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SPHINGOLIPIDS IN APOPTOSIS

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Forty years ago, the term "apoptosis" was introduced to describe a form of programmed cell death. Key players that mediate apoptosis at the molecular level such as caspases, death receptors, Bcl-2 family members have since been identified and their regulation remains a research focus of many laboratories. In 1993, approximately 20 years after the introduction of apoptosis, the sphingolipid ceramide was first linked to this form of cell death. Sphingolipids are bioactive components of cellular membranes that are involved in numerous physiological functions. In this paper, we discuss the inherent complexities of sphingolipid signaling and elaborate on how sphingolipids, primarily ceramide, influence apoptotic events such as death receptor aggregation in the plasma membrane and pore formation at the mitochondria. Possible roles of sphingolipids in other subcellular compartments, such as the nucleus, endoplasmic reticulum and lysosomes are also discussed. We conclude by summarizing the recent developments in sphingolipid based cancer therapy. This article is part of a Special Issue entitled "Apoptosis: Four Decades Later".

***Key Words*: sphingolipids, ceramide, apoptosis.**

HISTORICAL PERSPECTIVE

This year marks the 40th anniversary of the seminal publication by Kerr, Wyllie and Currie in the British Journal of Cancer in which the term "apoptosis" was introduced to describe a form of programmed cell death [1]. In the Greek language "apoptosis" means "dropping off" of leaves from trees or petals from flowers. The British scientists used the term for the morphological description of blebs that are pinched of the cell as it undergoes programmed cell death. Apoptosis is a carefully controlled signaling cascade that can be initiated through extrinsic signals or intrinsic stress. Although key players of apoptosis such as death receptors, caspases, and Bcl-2 family members have been identified, their regulation remains under intense investigation. This review focuses on the influence of sphingolipid signaling on apoptotic cell death.

Sphingolipids were first described by the German biochemist, J.L.W. Thudichum in 1884. For approximately 100 years sphingolipids were thought to function merely as structural components of cellular membranes. In 1993, a seminal paper published by the laboratory of Yusuf Hannun linked the sphingolipid ceramide to apoptosis [2]. Prompted by the observation that TNF treatment of U937 leukemia cells resulted in hydrolysis of sphingomyelin and generation of ceramide, they investigated whether ceramide itself can induce apoptosis. Indeed, exogenous ceramide but not other amphipathic lipids or structurally similar dihydro-ceramide induced DNA laddering, a classical hallmark of apoptosis. Other laboratories subsequently demonstrated a role for ceramide in response to various apoptotic stimuli in cells derived from diverse tissues [3–8]. Sphingolipid signaling has now taken center stage as cloning of enzymes, development of tools to study their roles, and methods of detection, have permitted insights into the complexity of sphingolipid biology.

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Abbreviations used: AC – acid ceramidase; AIF – apoptosis inducing factor; Apaf-1 – apoptotic protease activating factor 1; ASM – acid sphingomyelinase; CerS – ceramide synthase; CERT – ceramide transport protein; ER – endoplasmic reticulum; GCS – glucosylceramide synthase; NPC – nuclear pore complex; NSM – neutral sphingomyelinase; S1P – sphingosine-1-phosphate; SK – sphingosine kinase; SM – sphingomyelin; SMase – sphingomyelinase; SMS – sphingomyelin synthase; SPT – serine palmitoyl transferase; TNF – tumor necrosis factor; TRAIL – tumor necrosis factor receptor-like apoptosis inducing ligand; UPR – unfolded protein response.

THE COMPLEXITY OF SPHINGOLIPID SIGNALING

Sphingolipids have been shown to modulate apoptosis at multiple steps of the process, including clustering of cell surface receptors and involvement in permeabilization of the outer mitochondrial membrane. Here we will review the complexity of sphingolipid signaling and elucidate the role of metabolic enzymes and sphingolipids in various subcellular compartments in relationship to apoptotic signaling. Three main levels of complexity have to be considered when discussing sphingolipid signaling. First, a large variety of sphingolipids with diverse signaling capabilities can be generated by substrate utilization and modification of the sphingoid backbone. Second, numerous sphingolipid enzymes and enzyme families catalyzing opposing biochemical reactions function to maintain homeostasis by interconverting different sphingolipids. Finally, sphingolipid metabolism is highly compartmentalized.

Sphingolipid variety

Mammalian sphingolipids have an 18-carbon chain sphingoid backbone attached to a head group and are conjugated to an acyl group of varying carbon chain length [9]. Ceramide is the basic unit of sphingolipids that contains a sphingosine backbone, a fatty acid side chain and a hydrogen atom as the head group. Variety is achieved by incorporation of fatty acids that differ in chain length and substitutions in the head group.

