphocytes were stimulated with low doses of doxorubicin and ceramide on the cell surface was detected by employing monoclonal anti-ceramide antibodies (clone 15B4). Fluorescence microscopy studies showed a release of ceramide as well as the formation of ceramide-enriched membrane-platforms upon doxorubicin treatment (Figure 4.17).

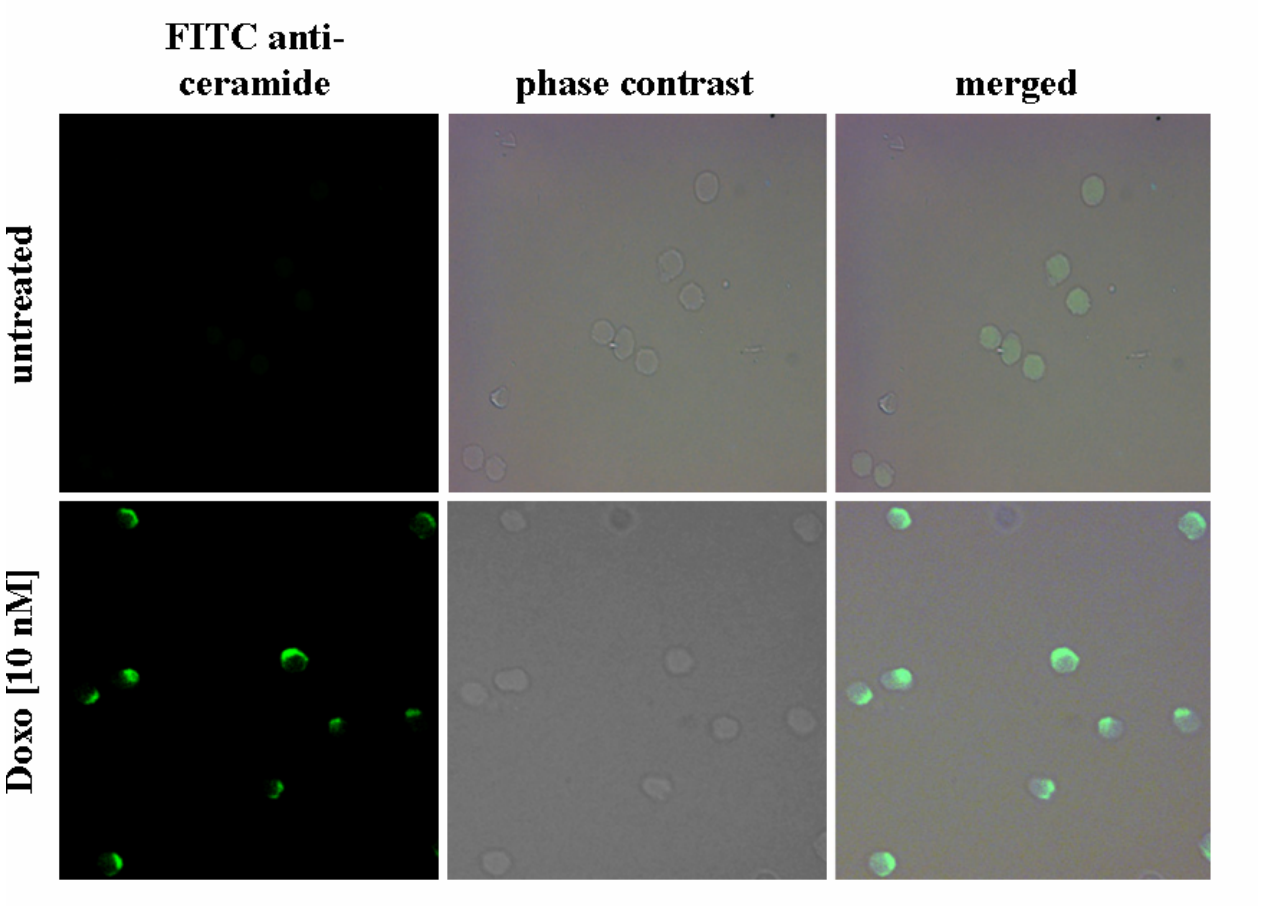


Figure 4.17. Doxorubicin treatment induces ceramide-enriched membrane platforms formation
Stimulation of BJAB lymphocytes with 10 nM doxorubicin for 30 min results in ceramide release and the formation of ceramide-enriched membrane platforms. Ceramide was determined by fluorescence microscopy after staining with FITC-coupled anti-ceramide 15B4 antibodies. The results are representative for 2 studies with analysis of each 200 cells/sample.

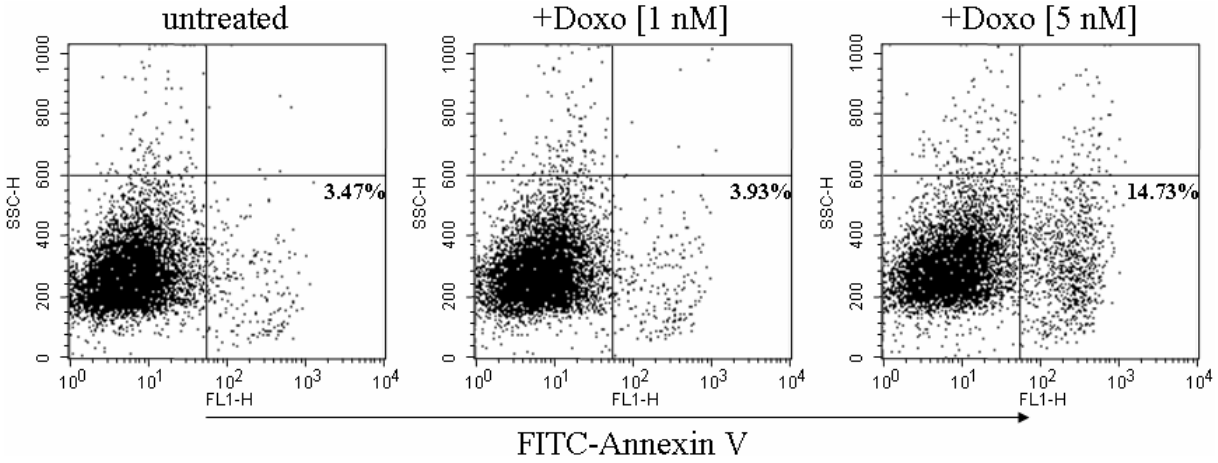
4.4.4. Doxorubicin-induced ceramide generation is ASM-dependant

Next, the pathway of doxorubicin-induced ceramide production was addressed. It was tested whether doxorubicin-induced ceramide production is mediated via the acid sphingomyelinase (ASM). For this purpose, ASM-positive and ASM-deficient T-splenocytes were employed. The subtoxic doses of doxorubicin were established by incubating ASM wild-type cells with increasing concentrations of the drug. The results indicate that a dose of 1 nM doxorubicin is not lethal for these cells, while at 5 nM doxorubicin, the splenocytes started to die (Figure 4.18a). The ASM wild-type and ASM-deficient splenocytes were then treated with these low doses of doxorubicin for different time intervals. The results reveal a rapid increase of ceramide in ASM-positive cells, while ASM-deficient splenocytes did not respond with an

increase of ceramide to doxorubicin stimulation (Figure 4.18b), indicating that doxorubicin triggers the release of ceramide via the ASM, at least in stimulated peripheral T-splenocytes.

The involvement of ASM in ceramide generation was confirmed by studies, which assessed the activity of the enzyme in cell lysates after doxorubicin treatment. Incubation of ASM-wild-type T-splenocytes and BJAB-lymphocytes with low doses of doxorubicin resulted in a rapid activation of the ASM, which correlated with the release of ceramide in these cells (Figure 4.18c).

a



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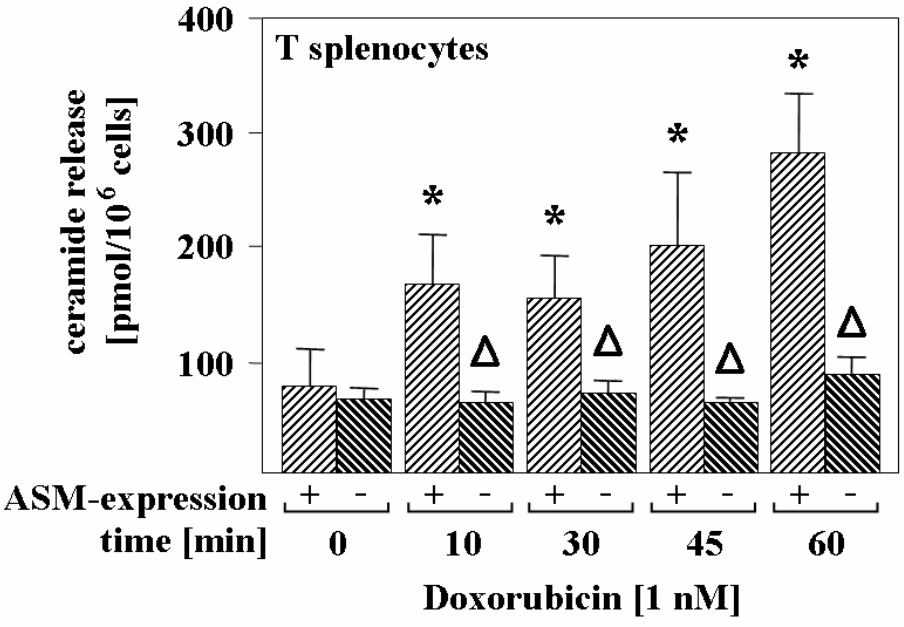


