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Glycosphingolipids and cell death

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Sphingolipids have been implicated in various cellular processes including growth, cell-cell or ligand-receptor interactions, and differentiation. In addition to their importance as reservoirs of metabolites with important signaling properties, sphingolipids also help provide structural order to plasma membrane lipids and proteins within the bilayer. Glycosylated sphingolipids, and sphingomyelin in particular, are involved in the formation of lipid rafts. Although it is well accepted that ceramide, the backbone of all sphingolipids, plays a critical role in apoptosis, less is known about the biological functions of glycosphingolipids. This review summarizes current knowledge of the involvement of glycosphingolipids in cell death and in other pathological processes and diseases.
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Introduction

Glycosphingolipids (GSL) are lipid components of membranes that are important for the proper development of vertebrates. They are involved in multiple processes, including cell type specific adhesion, cell-cell interaction, embryogenesis, and development and differentiation of neuronal cells and leukocytes [1]. GSL can also serve as binding sites for several viruses, bacteria, and bacterial toxins [2]. Different tissues display different GSL patterns on the cell surface which can be dramatically altered during development [1]. A further modulation can be seen during pathological processes such as tumor development. GM3/GD3, for example, is a melanoma-associated antigen involved in metastasis [3–5]. On the other hand, glucosylceramide (GlcCer) expression is associated with multidrug resistance in many cancer cells [6–8].

GSL are predominantly located at the plasma membrane and the early endosomes of the Golgi complex. In the plasma membrane, it has recently been shown that sphingolipid-derived molecules aggregate and form a less fluid and more ordered phase, referred to as membrane rafts, which are formed in the Golgi compartment and targeted to the plasma membrane. Rafts are considered to be small, mobile lateral assemblies of sphingolipids, particularly enriched in sphingomyelin and cholesterol, but also containing ceramide and GPI-anchored proteins. They have important roles in concentrating and modulating specific signaling molecules, such as Src-tyrosine kinase family members, growth receptors, and death receptors [9–12]. The role of rafts will not be discussed here as it has been the subject of recent excellent reviews [9,13]. Less complex sphingolipid-derived molecules, including ceramide, ceramide-1-phosphate, sphingosine, and sphingosine-1-phosphate (S1P), are known signaling molecules in diverse receptor and non-receptor-mediated signaling pathways. These bioactive lipid mediators are formed as a result of stimuli-induced metabolism of complex sphingolipids. Ceramide has mainly been implicated in signaling pathways leading to suppression of growth, cellular senescence, differentiation, and apoptosis, whereas ceramide-1-phosphate mediates cell survival and is involved in synaptic vesicular fusion in neuronal cells, as well as neutrophil phagolysosome formation [14]. S1P has many biological actions and, importantly, acts counter to ceramide to mediate cell growth and survival, as well as influencing directed cell movement [15,16]. The biological effects of sphingosine may vary among cell types but it has been associated with negative effects on cell growth and survival and has been implicated as an inhibitor of protein kinase C and other protein kinases [17,18].

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Biosynthesis and structure of glycosphingolipids

The *de novo* biosynthesis of GSL is initiated at the cytosolic surface of the endoplasmic reticulum (ER) by the condensation of L-serine and palmitoyl coenzyme A to form 3-ketosphinganine catalyzed by serine palmitoyltransferase (SPT), a pyridoxal phosphate-dependent enzyme [19,20]. SPT has lower activity

than the other enzymes involved in biosynthesis and is rate-limiting and seems to be a key enzyme controlling cellular sphingolipid content. In the ensuing NADPH-dependent reaction, 3-ketosphinganine is reduced to D-*erythro*-sphinganine by 3-ketosphinganine reductase. The enzyme sphinganine-*N*-acyltransferase (ceramide synthase) transfers a long-chain fatty acid to the amino group of 3-ketosphinganine, resulting in the formation of D-*erythro*-dihydroceramide. The latter enzyme shows selectivity for stearic acid and is also able to acylate sphingosine derived from the "salvage pathway" of sphingolipid catabolism [21]. A double bond is then introduced between carbon atoms 4 and 5 by a desaturase to form ceramide [22]. All four enzymes of ceramide biosynthesis are located at the cytosolic surface of the ER membrane [23,24]. Recently, major progress has been made in cloning the enzymes of the *de novo* pathway.