The activity of a "ceramide synthase" had been described biochemically but it was not until the early 2000's that a family of six enzymes with preferential sub-

strate specificity was identified at the genetic level [10, 11]. These ceramide synthases, also initially described as "LASS" for longevity assurance genes, generate ceramides with fatty acids ranging from 14 to 26 carbons. For example, ceramide with a 16-carbon fatty acid is known as C_{16}-ceramide, whereas incorporation of an 18-carbon fatty acid yields C_{18}-ceramide. The increased use of liquid chromatography-mass spectroscopy (LC-MS) to determine changes in specific ceramide species reveals that generation of C_{16}-ceramide maybe preferentially associated with apoptosis. Studies from the Amascato laboratory demonstrated that exposure of cells to FasL or radiation induced a specific increase in mitochondrial C_{16}-ceramide that closely paralleled the decrease in mitochondrial mass during apoptosis [12, 13]. Krosen and co-workers showed that crosslinking of the B-cell receptor results in increased C_{16}- but not C_{24}-ceramide during early apoptosis [14]. Similarly, we detected a preferential increase in C_{16}-ceramide during TRAIL-mediated apoptosis in sensitive but not resistant cells [15]. Recently, C_{16}-ceramide was linked to triacylglycerol-induced apoptosis in macrophages [16]. In addition to C_{16}-ceramide, C_{18}-ceramide is also important for apoptosis. The Ogretmen laboratory found that head and neck squamous cancer cells have a selective decrease in C_{18}-ceramide and that restoration to normal levels by overexpression of murine ceramide synthase 1 (CerS1) resulted in modulation of telomerase activity and induction of apoptosis resulting in 70–80% growth inhibition [17]. C_{18}-ceramide may also contribute to amyloid protein mediated apoptosis in neuronal cells in Alzheimer's disease [18]. In contrast, growth arrest may be more specifically associated with an increase in C_{24}-ceramide [19]. Taken together, these studies indicate that cellular responses can be influenced by changes in specific ceramide species.

Substitution of different head groups yields complex sphingolipids. For example, replacing the hydrogen head group with phosphocholine yields sphingomyelin, whereas substitution with sugars leads to generation of glycosphingolipids. Since these substitutions can occur on ceramides with different fatty acid chain lengths, sphingomyelin species also exist as C_{16}-SM, C_{18}-SM, C_{20}-SM etc. Sphingolipids can also be modified by phosphorylation. The combination of different fatty acids with head group substitution and further modification such as phosphorylation, yields a large number of sphingolipids that each may have its own importance in cell physiology. According to the LIPID MAPS structure database, nearly 4000 different sphingolipids that occur naturally in organisms from yeast to mammals as well as those generated synthetically have been described (http://www.lipidmaps.org/data/structure/index.html).

Metabolic flux

Second, sphingolipid metabolic enzymes function as a family to maintain homeostasis. Ceramide is considered the "hub" of sphingolipid metabolism and can be generated by multiple pathways. The entry point into sphingolipid metabolism is the condensation serine palmitoyl transferase, which catalyses the condensation of serine and palmitoyl CoA. The product is reduced, acylated and desaturated to generate ceramide via the *de novo* pathway. Alternatively, ceramide can be generated from hydrolysis of complex sphingolipids. For example, cleavage of sphingomyelin by sphingomyelinases results in phosphatidylcholine and ceramide. Lastly, sphingosine can be utilized by ceramide synthases to generate ceramide via a salvage pathway. Just as ceramide can be generated by multiple mechanisms, it can also be cleared by several metabolizing enzymes, including sphingomyelin synthases and ceramidases. Sphingosine, the product of ceramidase action, serves as a substrate for sphingosine kinase to generate sphingosine-1-phosphate (S1P). Hydrolysis of S1P into ethanolamine phosphate and hexadecenal by S1P lyase is the only route to exit sphingolipid metabolism. Virtually all metabolic reactions between entry and exit points of sphingolipid metabolism are interconvertible [9]. Consequently, sphingolipid metabolism is in constant flux with the balance between pro- and anti-apoptotic metabolites determining cellular fate. Over the last 10 years, this concept has become known as the "sphingolipid rheostat" [20, 21]. The metabolites ceramide and sphingosine have been associated with apoptosis, cell cycle arrest, senescence and differentiation whereas S1P plays an important role in suppression of apoptosis, survival, angiogenesis and inflammation. A simplified sphingolipid pathway and rheostat are shown in Fig. 1. A shift of sphingolipid metabolism (or the rheostat) towards increased production of ceramide is anti-proliferative while a shift towards S1P favors survival [22]. Thus enzymes participating in sphingolipid metabolism form an intricate network to maintain homeostasis and to dictate cellular physiology.

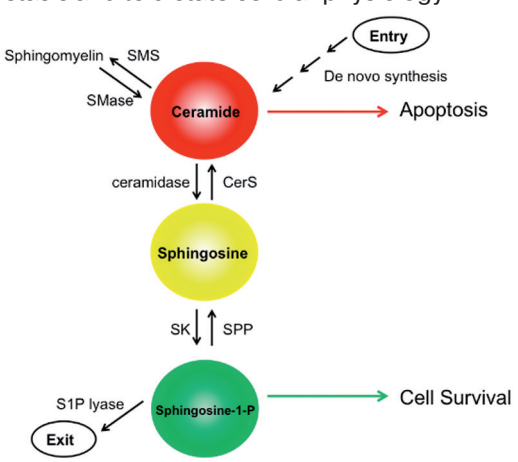

Fig. 1. Sphingolipid metabolism and homeostasis. Ceramide is synthesized *de novo* and can also be generated through breakdown of complex sphingolipids. Ceramide can also be further metabolized to sphingosine, which is then phosphorylated to generate S1P. Degradation of S1P by S1P lyase marks the exit from sphingolipid pathway. Ceramide has been linked to anti-proliferative responses including apoptosis while S1P is important for survival. Homeostasis is maintained through interconversion of ceramide and S1P though sphingosine. *Abbreviations used*: SMase — sphingomyelinase, SMS — sphingomyelin synthase, CerS — ceramide synthase, SK — sphingosine kinase, SPP — sphingosine-1-phosphate phosphatase, S1P lyase — sphingosine-1-phosphate lyase