c

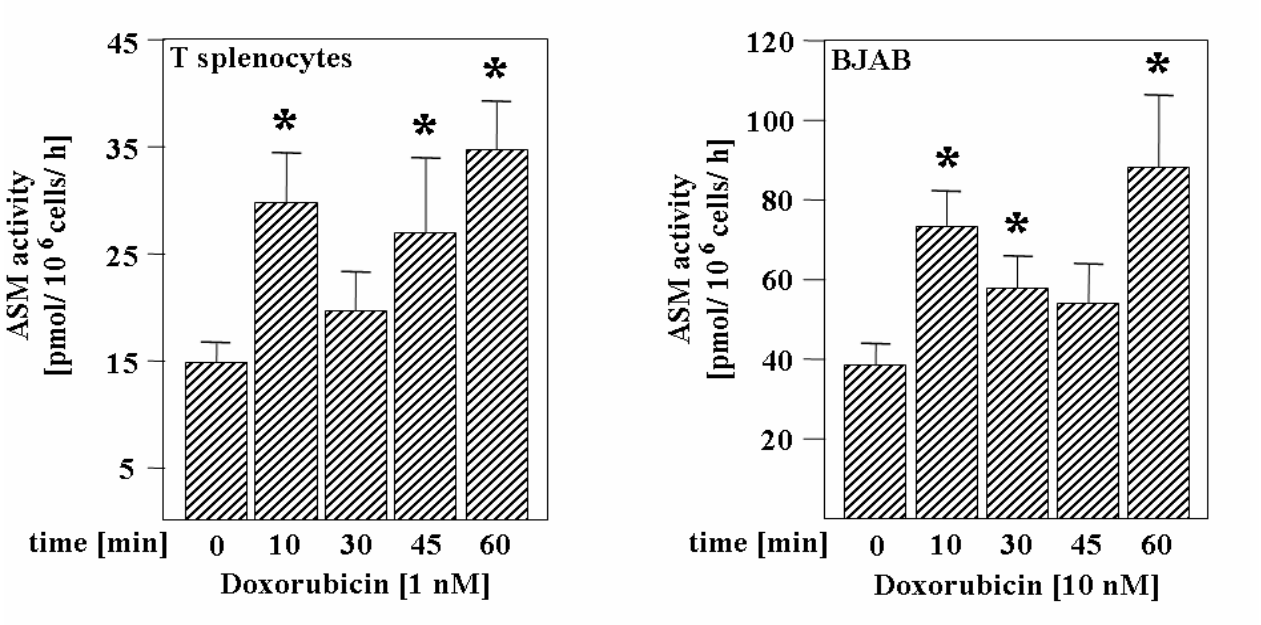


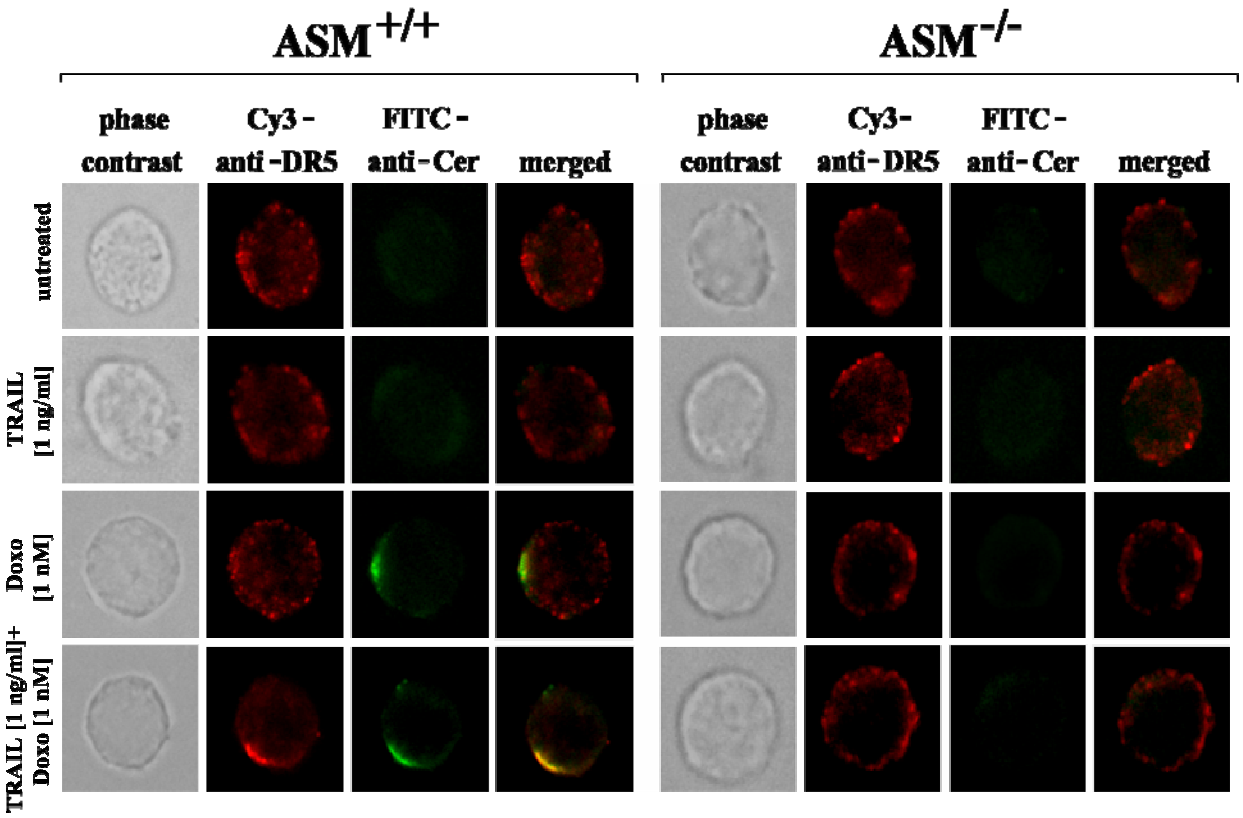
Figure 4.18. Doxorubicin treatment induces an ASM-dependent ceramide release
(**a**) ASM wild-type splenocytes were incubated with increasing doses of doxorubicin for 8 h. FACS analysis was performed after staining with FITC-Annexin V. The results are representative for 2 similar experiments.
(**b**) ASM wild-type and ASM-deficient splenocytes were treated with 1 nM doxorubicin and ceramide levels were assessed by DAG-assay. The data reflect the mean ± S.D. of 3 independent experiments.
(**c**) ASM wild-type T-splenocytes and BJAB cells were incubated with 1 nM or 10 nM doxorubicin, respectively, and activation of ASM was determined in both cell types. The data are the mean ± S.D. of 3 independent experiments.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated cells or between ASM-positive and -negative cells are indicated by an asterisk* or a delta Δ, respectively.

4.4.5. ASM mediates clustering of DR5 upon treatment with doxorubicin

Previous studies indicated that ceramide-enriched membrane platforms serve to re-organize and cluster activated receptor molecules in the plasma membrane, which is pre-requisite for efficient signaling via these receptors (Grassme *et al.,* 2001a, b; Grassme *et al.,* 2002b; Grassme *et al.,* 2003b; Cremesti *et al.,* 2001; Abdel-Shakor *et al.,* 2004).). In this part of the study it was investigated whether doxorubicin-induced ceramide release is able to mediate DR5 clustering in cells treated with doses of TRAIL that are too low to induce ceramide release and DR5-clustering by themselves. The results confirm this hypothesis and show clustering of DR5 within ceramide-enriched membrane platforms after combined stimulation with low doses of doxorubicin and TRAIL, while single stimulations were without effect (Figure 4.19a-b). ASM-deficiency prevented the release of ceramide upon treatment with doxorubicin and, consequently, also the clustering of DR5 after combined treatment with

doxorubicin/TRAIL (Figure 4.19a-b). Control experiments confirmed that low doses of TRAIL were insufficient to release ceramide (Figure 4.20).

a



b

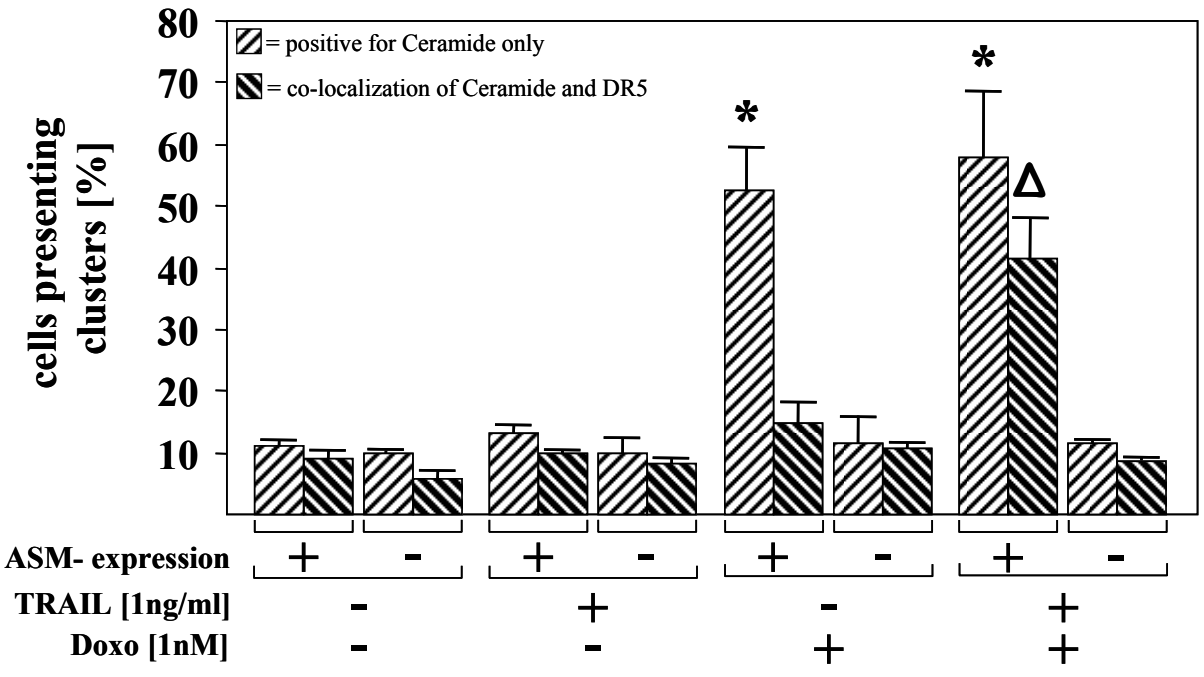


Figure 4.19. ASM mediates clustering of TRAIL upon treatment with doxorubicin
(**a**) ASM wild-type and ASM-deficient T-splenocytes were stimulated with 1 ng/ml TRAIL or 1 nM doxorubicin separately or in combination. Fluorescence microscopy analysis was performed using FITC-coupled anti-ceramide antibodies 15B4 and Cy3-coupled anti-DR5 antibodies. The results are representative for 3 independent experiments.
(**b**) Quantitative analysis of ASM wild-type and ASM-deficient splenocytes presenting ceramide platforms and/or DR5 clusters after stimulation with 1 ng/ml TRAIL and/or 1 nM doxorubicin.
Significant differences ($p \leq 0.05$, *t*-test) between unstimulated cells and cells presenting ceramide platforms or ceramide/DR5 cluster are indicated by an asterisk* or a delta Δ, respectively.