Ceramide is a precursor of both GSL and sphingomyelin. In the synthesis of sphingomyelin, a phosphocholine group is transferred from phosphatidylcholine to ceramide. Sphingomyelin synthesis occurs in several cellular compartments, although most is synthesized on the lumenal side of the Golgi complex [25–27]. In vertebrates, GSL synthesis is initiated by coupling a glucose [28] or galactose [29] residue in a β-glycosidic linkage to the C1-hydroxyl of ceramide. Specific glycosyltransferases catalyze [30,31] the transfer of additional single nucleotide activated sugars onto ceramide forming more complex GSL (Figure 1) [30–32]. Most of the GSL of vertebrates arise from glucosylation rather than galactosylation of ceramide. Glucosylation is rate-limiting for ganglioside biosynthesis. This glucosyltransferase seems to be crucial during embryogenesis as the knock out of the respective gene has been shown to be lethal [33].

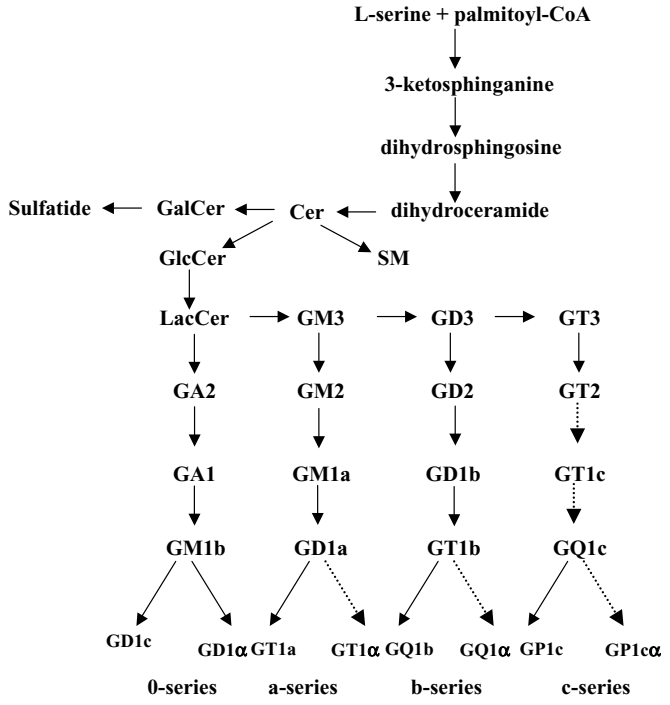

Figure 1. Simplified scheme of glycosphingolipid biosynthesis. For detailed descriptions of the biosynthetic pathways, see [38] and text.

Regarding topology of GSL synthesis, ceramide must be transported from the ER to the Golgi complex where it is glucosylated on the cytosolic surface of the Golgi-compartment [30,34–36]. Galactosylation of ceramide in the formation of glycosphingolipids of the galacto-series has been localized to the ER and Golgi. Transfer of a sulfate headgroup results in formation of sulfatides. The galacto-series gangliosides are found predominantly in the nervous system where they are important in development and normal functioning of the CNS [37–39]. Transport of ceramide from the ER to Golgi can occur by means of vesicular and non-vesicular mechanisms [40–42] and subsequent addition of sugar residues occurs on the lumenal face of the Golgi catalyzed by distinct glycosyltransferases [43].

Almost all gangliosides are structurally and biosynthetically derived from lactosylceramide which is formed by the transfer of a galactosyl residue to glucosylceramide. Sequential addition of one, two or three sialic acids to lactosylceramide results in formation of GM3, GD3 and GT3, respectively, precursors for more complex ganglio-series gangliosides. Sphingolipids are targeted to their cellular sites by both vesicular and non-vesicular trafficking [44].

