Glycoconjugate Journal 20, 579–588, 2004

Glycosphingolipids and mitochondria: Role in apoptosis and disease

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Glycosphingolipids (GSLs) comprise a class of lipids with important structural and signaling functions. Synthesized from ceramide in the Golgi, they are subsequently distributed to different compartments, most predominantly in the plasma membrane where they integrate signaling platforms. A recently characterized trafficking of ganglioside GD3 (GD3), a GSLs with two sialic-acid residues, to mitochondria has revealed a novel function of this lipid as a death effector. In addition to the interaction of GD3 with mitochondria recruiting these organelles to apoptotic pathways, GD3 disables survival paths dependent on NF-κB, thus favoring the balance towards cell death. The present review gathers the evidence documenting this emerging function of GSLs in cell death and their involvement in pathological states.
Published in 2004.

Keywords: **GD3, apoptosome, oxidative stress, cell death, NF-κB, cancer therapy**

Introduction

Glycosphingolipids (GSLs) are ubiquitous membrane constituents that, in addition to their structural role, are emerging as signaling lipids involved in several pathological states. GSLs constitute an amphipathic family of lipids made up of a ceramide lipid anchor linked to an oligosaccharide chain of variable length and complexity. Gangliosides are prominent members of GSLs that are distinguished by the presence of one or more sialic acid residues. The abundance and complexity of GSLs are of particular relevance in brain where they were discovered in 1942 by Klenk et al. [1], displaying a complex heterogeneity as demonstrated later on by Svennerholm [2]. These lipids, however, are found on plasma membranes from all mammalian cells, where they are concentrated in microdomains specialized for cell signaling [3]. Gangliosides have been implicated in fundamental cell processes such as growth, differentiation and adhesion. In addition to these functions, an emerging role of GSLs, particularly ganglioside GD3, as apoptosis regulators is increasingly recognized due to their ability to recruit mitochondria to cell death pathways [4]. In this review we will summarize the evidence provided recently documenting the role of GSLs, with particular focus on ganglioside GD3, in apoptosis through mitochondrial-dependent pathways and their role in disease.

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Ceramide as the source of GSLs and role in cell death

The biosynthesis of gangliosides involves several steps that occur in different intracellular compartments and imply the sequential addition of oligossacharides to ceramide. Ceramide is synthesized on the endoplasmic reticulum by the pyridoxal phosphate-dependent enzyme serine palmitoyl transferase, the rate-limiting step in ceramide synthesis from L-serine and palmitoyl coenzyme A [5]. After transport to the Golgi apparatus, specialized glycosyltransferases transfer a glucose [6] or galactose [7] residue in a β-glycosidic linkage to the C1-hydroxyl of ceramide to produce glucosylceramide or galactosylceramide. While the glucosylation of ceramide occurs on the cytosolic surface of the Golgi [8,9], its galactosylation can occur both in the endoplasmic reticulum and the Golgi. However, most of the GSL arises from the glucosylation rather than galactosylation of ceramide, a step catalyzed by the rate-limiting enzyme glucosylceramide synthase (GlcT), an enzyme essential for embryogenesis [10]. Glucosylceramide is then transferred to the lumenal leaflet of the Golgi, where it is modified by the addition of a galactose moiety to produce lactosylceramide from which most of gangliosides derive by the action of specific glycolipid-glycosyltransferases (GSL-GLTs) [11–13] (Figure 1). Indeed, sequential addition of one, two, or