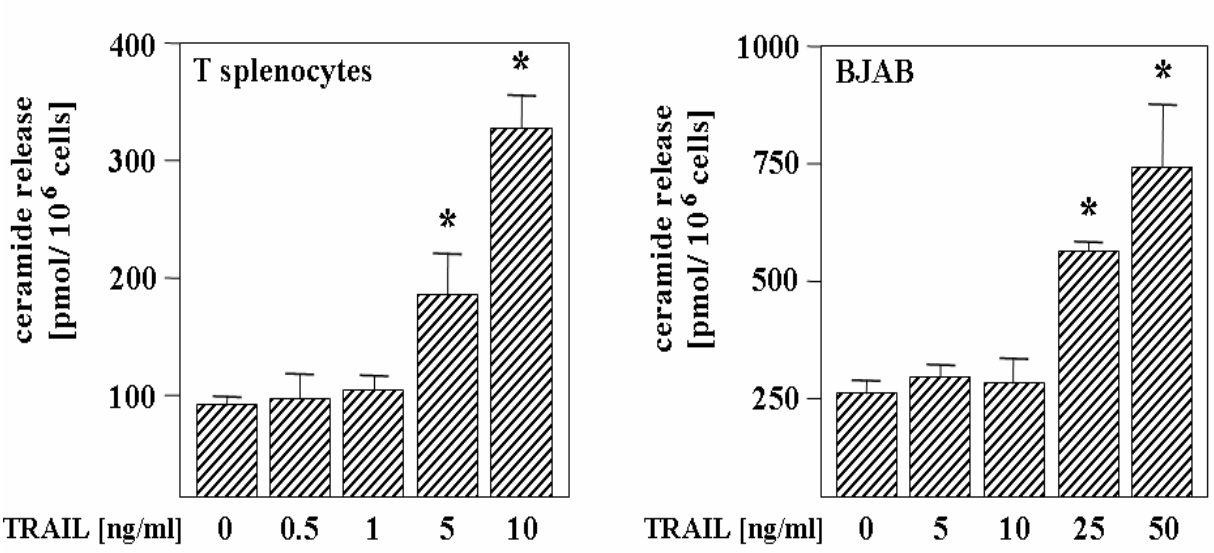


Figure 4.20. Low doses of TRAIL do not induce ceramide release.
ASM-positive splenocytes and BJAB cells were treated with different concentrations of TRAIL and ceramide levels were assessed by DAG-assay. The data show the mean ± S.D. of 3 independent experiments.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated cells are indicated by an asterisk*.

4.4.6. ASM mediates the amplification of TRAIL-induced apoptosis by doxorubicin

The data described above show that receptor clustering is a crucial step for induction of apoptosis via TRAIL/DR5. Next, it was determined whether doxorubicin-induced release of ceramide and clustering of DR5 is also sufficient to convert very low doses of TRAIL into a potent apoptotic stimulus. The results show that neither low doses of doxorubicin nor of TRAIL applied as single reagents were sufficient to induce apoptosis in splenocytes, while the combination of both triggered apoptosis in 43 ± 7% of the cells, already at 8 h after stimulation (Figure 4.21). Deficiency of the ASM prevented apoptosis following treatment with doxorubicin and TRAIL (Figure 4.21), indicating that the ASM and ceramide are central for the amplification of TRAIL induced cell death by doxorubicin.

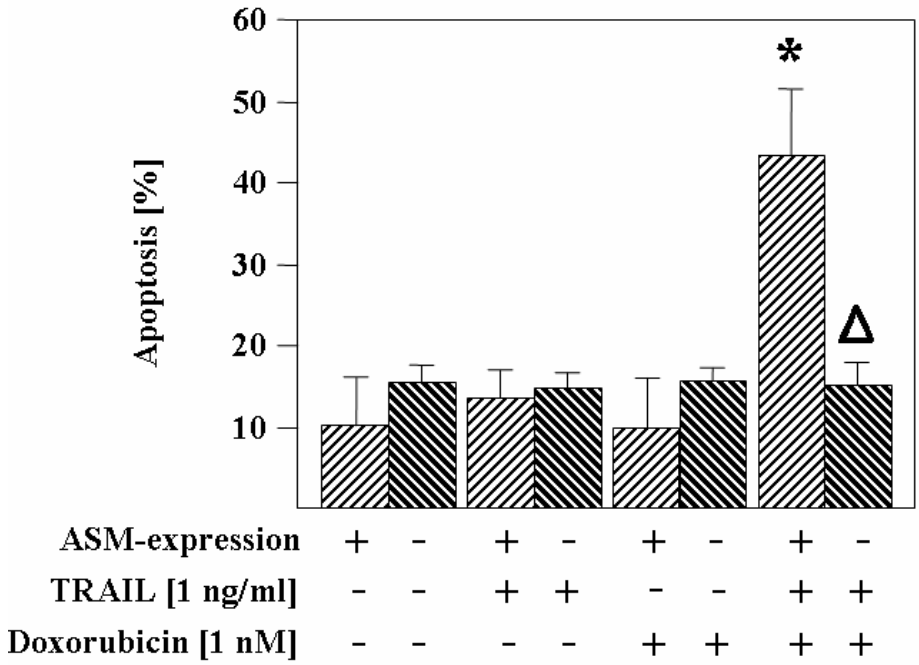


Figure 4.21. ASM mediates the amplification of TRAIL signalling by doxorubicin
ASM wild-type and ASM-deficient splenocytes were treated with either 1 ng/ml TRAIL, 1 nM doxorubicin, or both. Cells were stained with FITC-Annexin V 8 h post treatment and apoptosis was determined by FACS. The results show that ASM-positive, but not ASM-negative splenocytes are sensitive to combined treatment with TRAIL and doxorubicin. The data depict the mean ± S.D. of 4 independent experiments.
Significant differences ($p \leq 0.05$, *t*-test) between stimulated and unstimulated cells or between ASM-positive and -negative cells are indicated by an asterisk* or a delta Δ, respectively.

5. DISCUSSION

5.1. Discussion of the Methods

5.1.1. Ceramide reconstitution by addition of exogenous C_{16}-Ceramide

In the present study, C_{16}-Ceramide was used to reconstitute ceramide in ASM-deficient cells or tumour cells treated with very low doses of TRAIL that were unable to induce ceramide release by themselves. C_{16}-Ceramide (N-hexadecanoylsphingosine) mimics natural ceramide, since most physiological ceramides display C_{16}-through C_{26} chains. Moreover, mass spectrometry (MS) analysis identified C16:0 fatty acid as the predominant molecular species of ceramide generated upon early CD95 activation with lesser elevations of ceramides containing C24:0 saturated and C24:1 mono-unsaturated fatty acids compared to the control group (Kolesnick and co-workers, personal communication). Since natural, long-chain ceramide is not water-soluble and is difficult to insert from outside into a phospholipid bilayer, synthetic short-chain ceramides, particularly C_2-Ceramide (*N*-acetylsphingosine) and C_6-Ceramide (*N*-hexanoylsphingosine), which are water soluble (form micelles) and membrane-permeable, are widely used as experimental tools (Venkataraman *et al.*, 2000; Kolesnick *et al.*, 2000). However, use of these exogenous short chain ceramide analogues led to much of the current confusion in the literature on the role of ceramide in cellular signalling due to the fact that effects of short-chain ceramide cannot be extrapolated to natural ceramide species.

The main difference between C_2-Ceramide and natural ceramides refers to the different partitioning and behaviour in bio-membranes. C_2-Ceramide, similar to DAG, tends to form and to promote a hexagonal, non-bilayer, inverted micellar structure. It disorders (fluidizes) the membrane and causes lipid-packing defects in the bilayer (Simon *et al.*, 1998). This contrasts with natural long-chain ceramides, which have an ordering/packing (rigidizing) effect on the phospholipids in the membrane and stabilizes the gel phase (Veiga *et al.*, 1999; Holopainen *et al.*, 1997). Solely for this reason, C_2-Ceramide is suspected to have a different lateral distribution in the membrane, and is also likely to differ with regard to its distribution/partitioning into microdomains such as rafts and caveolae. Also important is the fact that natural ceramide stays relatively tightly bound to the membrane where it is generated

(Venkataraman *et al.*, 2000; Chatelut *et al.*, 1998) and any possible interaction with a target protein must occur in this membrane or in a membrane vesicle derived directly (by fission) from this membrane. C_2-Ceramide, on the other hand, due to its amphiphilic nature, can leave the (plasma) membrane and translocate, via the cytosol, to other membranes inside the cell.

C_2-Ceramide has also a pro-apoptotic effect that may be related to its facile intracellular diffusion. For example, exogenously applied C_2-Ceramide can reach the endoplasmic reticulum, where it inhibits the CDP-choline pathways for the biosynthesis of phosphatidylcholine (PC) and phosphatidylethanolamine (Allan. D, 2000; Bladergroen *et al.*, 1999; Ramos *et al.*, 2000; Ramos *et al.*, 2002). The blockade of phospholipid synthesis/turnover by C_2-Ceramide may severely impede intracellular vesicular trafficking (McMaster C.R., 2001) and is a direct cause of apoptosis in at least some cell types, since supplementation of, for example, PC by other means (such as acylation of lyso-PC) rescues the cells from apoptosis induction (Ramos *et al.*, 2000; van der Luit *et al.*, 2002). Other pro-apoptotic effects of short-chain ceramide that may depend on its easy intracellular diffusion are seen in mitochondria, on the translocation and activation of protein kinase C zeta (PKCζ) (Calcerrada *et al.*, 2002), and on the stimulation of formation of endogenous, long-chain ceramide.

Taken together, these data point out the importance of using C_{16}-Ceramide or other long-chain ceramides when the physiological effects of the natural ceramides are to be mimicked, as performed in the present study.

5.1.2. Ceramide measurement by DAG-kinase assay

The crucial role of ceramide in numerous cellular processes and particularly stress responses, has led to the necessity of developing rapid and quantitative assays for ceramide determination. Several methods have been developed for quantifying ceramide, among which normal phase HPLC analysis after derivatization with a fluorescent tag (Iwamori *et al.*, 1979; Previati *et al.*, 1996; Yano *et al.*, 1998; Couch *et al.*, 1997) or by evaporative light-scattering detection (McNabb *et al.*, 1999), HPTLC analysis (Motta *et al.*, 1994), or cell labelling with radioactive precursors (Tepper *et al.*, 2000; Allan D., 2000). Ceramide molecular species can be determined following hydrolysis and analysis of the liberated and derivatized sphingoid

bases by means of HPLC (Smith and Merril, 1995; Nishimura and Nakamura, 1985) and fatty acids by means of GC/MS (Samuelsson and Samuelsson, 1970). New quantitative analyses of ceramide molecular species have been developed and are based on HPLC or RP-HPLC separation of their fluorescent analogs prepared after derivatization with anthroyl cyanide (Yano *et al.*, 1998), benzoyl chloride (Couch *et al.*, 1997), or benzoic anhydride (Iwamori *et al.*, 1979). Moreover, mass spectrometry methodologies have been developed for the detection of ceramide molecular species (Watts *et al.*, 1999; Couch *et al.*, 1997; Allan D., 2000; Kalhorn and Zager, 1999; Mano *et al.*, 1997; Gu *et al.*, 1997; Liebisch *et al.*, 1999; Karlsson *et al.*, 1998). However, most of these methods require lengthy periods of processing and/or analysis.