Mechanisms of GD3-induced apoptosis

Ceramide is a well-known participant in the progression of pro-apoptotic signals initiated by Fas and tumor necrosis factor-α (TNF-α) through activation of their respective death receptors. The mitochondria also has a central role in ceramide-mediated cell death [45,46]. Generation of ceramide at the mitochondria, but not at other organelles, was shown to be involved in apoptosis of MCF-7 human breast cancer cells [47]. Fas cross-linking, TNF-α, and cell-permeable ceramide analogs all induce transient intracellular ceramide accumulation. An elegant study has shown that the intracellular ceramide accumulated due to Fas cross-linking is rapidly converted to GD3 by enhanced GD3 synthase activity in lymphoid and myeloid cell lines [48]. Antisense RNA against GD3 synthase prevents apoptosis, implying the need for newly-synthesized GD3. On the other hand, enforced expression of GD3 synthase was sufficient to trigger apoptosis. In this study, other gangliosides, such as GD1a, GT1b or GM1, failed to mimic GD3-induced cell killing [48]. Use of broad-spectrum caspase inhibitors revealed that caspases upstream of GD3 synthesis were crucial for Fas-induced apoptosis, suggesting modulatory interaction between GD3 and caspases. With respect to the mechanism by which GD3 induces apoptosis, changes in the mitochondrial membrane potential ($\Delta\Psi_m$) and increased reactive oxygen species (ROS) production have been demonstrated [48–51]. As a consequence of decreased $\Delta\Psi_m$, permeability of the inner mitochondrial membrane increases, causing the collapse of the ion gradient along the membrane and depolarization of the

mitochondria. ROS have been implicated in the initiation phase as well as in the execution phase of the apoptotic program, depending on cell type. Also, addition of ROS or depletion of endogenous antioxidants induces apoptosis that can be reversed by exogenous addition of antioxidants. The detailed mechanisms by which ROS function in cell death are not yet clear, but they are likely to be involved in activation of executionary caspases. In TNF-α-resistant hepatocytes, it was demonstrated that TNF-α was still able to induce synthesis of GD3. Only after depletion of mitochondrial glutathione, which is crucial for the maintenance of the cellular redox state, were cells sensitized to TNF-α- or GD3-mediated apoptosis [52]. This study underlines the importance of oxidative stress in TNF-α-mediated apoptosis in hepatocytes. By using inhibitors or antioxidants, it was shown that GD3 interacts with complex III of the mitochondrial electron transport chain causing an oxidative burst that precedes mitochondrial swelling. Cytochrome c and apoptosis inducing factor (AIF) are subsequently released, leading to activation of the caspase cascade and eventual DNA fragmentation [50,51]. *In vitro* studies performed with isolated mitochondria revealed that short-chain ceramides and glycosphingolipids, such as GlcCer, LacCer, GD1a, and GM1, are able to mimic GD3, while sphingosine and sphinganine failed to do so. Apparently, the *N*-acylsphingosine (ceramide) moiety is required for interaction of GD3 with the mitochondria rather than the carbohydrate component [50]. However, other groups showed that GD3-mediated effects on isolated mitochondria are very specific and could not be mimicked by C_2-ceramide, GM1, GM3, GD1a, or GT1b [53,54]. Future studies are needed to clarify this discrepancy and to determine the minimal structural requirement that enables GD3 to interact with and recruit mitochondria to the apoptotic signal transduction pathway.

GD3 can directly activate $\Delta\Psi_m$ independently of Ca^{2+}, although Ca^{2+} has been shown to act synergistically with GD3 [53]. It has been suggested that the effects of GD3 on mitochondria are mediated by the opening of the mitochondrial permeability transition pore (MTP), rather than by inhibition of the respiratory complex, as GD3-mediated effects could be prevented with the MTP blocker, cyclosporin A [54]. The MTP is a conductance channel formed by several different proteins, which is inserted into the mitochondrial membrane [55].