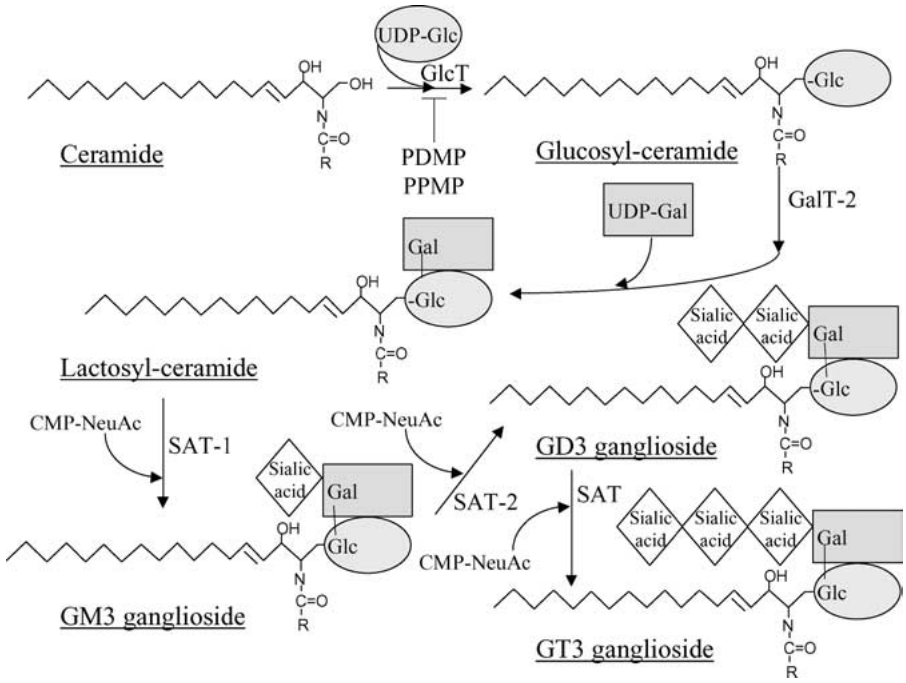

Figure 1. Glucosylation of ceramide as the initial step in the synthesis of sialic-acid gangliosides GM3 and GD3. GlcT catalyzes the transfer of a glucose residue to the carbon backbone of ceramide to yield glucosylceramide which then is transformed into lactosylceramide. The addition of several sialic acids residues produces GM3-GT3.

three sialic acids to lactosylceramide results in the formation of GM3, GD3, and GT3, respectively, whose carbon backbone derives from ceramide. Since the synthesis of gangliosides involves the trafficking of ceramide from the endoplasmic reticulum to the Golgi, the characterization of this process may be important in ganglioside-mediated signaling. The intracellular trafficking of ceramide has been suggested to occur by vesicle-dependent and—independent mechanisms [14,15]. However, recent findings provided a new insight in this process with the identification of a novel protein named CERT that mediates the ATP-dependent transport of ceramide from the endoplasmic reticulum to the Golgi in a vesicle-independent manner [12]. CERT is a cytoplasmic protein with a phosphatidylinositol-4-monophosphate-binding (PtdIns4P) domain and a putative domain for lipid transfer. Indeed, the lipid-transfer-catalysing domain of CERT is responsible for its ability to specifically extract ceramide from phospholipid bilayers, as the disruption of its PtdIns4P-binding activity impairs its Golgi-targeting function [16].

Since ceramide provides the carbon backbone of GSLs, their synthesis is also dependent on the availability of ceramide generation. In addition to the *de novo* synthesis of ceramide by serine-palmitoyl transferase or ceramide synthetase, ceramide can arise from hydrolysis of sphingomyelin-engaging sphingomyelinases (SMases) [17]. This pathway may be of significance in promoting specific macrodomain formation in the plasma membrane, allowing oligomerization of certain cell surface proteins such as ligated receptors (TNF family) [18]. Several SMases have been characterized of which two are of relevance in signaling. The membrane-bound neutral SMase (NSMase) with an optimum pH of approximately 7.5 and an acidic SMase (ASMase) with an optimum pH of about 4.8 further subclassified into an endosomal/lysosomal ASMase and a secretory Zn^{2+}-dependent SMase [17]. Apoptotic stimuli, such as death ligands (*e.g.*, Fas and TNF), chemotherapeutic agents, or ionizing radiation, activate these SMases, accounting for the ability of the inducing stimuli to generate ceramide with various kinetics and possibly at different intracellular locations. However, their individual contribution to apoptotic cell death is not clearly established and seems to depend on the kind of apoptotic stimuli used and the cell type studied. For instance, NSMase has been involved in the stress response mediating the cytotoxic effects exerted by chemotherapeutic agents [19]; ASMase has been shown to mediate ionizing radiation-induced cell death [20,21] as well as in the developmental death of oocytes [22]. On the other hand, although the factor associated with NSMase activation (FAN), an adaptor protein involved in NSMase activation, has been shown to contribute to TNF-induced fibroblast apoptosis and lipopolysaccharide and TNF-induced lethality [23,24], the role of NSMase ablation itself in TNF-induced apoptosis remains unknown. In contrast, ASMase has been shown to mediate Fas-induced cell death in hepatocytes [25,26]. Furthermore, ASMase has been recently shown to play a significant role in hepatocellular apoptosis and liver damage induced by TNF through a dual mechanism involving GD3 generation and downregulation of MAT1A [27,28]. Thus, as illustrated by these recent findings on TNF signaling, the role of ASMase-induced ceramide generation on apoptosis