Compartmentalization

Compartmentalization of sphingolipid metabolizing enzymes into different subcellular locations adds a third dimension of complexity. *De novo* ceramide generation occurs at the endoplasmic reticulum and is followed by modification to glycosphingolipids and sphingomyelin in the Golgi network. The ceramide transporter CERT shuttles ceramide from the endoplasmic reticulum to sphingomyelin synthase 1 in the Golgi for synthesis of sphingomyelin [23]. CERT is highly specific for ceramide [24] but it is not yet known whether CERT transfers ceramide to subcellular locations other than the Golgi or whether additional ceramide transport proteins exist. Once incorporated into complex sphingolipids within cellular membranes, ceramide can be liberated by hydrolysis but its range of activity is locally restricted by its hydrophobic nature. Only sphingosine is soluble and can be recycled via the salvage pathway to regenerate ceramide in other cellular compartments. Thus sphingolipid enzymes and products are largely restricted to act at the site of generation and different pools of ceramides may be important for specific functions in certain subcellular compartments.

SPHINGOLIPID-MEDIATED REGULATION OF APOPTOSIS PROXIMAL TO THE PLASMA MEMBRANE

Apoptosis can be initiated through extracellular stress, which involves propagation of an extrinsic stimulus such as binding of a ligand (FasL, TNF, TRAIL) to their respective death receptors on the cell surface. Alternatively, extrinsic apoptosis can be initiated through dependence receptors (i.e. netin receptors) when ligands fall below a critical threshold [25]. Death receptors are found in the plasma membrane, where they likely exist as timers in so-called pre-ligand assembly domains [26]. Ligand binding results in receptor stabilization and conformational changes in its cytoplasmic domain to permit binding of intracellular signaling molecules. In FasL and TRAIL-mediated apoptosis, the adaptor protein FADD and pro-caspase-8 (-10) are recruited to form the death inducing signaling complex (DISC) in which caspase-8 is cleaved into an active tetramer. In TNF-mediated apoptosis, caspase-8 is activated in a two-step manner via complex I (TNF-R1/RIP1/TRAF2/TRADD) and complex II (RIP1/TRAF2/TRADD/FADD/caspase-8).

Sphingolipids have important roles in initial apoptotic events at the plasma membrane. According to the classic fluid mosaic model that was proposed by Singer and Nicolson phospholipids and proteins were uniformly distributed in membranes [27]. However, Simons and Ikonen subsequently suggested the presence of specific structures called lipid rafts that are enriched in sphingolipids [28]. Indeed, ceramide has been shown to increase rapidly following engagement of surface Fas, suggesting that it was liberated by activation of sphingomyelinases. Several sphingomyelinases exist in mammals including acid sphingomyelinase (ASM) and three neutral sphingomyelinases (NSM1, NSM2 and NSM3). Both ASM and NSM2 have been linked to apoptosis [29, 30]. Ceramide generated at the plasma membrane following ASM-mediated hydrolysis of sphingomyelin is thought to be instrumental in reorganization of signaling molecules into ceramide-enriched platforms.

Acid sphingomyelinase

Acid sphingomyelinase (ASM) is an enzyme located within the lysosomal/endosomal compartment whose lack of function was initially known to cause the lysosomal storage disorder Niemann-Pick disease [31]. Joint efforts of several research groups have established ASM as an important stress activated enzyme following ligation of death receptors as well as non-receptor stimuli [29]. ASM acts on sphingomyelin to liberate ceramide by cleavage of the phosphocholine head group. A role for ASM in apoptosis was discovered when cells from Niemann-Pick patients were evaluated for radiation responsiveness. Interestingly, Niemann-Pick lymphoblasts resisted radiation-induced apoptosis, a phenotype that was reversible upon restoration of ASM expression [32]. Mouse embryo fibroblasts from ASM-deficient mice were also resistant to radiation-induced apoptosis and partially resistant to TNF or serum withdrawal induced apoptosis [33]. In a model of Fas-induced apoptosis, ceramide generation was attributed to activation of both neutral and acid sphingomyelinase, yet only ASM activation was linked to propagation of the apoptosis signal [34]. Taken together, these studies clearly defined a role for ASM in apoptosis.

ASM-mediated formation of ceramide-enriched platforms

An important early event at the plasma membrane following initiation of death receptor mediated apoptosis involves clustering of the receptors. Cremesti *et al*. demonstrated rapid formation of receptor signaling platforms into “caps” following stimulation of Fas. This event was ASM-dependent and a defect in “cap” formation could be overcome by providing cells with exogenous C_{16}-ceramide [35]. Grassme and Gulbins first proposed a model in which ASM translocates to the plasma membrane, flips to the outer side of the cell and hydrolyzes sphingomyelin in the outer leaflet of the membrane to generate ceramide, which then facilitates clustering of receptors into signaling platforms [36]. Several studies have shown that ASM activation following stimulation of Fas is dependent on the activity of the initiator caspase-8 and that ceramide functions to amplify caspase-8 activity [37–39]. In contrast, radiation-induced apoptosis was independent of caspase activation [39]. Although initial studies were conducted in models that involved stimulation of Fas, it was subsequently shown that chemotherapeutic agents, including doxorubicin and cisplatin, also activate ASM and facilitate death receptor-mediated apoptosis [40, 41]. Both Fas and the related death receptor ligand TRAIL activate ASM via a redox dependent mechanism [42], suggesting the possibility that induction of oxidative stress, common to both chemotherapy and death receptor-mediated apoptosis, may be involved in this

process. However, the exact mechanism by which non-receptor ligands induce ceramide-rich signaling platforms within plasma membranes remains unclear.