The DAG-kinase assay (or DG kinase assay) has become the most common method for the quantification of ceramide. The primary advantages of the DAG kinase assay are the measurement of total mass levels of ceramide; the use of crude lipid extracts in the assay; and the ability to process a large number of samples in a rapid manner. DAG kinase activity was originally reported by Hokin and Hokin (1959). The enzyme was validated as an analytical tool in measuring diglyceride levels by the demonstration of a linear relationship between the amount of diglyceride added to an *in vitro* assay and the amount of product (phosphatidic acid) formed. Ceramides share structural similarities with diglycerides, and Schneider and Kennedy reported that bacterial DAG kinase can utilize ceramide as a substrate with a K_m nearly five times greater than that for diglyceride (Schneider and Kennedy, 1973). Early attempts to use DAG kinase to quantify ceramide revealed a linear but non-quantitative relationship between substrate added and product formed. Further modification of the assay demonstrated that DAG kinase could also be used for quantitative conversion of ceramide to ceramide-1-phosphate over a range of 25 pmol to 2 nmol (van Veldhoven *et al.*, 1995). These refinements have required special emphasis on the protocol of lipid extraction, purity of the reagents used for the preparation of the mixed micelles and on the development of high levels of recombinant DAG kinase (see below).

The nonpolar properties of ceramide require that it be extracted from cells in organic solvents. This is accomplished by lysing the cells in a solution containing chloroform and methanol. Acidification of the lysate by using hydrochloric acid helps to extract also shorter acyl chain ceramide-1-phosphates or hydroxylated ceramides, thus being important to gain optimal usage of the exogenously added ceramides or internal standards. In the DAG kinase reaction, presentation of the substrate in a soluble form to the enzyme is critical for its optimal

conversion to product. Mixed micelles containing a non-ionic detergent, such as n-octyl-ß-D-glucopyranoside, and a phospholipid, such as cardiolipin, are utilized for this purpose. Of particular importance is the level of ceramide conversion to ceramide-1-phosphate. For the DAG kinase assay to yield reliable quantitative results, the reaction must go to completion with total conversion of DAG and ceramide. Otherwise, the results become sensitive to the effects of the efficiency of the reaction (K_m and V_{max} consideration of the DAG kinase) and to possible 'competition' between DAG and ceramide as substrates. In the present study, an excess of enzyme and ATP was used which allowed linear and quantitative conversion and was sufficient for the phosphorylation of cellular ceramides as well as exogenously added ceramide.

5.1.3. Determination of acid sphingomyelinase (ASM) activity in cell lysates

In determining sphingomyelinase enzymatic activity two different situations must be distinguished: assays of pure enzyme and assays of cell extracts. When purified enzyme preparations are available, no labelled substrate is required. Natural or synthetic sphingomyelin is prepared, pure or mixed with other lipids, in the form of extruded large unilamellar vesicles (LUV) cca. 100 nm in diameter (Richards *et al.*, 1986). When LUV are assayed with sphingomyelinase, ceramide production in the bilayers leads to vesicle aggregation, which in turn produces an increase in turbidity or light scattering in the suspension. Thus the reaction can be followed in real time just by measuring the increase in turbidity (absorbance at 500 nm) or in light scattering (e.g. with a fluorometer with both the excitation and emission monochromators adjusted at 500 nm).

Assaying sphingomyelinase activity in cell lysates requires that sphingomyelin be labelled, radioactively, fluorescently or otherwise. In the present study, the enzymatic activity was measured as the degradation of radioactive [^{14}C]sphingomyelin to ceramide and phosphorylcholine. Since the choline group is soluble in water and contains the radioactive label, released radioactivity can be easily separated from the substrate, which partitions into the organic phase following extraction. Sphingomyelin can serve as substrate for three forms of sphingomyelinases that manifest acid, neutral or basic pH optima for maximal enzyme activity (Hannun Y.A., 1996). In the present study acid sphingomyelinase (ASM) activity was discriminated from the neutral or basic sphingomyelinase activity by performing the assay at

pH 5.0. Because of the difficulty in bringing together the enzyme and substrate molecules in the presence of cell homogenates, a suitable detergent, such as Triton X-100 was used. It should be noted that, apart from emulsifying the substrate, the detergents bind and modify the enzyme activity, thus detergent concentration and initial detergent:substrate ratio was kept constant for reproducibility of assays. Finally, it has been suggested that the ASM may be located in detergent-resistant/insoluble fractions; thus, for determination of ASM activity in whole-cell lysates, no centrifugation step was performed after lysis and sonication of the cells to prevent pelleting and loss of the ASM.

5.1.4. Analysis of aggregated molecules and co-localized signals

Of particular importance for the present study was the detection of clustered DR5 receptors and /or ceramide platforms on the surface of stimulated cells. This was achieved by fluorescent microscopical analysis of receptor and ceramide molecules after staining with the respective fluorochrome-labeled antibodies. The major problem in detection of aggregated molecules onto the plasma membrane is the possibility of superposition of two or more membranes within the microscopical field of interest. This would lead to an amplification of the fluorescent signal and a false-positive result. To avoid this artefact, single-cell suspensions were obtained and cell clumps were disregarded during counting of cells.

Many of the present experiments involved, however, analysis of clustered DR5 receptors which co-localized with ceramide platforms. When two or more stainings are performed simultaneously, the fluorescence signals might be recorded in one detection channel and might no longer be separated in the images. This is called ‘cross-talk’ or ‘bleeding-through’ of the fluorescence signals. Apart from preparing single-stained specimens as controls, ‘sequential image analysis’ of the respective samples by confocal microscopy was employed to rule out these false-positive data. The advantage of sequential analysis regarding multiple stainings lies in the ability to record a certain optical section using an instrument parameter settings (detection channel and excitation wavelength) adapted to one fluorochrome, before the system switches to different instrument parameter settings adapted for another fluorochrome. Therefore, this method is particularly useful to exclude the cross-talk between multiple fluorescent signals.

5.1.5. ASM-deficient animals

To determine the importance of ASM in the present study, splenocytes from ASM-deficient mice were used. The ASM knock-out mouse was originally generated in the laboratory of Dr. Kolesnick (Memorial Sloan-Kettering Cancer Center, NY, USA) and has proven to be a valuable tool in investigating the role of ASM in different cellular processes. However a study from Nix and Stoffel (2000) reported marked biochemical alterations and membrane dysfunction in cells derived from their line of ASM knock-out mice such as: increase of sphingomyelin and gycosphingolipids in the plasma membrane of hepatocytes, reduction of caveolin levels in embryonic fibroblasts, reduced signalling through tyrosine kinases in T lymphocytes, lymphopenia, the absence of proliferation of T cells in response to anti-CD3, reduced expression of the anti-apoptotic adapter FLIP, and a paradoxical increase in apoptosis of anti-CD3 pre-treated splenocytes upon activation of CD95 (Nix and Stoffel, 2000). Therefore, the authors concluded that the previously reported apoptotic abnormalities in ASM-deficient cells and tissues (Santana *et al.*, 1996; Zundel and Giaccia, 1998; Zundel *et al.*, 2000; Cifone *et al.*, 1995; Lin *et al.*, 2000; Pena *et al.*, 2000; Perez *et al.*, 1997; Morita and Tilly, 2000) did not result merely from ASM deficiency, but rather were impacted by disruption of membrane microdomains in response to altered sphingolipid metabolism (Nix and Stoffel, 2000).

However, Lozano and co-workers pointed out that the phenotype of ASM- deficient mouse line used in the study of Nix and Stoffel was different from the mouse line generated in the laboratory of Dr. Kolesnick, which displayed, up to a certain age (12-16 weeks), only a minimal increase in sphingomyelin content, unchanged levels of caveolin-1, normal MAP-kinase signalling and tyrosine phosphorylation patterns, no lymphopenia, normal T cells proliferation and no decrease in FLIP levels (Lozano *et al.*, 2001). Furthermore, the life expectancy of around 9-10 months (Santana *et al.*, 1996; Pena *et al.*, 2000) was in contrast with that of the mice generated by Stoffel and co-workers, who reported that the life span of their ASM- deficient mice was maximally 4 months, with mice succumbing to advanced Niemann-Pick disease type A (Otterbach and Stoffel, 1995).

The mice used in the present study show the earliest clinical manifestation of Niemann-Pick disease type A between 12-16 weeks of age; therefore, all the experiments involving cells from ASM-deficient mice were carried out with animals younger than 12 weeks of age, before any biochemical, histological or clinical manifestations of Niemann-Pick disease type

A were apparent. This excluded that the effects observed in the ASM-deficient cells were due to altered cellular processes (as described above) but instead, were completely dependent on the lack of ASM.

5.2. Discussion of the Results

5.2.1. The TRAIL/DR5 system

In the present study new key events in TRAIL-induced apoptotic pathway upon binding of the ligand to the DR5 receptors are characterized. Although TRAIL can induce apoptosis also upon binding to DR4 receptors, the interaction of TRAIL with the DR5 receptors seems to play the most important role for TRAIL-induced apoptosis. Studies which investigated the affinity of TRAIL for its receptors showed that although DR4 and DR5 have similar affinities for TRAIL at 4°C, the affinities are substantially different at physiological temperature (37°C), with DR5 having the highest affinity ($K_D \leq 2$ nM), while DR4 has a much lower affinity ($K_D \leq 70$ nM) (Truneh *et al.*, 2000). Recently, Kelley *et al.* (2005) generated death receptor-selective TRAIL variants using a novel approach that enables phage display of mutated trimeric proteins. The results showed a markedly reduced ability of DR4-selective TRAIL variants to trigger apoptosis, whereas the DR5-selective variants had minimally decreased or slightly increased apoptosis-inducing activity, suggesting that DR5 may contribute more than DR4 to TRAIL-induced apoptosis in cells expressing both death receptors. Another recent study, by Lee and co-workers (2005), proposed that TRAIL receptors (both death and decoy receptors) are assembled in homomeric and heteromeric complexes prior to TRAIL binding. Interestingly, their study showed that TRAIL decoy receptors are able to form complexes with DR4, but not with DR5 and the authors suggested that in contrast to homomeric complexes of death receptors, heteromeric complexes of DR4-decoy receptors might not induce apoptosis due to incomplete interactions of the intracellular death domains necessary for the formation of the DISC. This finding supports the hypothesis of DR5 being a more efficient death-inducing receptor than DR4 at least in cells that also express decoy receptors.

