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Chapter 12

Acid Sphingomyelinase-derived Ceramide Signaling in Apoptosis

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1. INTRODUCTION

Biological membranes of eukaryotic cells primarily consist of glycerophospholipids, sphingolipids and cholesterol. Sphingomyelin, which is comprised of a highly hydrophobic ceramide moiety and a hydrophilic phosphorylcholine headgroup, is the most prevalent cellular sphingolipid (Figure 1). Ceramide is the amide ester of the sphingoid base D-erythro-sphingosine and a fatty acid usually of C_{16} through C_{26} chain length (Figure 1). Sphingomyelin is synthesized predominantly on the luminal side of the Golgi apparatus, but also in the plasma membrane (Jeckel *et al.*, 1990; Futerman *et al.*, 1990; Andrieu-Abadie *et al.*, 1998). The two pools are connected by vesicular transport. Sphingomyelin is almost exclusively located in the anti-cytosolic leaflet of biological membranes resulting in lipid bilayer asymmetry (Emmelot and Van Hoeven, 1975). Hydrogen bonds and hydrophobic van der Waal interactions mediate the tight interaction between the cholesterol sterol ring system and the ceramide moiety of sphingomyelin. The lateral association of sphingolipids in the cell membrane is further promoted by hydrophilic interactions between the sphingolipid headgroups.

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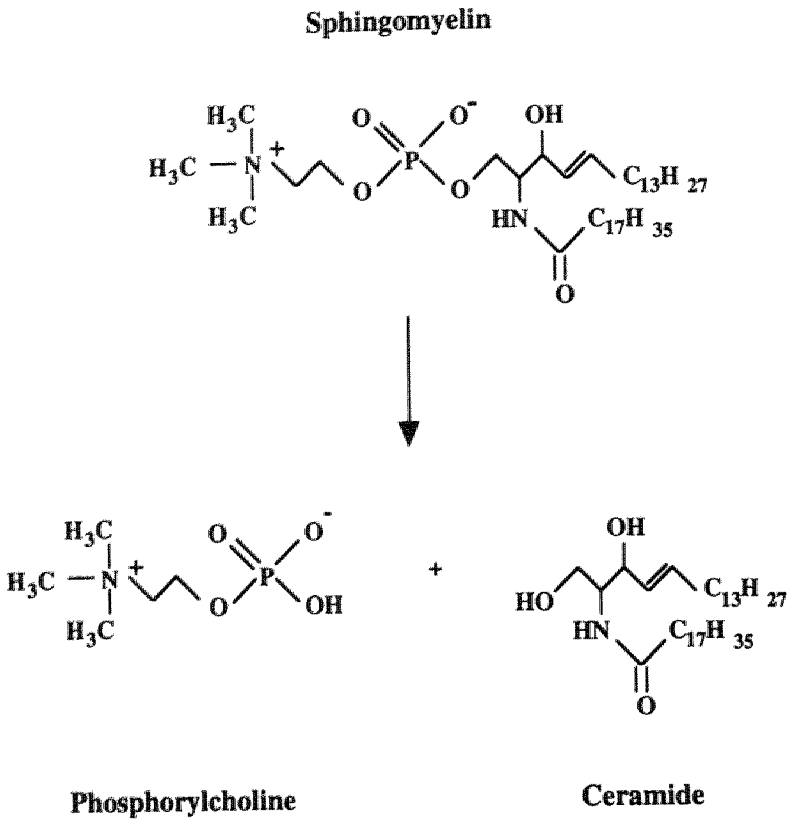

Figure 1. Displayed are the structures of sphingomyelin and ceramide. Sphingomyelinases function as phospholipases C to hydrolyze sphingomyelin.

The association of these lipids and the high local concentration result in their separation from phospholipids within the bilayer and the transformation of these sphingolipid- and cholesterol-rich membrane domains into a distinct liquid ordered phase (Harder and Simons, 1997; Brown and London, 1998; Andersen, 1998). The tightly packed sphingomyelin/cholesterol domains have been compared to rafts floating within the phospholipid portion of the cell membrane. Cholesterol seems to function in rafts as a hydrophobic spacer between the bulky sphingolipids required for the generation of the liquid ordered phase (Harder and Simons, 1997; Brown and London, 1998). In accordance with this concept, pharmacological extraction of cholesterol destroys these membrane rafts. This highly ordered biophysical phase results in a relative resistance of these sphingolipid-rich rafts to detergents and,

thus, these glycosphingolipid-enriched membrane (GEM) domains have also been named detergent-insoluble glycolipid enriched membranes (DIGs) (Harder and Simons, 1997; Brown and London, 1998).