Bcl-2 is a proto-oncogene known to suppress cell death by diverse stimuli [55]. One mechanism by which Bcl-2 protects cells is suppression of the formation of ROS by acting as an antioxidant [55,56]. Because several studies have shown that ROS production in the mitochondria is a key target for apoptogenic GD3, it is conceivable that Bcl-2 might be able to modulate this pathway as well. Indeed, in T cell lymphoma CEM cells stably overexpressing Bcl-2, GD3 failed to induce mitochondrial changes or release of cytochrome c, AIF and activate caspase-9 [51]. Similar observations were made in oligodendrocytes where GD3-induced increase in $\Delta\Psi_m$ and cytochrome c release could be partially blocked by enforced Bcl-2 expression [57]. How Bcl-2 blocks GD3-induced cell death is not known yet. Several models have been proposed to explain how Bcl-2 might exert its anti-apoptotic function. It could prevent pore formation induced by other pro-apoptotic Bcl-2 family members, such as Bax/Bak, via increased heterodimerization of these proteins, or it could inhibit the opening of the MTP [58,59]. Moreover, Bcl-2 family proteins appear to regulate voltage-dependent anion channel (VDAC) function [60,61]. Further studies are needed to identify the relevant GD3 targets which are under Bcl-2 control. However, pretreatment of isolated mitochondria with cyclosporin completely suppressed GD3-induced swelling and release of apoptogenic factors, indicating that GD3 acts at the level of the MTP. Whether this is due to a direct interaction with any of the MTP components remains to be established [51].

Ceramide can be generated from degradation of sphingomyelin by either acidic sphingomyelinase (aSMase) or neutral sphingomyelinase (nSMase). Alternatively, ceramide generated by *de novo* synthesis has also recently been implicated in apoptosis [62]. Furthermore, it has been reported that ceramide generated by aSMase, and not nSMase, is involved in Fas and TNF-α signaling pathways activated in GD3 mediated cell death even though nSMase is active and contributes to the increase in ceramide in human colon cells [63]. These results were further confirmed with Niemann-Pick-derived lymphoblastoid cells that are devoid of aSMase but display normal nSMase activity [64]. In these cells, Fas failed to initiate the apoptotic program. Reconstitution of aSMase activity or addition of exogenous aSMase, however, caused GD3 accumulation and efficiently triggered the apoptotic program after Fas cross-linking or γ-irradiation.

Gangliosides are distributed predominantly on the plasma membrane and in the early Golgi compartment where they are synthesized. GD3 synthase (α2,8-sialyltransferase), which resides in the Golgi, adds a second sialic acid to GM3 to produce GD3. Just as is the case with ceramide, there seems to be a dichotomy in the signaling properties between newly synthesized GD3 and GD3 formed from degradation of other complex gangliosides. It appears in this case that newly-synthesized GD3 is involved in regulating apoptosis [65]. The question arises as to how newly formed GD3 is targeted to mitochondria where it executes its function in cell death. Different pathways might come into play. First, mitochondria might be in close physical contact with the ER/early Golgi to form a functionally interconnected network which has been described recently [66–68]. Second, and more likely, GD3 might be redistributed to mitochondria by actin-dependent endosomal vesicles. Indeed, disruption of actin cytoskeletal organization prevents release of GD3 from plasma membrane and co-localization with mitochondria [65]. Also, GD3 was shown to co-localize and associate with the actin cytoskeletal protein ezrin upon Fas cross-linking [69]. Moreover, pretreatment of cells with inhibitors of vesicular transport, such as monensin or mannose-6-phosphate, abolished localization of GD3 with mitochondria in hepatocytes treated with TNF-α [65]. Trafficking of GD3 was monitored over time and co-localization of GD3 was seen with markers specific for