An additional reason for investigating the TRAIL/DR5 system in this study was the necessity of using murine cells, which express only one TRAIL death receptor that is homologous with human DR5 (Wu *et al.*, 1999b).

5.2.2. Role of ASM and ceramide for TRAIL-induced apoptosis

The data presented here indicate that ASM is a novel key molecule for the induction of apoptosis by TRAIL/DR5. The results show that TRAIL very rapidly induces a stimulation of the ASM to release ceramide that forms large ceramide-enriched membrane domains serving to trap and cluster DR5. The reorganization of DR5 in the cell membrane into ceramide-enriched membrane domains seems to be required for the induction of apoptosis by DR5, as cells lacking ASM and, thus, unable to release ceramide after stimulation, are resistant to TRAIL/DR5-induced apoptosis, while addition of natural ceramide restored apoptosis. Furthermore, very low doses of TRAIL that are insufficient to trigger cell death by themselves are converted into potent inducers of apoptosis upon formation of ceramide-enriched membrane platforms after treatment of normal or tumour cells with C_{16}-Ceramide. Because sphingomyelin constitutes 10 to 15% of total plasma membrane phospholipids, it serves as a critical modulator of membrane fluidity (Barenholz and Thompson, 1980), and it was suggested that depletion of sphingomyelin via activation of sphingomyelinases can directly increase cell damage by fluidization of the plasma membrane (Zager *et al.*, 2000). However, the experiments employing ceramide reconstitution in the absence of ASM-induced sphingomyelin hydrolysis indicate that the release of ceramide and not just the consumption of sphingomyelin is required for TRAIL/DR5-induced apoptosis.

It is interesting to note that although the activity of the ASM and the release of ceramide were lower in T splenocytes than in the tested cell lines, the number of cells presenting ceramide-enriched membrane platforms and DR5 cluster induced by TRAIL were very similar. This suggests that a threshold of ceramide in the plasma membrane is sufficient to mediate DR5 clustering and apoptosis. The present data indicate that the central function of ASM and ceramide for the initiation of apoptosis is not restricted to CD95 and TNF, but is also required for death by DR5 and, very likely, other pro-apoptotic members of the TNF/NGF-factor receptor family. This suggests that the formation of ceramide-enriched membrane platforms

upon ASM activation is a general mechanism to initiate signalling of the TNF/NGF-factor receptor family.

Recent data by Voelkel-Johnson and co-workers (2005) confirmed the notion that ceramide is involved in TRAIL-induced death. However, these studies on colon cancer cells revealed a later increase of ceramide by TRAIL, which is consistent with a reduced sensitivity and a delayed time course of apoptosis (only 20% apoptosis 24 h after stimulation with 10 ng/ml TRAIL) in these cells compared to lymphocytes (Voelkel-Johnson *et al.,* 2005).

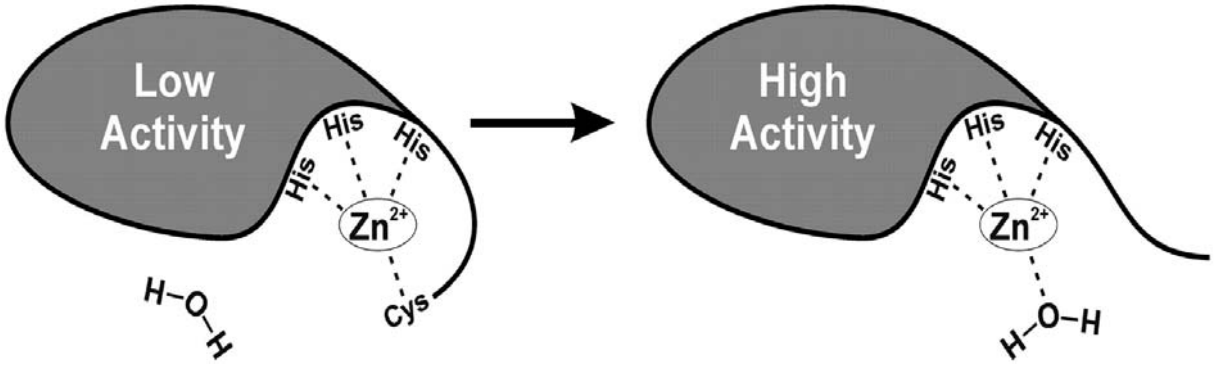
The experiments further suggest that clustering of DR5 receptors in ceramide-enriched membrane platforms is required for induction of apoptosis by TRAIL/DR5. This concept is consistent with findings on CD95 that demonstrated an activation of the ASM and the formation of ceramide-enriched membrane platforms before formation of the DISC and execution of apoptosis (Grassme *et al.*, 2003b).

5.2.3. Mechanism of TRAIL-induced ASM activation

In the present study, a new molecular mechanism of ASM activation *in vivo* is characterized, which involves a direct or indirect regulation of the enzyme via a redox mechanism. The results suggest that release of free radical oxygens (ROS) mediate activation of the ASM upon stimulation via death receptors. This notion is consistent with a previous biochemical study (Qiu *et al.*, 2003) indicating that oxidation of purified recombinant ASM (rhASM) results in activation of the enzyme via dimerization. The authors demonstrate a critical role for the C-terminal cysteine (Cys^{629}) in the enzymatic activity of rhASM. Particularly, it appears that any change that causes a loss of the free sulfhydryl group on this amino acid also results in activation of the enzyme, i.e. copper-promoted dimerization of rhASM via the C-terminal cysteine, thiol-specific chemical modification of this cysteine to form a mixed disulfide bond or a sulphur-carbon linkage, deletion of this cysteine by carboxypeptidase or recombinant DNA technology, and site-specific mutation to change the cysteine to a serine residue.

Based on the fact that zinc is required for the activity of the ASM, the authors proposed a model which explains the effect of C-terminal cysteine modification regarding the activation of the ASM. In the low activity form, the free C-terminal cysteine is involved in the active site zinc coordination, either by competing with a water molecule for coordination with zinc or by

forming a non-optimal five-ligand coordination structure (Figure 5.1). This decreases the ability of zinc to ionize water for the nucleophilic attack and leads to decreasing enzymatic activity. As thiol is a better zinc ligand than water, the non-optimal structure may be energetically favourable as long as the cysteine is freely available. In the high activity form of rhASM, however, the free cysteine is lost by either chemical modification or deletion and is no longer available for coordination. As a result, zinc coordinates with a water molecule, resulting in an optimal structure for catalysis (Figure 5.1). This model is essentially identical to the "cysteine switch" activation mechanism described previously for the matrix metalloproteinase family (van Wart and Birkeda-Hansen, 1990).



Qiu et al., J Biol Chem, 2003

Figure 5.1. Proposed model for rhASM activation through availability of the C-terminal cysteine residue

In the present study we show that stimulation of cells with TRAIL induces a rapid ROS release which mediates ASM activation, since neutralization of ROS inhibits TRAIL-induced ASM activation and subsequent events. However, whether the dimerization and/or further molecular events are required for stimulation of the ASM *in vivo*, needs to be addressed in future studies. It should be mentioned that although Tiron and N-acetylcysteine did not completely block ASM activation and TRAIL-induced apoptosis, this is most likely caused by an incomplete neutralization of ROS that immediately act with biomolecules in the very close vicinity.

Recently, experiments on hepatocytes demonstrated that an inhibitor of the ASM blocks the release of ROS suggesting that ROS functions downstream of the ASM (Reinehr *et al.*, 2006).

However, this is not necessarily in contrast to the present data. It has been recently shown for CD95, which releases and requires ROS for induction of apoptosis (Gulbins *et al.*, 1996; Um *et al.*, 1996, Reinehr *et al.*, 2006), that ligation of the receptor primarily induces a very weak recruitment of FADD and stimulation of caspase 8, which reaches approximately 1% of the levels that are observed for maximal activation of caspase 8. This weak activation of caspase 8 is even observed in ASM-deficient cells, but it is not sufficient to trigger apoptosis. However, the low activity of caspase 8 is sufficient to trigger the translocation and activation of the ASM within seconds, with the subsequent formation of ceramide-enriched membrane platforms that cluster CD95 (Grassme *et al.*, 2003b). Receptor clustering leads to DISC formation and full caspase 8 activation. Thus, the ASM functions in a feed forward loop to amplify signalling via death receptors and a blockade of this forward loop by inhibition of the ASM would also prevent the release of ROS.

5.2.4. Generation and function of ceramide-enriched membrane platforms.

Several studies in the last years linked the formation of ceramide-enriched membrane platforms to the induction of cell death. For instance, apoptosis triggered by cisplatin or doxorubicin requires functional expression of the ASM, the release of ceramide and the formation of ceramide-enriched membrane platforms (Andrieu-Abadie *et al.*, 1999; Delpy *et al.*, 1999; Morita *et al.*, 2000; Lacour *et al.*, 2004). Very similar results were described for induction of apoptosis by CD95 as shown by many groups employing a variety of different cells (Cremesti *et al.*, 2001; Grassme *et al.*, 2001a, b, 2003; Fanzo *et al.*, 2003). Furthermore, developmental apoptosis of oocytes and neutrophils is delayed by deficiency of the ASM (Morita *et al.*, 2000; Scheel-Toellner *et al.*, 2002, 2004a, b) and the formation of ceramide-enriched membrane platforms constitutes one of the earliest events linked to the induction of apoptosis in these cells. It has been shown that infection of mammalian epithelial and endothelial cells or fibroblasts with *S. aureus* or *P. aeruginosa*, respectively, induces apoptosis of the host cells via formation of ceramide-enriched membrane platforms that may serve to reorganize cellular receptors and signalling molecules involved in the induction of apoptosis by the pathogens (Grassme *et al.*, 1997, 2003a; Esen *et al.*, 2001).