Metabolites derived from sphingolipids constitute a novel class of cellular signaling molecules involved in apoptosis, cell proliferation and differentiation. Members of this group of signaling molecules include ceramide (Figure 1), sphingosine, sphingosine-1-phosphate, and perhaps ceramide 1-phosphate. In the present overview, we will focus on the function of ceramide in apoptosis. Death of cells by apoptosis is central in the development of the organism to delete excess cells, but also in the physiological turnover of normal tissues to remove aged or damaged cells. Apoptosis, reviewed extensively recently (e.g. Hengartner, 2000), is characterized by the ordered disintegration of intracellular organelles and their packaging into apoptotic bodies, while integrity of the plasma membrane is maintained until late in the process. These events are the result of activation of a family of cysteine aspartate proteases known as caspases.

Ceramide, released by the acid sphingomyelinase or synthesized *de novo* via the ceramide synthase pathway, is critically involved in many forms of apoptosis. This includes apoptosis triggered by receptor molecules, e.g. CD95 or the tumor necrosis factor receptor, during development, or upon induction by environmental stress, e.g. irradiation, heat shock or UV light. These stimuli initiate rapid activation of acid sphingomyelinase and concomitant release of ceramide. Cellular effector mechanisms for ceramide involve direct or indirect regulation of the activity of several proteins, e.g. kinase suppressor of Ras, cathepsin D or phospholipase A_2. This chapter will consider the recently advanced concept suggesting that ceramide modifies distinct domains in the membrane to create rafts, enabling activated receptors to concentrate in clusters. Clustering of receptor and signaling molecules in these rafts seems required for initiation of receptor- and cell type-specific signaling.

2. CERAMIDE AND ACID SPHINGOMYELINASE IN APOPTOSIS

Diverse stimuli have been shown to trigger apoptosis. In general, at least three types of pro-apoptotic stimuli are identified. First, many receptors have been shown to initiate apoptosis. The most important of these belong to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily and include CD95, the Trail-receptor DR4, and the TNF receptor (TNF-R) (Walczak and Krammer, 2000; Holtzman *et al.*, 2000). These receptors are constitutively present or up-regulated on the cell surface

upon cellular maturation. Interaction of the cognate receptor with its ligand initiates apoptosis. For example, expression of CD95 on stimulated lymphocytes seems critically involved in regulation of the immune response, since genetic deficiency of CD95 or its ligand in *lpr* or *gld* mice, respectively, results in accumulation of atypical lymphocytes and in the development of an autoimmune-like disorder (Nagata and Suda, 1995). Second, apoptosis can be induced by various pathophysiologic stress stimuli including γ-irradiation, ultraviolet (UV) light, heat shock, cytotoxic drugs, H_2O_2, toxins, bacteria and viruses (for review see Mathias *et al.*, 1998). Third, survival of many cells critically relies on the supply of growth factors and their withdrawal results in apoptosis. For example, interleukin-2 is necessary to prevent apoptosis in lymphocytes, while NGFs are necessary in neurons. These cells require continuous external survival signals to balance pre-existing intracellular death-signaling pathways. Depletion of growth factors very likely also plays a fundamental role in apoptosis during development. Cells provided with the correct mix of survival factors will be maintained, while cells lacking the required growth factors undergo apoptosis and are deleted in the developmental process. Ceramide appears to be involved in all three forms of apoptosis, however, most data exist on the role of ceramide in receptor and stress-mediated apoptosis.

Cellular ceramide is generated either by hydrolysis of sphingomyelin or by *de novo* synthesis. Sphingomyelin hydrolysis is catalyzed by at least three different sphingomyelinases that are discriminated by their optimal pH: acid, neutral, and alkaline sphingomyelinases. In the present manuscript we will focus on acid sphingomyelinase (ASM)-released ceramide.