How does an enzyme, which is primarily located in the lysosomal compartment with optimal function at an acidic pH, hydrolyze sphingomyelin in the outer leaflet of the plasma membrane? While earlier studies employing immunohistochemistry demonstrated that ASM relocates to the plasma membrane in response to stress stimuli, a mechanism was eluded only recently. Using a UV stress model, Zeidan *et al*. demonstrated that PKC -mediated phosphorylation of ASM on Ser^{508} was required for translocation of ASM to the plasma membrane [43]. A recent study confirmed a role for PKC in translocation of ASM to the plasma membrane. Tsukamoto and coworkers found that phosphorylation of PKC on Ser^{664} was required for ASM translocation to the plasma membrane of multiple myeloma cells that had been exposed to the green tea polyphenol EGCG [44]. While generation of ceramide was not determined, it may have contributed to lipid raft clustering of the laminin receptor thereby promoting apoptosis [44]. While ASM is partially active at neutral pH [45], the possibility that phosphorylation improves activity at physiological pH has not yet been investigated.

Although several investigators demonstrated that ASM plays an important role in apoptosis, its activity is not universally required. For example, ASM-deficient cells can still undergo staurosporine-induced apoptosis, which is independent of ceramide formation [33]. In addition, the Blitterswijk group found that ceramide generation upon Fas stimulation occurs within hours rather than minutes and is independent of ASM [46, 47]. Our laboratory has studied ceramide generation in response to TRAIL using the isogenic colon cancer cell lines SW480 and SW620, which are TRAIL sensitive and resistant, respectively. Similar to the Blitterswijk group, we were unable to detect a rapid increase in ceramide generation, although ceramide did increase at later time points paralleling caspase activation [15]. In our hands, clustering of TRAIL receptor 2 (DR5) correlated with apoptotic susceptibility in the SW480/SW620 model but disruption by pretreatment with nystatin did not impact effector caspase activity in SW480 cells (White-Gilbertson and Voelkel-Johnson, unpublished data). These results suggest that the requirement for ASM in apoptosis may be stimulus and cell type-specific. The observation that apoptosis resistance in ASM-deficient cells can be overcome by exogenous ceramide, suggests that ceramide, not necessarily ASM, it critical for apoptosis [33].

SPHINGOLIPID-MEDIATED REGULATION OF APOPTOSIS AT THE MITOCHONDRIA

Mitochondria play a central role in the extrinsic as well as intrinsic pathway of apoptosis. In extrinsic apoptosis, mitochondrial events serve as an amplification loop. Caspase-8, activated in response to death receptor ligands, not only cleaves downstream executioner caspases but also Bid, a pro-apoptotic BH3-only protein of the Bcl-2 family. Truncated Bid facilitates oligomerization of Bax (and/or Bak) leading to pore formation, permeabilization of the outer mitochondrial membrane, and leakage of proteins such as cytochrome c, SMAC/Diablo, and AIF, and other apoptogenic factors into the cytosol. Cytochrome c together with pro-caspase-9 and Apaf-1 forms the apoptosome, the complex in which caspase-9 is activated to cleave the executioner caspase-3. SMAC/Diablo relieves the inhibitory function of IAP's on executioner caspases and AIF translocates to the nucleus where it is involved in DNA fragmentation [48]. These post-mitochondrial events are also initiated when stressors such as chemotherapeutic agents or radiation trigger apoptosis intrinsically, except that BH3-only proteins other than Bid promote Bax (or Bak) mediated pore formation in the outer mitochondrial membrane [49].

Ceramide plays an important role in these mitochondrial events. Zhang *et al*. demonstrated that hydrolysis of SM and generation of ceramide following TNF stimulation occurs in a compartment distinct from the plasma membrane [50]. In support, transfection of but not exogenous treatment with bacterial SMase recapitulated ceramide-mediated apoptosis, indicating that intracellular ceramide generation is required for the response. In a subsequent study, targeted expression of bacterial SMase resulted in elevated ceramide levels in the respective subcellular compartments, yet apoptosis occurred only when bacterial SMase was targeted to the mitochondria [51]. Mitochondrial ceramide plays an important role in Bax oligomer formation following exposure to TNF [52]. Importantly, ceramide and Bax synergize to induce permeabilization of the outer mitochondrial membrane [53].

Ceramide may facilitate apoptosis via formation of channels in the mitochondrial membrane. Mark Columbini's group first demonstrated the formation of ceramide channels in cell-free phospholipid membranes [54] and subsequently confirmed these results in isolated rat liver mitochondria [55]. Ceramide channels permitted the release of proteins smaller than 60kDa, such as cytochrome c, from the intermembrane space of the mitochondria [55]. Of significance is that ceramide channel formation appears to be unique to the outer mitochondrial membrane and occurs in response to ceramide concentrations that are physiologically relevant [56]. Furthermore, the formation of these channels is a regulated process, since disassembly can occur by the action of anti-apoptotic proteins like Bcl-x_L [57] and since channel formation in isolated mitochondria and liposomes can be inhibited by the sphingolipid dihydro-ceramide, which until recently had been believed to be biologically inactive [58]. Lastly, while the ceramide metabolite sphingosine can also form channels in isolated mitochondria, these channels are smaller than those formed by ceramide, and do not allow passage of pro-apoptotic proteins out of the mitochondria [59]. In fact, sphingosine may also play a role in disassembly of ceramide chan-

nels, suggesting the existence of a positive feedback mechanism that regulates ceramide channels [60].