seems to be mediated by its conversion to GD3 as its downregulation by inhibition of GlcT with PDMP protects *ASMase*$^{+/+}$ hepatocytes from TNF despite enhancement of TNF-stimulated ceramide formation. In addition to providing GD3, ceramide generated from ASMase is required for the efficient activation of lysosomal cathepsin D by TNF [29], thus linking TNF receptor signaling to acidic compartments engagement.

GD3 and mitochondria: A dangerous interaction

Consistent with the relevance of mitochondria in the regulation of apoptosis [30–32], and the recognized role of ceramide in the stress response and cell death [17,33], early studies documented a direct effect of ceramide on isolated mitochondria from rat liver [34]. These findings were subsequently confirmed indicating that ceramide disrupts electron flow at complex III of the respiratory chain, resulting in enhanced reactive oxygen species (ROS) generation, release of cytochrome c, and caspase activation [34–37]. In addition, the *in situ* generation of ceramide within mitochondria by enforced mitochondrial targeting of bacterial sphingomyelinase induces apoptosis in MCF7 cells [38]. As mentioned above and consistent with the structural features shared by ceramide and GD3, the ability of ceramide to directly interact *in vitro* with isolated mitochondria was reproduced with GD3 (Figure 2) [39–43]. Although GD3 clearly induced the release of cytochrome c, Smac/Diablo, and AIF engaging the apoptosome in a cell-free system [39,41,43], the underlying mechanisms responsible for the GD3 effects are not completely known. The ability of GD3 to evoke the release of cytochrome c from mitochondria was mimicked by GD1a, GD1b, GT1b, and GQ1b along with synthetic GD3 mimetics, indicating the critical role of two sialic acid residues in this effect [44]. According to the evidence provided, it appears that GD3-stimulation of ROS was independent of Ca^{2+} and preceded mitochondrial membrane permeabilization that mediates the release of cytochrome c and caspase 3 activation [39]. Consistent with this sequence of events, antioxidants and cyclosporin A prevented GD3-mediated effects in isolated mitochondria [39,43]. Moreover, the consequences of GD3-elicited changes in mitochondria such as mitochondrial membrane permeabilization and subsequent release of proapoptotic proteins from the intermembrane space of mitochondria were controlled by the levels of mitochondrial GSH independent of the fluidity state of mitochondrial membranes [43]. These observations further support the view that GD3 induces a burst of ROS from the respiratory chain. An interesting study indicated that the apoptotic potential of GD3 is modulated by acetylation so that acetylated GD3 (9AcGD3) is unable to elicit the change reported for GD3 [45]. These provocative findings raise the question as to why GD3 is active within mitochondria but not other gangliosides with closely related structures such as 9AcGD3, implying the existence of a GD3 receptor or flippase protein that recognizes the structure of GD3, including the outer sialic acid residue that becomes *O*-acetylated in 9AcGD3. In this regard, truncated Bid, a proapoptotic cytosolic factor of the Bcl-2 family, displays high affinity toward acidic phospholipids and is thought to be involved in membrane lipid transfer to mitochondria [46]. It is thought that Bid affects the structural