Most of these studies suggested that the formation of ceramide-enriched membrane platforms is initiated within small cholesterol- and sphingolipid-enriched membrane rafts. The notion

that rafts are important for the induction of apoptosis is supported by several recent studies demonstrating that destruction of these membrane rafts prevents CD95 clustering, recruitment of FADD and caspase 8 and apoptosis by CD95 (Cremesti *et al.*, 2001; Grassme *et al.*, 2001b; Hueber *et al.*, 2002; Scheel-Toellner *et al.*, 2002). Recent studies on CD95 and CD40 demonstrated some insights into the role of lipids in receptor clustering (Grassme *et al.*, 2002a, b; Grassme *et al.*, 2001a, b): stimulation of lymphocytes via CD95 or CD40 using the cognate ligand or stimulatory antibodies induced a translocation of the ASM onto the extracellular leaflet of the cell membrane (Figure 5.2) that was detected by fluorescence microscopy and biochemical techniques. The surface translocation of the ASM occurred within seconds to minutes, suggesting that this process might be involved in the generation of the signal via the cognate receptor (Grassme *et al.*, 2002; Grassme *et al.*, 2001a, b). A surface localization of the ASM is consistent with previous findings on signalling of IL-1 (Liu and Anderson, 1995) and p75 NGF receptors (Bilderback *et al.*, 1997) that demonstrated a release of ceramide in distinct membrane domains. The directed transport of the ASM onto the extracellular leaflet of sphingolipid-enriched rafts results in close proximity of the enzyme to cellular sphingomyelin, which is contained predominantly within the outer leaflet of the plasma membrane. Consumption of sphingomyelin in rafts by the ASM was shown to trigger a release of ceramide in the outer leaflet of the cell membrane and an accumulation of ceramide in rafts (Figure 5.2) (Grassme *et al.*, 2002a, b; Grassme *et al.*, 2001a. b). These studies employed monoclonal anti-ceramide antibodies to detect ceramide in the cell membrane by fluorescence microscopy. Probably due to ceramide tendency to self-associate (Nurminen *et al.*, 2002), generation of ceramide in rafts resulted in the formation of large ceramide-enriched membrane domains that were visualized in fluorescence microscopy studies as ceramide-enriched membrane platforms (Grassme *et al.*, 2002a, b; Grassme *et al.*, 2001a, b). These platforms were already detected seconds to minutes after stimulation of CD95 and CD40 using monoclonal anti-ceramide-antibodies and fluorescence microscopy methods to analyze the cells. Staining of cells with fluorescently labelled cholera toxin (B-subunit), a typical raft marker, revealed a co-localization of cholera toxin and ceramide within the membrane platforms after stimulation via CD95 or CD40 (Grassme *et al.*, 2002a, b; Grassme *et al.*, 2001a, b), suggesting that membrane rafts and ceramide-enriched membrane platforms share at least some molecules (Figure 5.2). Furthermore, ASM on the cell surface also co-localized with ceramide and cholera toxin supporting a role of the ASM in the formation of ceramide-enriched membrane platforms. The central role of the ASM in ceramide-enriched platforms formation was finally shown in cells lacking the ASM that fail

to form cholera toxin- and ceramide-positive membrane platforms after CD95 or CD40 stimulation (Grassme *et al.*, 2002; Grassme *et al.*, 2001a, b). The function of these platforms was indicated by the finding that stimulation via CD95 or CD40 resulted in rapid clustering of these receptors in ceramide-enriched membrane platforms (Figure 5.2) (Grassme *et al.*, 2002a, b; Grassme *et al.*, 2001a, b), an observation that was recently confirmed by six independent groups studying CD95 clustering in a variety of cell types (Fanzo *et al.*, 2003). Cells lacking the ASM failed to cluster the receptor, to initiate signalling via CD95 or CD40 *in vitro* and *in vivo* (Grassme *et al.*, 2002; Grassme *et al.*, 2001a, b; Kirschneck *et al.*, 2002) and were defective in the induction of apoptosis via CD95 or the initiation of CD40 signalling. Artificial, forced cross-linking of the receptor molecules by addition of very high concentrations of stimulatory antibodies restored at least partially the signalling initiated by these receptors even in cells lacking the ASM (Grassme *et al.*, 2002b; Grassme *et al.*, 2001a, b; Kirschneck *et al.*, 2002) indicating that the cross-linking event functions as a requirement for signal initiation by these receptors (Figure 5.2). Very similar results were obtained with reagents that destroy sphingolipid-enriched rafts (Grassme *et al.*, 2002a, b; Grassme *et al.*, 2001a, b; Kirschneck *et al.*, 2002). This certainly does not exclude that other receptors employ different mechanisms to cluster and/or transform rafts into a larger membrane platform.

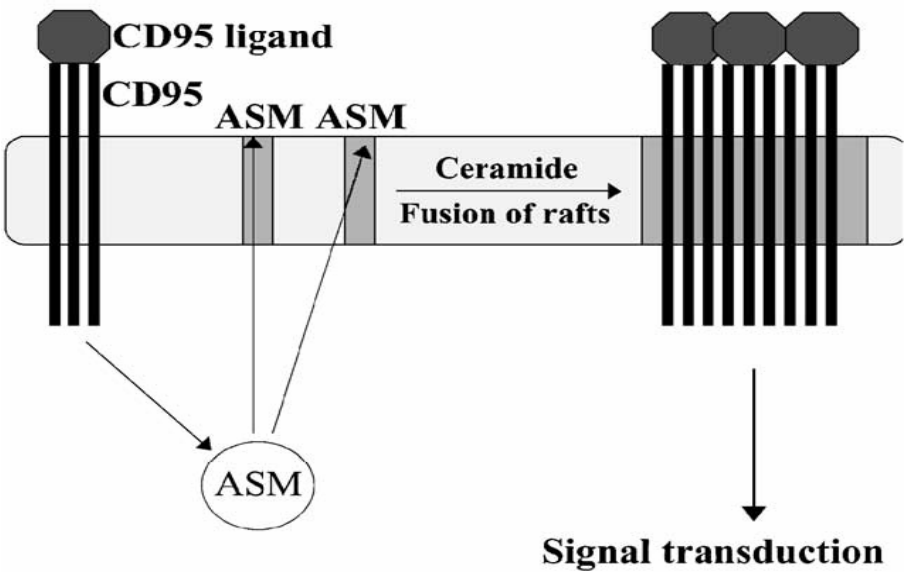


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Figure 5.2. Model of ASM and ceramide-mediated receptor clustering in membrane platforms

5.2.5. Mechanism of death receptors clustering in ceramide-enriched membrane platforms.

Although many receptors have been shown to cluster in detergent-resistant membrane domains, molecular mechanisms mediating the preferential partitioning of receptors, including DR5, in those domains require definition. Recently, Bock and Gulbins (2003) proposed a mechanism of clustering for CD40, which might explain also the ability of DR5 to cluster in ceramide-enriched membrane platforms upon TRAIL ligation. These studies employed chimeric proteins that consisted of the extra- and intracellular domain of CD40 and the trans-membranous domain of CD45. CD45 is known to localize outside the rafts, at least under most circumstances. The results identified the trans-membranous domain of CD40 as an important element of the receptor to interact with sphingolipid-enriched rafts and to cluster in membrane platforms upon stimulation, since CD40 receptors with mutated trans-membranous domain (CD40/CD45 mutants) failed to cluster. According to this model, the failure of the CD40/45 mutant to cluster was caused by neither inactivity of the mutant protein, nor a large difference in the length of the trans-membranous domains of CD40 and CD45. Although the individual amino acid residues composition of the respective domains (i.e. number and/or location of the hydrophilic amino acids) might mediate the different abilities of the receptor molecules to cluster within lipid rafts, the authors also suggested that the release of ceramide in rafts not only triggers fusion of those rafts to larger platforms, and alters the hydrophobicity of the membrane domain, but may also alter the diameter of the cell membrane in the platform area. Binding of the ligand may change the conformation of the receptor to fit into a ceramide-enriched membrane domain, while the presence in membrane parts with other diameters might be energetically unfavourable for the ligand-receptor complex.

Clustering of receptor molecules within ceramide-enriched membrane platforms might have several functions: Receptor clustering in ceramide-enriched signalling platforms might function to induce a close proximity of many receptor molecules. The high receptor density may greatly facilitate trans-activation of signalling molecules associating or interacting with a receptor and, thus, permitting specific signalling of the activated receptor. This density model is reminiscent to blood coagulation that is initiated in a small area by the concentration of pro-coagulatory enzymes and the concomitant exclusion of inhibitory enzymes. In addition to induction of a high receptor density, ceramide-enriched platforms might also facilitate the contact between a receptor and intracellular signalling molecules, which are constitutively

present in rafts, e.g. small G proteins or Src-like tyrosine kinases (Field *et al.*, 1995). Further, the presence of receptors in and the interaction with ceramide may alter the conformation of a receptor to increase the affinity/avidity of the receptor for its ligand and, thus, stabilize the receptor/ligand interaction. Finally, raft-ceramide might directly activate intracellular signalling molecules and, thus, further modify the signalling of an activated receptor. Those signals might be generated directly by ceramide or upon metabolism of ceramide. For instance, it has been recently demonstrated that ceramide is involved in the recruitment of caveolin 1 to the cell membrane upon cellular stimulation (Zundel *et al.*, 2000). In addition, ceramide in rafts might be metabolized to secondary messengers that alter the activity of enzymes or organelles involved in cellular signalling of the specific receptor. In this context, De Maria *et al.* (1998) demonstrated synthesis of GD3 from ceramide upon CD95 stimulation that was transported to and acts in mitochondria to induce apoptosis.

5.2.6. Possible additional roles of ceramide as activator of intracellular molecules for TRAIL-induced apoptosis

Ceramide has been commonly stated to be also second messenger in apoptotic signalling. Several direct or indirect intracellular targets for ceramide have been proposed, such as JNK (Westwick *et al.*, 1995; Brenner *et al.*, 1997), PKC (Bourbon *et al.*, 2000; Lozano *et al.*, 1994; Muller *et al.*, 1995; Procyk *et al.*, 2000), KSR (Joseph *et al.*, 1993; Liu *et al.*, 1994; Mathias *et al.*, 1991; Zhang *et al.*, 1997), cathepsin D (Heinrich *et al.*, 1999) or PP2A (Dobrowski *et al.*, 1993; Law *et al.*, 1995). Ceramide can activate JNK via Rac-1 (Brenner *et al.*, 1997), PKC ζ (Boubon *et al.*, 2000; Lozano *et al.*, 1994), or TAK-1 (Shirakabe *et al.*, 1997). The exact role for JNK in ceramide-induced cell death pathways is still unclear; however, the identification of novel targets for JNK including c-jun (Jarvis *et al.*, 1996; Basu *et al.*, 1998b; Verheij *et al.*, 1996), Bcl-2 (Ruvolo *et al.*, 1999; Ruvolo *et al.*, 2002), and the transcription factors AP-1 and GADD153 (Basu *et al.*, 1998b; Brenner *et al.*, 1997; Sawai *et al.*, 1995) suggest possible mechanisms how ceramide activation of JNK may promote cell death. Interestingly, Bcl-2 has been shown to be a negative regulator of TRAIL-induced intrinsic apoptotic pathway (Wen *et al.*, 2000; Burns and El-Deiry, 2001) and inactivation of Bcl-2 by JNK (Maundrell *et al.*, 1997; Yamamoto *et al.*, 1999) could promote TRAIL-induced apoptosis at least in type II cells.