The source of mitochondrial ceramide is largely unknown, but several enzymes involved in sphingolipid metabolism are localized to the mitochondria and may play a role in generation of a local pool of ceramide that facilitates apoptosis. For instance, the Gudz group showed that CerS1 and CerS6 partially localize to the brain mitochondria upon ischemia reperfusion injury and elevate C_{18}- and C_{16}-ceramides respectively [61]. The Kolesnick group described mitochondrial ceramide-rich macrodomains that are sensitive to ceramide synthase inhibition and are involved in Bax membrane insertion and mitochondrial apoptosis in HeLa cells [62]. While providing the ceramide synthase substrate sphingosine to isolated mitochondria resulted in ceramide generation, this response was not impacted by the ceramide synthase inhibitor fumonisin B1 but was partially blocked by ceramidase inhibition [59]. A later study found that mitochondria from neutral ceramidase knockout mice had a reduced ability to generate ceramide from sphingosine implicating that the reverse activity of this enzyme contributes to mitochondrial ceramide generation [63]. Recently, Wu *et al*. characterized a novel mitochondria associated neutral sphingomyelinase [64] but whether this enzyme contributes to apoptosis has not yet been investigated.

SPHINGOLIPIDS AND NUCLEAR APOPTOTIC EVENTS

Sphingomyelin is present not only in the plasma membrane but also in the nuclear envelope and intranuclear sites. Several laboratories have demonstrated a role for nuclear ceramide in apoptosis. Tsugane and co-workers found that hepatocyte apoptosis following portal vein ligation in an *in vivo* model was preceded by an increase in neutral sphingomyelinase (NSM) activity and subsequent generation of ceramide and sphingosine [65]. A French group found that ionizing radiation failed to induce ceramide generation in nuclei-free lysates and cytoplasts, although cytoplasts did respond to Fas by externalization of phosphatidylserine [66]. Activation of NSM, ceramide generation and features of apoptosis, such as PARP and DNA cleavage, were observed in highly purified nuclei [66]. Watanabe *et al*. found that Fas stimulation of Jurkat T cells resulted in increased nuclear ceramide though the activation of a putative nuclear NSM and inhibition of sphingomyelin synthase (SMS) [67]. The caspase-3 inhibitor, Ac-YVAD-cmk, prevented Fas-induced apoptosis, generation of ceramide and stimulation of NSM activity, suggesting that these events occur downstream of executioner caspase activation [67]. Similarly, serum deprivation of hippocampal cells resulted in activation of a putative nuclear NSM and inhibition of SMS [68]. Stimulation of NSM, inhibition of SMS and increased ceramide is also observed in response to radiation of nuclei isolated from proliferating cells [69]. Taken together, these studies suggest activation of a nuclear NSM and generation of nuclear ceramide play an important role in a variety of apoptosis models. However, the role of nuclear ceramide in apoptosis remains to be addressed. One possibility is that ceramide affects nucleocytoplasmic trafficking.

Numerous proteins translocated to and from the nucleus during apoptosis. One of the hallmarks during apoptosis is DNA fragmentation, which is mediated by AIF, EndoG and CAD [70, 71]. These proteins translocate from the mitochondria to the nucleus (AIF, EndoG) or are activated by caspase-mediated cleavage of an inhibitory unit (CAD). Other proteins that enter the nucleus during apoptosis include mitochondrial proteins (AIF, WOX1, EndoG) and death-fold proteins, which are directly or indirectly involved in caspase activation (DEDD, TRADD, FADD, Apaf-1, PEA-15) [72, 73]. Proteins that are released from the nucleus during apoptosis include p53, which interacts with Bcl-2 at the mitochondria and histone H1.2, which appears to be involved in oligomerization of Bax at the mitochondria [72]. Nucleo-cytoplasmic exchange occurs through nuclear pore complexes (NPC), which consist of about 30 different proteins that make up the cytoplasmic fibrils, the central framework and the nuclear basket [74]. Of the 28 nucleoporins in the NPC only 7 are caspase substrates [75]. Passive transport into the nucleus is restricted to small molecules and timing of nucleoporin degradation suggests that active transport is required for exchange of proteins between the nucleus and the cytoplasm during apoptosis. For example, active caspase-3 is found in the nucleus during apoptosis and is transported into the nucleus by a specific but yet unidentified mechanism [76]. A-kinase-anchoring protein 95 (AKAP95) has been identified as a potential carrier of active caspase-3 [77].

Faustino and co-workers recently addressed the hypothesis that ceramide inhibits cell growth by impacting nucleo-cytoplasmic trafficking and demonstrated that treatment of vascular smooth muscle cells with exogenous ceramide altered the distribution of importin-α and the RanGTP bound exportin Cellular Apoptosis Susceptibility (CAS) protein via a p38-mediated mechanism [78]. Whether endogenous ceramide induces similar changes has not yet been investigated. Additionally, it is not yet known whether ceramide affects the redistribution of proteins involved in nucleo-cytoplasmic transport during apoptosis and whether a nuclear pool of ceramide specifically contributes to these events. It has been established that nucleo-cytoplasmic transport is affected at an early stage of apoptosis independent of caspase activity. The redistribution of Ran, importin-α and importin-β upon induction of apoptosis occurs in the absence or presence of the pan-caspase inhibitor ZVAD [79]. Cytoplasmic Ran also accumulates in response to cisplatin, hydrogen peroxide, and UV irradiation and may represent a general early event during apoptosis [80]. Permeability to larger size dextrans increases during this phase of apoptosis but the mechanism mediating increased NPC permeability is unknown [80]. Given that ceramide can influence the behavior of proteins

in the plasma membrane and contributes to the formation of mitochondrial channel formation during apoptosis, it is tempting to speculate that ceramide may also play a role in the dilation of the NPC.