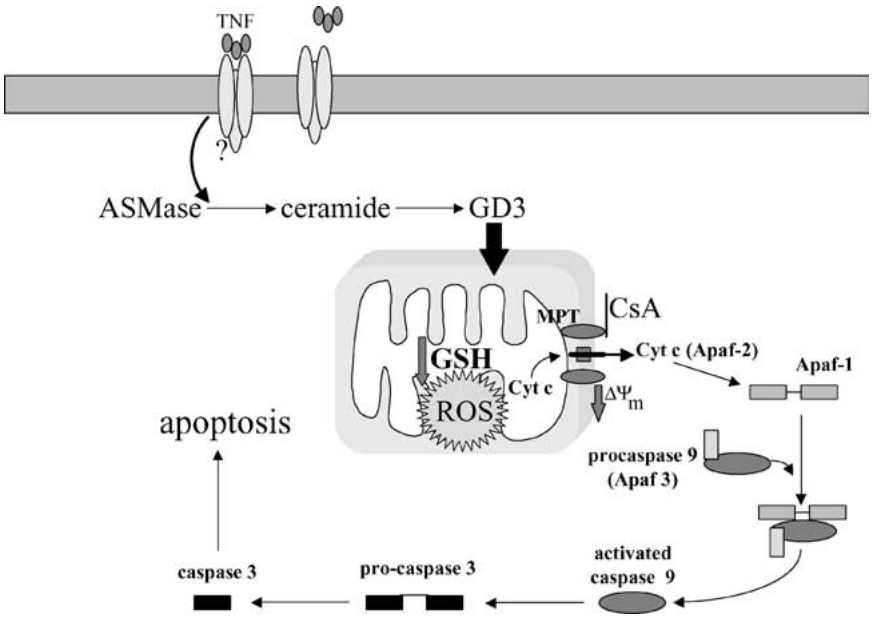

Figure 2. Role of GD3 in apoptosis. The interaction of GD3 with mitochondria elicits the mitochondrial apoptosome resulting in activation of executioner caspase 3. The upregulation of ASMase by TNF elicits ceramide generation which can be converted into GD3. The exact mechanism of ASMase by TNF is not fully understood at present, nor the route responsible for the trafficking of GD3 to mitochondria.

state of multidomain antiapoptotic Bcl-2 proteins in the outer membrane by changing the lipid environment in mitochondria. Whether truncated Bid or another Bid-like proapoptotic protein functions as a GD3 flippase remains unknown. It would be interesting to test if mitochondria isolated from *Bid*$^{-/-}$ mice respond to a GD3 challenge to elicit the described effects on rat liver mitochondria.

GD3 trafficking to mitochondria

While these studies clearly established a direct effect of GD3 on isolated mitochondria, the relevant question was whether the interaction between GD3 and mitochondria occurs in intact cells thus contributing to apoptosis. The first suggestion for this event was provided by DeMaria *et al.* examining the fate of myeloid and lymphoid cell lines in response to Fas and ceramide [47]. These pioneering studies demonstrated that the ceramide increase in response to Fas was rapidly converted into GD3 by GD3 synthase so that its knockdown by antisense RNA prevented Fas-induced apoptosis. Although, the accumulation of GD3 in response to Fas was accompanied by dissipation of mitochondrial membrane potential, these data did not demonstrate the interaction of GD3 with mitochondria [47]. Recent studies using immunoelectron and laser confocal microscopy showed the physical interaction and accumulation of GD3 in mitochondria from human lymphoblastoid CEM cells [48], intact hepatocytes [49], or human colon HT-29 cells [50] exposed to C_2-ceramide or TNF. The trafficking of GD3 to mitochondria was observed in hepatocytes in response to various apoptotic inducers and was preceded by a gradual disappearance of GD3 from the plasma membrane and its co-localization with Rab5 and Rab7 in early and late endosomes via coordinated secretory/endocytic vesicular trafficking [49]. The disruption of this pathway by actin-disrupting agents, *e.g.* latrunculin A, prevented the interaction of GD3 with mitochondria sparing sensitized hepatocytes to TNF exposure. In line with these findings, the colocalization of GD3 with ezrin, an actin cytoskeleton protein, mediating Fas-induced apoptosis in CEM cells has been recently described [51]. Furthermore, inhibition of GlcT by PDMP, disruption of microtubules, or plasma membrane cholesterol extraction by nocodazole and filipin, respectively, prevented the redistribution of GD3 from the plasma membrane to mitochondria [49], indicating that the newly synthesized GD3 undergoes a regulated trafficking to the plasma membrane and from here to mitochondria. The exact route followed by GD3 to target mitochondria is presently unknown. Although these observations in hepatocytes support the involvement of endosomal vesicle movement in the targeting of GD3 to mitochondria, a direct targeting of GD3 to mitochondria resulting from the continuity and contact between the Golgi/endoplasmic reticulum network with mitochondrial membranes cannot be ruled out at present [52,53]. Since GD3 is synthesized within the lumen of the Golgi, being then embedded in the outer leaflet of the plasma membrane, either of the above delivery systems would cause GD3 incorporation into the inner leaflet of the outer mitochondrial membrane [54]. Thus, the trafficking of endosomal vesicles through actin cytoskeleton may be part of the TNF/Fas multicomponent signaling complex delivering death signals, *e.g.*, GD3, to mitochondria.