ASM is the best characterized sphingomyelinase and many studies implicate this enzyme in apoptosis. Upon synthesis, ASM is post-translationally modified by glycosylation, a requirement for functionality (Newrzella and Stoffel, 1996). A lysosomal ASM and a secretory ASM have been distinguished (Schissel *et al.*, 1996). They are derived from the same gene, but differentially processed at the NH2-terminus and display a different glycosylation pattern (Schissel *et al.*, 1998). The glycosylation pattern very likely determines the targeting of the ASM to acidic compartments (lysosomes and endosomes) to regulate sphingomyelin turnover, or into secretory vesicles for secretion into the extracellular space. Recently, we identified a third form of ASM, which binds to the cell surface upon application of the appropriate stimulus (Grassmé *et al.*, 2001). At present, it is unknown whether this surface ASM represents a third isoform or a specialized form of the secretory or lysosomal ASM. Even if the pH at the cell surface or the extracellular space is only slightly acidic, it is very likely that both the secretory and the surface ASM are active: The increase of the pH from 4.5 to neutral values does not alter the activity [V_{max}] of the

enzyme, but increases the K_m value, decreasing substrate affinity (Callahan *et al.*, 1983). Since the activity of the ASM is modified by environment (Schissel *et al.*, 1998), the reduction of substrate affinity might be compensated by the presence of extracellular factors such as LDL or by membrane co-factors, restoring ASM activity even at neutral pH values.

ASM is activated by several pro-apoptotic receptors, in particular the TNF-R (Schutze *et al.*, 1992), IL-1 receptor (Mathias *et al.*, 1993) and CD95 (Cifone *et al.*, 1994; Gulbins *et al.*, 1995; Brenner *et al.*, 1998), but the enzyme is also stimulated by non-apoptotic receptors, e.g. CD40 (Grassmé *et al.*, 2001b) or CD28 (Boucher *et al.*, 1995) or by internalization of some bacteria into mammalian cells (Grassmé *et al.*, 1997), an observation discussed in the last part of this review. Most of these stimuli trigger only a 2-4 fold activation of the enzyme. However, since only a small portion of total cellular ASM appears stimulated upon ligation of a cognate receptor, whereas the bulk of the lysosomal ASM might not be regulated, it is likely that the effective local increase in ASM activity is much greater than the measurement of total cellular ASM activity suggests.

CD95 and the TNF-R activate ASM to release ceramide within seconds to minutes upon ligation suggesting that ASM functions in the early signaling of apoptosis. Several genetic studies have provided clear evidence that ASM plays an important role in TNF-R1- and CD95-triggered death, at least in some cell types (Lozano *et al.*, 2001; De Maria *et al.*, 1998; Paris *et al.*, 2001a; Kirschnek *et al.*, 2000). These studies employed B lymphocytes from Niemann-Pick disease Type A (NPDA) patients, which suffer from an inborne defect of ASM, or cells from ASM knock-out mice. Thus, human B-lymphocytes from NPDA patients are resistant to CD95-triggered ceramide generation and apoptosis (Grassmé *et al.*, 2001a; De Maria *et al.*, 1998). Furthermore, recent studies showed a 10-fold reduction of apoptosis in hepatocytes isolated from ASM-deficient mice upon CD95 stimulation compared to normal hepatocytes (Paris *et al.*, 2001a; Kirschnek *et al.*, 2000). Addition of C_{16}-ceramide restored CD95-induced apoptosis providing evidence that ceramide is central for CD95-triggered apoptosis in this population. This notion is supported by *in vivo* studies employing a CD95-dependent autoimmune hepatitis and CD95-dependent death of lymphocytes after anti-CD4 antibody injection (Kirschnek *et al.*, 2000). Both types of CD95-triggered death were deficient in mice lacking ASM. Additional studies show, however, that the ASM is dispensible for apoptosis in some cell types as CD95-triggered apoptosis of thymocytes is normal in ASM-deficient animals consistent with the low ASM content of these cells (Lin *et al.*, 2000).

The function of ASM-released ceramide is not restricted to receptor-initiated signaling, but has been also demonstrated to play a pivotal role in

developmental apoptosis in at least one specific cell type. It has been shown that ASM is required for the normal development of the ovaries (Morita *et al.*, 2000). The normal pattern of development of the ovary involves deletion of as many as 80% of all oocytes before birth of the mouse. A similar process occurs in the human female. Genetic deficiency of ASM prevented developmental apoptosis of oocytes resulting in oocytes hyperplasia at birth. Further, oocytes from *asmase*$^{-/-}$ mice, when superovulated from adult females, were resistant to therapeutic levels of daunorubicin, in contrast to wild type littermates. These investigations provide clear genetic evidence for a central role for ASM in developmental and stress-induced deletion of oocytes.