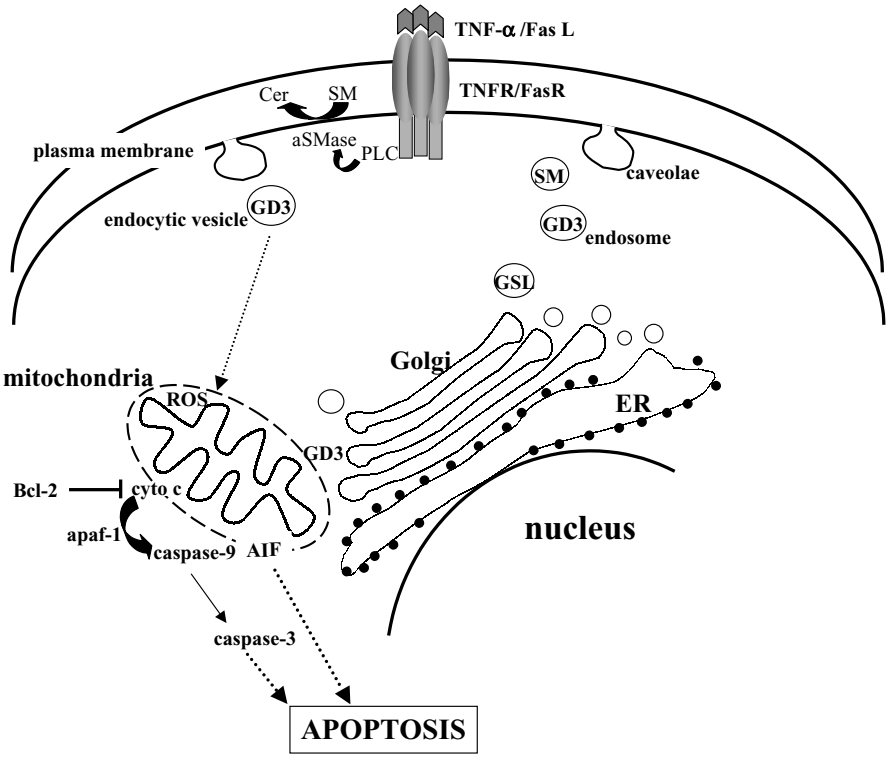

Figure 2. Signaling pathway of GD3-induced apoptosis. After oligomerization of the Fas or TNF-α receptors, aSMase is activated in a PC-PLC-dependent manner. Ceramide accumulates and activates GD3 synthesis. GD3 is targeted from the plasma membrane by vesicle transport or alternatively by physical redistribution from the Golgi to the mitochondria. There, GD3 perturbs the mitochondrial membrane leading to release of cytochrome c and AIF and caspase-9 activation, which activate the execution phase of the apoptotic program leading to demise of the cell.

plasma membrane, early endosomes, late endosomes, and finally with mitochondria [65]. GD3 ganglioside on the plasma membrane is localized, most likely, in specialized rafts, known as caveolae, where it can be internalized through endocytosis and trafficked to mitochondria (Figure 2). Co-localization of GD3 with caveolin-1 has been described previously [70].

As mentioned above, only ceramide generated in specific compartments, such as mitochondria or at the plasma membrane, has been shown to be involved in programmed cell death [47,71,72]. Furthermore, depending on cell type and/or agonist, aSMase and/or nSMase contribute to ceramide generation. Cells derived from aSMase null mice are defective in Fas-, radiation-, and TNF-α-induced cell death [63,64,73,74]. aSMase is active mainly in acidic compartments, such as recycling endosomes, and soluble aSMase is taken up by endocytosis and transported to acidic compartments. Membrane-bound forms of aSMase have also been detected in caveolae microdomains enriched in sphingomyelin that can be activated by various stimuli resulting in formation of ceramide [75,76]. Translocation of aSMase from intracellular compartments to plasma membrane rafts has been demonstrated after Fas stimulation [77].