GSLs as resourceful proapoptotic lipids: Role in cancer therapy

Cell death/survival is a dynamic process that reflects the balance of opposing signals promoting death or survival pathways. NF-κB is a transcription factor that besides to its role as a master regulator of the inflammatory and immune responses it is also known to induce the expression of proteins promoting cell survival [55–57]. Indeed activation of NF-κB promotes cell survival through induction of antiapoptotic genes including Bcl-XL, c-IAP1, c-IAP2, A1/Bfl1, or modulation of tumor suppressor PTEN [58–63]. NF-κB is usually kept inactive in the cytoplasm through association with an endogenous inhibitor protein of the IκB (inhibitor of NF-κB) family. The most common pathway leading to NF-κB activation involves the phosphorylation of IκB at specific serine residues that targets its subsequent degradation by the proteasome [55,56]. The released subunits of NF-κB then translocate to the nuclei where they bind to specific sites in the promoter/enhancer region of target genes. Thus accordingly, suppressing the activation of the survival pathway dependent on NF-κB may have profound consequences in the response of cancer cells to therapy. In this regard, prior studies reported the ability of GD1a and GM1 to suppress the activation of NF-κB that blunts antitumor immune responses [64]. In addition, GD3 was shown to interfere with the nuclear translocation of active NF-κB members to the nuclei thus rendering rat hepatocytes susceptible to TNF-mediated cell death [42]. Furthermore, gangliosides expressed in renal cell carcinomas promoted the degradation of RelA/p50 dimers in T cells [65], and the modulation of cell cycle and survival of keratinocytes by GT1b was mediated by its direct inhibition of Akt signaling [66]. Based on this novel function of gangliosides in abrogating the induction of survival signals, GSLs may have an emerging role in cancer therapy. For instance, recent observations reported the role of GD3 to sensitize human hepatoblastoma cells HepG2 to cancer therapy that was dependent on its ability to blunt NF-κB-mediated survival signals induction [67]. While ionizing radiation and daunorubicin induced NF-κB activation and transactivation, preincubation of HepG2 cells with a sublethal dose of GD3 abrogated the nuclear translocation of RelA/p50, thus blunting NF-κB-mediated gene induction. In this paradigm, cells were sensitized to radiotherapy due to overaccumulation of ROS/RNS generated from mitochondria [67]. In comparing the action of various GSLs in promoting ROS generation from mitochondria and NF-κB inactivation, it appears that the presence of carbohydrate residues is required for the interference with the nuclear translocation of NF-κB [42]. In addition, GM3 overexpression was shown

to reduce malignant potential in murine bladder cancer through enhanced apoptosis [68]. Furthermore, GM3 and GD3, purified from human melanoma tumors, inhibit the phenotypic and functional differentiation of monocyte-derived dendritic cells induced by CD154 in a dose-dependent manner. Additions of GM3 and GD3 decrease the viable cell yield and induce significant monocyte-derived dendritic cell apoptosis, decreasing interleukin-2 (IL-2) and increasing interleukin-10 (IL-10) concentration in monocyte-derived dendritic cells. This cytokine pattern may hamper an efficient antitumor immune response [69]. Similar to the effect elicited by GD3 on HepG2 cells, exogenous GM1 was shown to induce thymocyte apoptosis, involving suppression of NF-κB [70]. Hence, the current data uncover gangliosides as multifunctional signaling lipids that induce apoptosis not only by a direct effect on mitochondria but also by suppressing survival genes, a dual personality of relevance to overcome cancer therapy resistance.