In addition to the function of the ASM in receptor-triggered and developmental apoptosis, the enzyme has a central function in the cellular response to environmental stress stimuli. This review will focus on the examples of ischemia, ultraviolet (UV) light exposure and, in particular, γ-irradiation.

Ischemia is a major cause of some important clinical problems including myocardial infarction and stroke. A recent study on stroke showed that ischemia results in activation of ASM and ceramide release (Yu *et al.*, 2000). The significance of this finding is indicated by the resistance of *asmase*$^{-/-}$ mice to focal cerebral ischemia. Those mice were protected against the middle cerebral artery stroke syndrome showing smaller infarct sizes and less behavioral changes than their wild type littermates. Consistent with these data, hippocampal neurons isolated from *asmase*$^{-/-}$ mice were resistant to hypoxic and excitotoxic stress compared to their wild type siblings. These genetic data suggest that activation of neuronal ASM is central to stress-induced apoptotic death of hippocampal neurons.

In addition, several studies identified ASM involvement in the stress response leading to apoptosis induction upon UVA exposure (Huang *et al.*, 1997). In contrast to wild type B blasts, MS1418 B blasts derived from a patient with Niemann-Pick disease were defective in the ASM activation, ceramide generation and c-Jun N-terminal kinase (JNK) activation that occurred within minutes of UVA exposure, and were defective in apoptosis induction. UVB and UVC responses were normal in MS1418 cells indicating specific utilization of the ASM-mediated signaling mechanism for UVA-induced apoptosis. That JNK was required for UVA-induced apoptosis was derived from studies using *jnk1*-/- and *jnk2*-/- cells which still activated ASM but were found resistant to UVA-induced apoptosis compared to wild type counterparts (Zhang, 2001).

Finally, ASM may be central to the response to γ-irradiation *in vitro* and *in vivo*, in some cells. A requirement for ASM for release of ceramide upon irradiation has been observed in T- and B-lymphocytes (Santana *et al.*,

1996), murine embryonic fibroblasts (Lozano *et al.*, 2001) and endothelial cells (Pena *et al.*, 2000; Paris *et al.*, 2001a). ASM deficiency prevented ceramide release and resulted in resistance of these cells to the ionizing radiation while re-constitution of ASM restored irradiation-triggered ceramide generation and cell death. The significance of ASM for radiation-induced cell death is clearly defined in experiments on ASM knock-out mice (Santana *et al.*, 1996; Pena *et al.*, 2000; Paris *et al.*, 2001b). Mice exposed to γ-irradiation failed to generate ceramide in endothelial cells in the lung (Santana *et al.*, 1996), small intestine (Paris *et al.*, 2001b) or brain (Santana *et al.*, 1996) and were resistant to radiation-induced damage, events readily observed in normal mice. In fact, *asmase*$^{-/-}$ mice were resistant to the GI syndrome, a major limiting toxicity for use of chemotherapy and of radiation to the abdomen (Paris *et al.*, 2001b). Likewise, oocytes within the ovaries of *asmase*$^{-/-}$ mice resisted irradiation if pre-treated with sphingosine 1-phosphate, a ceramide metabolite and antagonist, whereas vehicle-treated oocytes died (Morita *et al.*, 2000). However, in the murine thymus, irradiation-triggered cell death does not seem to involve ASM, since the thymus of *asmase*$^{-/-}$ mice were as sensitive to irradiation as *asmase*$^{+/+}$ littermates (Santana *et al.*, 1996). This indicates different cells employ different mechanisms to trigger apoptosis in response to a single stress.