Two different cytoplasmic domains have been described in the 55 kDa TNF receptor. One domain is able to activate nSMase and the other activates aSMase with no apparent crosstalk between them. The aSMase activation domain resides in the so-called death domain of the TNF receptor responsible for the cytotoxicity of TNF-α [78]. A phosphatidylcholine-specific phospholipase C activity was also required for aSMase activation [79,80].

It is assumed that intracellular ceramide concentrations regulate sphingolipid and glycosphingolipid metabolism; and, hence, ceramide should be targeted to the Golgi complex. Because aSMase has been shown to reside in caveolae, decreased sphingomyelin and concomitant ceramide production can lead to structural changes of the plasma membrane which can somehow stimulate endocytosis and trafficking of ceramide to the ER and Golgi, thereby enhancing GD3 synthesis. This might also explain why ceramide generated via aSMase, but not nSMase, is able to activate *de novo* GD3 synthesis.

Glycosphingolipids with anti-apoptotic properties

Difficulties in effective chemotherapy correlate with defective activation of programmed cell death on several distinct levels in many types of tumors [81]. Chemotherapeutic agents often exert some of their effects through generation of ceramide even though their mechanisms of action might differ.

Some stimulate *de novo* ceramide synthesis whereas others induce sphingomyelin hydrolysis or block ceramide degradation. Multidrug resistance, defined as cross-resistance to a variety of chemotherapeutic substances, is a common phenomenon in the treatment of various cancers. Besides accelerated removal of the drug (*i.e.* enhanced drug efflux via the P-glycoprotein pump), their intracellular effects may be altered [82,83]. It is also possible that multidrug resistance could result from modulation of ceramide metabolism whereby ceramide accumulation is prevented or it is converted to less toxic molecules [6,7,84]. Yet another way to keep endogenous ceramide low is by GlcCer synthase catalyzed conversion to GlcCer which has been shown to have growth stimulatory and anti-apoptotic effects [85]. Studies with exogenous administration of GlcCer revealed that it is able to stimulate growth of keratinocytes even in aged murine epidermis where epidermal growth is normally reduced [86]. Furthermore, GlcCer is consistently increased in several multidrug-resistant cancer cell lines [83,87]. In this regard, it was demonstrated that some sensitive cells acquire drug resistance by overexpressing GlcCer synthase [88]. Conversely, blocking glycosylation of ceramide with different agents, such as verapamil, tamoxifen, cyclosporin A or PDMP, in multidrug resistant MCF-7 breast cancer cells, sensitized them to adriamycin [89]. In addition, GlcCer synthase antisense RNA rendered otherwise resistant cells sensitive to drug treatment [88–91]. Reduced tumorigenicity and metastatic potential of melanoma cells was also observed *in vivo* with GlcCer synthase antisense RNA [92].

Another ganglioside that has been implicated in protection of cells from apoptosis is the monosialylganglioside GM1. GM1 has been shown to prevent apoptotic cell death in growth factor-deprived neuronal PC12 cells [93]. GM1 acts by promoting nerve growth factor (NGF)-induced TrkA dimerization. It has also been demonstrated that NGF signaling can activate sphingosine kinase to form S1P that acts as a pro-survival signal [93,94]. Similar results were obtained in a study conducted in rat heart fibroblasts where GM1 was shown to act like S1P and protect cells from C_2-ceramide or staurosporine-induced cell death [95]. It was also demonstrated in this study that GM1 enhanced S1P production by activating sphingosine kinase [95]. In a more physiologically relevant study, application of GM1 also protected the mouse heart from hypoxic cell death. Again, sphingosine kinase-dependent activation by protein kinase Cε was suggested [96]. These results have relevance to human physiology and there are ongoing clinical trials using GM1 ganglioside as a therapeutic agent for promoting nerve regeneration in Alzheimer's disease [97]. Furthermore, autoantibodies against various glycosphingolipids have been detected in patients with different neurological disorders, and have been suggested to play a critical role in development of diseases of the nervous system. In contrast, Le(y) antigen expression is correlated with apoptosis [98].