SPHINGOLIPID-MEDIATED REGULATION OF APOPTOSIS IN OTHER SUBCELLULAR COMPARTMENTS

So far we have discussed the role of ceramide in aggregation of death receptors into signaling platforms at the plasma membrane, in channel formation of the outer mitochondrial membrane, and the existence of a putative sphingomyelin cycle in the nucleus. However, other compartments such as the lysosomes and endoplasmic reticulum are also involved in apoptosis and some links to sphingolipid metabolism have been established.

Lysosomes

In 1998, Monney and co-workers reported that an "acidic compartment" contributes to TNF-induced apoptosis [81]. It was subsequently shown that the ceramide metabolite sphingosine is lysosomotrophic and its accumulation in the lysosome results in partial rupture prior to caspase activation and loss of mitochondrial function [82]. Lysomotropic acid ceramidase inhibitors have also been shown to induce apoptosis by modulating ceramide metabolism [83]. Cathepsin D, an aspartic protease found in the lysosome that can contribute to death receptor mediated apoptosis, has been identified as a direct target of ceramide, which is generated following activation of ASM [84, 85]. As discussed above, chemotherapeutic agents can mediate apoptosis via an ASM/ceramide-dependent pathway. Dumitru *et al*. demonstrated that clinically relevant concentrations of gemcitabine induced apoptosis in glioma cells via activation of ASM, which resulted in lysosomal accumulation of ceramide, activation of cathepsin D and insertion of Bax into the mitochondrial membrane ultimately leading to cell death [86]. Camptothecin-mediated apoptosis in U937 myeloma cells was mediated by activation of PKCΔ, which translocated to the lysosomal compartment to phosphorylate and stimulate the activity of ASM [87]. In summary, both extrinsic and intrinsic apoptotic pathways have been linked to lysosomal sphingolipid metabolism.

Endoplasmic reticulum (ER)

Stress caused by changes in cellular ATP, redox states or calcium concentration results in reduced ability of the ER to chaperone protein folding, thereby activating the Unfolded Protein Response (UPR) [88]. Prolonged UPR may trigger apoptosis via three different signaling pathways, involving inositol-requiring protein 1α (IRE1α), protein kinase RNA-like endoplasmic reticulum kinase (PERK) and activating transcription factor 6 (ATF6) [89]. These may activate caspases directly or through activation of mitochondrial apoptosis [88, 90–92]. Since *de novo* ceramide synthesis takes place in the ER links between sphingolipid metabolism and ER stress-induced apoptosis have been explored.

Ceramide synthases are ER resident enzymes involved in the *de novo* pathway of sphingolipid metabolism. Several studies indicate a possible role for CerS6 in ER stress. For example, in renal cancer cells, MDA7/IL-24 induced apoptosis via a Fas-mediated mechanism that was dependent on the expression of CerS6 [93]. Ceramide plays an important role in MDA7/IL-24 induced apoptosis and may mediate its effects via interaction with Beclin 1 as well as calpain-mediated cleavage of ATF5, thereby switching the physiological response from autophagy and to apoptosis [94, 95]. Another study demonstrated that geldanamycin (17AAG) results in loss of GRP78/BiP function and induced *de novo* ceramide synthesis [96]. CerS6 was implicated in this response, since SW620 colon cancer cells overexpressing the enzyme were more susceptible to 17AAG than control cells [96]. CerS6 may also contribute to apoptosis in yeast and rat pancreatic INS-1E cells in which activation of the UPR resulted in transcriptional activation of CerS6 and generation of C_{16}-ceramide [97]. On the other hand, in squamous cell carcinoma cells downregulation of CerS6/C_{16}-ceramide was required to enhance ER stress induced apoptosis via activation of ATF6 activation [98]. Thus contribution of CerS6 activity towards ER stress and apoptosis may be context-specific. Studies in HEK293 cells indicate that CerS1, which preferentially generates C_{18}-ceramides, translocates from the ER to the Golgi following treatment with cisplatin [99] while apoptosis in response to serum starvation increased the proportion of the pro-apoptotic sphingosine kinase 2 in the ER [100]. Therefore, apoptotic stimuli not only affect the activity of sphingolipid enzymes but can also alter their distribution to and from the ER.

INDUCTION OF APOPTOSIS BY MODULATING SPHINGOLIPIDS IN CANCER THERAPY

Strategies to eliminate malignant cells typically induce apoptosis via the intrinsic or extrinsic pathway and frequently impact sphingolipid metabolism. Whether sphingolipid metabolism is targeted intentionally through recently designed sphingolipid analogs and antibodies or whether it is impacted by radiation and chemotherapy, a shift in the sphingolipid rheostat towards increased ceramide and decreased S1P is a common denominator. We have summarized therapies that increase apoptosis through promoting ceramide generation, inhibiting ceramide clearance, or interfering with S1P generation and signaling (see Table).