3. ASM ACTIVATION METHODS

Molecular mechanisms mediating activation of ASM by CD95 or the TNF-R are still only poorly understood. Both receptors contain a short intracellular domain, the death domain (Itoh and Nagata, 1993), which is required for apoptosis (Itoh and Nagata, 1993), and crucial for ASM activation (Jekle *et al.*, 2001). This is consistent with the finding that caspases, which interact with the death domain of CD95 or the TNF-R via the adapter proteins FADD and TRADD, respectively, are involved in ceramide release (Brenner *et al.*, 1998; Schwandner *et al.*, 1998; Jekle *et al.*, 2001; Grullich *et al.*, 2000). Thus, overexpression of FADD or caspase 8, or transfection of a constitutively active caspase 8 mutant, resulted in increased ceramide release suggesting that FADD and caspase 8 regulate ceramide release during some forms of apoptosis (Schwandner *et al.*, 1998; Grullich *et al.*, 2000). A role for caspase 8 in ASM activation and ceramide release upon CD95 triggering is also suggested by the finding that transfection of Crm A, a viral protein blocking some caspases, or treatment of cells with Ac-YVAD-cmk, a pharmacological inhibitor of caspases, prevent ASM stimulation and ceramide release by CD95 (Brenner *et al.*, 1998). However, caspase 8 seems not to be involved in TNF-R-triggered ASM stimulation,

which appears mediated by a yet unknown initiator caspase (Schwandner *et al.*, 1998). At present, the intermediates between caspases and ASM are unknown.

Recent studies showed that ASM specifically associates with phosphatidylinositol-3-kinase (PI-3-K) upon NGF stimulation of PC12 cells via TrkA (Bilderback *et al.*, 2001). Association between the two molecules was mediated by the regulatory p85 subunit of PI-3-K and was restricted to membrane rafts. Activation of PI-3-K by NGF triggering resulted in an approximately 50% reduction of ASM activity pointing to a negative regulation of ASM by this mechanism.

4. NEUTRAL AND ALKALINE SPHINGOMYELINASES

In addition to ASM, neutral sphingomyelinase (NSM) is activated by the TNF-R and CD95 (Cifone *et al.*, 1995; Chatterjee, 1994). However, at least for CD95, the NSM functions at a later phase of the apoptotic process, and ceramide released by NSM seems to have a completely different role than ceramide released by ASM. It is likely that NSM, which resides in the cytosol, gains access to sphingomyelin only in the late phase of apoptosis, and may be involved in an amplification loop for the apoptotic process. In addition to its activation by the TNF-R and CD95, NSM is also stimulated by neurotrophic factors, CD40 ligand, L-selectin, daunorubicin, dexamethasone, D-cytosine arabinoside, cell cycle arrest, serum deprivation and cell senescence (for review see Ferlinz *et al.*, 2000). Thus, ceramide released by NSM may serve diverse functions depending on cell type or the context in which the lipid is acting. The tight regulation of the NSM by glutathione suggests the enzyme to be involved in sensing oxidative stress (Liu and Hannun, 1997). A recently identified human alkaline sphingomyelinase (Nyberg *et al.*, 1996) from bile with an enzymatic optimum activity at pH 9.0 does not seem to be involved in cellular signaling.

5. CELLULAR TARGETS OF CERAMIDE IN APOPTOSIS

Ceramide has been shown to regulate directly or indirectly kinase suppressor of Ras (KSR; identical to ceramide-activated protein kinase) (Basu *et al.*, 1998), a ceramide-activated protein phosphatase (Dobrowsky

and Hannun, 1993), protein kinase C (Muller *et al.*, 1995), c-Raf-1 (Yao *et al.*, 1995), the small G-proteins Ras and Rac (Gulbins *et al.*, 1995; Brenner *et al.*, 1997), Src-like tyrosine kinases (Gulbins *et al.*, 1997), phospholipase A_2 (Huwiler *et al.*, 2001), cathepsin D (Heinrich *et al.*, 1999), Jun-N-terminal kinases (Westwick *et al.*, 1995) and the ion channels Kv1.3 and CRAC (Szabo *et al.*, 1996; Lepple-Wienhues *et al.*, 1999) (Table 1).

Table 2. Shown is a summary of direct or indirect targets of cellular ceramide indicating the multiple functions of this lipid mediator.