Gangliosides can also regulate cell signaling by altering growth factor receptor functions [99]. High GM3 ganglioside expression on keratinocytes has been correlated with inhibited cell growth and low expression has been reported in several hyperproliferative skin disorders, including psoriasis and squamous cell carcinoma [100,101] where programmed cell death is aberrant. Ganglioside GM3 was shown to interfere with binding of EGF and activation of its receptor which is required for proliferation [102].

Surprisingly, GM3 is also pro-apoptotic in certain types of cells, particularly in the presence of metastasis-suppressing gene product CD82 and its analogue CD9. It was shown that the malignancy-suppressing effect of CD82 or CD9 is based partially on cell motility inhibition and apoptosis induction promoted by concurrent GM3 synthesis and N-glycosylation [103]. GM3 in various colorectal carcinomas may also promote apoptosis, since enhancement of endogenous sialidase promotes tumor malignancy and metastasis through inhibition of Bcl-2 [104]. These dual actions of GM3 merit further study.

Significance of gangliosides in pathological processes

It has long been known that tumor cells display a different pattern of cell surface glycosphingolipids than corresponding untransformed cells [1,105]. Predominant expression of specific gangliosides, GD3, GM2, or GD2, has been observed on several types of tumor cells including melanoma, neuroblastoma, lymphoma, and ovarian cancer cells [105,106]. Thus, antibodies against specific gangliosides have received consideration as immunotherapeutic agents and clinical trials have been initiated [4,106–108].

Augmented GSL shedding, which is the release of cell surface components, is a characteristic of cancer cells. Shedding seems to be important for infiltration and metastasis of the tumor as well as for suppression of the immune system [109]. The underlying mechanism by which the released components evoke these biological effects is not yet fully understood. Gangliosides are among the main constituents of the released molecules. *In vitro,* and more importantly, *in vivo* effects of gangliosides shed from T cell lymphoma on bone marrow cells (BMC) have been documented [110]. These gangliosides not only impaired cell viability but also induced apoptosis of BMC. The shed gangliosides activated NF-κB and elevated expression of p53 and Bax, both of which have been described as components of pro-apoptotic signaling pathways. The apoptosis effects were ascribed to GD3 by investigations with antibodies against the major ganglioside species produced by T cell lymphomas. GD3 exogenously applied to BMC effectively induced apoptosis, further confirming this finding [110].

Another disease in which GD3 seem to be involved in is the progression of pathogenesis in Farber Disease, a lysosomal storage disorder which results from an acid ceramidase deficiency. As a consequence, ceramide accumulates in lysosomes leading to tissue damage, although the detailed mechanisms of tissue destruction are not well known. Histochemical analyses of tissues from affected patients revealed a high apoptotic rate

that correlated with concomitant elevated levels of GD3 and activated caspase-3 [111].

Conclusions

There is now abundant evidence documenting the importance of sphingolipid-derived signaling molecules. Although some sphingolipids have been well established as second messengers, *i.e.* ceramide, sphingosine and S1P, others await more detailed investigations. One difficulty in identifying the specific sphingolipid involved in a particular signaling pathway is their complex interconversion. For example, functions attributed to sphingosine might actually result from its conversion to S1P. Moreover, some effects of ceramide might result from its conversion to ceramide-1-phosphate, GlcCer, or even sphingosine and S1P. Also rapid degradation of gangliosides to ceramide has been described and this might confuse distinctions between ceramide and ganglioside-mediated effects [112]. As found for GM1, one GSL might also be able to stimulate the generation of another sphingolipid [95]. Since GD3 has mainly been associated with cell death and GM1 with survival, it will be very important to determine exactly what structural feature of these molecules is required for initiation of particular signaling pathways. This further emphasizes how important it is to elucidate the mechanism by which a molecule produces a certain biological response in order to design drugs for therapeutic applications.

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