GSLs and multidrug resistance

Despite observations in the early 1980s indicating differences in GSLs composition of drug-induced resistant cells and their inability to synthesize complex gangliosides [71], the involvement of GSLs in drug-induced cancer cell death is not fully established and relates to the ultimate intermediate responsible for cell death ceramide *vs* GSLs. The modulation of ceramide levels induced by cancer therapy may determine the response of cancer cells to therapy. GlcT, the enzyme that transfers a glucose residue to the carbon backbone of ceramide [6] (Figure 1), yields glucosylceramide diminishing the availability of free ceramide. In multridrug resistant cells, ceramide glucosylation contributes to drug resistance and the inhibition of GlcT sensitizes cells to various drug treatments [72–74]. However, recent observations showed that melanoma cells lacking GlcT activity were equally sensitive to chemotherapy [75] indicating that the involvement of GlcT in drug resistance is not universal. Interestingly, recent data showed a cytoprotective role of GlcT inhibition against daunorubicin-induced apoptosis in human leukemic cell lines [76]. While GlcT inhibitors blocked drug-induced apoptosis, galactosylation was associated with drug resistance. Thus, the generation of ceramide serves as the precursor for antagonizing GSLs; while GD3 promoted apoptosis, galactosylceramide elicited an antiapoptotic role. In line with this, GlcT inhibition by antisense knockdown and the classic inhibitor PDMP [77] was also shown to drastically decrease the apoptosis induced by *N*-(4-hydroxyphenyl)retinamide in neuroepithelioma cells [78]. Hence, although the multidrug-resistance in cancer cells is a complex phenomenon involving the interplay of different molecular mechanisms and multistep alteration of the sphingolipid metabolism [79], the current evidence indicates that gangliosides may function as sensitizing agents enhancing the anti-cancer properties of currently used therapy.

GSLs in disease

The emerging role of GSLs in cell signaling and in the regulation of apoptosis impinges on the development of pathological processes. This is of particular significance in states in which overproduction of TNF is essential for the progression of the disease. As alluded above, the generation of GSLs, *e.g.* GD3, is modulated by the availability of ceramide and TNF is known to increase cellular ceramide content [17,27,34]. In alcohol-induced liver disease (ALD), the pathogenic role for TNF has been established [80,81] and ASMase has been described to contribute to TNF-mediated hepatocellular apoptosis and liver damage [27,28]. Recent studies indicated that alcohol feeding to mouse enhanced the activity of ASMase induced by LPS administration resulting in elevated tissue content of ceramide [82]. In addition, previous findings have shown that alcohol feeding potentiates the mitochondrial membrane permeabilization induced by GD3 [83] and the treatment of mice with LPS induced the expression of GlcT mRNA leading to enhanced levels of glucosylceramide and GM3 in the liver [84]. On the other hand, since alcohol feeding depletes the mitochondrial GSH content [85–88], which controls the ability of GD3 to induce mitochondrial membrane permeabilization [41,43], it is conceivable that alcohol-stimulated GD3 may play an important role in the progression of ALD, a line of research currently under investigation.

Liver natural killer T cells (NKT) are specifically stimulated by α-galactosylceramide and mediate intrahepatic immunity to several infections and certain hepatic disorders (*e.g.*, viral-induced hepatitis) [89–91]. α-galactosylceramide binds to CD1d, which in turn up-regulates Fas ligands on the surface of liver NKT and induces hepatocyte apoptosis through the Fas-Fas ligand signaling pathway [92–94]. In addition, a recent study reported that mice immunized with the human melanoma cell line SK-MEL-28 (GD3+ GM2− CD1−) results in a GD3-reactive natural killer T (NKT) cell response, indicating the cross-presentation of GD3 to NKT cells [95]. Thus, upregulation of specific GSLs, *e.g.*, GD3 levels, may play a key role in liver injury mediated by both an expansion of liver NKT cells and up-regulation of hepatocyte Fas antigen.

GD3 may play also a key role in liver fibrogenesis. The deposition of collagen in the liver is a dynamic process mediated by activation of stellate cells (HSC) [96]. The activation of these cells occur in response to a wide variety of stimuli (viral infection, alcohol, etc.) so that liver fibrogenesis is an integrated response to liver injury and a characteristic stigma of liver diseases. Although the molecular mechanisms responsible for liver fibrogenesis are not completely understood, an important aspect in the regulation of this process is the resistance of activated HSC to apoptosis induction, and thus the characterization of agents that induce apoptosis of HSC may be of relevance in liver fibrogenesis. In this regard, using a large scale sequencing of a 3′-directed cDNA library, the upregulation of *O*-acetyl

disialoganglioside synthase was detected both in activated rat HSCs and human cirrhotic livers [97]. Consistent with the loss of apoptogenic potential of acetylated 9OAcGD3 [45], it is conceivable that the upregulation of *O*-acetyl disialoganglioside synthase may constitute a critical step favoring perpetuation of activated HSC and hence fibrogenesis. The ability of exogenous addition of GD3 to activated HSCs to induce apoptosis remains to be investigated.