Targets of ceramide
Kinase suppressor of Ras (KSR = ceramide-activated protein kinase)*
Ceramide-activated protein phosphatase
Protein kinase C isoforms alpha, delta and zeta*
c-Raf-1*
The small G-proteins Ras and Rac
Src-like tyrosine kinases
Phospholipase A_2*
Cathepsin D*
Jun-N-terminal kinases
Ion channels (Kv1.3 and CRAC)

* indicates putative direct targets of ceramide

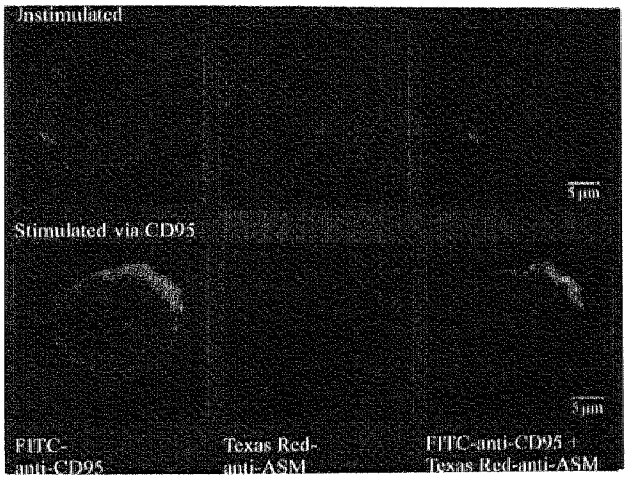

Figure 2. The confocal microscopy results show the clustering of CD95 upon stimulation. Clustered CD95 co-localizes with ASM in the cell membrane. ASM is transported to the cell membrane most likely with intracellular storage vesicles, which fuse with the cell membrane upon CD95 stimulation. Unstimulated cells show a homogenous distribution of CD95 in the cell membrane. Cells were stimulated via CD95 for 2 min or left unstimulated, fixed, permeabilized, stained with a FITC-labeled anti-CD95 and a Texas Red anti-ASM antibody and analyzed by confocal microscopy. The right pictures show the overlay.

The membrane-associated kinase KSR plays an important role in ceramide-mediated regulation of BAD, a pro-apoptotic protein belonging to the Bcl-2-like protein family (Basu *et al.*, 1998). Ceramide indirectly activates BAD via a pathway involving KSR, Ras, c-Raf-1, and MEK-1. Stimulation of this pathway results in Akt inactivation. Since the kinase activity of Akt maintains Bad in the inactive form, inhibition of Akt in turn releases BAD and, finally, permits BAD-triggered cell death.

While the exact mechanism of ceramide-mediated regulation of most of the above proteins is still unknown, ASM-released ceramide binds directly to PLA_2 and cathepsin D (Huwiler *et al.*, 2001; Heinrich *et al.*, 1999). Binding of ceramide to cathepsin D in endosomes triggers autocatalytic cleavage of cathepsin to its active form (Heinrich *et al.*, 1999). However, the physiological role of ceramide binding to cathepsin D and PLA_2 for apoptosis still remains to be determined.

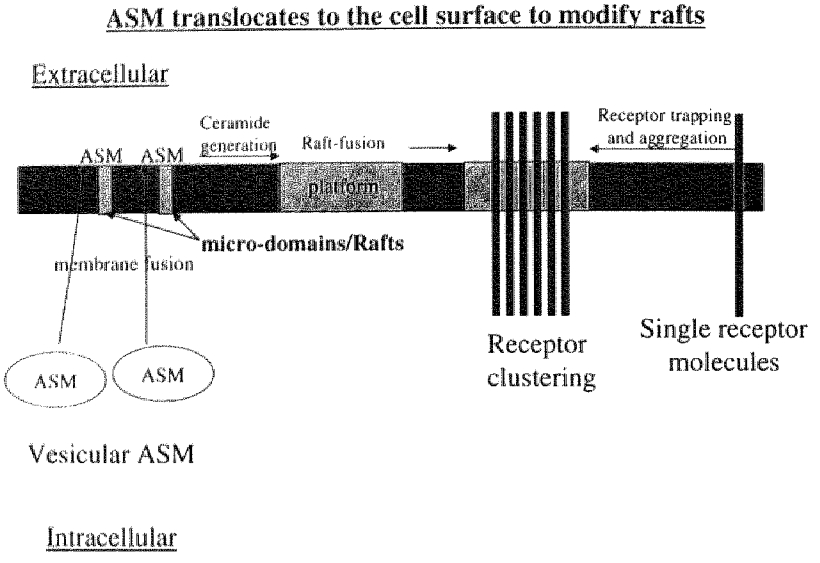

Figure 3. Primary stimulation of CD95 is insufficient to trigger apoptosis but sufficient to initiate ASM transport to the outer leaflet of the cell membrane. Surface ASM releases ceramide from sphingomyelin in the outer membrane leaflet. Ceramide alters pre-existing micro-rafts to fuse to larger platforms and to trap activated CD95. This clustering event of CD95 permits efficient signaling via the receptor possibly by recruiting intracellular signaling molecules to CD95, bringing intracellular signaling molecules into close proximity to each other, excluding inhibitory pathways, altering the affinity/avidity of the receptor for its ligand and/or by increasing the stability of CD95/CD95 ligand interactions.