Stimulation of ceramide synthesis

Ceramide can be generated through increased ceramide synthesis or breakdown of complex sphingolipids. In many models, the apoptotic response is sensitive to inhibition by myriocin and/or fumonisin B1, indicating a requirement for the activity of SPT and/or CerS [101–111]. Of the ceramide synthases, CerS1 and CerS6 have been primarily associated with apoptosis [111–118]. Strategies that facilitate the breakdown of sphingomyelin into ceramide via sphingomyelinase activation include radiation and well-known chemotherapeutics such as cisplatin and

camptothecin [4, 40, 87]. More recently, naturally occurring compounds such as the plant extract evodiamine, capsaicin an active component of chili peppers, the green tea extract EGCG, the vitamin E derivative α-TEA and cyclopamine, a naturally occurring alkaloid that inhibits hedgehog signaling were found to promote apoptosis via sphingomyelinase activity [44, 119–122]. MDA7/IL-24 requires both CerS6 and ASM for induction of apoptosis in prostate and renal cell carcinoma cell lines [93, 123] and has shown efficacy against renal cell tumor growth *in vivo* [124].

Inhibition of ceramide metabolism

Intracellular ceramide may also be increased through prevention of subsequent metabolic conversion into sphingosine and S1P or into complex sphingolipids. Development of ceramide analogs that interfere with acid ceramidase have been the most prominent approach. The prototype drug B13 effectively inhibited the growth of hepatic cancer xenografts [125], thereby prompting further modification and design of this drug [126–128]. B13-based drugs have been successfully combined with apoptin and FasL gene therapy in preclinical models of prostate and head and neck cancers [129, 130]. AD-2646, which also inhibits acid ceramidase activity, induces apoptosis in TSU-Pr1 prostate cancer cells and xenograft models [131].

Strategies to prevent metabolism of ceramide into complex sphingolipids have primarily focused on glucosylceramide synthase (GCS). Inhibition of GCS may result in a two-pronged effect of maintaining intracellular ceramide while also inhibiting the multidrug resistance gene MDR1 or P-glycoprotein (P-gp) to prevent drug efflux from the cell. For instance, the mixed backbone oligonucleotide designed against GCS (MBO-asGCS) sensitized multi-drug resistant NCI/ADR-RES xenografts to doxorubicin by increasing C_{18}-cer [132] and also reduced P-gp induced drug efflux in tumors [133]. GCS inhibition coupled with Bcr-Abl inhibition has also proven effective against primary cells isolated from T3151 mutant CML patients [134].

Ceramide and its analogs

Short chain ceramides such as C_6-cer have also been combined with inhibitors of phosphatidylinositol 3-kinase [135], the acid ceramidase inhibitor DM102 [136], HDAC inhibitors [137], and doxorubicin [138] to enhance cancer cell apoptosis. Delivery of short chain ceramides can be enhanced using transferrin liposomes that enable lysosomal internalization of ceramide resulting in cathepsin mediated apoptosis of A2780 ovarian carcinoma cells *in vitro* and *in vivo* [139]. Another strategy to use short chain ceramide therapeutically is incorporation of the lipid into pegylated nanoliposomes [140–143]. Finally, analogs that mimic the function of ceramide can also facilitate apoptosis. For example, combination of LCL30 with photodynamic therapy induces apoptosis in SCCVII mouse squamous carcinoma cells [144] while LCL29, a mitochondrion-targeted ceramide analog

Table. Overview of therapeutic strategies to induce apoptosis by sphingolipid modulation

	Therapeutic Approach	Enzyme	Model System	Reference
(A)	Curcumin	SPT	Colon cancer cells	[101]
	γ-Tocopherol	CerS	Androgen sensitive prostate cancer cells	[102]
	Fenretinide (4-HPR)	SPT/CerS	HL60 acute myeloid leukemia cells (AML), MCF7 multi-drug resistant breast cancer cells, HT29 colon cancer cells	[103, 104]
	Cannabinoids	CerS	Prostate, colon, cervical, lung and pancreatic cells as well as *in vivo* models of prostate and pancreatic cancer	[105–108]
	Carbonic anhydrase inhibitors	CerS	CA IX-positive HeLa and 786-O cells and CA IX-negative 786-O/von Hippel-Lindau (VHL) cells	[109]
	Ursolic acid	CerS	T24 bladder cancer cells	[110]
	Celecoxib	CerS6	Colon cancer cells and xenografts	[111]
(B)	MDA7/IL-24	ASM/CerS6	Renal and prostate cancer cells	[93, 123, 124]
	Radiation	ASM/CerS	Endothelial cells, Jurkat T cells, HeLa cells, MCF7 cells	[4, 39, 43, 115]
	Cisplatin	ASM	HT29 colon cancer cells	[40]
	20-S-camptothecin lactone	ASM	U-937 lymphoma cells	[87]
	Evodiamine	ASM/NSM	SGC-7901 gastric cancer cells	[119]
	Capsaicin	NSM	PC3 prostate cancer cells	[120]
	EGCG	ASM	Multiple myeloma cells and xenograft model	[44]
	α-TEA	ASM	MDA-MB-231 breast cancer cells and xenograft models	[121]
	Cyclopamine	NSM	Daoy medullobastoma cells	[122]
(C)	B13	AC	Colon cancer cells and xenografts	[125]
	LCL204	AC	Prostate and head and neck cells and xenografts	[129, 130]
	AD-2646	AC	TSU-Pr1 prostate cancer cells and xenografts*	[131]
	MBO-asGCS	GCS	Drug resistance breast, ovary, cervical and colon cancer cells and breast xenografts	[132, 133]
	PDMP	GCS	CML – T3151 mutant cells, *in vivo* transplants, primary cells from T3151 patients	[134]
(D)	C6-cer in combination		Ovarian, breast, pancreatic, melanoma cell lines and pancreatic and ovarian xenografts	[135–138]
	C6/C16-cer transferrin liposomes		Ovarian cancer cells and xenografts	[139]
	C6-cer nanoliposomes		Pancreatic and hepatocellular carcinoma cells and xenografts, sygeneic model of leukemia	[140–143]
	LCL29		MCF7 breast cancer cells	[145]
	LCL30		SCCVII mouse squamous carcinoma cells	[144]
(E)	FTY720	SK1	Hormone refractory prostate cancer cells and PC3 xenografts	[146, 147]
	ABC294640	SK2	Multiple cells lines and breast cancer xenografts	[148, 149]
	SK I-II	SK1, SK2	Breast cancer cells	[150]
	Sonepcizumab		Clinical trial including various solid malignancies	[151, 152]