Ceramide has also been shown to regulate apoptosis via PKCs. In particular, ceramide-induced PKC ζ activation (Bourbon *et al.*, 2000; Lozano *et al.*, 1994; Muller *et al.*, 1995; Procyk *et al.*, 2000) can modulate apoptosis through multiple pathways. PKC ζ has been shown to inhibit Akt/PKB (Doornbos *et al.*, 1999), which is another negative regulator of TRAIL-induced apoptosis (Beresford *et al.*, 2001; Nesterov *et al.*, 2001). Ceramide activation of PKC ζ appears to be essential also for SAPK/JNK pathways in some cell types since a dominant negative PKC ζ protein can block SAPK activation and inhibit anti-proliferative responses when cells are treated with ceramide (Bourbon *et al.*, 2000). Ceramide appears to directly activate PKC ζ, perhaps by binding to the putative ceramide-binding region of the protein, the cysteine-rich domain (Bourbon *et al.*, 2000; Hurley *et al.*, 1997). Since PKC ζ is a direct target of ceramide but is unable to act on Rac-1 directly (Uberall *et al.*, 1999), it is very possible that ceramide-mediated SAPK signalling cascades involving PKC ζ are distinct from pathways initiated by Rac-1.

Ceramide has been reported to stimulate the activity of the guanine nucleotide-exchange factor Vav in a protein kinase independent manner (Gulbins *et al.*, 1994). Vav activation of the small GTPases appears to have a role in ceramide-mediated apoptosis (Esteve *et al.*, 1998). It is possible that this mechanism may involve also the activation of Rac-1-mediated signalling cascades since introduction of dominant-negative N17Rac-1 into Jurkat cells prevents SAPK activation and suppresses apoptosis (Brenner *et al.*, 1997).

Phosphatase A2 (PPA_2) is a major protein serine/threonine phosphatase that regulates many signalling pathways in mammalian cells (Mumby and Walter, 1993). PPA_2can negatively regulate pro-growth kinases such as Akt/PKB (Schubert *et al.*, 2000; Salinas *et al.*, 2000) or anti-apoptotic molecules such as Bcl-2 (Ruvolo *et al.*, 1999; Ruvolo *et al.*, 2002). Since ceramide is a potent apoptotic agent and, in general, is antagonistic to pro-growth signalling pathways (Smyth *et al.*, 1997; Hannun YA., 1996; Ruvolo PP., 2001) it is not surprising that ceramide has been found to activate PPA_2 (Dobrowsky *et al.*, 1992; Dobrowsky *et al.*, 1993a, b; Ruvolo *et al.*, 1999). Ceramide promotes PPA_2 de-phosphorylation of Bcl-2 resulting in the loss of Bcl-2 anti-apoptotic function (Ruvolo *et al.*, 1999). Since expression of S70E Bcl-2, a non-phosphorylable, activated form of Bcl-2, can protect cells from ceramide-induced apoptosis, one likely mechanism by which ceramide induces cell death is by functionally inactivating Bcl-2 via de-phosphorylation (Ruvolo *et al.*, 1999).

The kinase suppressor of Ras (KSR) was one of the first non-SAPK enzymes to be identified as a ceramide-activated protein kinase (CAPK) (Joseph *et al.*, 1993; Liu *et al.*, 1994; Mathias *et al.*, 1991; Zhang *et al.*, 1997). Experiments using dominant-negative mutants suggest that KSR is required in both TNF alpha and ceramide induced ERK1/ERK2 pathway and involves the activation of Raf-1 (Yan and Polk, 2001). Ceramide has been shown to activate Raf through Ras (Salinas *et al.*, 2000; Huwiler *et al.*, 1996). Although in many systems activation of the Ras, Raf-1 and MAPK pathway is mitogenic and anti-apoptotic (Xia *et al.*, 1995; Cuvillier *et al.*, 1996), recent studies demonstrated that ceramide can induce apoptosis via KSR, Ras, and Raf-1 when the pro-apoptotic Bcl-2 family member, BAD, is present (Basu *et al.*, 1998a). The KSR/Ras/Raf-1 pathway linked to BAD de-phosphorylation by prolonged inactivation of Akt/PKB (Basu *et al.*, 1998a). Since TRAIL-induced apoptosis has been shown to be inhibited by serum via an Akt-dependant BAD phosphorylation (Kang *et al.*, 2004) and ceramide can block the Akt/PKB pathway (Basu *et al.*, 1998a; Zhou *et al.*, 1998, Summers *et al.*, 1998), it is not excluded that ceramide might regulate TRAIL-induced apoptosis via BAD de-phosphorylation.

Ceramide has been shown to signal an increase in the levels of cytosolic phospholipase A_2 ($cPLA_2$) (Hayakawa *et al.*, 1996). A recent study by Klapisz and co-workers (2000) demonstrated by *in situ* and *in vivo* experimental approaches that membrane microdomains enriched in sphingomyelin and cholesterol modulate the activity of $cPLA_2$ via ceramide, since conversion of sphingomyelin to ceramide by *S. aureus* sphingomyelinase enhanced $cPLA_2$ activation and arachidonic acid production (Klapisz *et al.*, 2000). $cPLA_2$ catalyzes the hydrolysis of the *sn*-2 position of glycerophospholipids to release free arachidonic acid, which in turn is metabolized to prostaglandins by the cyclooxygenase pathway and to leukotrienes by the 5-lipoxygenase pathway (Shimizu and Wolfe, 1990). Although $cPLA_2$ and/or arachidonic acid have been linked to induction of apoptosis (Rizzo *et al.*, 1999; Monjazeb *et al.*, 2006; Kirschnek and Gulbins, 2006), the mechanisms mediating induction of apoptosis are not completely clear. It has been suggested that arachidonic acid may induce apoptosis via reactive oxygen species (Kwon *et al.*, 2005; Wie *et al.*, 1999) or activation of transcription factor c-jun (Monjazeb *et al.*, 2006). Whether these mechanisms may play a role in TRAIL-induced apoptosis remains to be determined.

A novel intracellular target for ceramide has been identified as the aspartic protease cathepsin D (Heinrich *et al.*, 1999). The authors show that an ASM-derived ceramide specifically binds to and induces cathepsin D proteolytic activity in endolysosomal compartments, since ASM-

deficient cells derived from Niemann-Pick patients show decreased cathepsin D activity (Heinrich *et al.*, 1999). Recently, the same group demonstrated also a TNF-induced activation of cathepsin D via the ASM (Heinrich *et al.*, 2004). The authors propose a model of TNF signalling which involves receptor internalization and caspase-8-dependant activation of the ASM in endolysosomal compartments. Ceramide generated by the ASM, in turn, activates cathepsin D which translocates to the cytosol and cleaves Bid. The resulting tBid induces cytochrome c release from mitochondria and activation of caspase-9 and-3, leading to apoptotic cell death by the intrinsic pathway.

The studies summarized above indicate that ceramide could potentially regulate TRAIL signalling at different levels of the apoptotic pathway. However, most of these mechanisms seem to involve the intrinsic apoptotic pathway, which was shown to play a relevant role only in some cell types that need the mitochondria-initiated pathway for successful induction of apoptosis (type II cells). Furthermore, the experiments employing forced cross-linking of DR5 receptors in the absence of ceramide support the crucial role of receptor clustering for TRAIL-induced apoptosis.

5.2.7. Clinical relevance of ceramide-mediated TRAIL-induced apoptosis

The present study suggests a novel mechanism how the pro-apoptotic potential of TRAIL might be employed to kill tumour cells without affecting normal cells. The results show that neither low doses of TRAIL nor of C_{16}-Ceramide are able to kill tumour cells or *ex vivo* stimulated T splenocytes. In contrast, the combination of both stimuli was able to kill a panel of tumour cells with extremely high efficiency and almost all tumour cells were killed within 24 h after combined treatment. The transfer of this concept to a clinical situation predicts that very low doses of TRAIL and an inducer of local ceramide are sufficient to kill tumour cells, while TRAIL at this dose will be without effect in other organs. Candidates for local production of ceramide are ionizing radiation, UV-light, locally applied cytostatic drugs or heat.

The potential of the cytostatic drug doxorubicin to sensitize cells to non-lethal doses of TRAIL via the formation of ceramide-enriched membrane platforms was investigated in the last part of this study. The data demonstrate that treatment of BJAB lymphoma cells and T-

splenocytes with subtherapeutic doses of doxorubicin triggers release of ceramide, thus, permitting the induction of apoptosis by non-lethal doses of TRAIL that are by themselves also too low to release ceramide and kill the cells. The results presented earlier showed that, with the exception of using very high doses of aggregated ligand, successful induction of apoptosis by TRAIL requires clustering of DR5 receptors mediated by ceramide-enriched membrane platforms, a mechanism described for other receptors, such as CD95 (Cremesti *et al.,* 2001; Grassme *et al.,* 2001a, b) or CD40 (Grassme *et al.,* 2002a, b). The present data demonstrates that ceramide generated after treatment with subtoxic doses of doxorubicin accumulates in large ceramide-enriched membrane domains serving to trap and aggregate DR5 receptors when activated by low doses of TRAIL.

Many studies indicated ceramide release in response to different stress-inducing stimuli including chemotherapeutic drugs (Lacour *et al.,* 2004; Modrak *et al.,* 2004; Lovat *et al.,* 2004). Although there seems to be an agreement regarding an increase in ceramide levels after doxorubicin treatment, the pathway of ceramide generation remains controversial (Morita *et al.,* 2000; Andrieu-Abadie *et al.,* 1999; Gouaze *et al.,* 2001; Mercier *et al.,* 2003). In the present study, we demonstrate the involvement of the ASM in ceramide release after treatment with low doses of doxorubicin. The mechanism by which doxorubicin activates ASM is presently not known. The present study demonstrates the involvement of oxidative reactions in ASM activation, a mechanism that is supported also by the studies of Qiu and co-workers (2003) and Reinehr and co-workers (2006). It is tempting to speculate that doxorubicin, previously shown to induce free radical oxygen species (Sinha *et al.,* 1989; Ubezio and Civoli, 1994; Cervantes *et al.,* 1988), may activate the ASM via this mechanism. Interestingly, Andrieu-Abadie and co-workers, showed that doxorubicin-induced ASM/ceramide pathway is inhibited upon treatment with L-carnitine (Andrieu-Abadie *et al.,* 1999). L-carnitine is known to have anti-oxidant properties (Arockia and Panneerselvam, 2001; Izgut-Uysal *et al.,* 2001; Luo *et al.,* 1999; Packer *et al.,* 1991; Sener *et al.,* 2004), therefore, this study may provide an indirect proof of doxorubicin-induced ROS involvement in ASM/ceramide pathway (Andrieu-Abadie *et al.,* 1999).