We have recently suggested a novel concept for the function of ceramide (Figures 2 and 3). Stimulation via CD95 or CD40 with the cognate ligand results in translocation of ASM onto the extracellular leaflet of the cell membrane (Grassmé *et al.*, 2001a; Cremesti *et al.*, 2001). Surface ASM, now in the proximity of sphingomyelin which predominantly resides in the outer plasma membrane of mammalian cells, releases extracellularly-oriented ceramide within sphingolipid rich rafts. The notion of a function of ASM in lipid rafts is consistent with the findings of other groups that demonstrated an ASM-mediated consumption of sphingomyelin and release of ceramide in rafts upon stimulation of several receptors including the IL-1 and p75 NGF receptor (Liu and Anderson, 1995; Bilderback *et al.*, 1997). Accumulation of ceramide in rafts may alter the rafts in several respects based on data derived from biophysical studies using model membrane systems: First, ceramide has the tendency to spontaneously self-aggregate into microdomains (Huang *et al.*, 1999; Veiga *et al.*, 1999; ten Grotenhuis *et al.*, 1996) and, second, ceramide-rich microdomains are capable of fusing into larger platforms (Holopainen *et al.*, 1998). Thus, generation of ceramide within rafts may result in their fusion into larger platforms with altered biophysical properties. Those ceramide rich raft-platforms may then serve to cluster liganded receptor molecules, e.g., CD95 or CD40. Clustering results in high receptor density and may facilitate trans-activation of intracellular signaling molecules associating with the receptor. In addition, trapping of activated receptors in rafts may stabilize the interaction with ligand. Finally, ceramide-enriched rafts may recruit intracellular signaling molecules to the activated, clustered receptor, exclude inhibitory pathways and/or directly alter the affinity/avidity of the receptor for its ligand. A modification of intracellular signaling molecules by ceramide generated in rafts has been recently demonstrated for PI-3-K (Zundel *et al.*, 2000). Those studies identified an ASM- and ceramide-dependent recruitment of caveolin 1 to PI-3-K-receptor complexes within rafts, which blocked the activity of PI-3-K. The inhibition of anti-apoptotic PI-3-K by ceramide through caveolin 1, then sensitized cells to apoptotic stimuli.

The concept of ceramide activity in and modification of rafts also provides an explanation for the activation and function of ASM and ceramide in non-apoptotic signaling pathways that might require clustering of specific receptors. Thus, preliminary studies suggest that ASM signaling in rafts might mediate clustering of CD40, CD28, CD48 or the CFTR molecule.

Further, rafts altered by stimulated ASM activation may be involved in non-receptor-mediated signaling. For instance, UVC triggers aggregation of cell surface CD95 (Rehemtulla *et al.*, 1997), which might be sufficient to initiate signaling of CD95 at least under stress conditions. Likewise, it has

been shown that stress stimuli are able to trigger aggregation of the TNF-R to initiate cell death (Boldin *et al.*, 1995). Activation of both receptors by stress stimuli seems to occur in the absence of the CD95-ligand or TNF, respectively. Thus, it is possible that some stress stimuli alter the conformation of CD95 or change the composition of sphingolipid-rich rafts permitting trapping of unliganded CD95 molecules. This may result in low level receptor activation, which even if inefficient, may be sufficient to signal apoptosis, if persistent. This notion is supported by the finding that dominant negative FADD blocks, at least in part, UV-induced apoptosis (Chatterjee and Wu, 2001). The relationship, if any, of CD95 clustering to the aforementioned JNK activation requirement for UVA-induced apoptosis is unknown.

6. CONCLUSIONS

A variety of studies demonstrate a central role for ASM and ceramide in several forms of apoptosis. Ceramide seems to regulate the activity of certain proteins and, thus, may function, in some circumstances, as a second messenger. In addition, the concept of raft modification by ceramide provides a comprehensive model for cellular effects of ceramide, and perhaps a biophysical explanation for the diverse functions of this lipid.

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