(A) Stimulation of ceramide synthesis. (B) Hydrolysis of sphingomyelin. (C) Inhibition of ceramide utilization. (D) Direct use of ceramide and ceramide analogs. (E) Inhibition of S1P signaling. *Abbreviations used*: SPT – serine palmitoyl-CoA transferase, CerS – ceramide synthase, ASM – acid sphingomyelinase, NSM – neutral sphingomyelinase, AC – acid ceramidase, GCS – glucosylceramide transferase, SK – sphingosine kinase. *Although TsuPr1 was initially described as a prostate cancer cell line, it was later shown to be a derivative of T24 bladder cancer cells.

induces Bid-independent apoptosis in MCF7 breast cancer cells [145].

Inhibition of S1P signaling

The ceramide metabolite sphingosine serves as a substrate for sphingosine kinases, which generate S1P, thereby shifting the sphingolipid rheostat away from apoptosis (Fig. 1). Interfering with SK or its product S1P is an effective therapeutic strategy [146]. For example, the sphingosine analog FTY720, which affects several targets within the sphingolipid network, was shown to inhibit SK1 in prostate cancer cells and sensitized orthotopic prostate tumors to radiation [147]. The orally bioavailable SK2 inhibitor ABC294640 induced apoptosis in endocrine therapy-resistant MDA-MB-231 and chemoresistant MCF-7TN-R cells, and reduced tumor volume in preclinical models [148, 149]. Simultaneous inhibition of both SK isoforms by the SKI-II inhibitor induced apoptosis in multi-drug resistant breast cancer cells [150]. Lastly, given the complexity of sphingolipid signaling, it is remarkable that a monoclonal antibody against S1P known as Sonepcizumab yielded promising results against several solid tumors in Phase I clinical trials and is now entering Phase II trials [151, 152]. However, whether the efficacy of Sonepcizumab is primarily mediated through induction of apoptosis or through inhibition of S1P-mediated inflammation and angiogenesis may be difficult to determine.

CONCLUSION

Inducing apoptosis via modulation of sphingolipid metabolism is a viable therapeutic strategy. As shown in Fig. 2, depending on the subcellular location of ceramide generation, apoptosis may be facilitated through events like formation of ceramide-enriched platforms in the plasma membrane, permeabilization of the outer mitochondrial membrane or other less well-defined mechanisms.

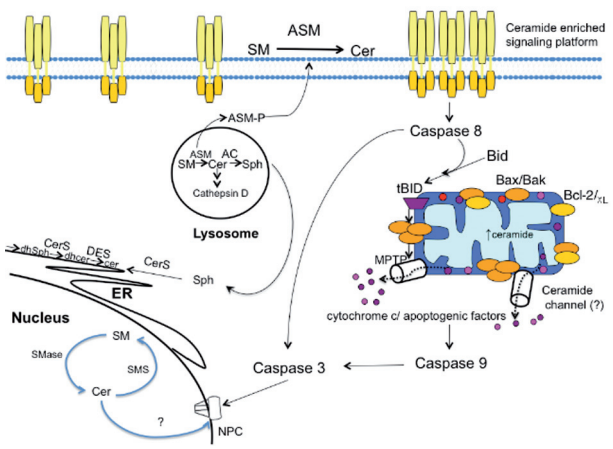

Fig. 2. Contribution of sphingolipids towards apoptotic signaling. Ceramide generated that the plasma membrane, mitochondria, lysosomes and nucleus may be required to enhance or promote the apoptotic signal. Details are described in the text. *Abbreviations used*: SMase — sphingomyelinase, ASM — acid sphingomyelinase, ASM-P — phosphorylated acid sphingomyelinase, SM — sphingomyelin, Cer — ceramide, Sph — sphingosine, CerS — ceramide synthase, DES — desaturase, dhSph — dihydrosphingosine, dhcer — dihydroceramide, SMS — sphingomyelin synthase, MTMP — mitochondrial permeability transition pore, ER — endoplasmic reticulum, NPC — nuclear pore complex

Generation of ceramide can occur in a tissue- and stimulus-dependent manner. Therefore, it will be important to further elucidate the physiological roles of ceramides with specific chain lengths and the contribution of specific subcellular pools of sphingolipids towards apoptosis in order to develop optimally designed therapeutic strategies to target various types of cancer.

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