Several recent reports described the ability of subtoxic concentrations of chemotherapeutic drugs to sensitize tumour cells that are resistant to TRAIL (Gibson *et al.,* 2000; Keane *et al.,* 1999; Bonavida et al, 1999; Ciusani *et al.,* 2005; Shankar *et al.,* 2005). The synergistic cytotoxic effect of genotoxic drugs and TRAIL, was proposed to be mediated by an up-regulation of TRAIL death receptors after pretreatment with cytotoxic drugs for several hours

(Sheikh *et al.,* 1998; Gibson *et al.,* 2000; Shankar *et al.,* 2005) and to be p53 dependent (Wu *et al.,* 1997; Wu *et al.,* 1999a). Up-regulation of the death receptors has been studied either at the mRNA level (Sheikh *et al.,* 1998; Gibson *et al.,* 2000; Wu *et al.,* 1997; Wu *et al.,* 1999a) or by immunoblotting (Gibson *et al.,* 2000). This drug-induced receptor up-regulation was not confirmed in the present study in which the expression of DR5 was analyzed at the plasma membrane by flow cytometry and the exposure to doxorubicin was much shorter. This is consistent with the study of Lacour and co-workers (2001), who showed, in addition, that lack of modulation of TRAIL receptor membrane expression upon exposure to DNA-damaging agents did not depend on p53 status because it was observed in both p53 wild-type and p53-mutated cell lines (Lacour *et al.,* 2001). The concentration of cytotoxic drugs used to sensitize tumour cells to TRAIL-induced cell death could account for the discrepancies observed with other studies. High concentrations of etoposide that are not clinically relevant were shown to up-regulate DR4 and DR5 mRNA and protein levels in breast cancer cell lines (Gibson *et al.,* 2000), whereas lower, more clinically relevant concentrations of doxorubicin had no effect on the expression of the studied receptors in these cell lines (Keane *et al.,* 1999). Thus, it cannot be ruled out that higher concentrations of doxorubicin could have enhanced the expression of TRAIL receptors at the surface of the tested cells. Discrepancies between studies could also depend on the choice of the cytotoxic drug because low concentrations of doxorubicin were shown to sensitize breast cancer cells to TRAIL, whereas high concentrations of 5-fluorouracil were required for obtaining this effect (Keane *et al.,* 1999).

Here, a novel mechanism mediating the synergistic effect of subtoxic doses of doxorubicin and TRAIL, respectively, is presented. This mechanism consists in trapping and clustering of activated DR5 receptors within ceramide-enriched membrane platforms induced by low doses of doxorubicin. At present it can not be excluded that doxorubicin treatment has also other effects amplifying TRAIL-induced signalling and apoptosis; however, the data on ASM-deficient cells demonstrate that these effects would also have to be mediated by ASM and ceramide.

Consistent with the present data, TRAIL or DR5-antibodies showed only modest effects on experimental tumours, while recent studies combining TRAIL with radiation or chemotherapeutic drugs, e.g. alkylphosphocholines or DNA-damaging agents (Jendrossek *et al.*, 2003; Jin *et al.,* 2004; Shankar *et al.,* 2005; Marini *et al.*, 2005; Straughn *et al.,* 2006; Marini *et al.*, 2006) revealed a very effective induction of apoptosis and suggest that TRAIL

or anti-DR5 antibodies should be used in combination with chemotherapeutic drugs and/or irradiation.

The concept of amplifying apoptosis induced by low doses of TRAIL via ceramide increase could have clinical relevance by combining application of a locally active stimulus that generates ceramide in the tumour, for instance irradiation or antibody-coupled activators of ASM, and low doses of TRAIL injected systemically, that by themselves have no effects (to both malignant and non-malignant cells and tissues, respectively), but in the tumour they will be converted into a potent apoptotic stimulus by locally induced/released ceramide. A timely and locally coordinated production of ceramide in the tumour tissues with the application of very low doses of systemic TRAIL may permit an efficient treatment of at least some human malignancies.

6. SUMMARY

The pro-apoptotic TNF-family ligand TRAIL is currently one of the most promising novel anti-cancer agents. Although TRAIL has been intensively studied in the past decade, the signalling cascades which lead to and regulate TRAIL-induced apoptosis are still not completely elucidated. Furthermore, since *in vivo* treatment with TRAIL alone did not reveal very spectacular outcomes, particular importance is given to characterizing mechanisms that may amplify TRAIL-induced apoptosis.

The present study investigated the role of acid sphingomyelinase (ASM) and ceramide-enriched membrane platforms for the apoptotic pathway triggered by TRAIL upon ligation of DR5 receptors. The major findings of the study are:

- ASM is activated by TRAIL and plays a crucial role in apoptosis induced by physiological doses of TRAIL.
- TRAIL activates the ASM via a direct or indirect redox mechanism.
- TRAIL-induced ASM activation leads to generation of ceramide-enriched membrane platforms on the outer leaflet of the plasma membrane.
- Ceramide-yenriched membrane platforms are essential for TRAIL-induced apoptosis.
- Ceramide-enriched membrane platforms serve to trap ligated DR5 receptors leading to a massive aggregation of the receptors (receptor clustering).
- Receptor clustering is essential for successful induction of apoptosis by TRAIL via amplification of the primary signal.

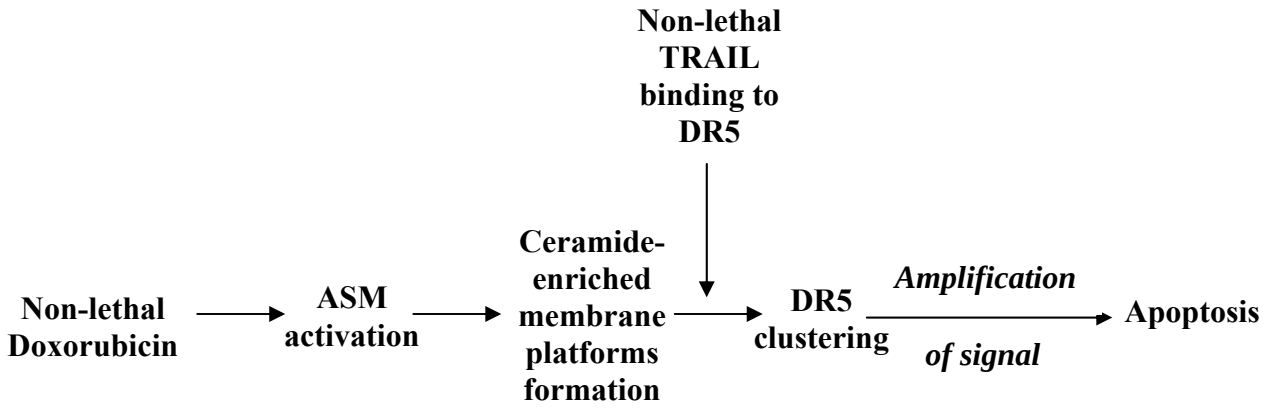
Therefore, the following signalling pathway is proposed:

TRAIL binding to DR5 receptors → **ROS release** → **ASM activation** → **Ceramide-enriched membrane platforms formation** → **DR5 clustering** —***Amplification of signal***→ **Apoptosis**

Furthermore, the present study identifies a novel mechanism of amplification of TRAIL-induced apoptosis via ceramide-enriched membrane platforms. Since ceramide platforms are able to amplify the killing potential of very low doses of TRAIL, this mechanism may prove of relevance for the *in vivo* treatment with TRAIL. The results demonstrate:

- Non-lethal doses of doxorubicin and TRAIL have a synergistic apoptotic effect.
- Non-lethal doses of doxorubicin are able to induce ceramide-enriched membrane platforms.
- Doxorubicin-induced ceramide platforms are generated via activation of the ASM.
- Doxorubicin-induced ceramide platforms trap and cluster the ligated DR5 receptors leading to successful induction of apoptosis.

Therefore, the following mechanism of amplification is proposed:



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PUBLICATIONS

- C.A. Dumitru, S. Dreschers and E. Gulbins (2006): **Rhinoviral infections activate p38MAP-Kinase via membrane rafts and RhoA.** *Cellular Physiology and Biochemistry*, 17:159-166.

- C.A. Dumitru and E. Gulbins (2006): **TRAIL activates acid sphingomyelinase via a redox mechanism and releases ceramide to trigger apotosis.** *Oncogene*, in press.

- S. Dreschers, C.A. Dumitru, C. Adams and E. Gulbins (2006): **The cold case – are Rhinoviruses perfectly adapted pathogens?** (review). *Cellular and Molecular Life Sciences*, in press.

- C.A. Dumitru, A. Carpinteiro, T. Trarbach, U.R. Hengge and E. Gulbins (2006): **Doxorubicin enhances TRAIL-induced cell death via ceramide-enriched membrane platforms**. *Oncogene*, submitted.

POSTERS and PRESENTATIONS

- **Acid sphingomyelinase and ceramide are critical for TRAIL- induced apoptosis** - oral presentation. *9th Joint Meeting of the Signal Transduction Society (STS)*, November 10-12, 2005, Weimar, Germany.

- **Rhinoviral infection activate p38 MAPK via membrane rafts and RhoA** - poster presentation. *9th Joint Meeting of the Signal Transduction Society (STS)*, November 10-12, 2005, Weimar, Germany.

- **Ceramide enables DR5 to cap and kill** - poster prsentation. *84th Annual Meeting of the Deutsche Physiologische Gesellschaft (DPG)*, March 6-9, 2005, Göttingen, Germany

- **Acid sphingomyelinase mediates TRAIL - induced DR5 clustering and is involved in TRAIL-induced apoptosis**. Awarded with Poster Prize and Presentation Prize at *Tag der Forschung der Medizinischen Fakultät*, November 19, 2004, Essen, Germany

- **Acid sphingomyelinase mediates TRAIL - induced DR5 clustering and is involved in TRAIL-induced apoptosis** - poster presentation. *10th International TNF Superfamily Conference*, September 29 - October 2, 2004, Lausanne, Switzerland

- **Ultrastructural evaluation of the maturation competence of oocytes from different mammals** - oral presentation. Awarded with the 2nd Prize at *Professors and Students Scientific Session of Communications*, April 13-15, 2002, Constanta, Romania

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Erklärungen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Fachbereiche 6 und 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Regulation and function of acid sphingomyelinase (ASM) and ceramide in TRAIL-induced apoptosis“ zuzuorden ist, in Forschung und Lehre vertrete und den Antrag von Frau Claudia-Alexandra Dumitru befürworte.

Essen, den 09.10.2006

Prof. Dr. Eich Gulbins

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Fachbereiche 6 und 9 zu Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 09.10.2006

Claudia-Alexandra Dumitru

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Fachbereiche 6 und 9 zu Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät abgelehnt worden ist.

Essen, den 09.10.2006

Claudia-Alexandra Dumitru