GM3 represents the simplest ganglioside oligosaccharide in the synthesis of gangliosides and is the most widely distributed ganglioside among tissues and serves as a precursor for most of the more complex ganglioside species. In addition to its structural role, GM3 has been found to inhibit intrinsic tyrosine kinase activity of soluble receptors [98,99], and GM3 depressed insulin-mediated signaling in cultured cells. In models of insulin resistance, an overexpression of GM3 synthase has been described [100], and enhanced insulin sensitivity in mice lacking GM3 has been reported [101]. Interestingly, these mutant mice were protected from high-fat diet-induced insulin resistance, demonstrating that GM3 has an important role in the regulation of insulin sensitivity. Thus, through impairment of insulin receptor phosphorylation GM3 may contribute to non-alcoholic steatohepatitis, a liver disease characterized by steatosis and insulin insensitivity [102].

The brain is particularly enriched in GSLs and disregulation of GSLs metabolism contributes to neurological diseases [103]. In the healthy brain, GD3 is found preferentially in early oligodendrocyte precursors, microglia, and some defined neuronal cell types, and the pattern of ganglioside composition and distribution changes during the progression of neurologic diseases [104–107]. Microglia increase their GD3 content upon activation, and elevated cerebrospinal fluid GD3 levels have been reported in multiple sclerosis and leukoaraiosis [108,109]. Moreover, the GD3 content is increased in multiple sclerosis plaques in comparison to healthy white matter [110], while GM1, the most abundant ganglioside in normal brain, decreases [111]. Recent findings reported that activated microglia cells in culture by LPS or bacteria exposure induced the synthesis and secretion of GD3 into the culture medium, resulting in selective toxicity to primary oligodendrocytes with no significant effects on microglia, astrocytes, or primary neurons (cerebellar granule cells) [112]. Consistent with the earlier reports on the effect of GD3 on rat liver mitochondria, GD3 induced morphological and functional changes in oligodendrocyte mitochondria resulting in the release of cytochrome c and caspase activation. Thus, these findings may have implications in several neurologic disorders in which migroglia cells become activated, *e.g.*, white matter degeneration, HIV-induced neurodegeneration, or medulloblastoma [107,109,113], functioning as the source of GD3 which then acts on specific populations of cells within the brain. Thus, the preceding studies illustrate the relevance of GSLs as modulators of cell growth and signaling pathways [114].

Concluding remarks

The scenario of GSLs in biology has changed in recent years. In addition to their predominant role as structural components of membranes, the available evidence indicates they play a regulatory role in signaling pathways. Notably, sialic acid-containing GSLs, best characterized for GD3, exert a dual role in apoptosis regulation by interacting with and recruiting mitochondria to apoptotic pathways, while suppressing the activation of survival pathways. In addition the current evidence points to GD3 as a signaling lipid mediating the apoptotic effect of death ligands *e.g.*, Fas or TNF. Combined, these novel functions of GSLs may have important consequences in cancer therapy and may prompt the search of strategies aimed to increase selectively the GD3 content of tumor cells to maximize cancer therapy. In the future we will witness a progression of knowledge in this area, in which the role of specific GSLs in disease progression will be unveiled, thus providing the basis to interfere with or halt disease pathogenesis with pharmacologic or genetic approaches to regulate GSLs metabolism.

Acknowledgments

The work presented was supported in part by the Research Center for Liver and Pancreatic Diseases (P50 AA11999) and Grant 1R21 AA014135-01 funded by the US National Institute on Alcohol Abuse and Alcoholism; Plan Nacional de I + D Grants SAF2001-2118, SAF 2002-3564, SAF 2003-4974, Fondo de Investigaciones Sanitarias 2002-3057, 2003-0426, and Red Tematica de investigacion Cooperativa G03/015 and Red de Centros C03/02 supported by Instituto Salud Carlos III. AM, AC and MM are Ramon y Cajal Investigators.

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