

O-acetylation of GD₃: An Enigmatic Modification Regulating Apoptosis?

Helen Y. Chen and Ajit Varki

Glycobiology Research and Training Center, Department of Medicine and Department of Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA 92093

Glycosphingolipids (GSLs) are amphipathic molecules with a polar glycan chain as a head group and a hydrophobic sphingosine-containing ceramide tail, which is typically embedded in the outer leaflet of the plasma membrane (1). GSLs are no longer thought to be mere physical components of the membrane. They often cluster in “glycosignaling domains” (GSDs; reference 2), sometimes along with other sphingolipids, glycosylphosphatidylinositol (GPI)-anchored proteins, and cholesterol, forming “lipid rafts” (3). These microdomains are thought to regulate signal transduction via cis interactions with signal transducer molecules. A GSL called GD₃ has recently been shown to be involved in Fas-mediated apoptosis in hematopoietic cells, causing the loss of mitochondrial potential and the release of apoptotic factors (for reviews, see references 4 and 5). In this issue, Malisan et al. (6) show that a naturally occurring modification of GD₃ (O-acetylation) can reverse its apoptotic effects, suggesting a way for cells to avoid the fate of GD₃-induced apoptosis. To understand this interesting story better, it is first necessary to review some general information about GD₃ and O-acetylation.

Structure, Biosynthesis, and Tissue Distribution of GD₃. Gangliosides are GSLs with one or more sialic acid (Sia) residues. GD₃ is a ganglioside with two Sias linked to a lactosylceramide core common to many GSLs (see Fig. 1). Sia is a generic name for members of a family of 9-carbon sugars typically found at the termini of glycan chains on vertebrate glycoproteins and glycolipids. Many endogenous or exogenous receptors recognize Sias, mediating or modulating processes such as cell adhesion, differentiation, signal transduction, pathogen invasion, or toxin action (for a review, see reference 7). The biosynthesis of GD₃ is traditionally thought to occur in the ER-Golgi pathway, by the sequential addition to ceramide of a glucose, a galactose and two Sia residues, each step being catalyzed by distinct glycosyltransferases (see Fig. 1). The last three of these enzymes have their active sites oriented toward the lumen of Golgi compartments. Such newly synthesized gangliosides

are delivered to the outer leaflet of the plasma membrane and eventually turned over via endocytosis and lysosomal degradation (1; see Fig. 1).

GD₃ is expressed at high levels in the embryonic brain, with marked decreases during postnatal development (8). In normal adult humans, GD₃ is only detectable in a few tissue types (9), and on some T lymphocytes (10). In contrast, GD₃ expression is highly elevated in melanomas, neuroblastomas, small cell carcinomas, and certain leukemias (11, 12).

Modified Forms of GD₃. The biological specificity of Sia can be modulated by substitutions or modifications at the 1, 4, 5, 7, 8, or 9 positions (7). O-acetyl esters are most commonly found at the 9-carbon position. Such ester groups can first be added at the 7-position and then slowly migrate to the 9-position under physiological conditions (7). The outermost Sia residue of GD₃ can be 9(7)-O-acetylated to a varying extent in various cell types. 9-O-Acetyl-GD₃ (9AcGD₃) was first discovered as a surface marker for germinal cells of the central nervous system (13), and then structurally characterized from human melanoma cells (11). Interestingly, the distribution of GD₃ and 9AcGD₃ is not identical in some regions of the developing nervous system (13–15). Elimination of 9AcGD₃ expression in the retina and adrenals of transgenic mice expressing an Influenza C Sia-specific 9-O-acetyltransferase gave variable abnormalities in development (16). While many biological roles for 9AcGD₃ have been proposed, the evidence is indirect, consisting of the effects of the viral 9-O-acetyltransferase (16, 17), or of anti-9AcGD₃ antibodies (18, 19).

Postnatally, 9AcGD₃ expression becomes restricted to the retina and cerebellum, and the only nonneural expression known is in the adrenal medulla, kidney glomeruli in rats, and some human lymphoid cell types (9, 10). High expression of 9AcGD₃ is also seen in human basal cell carcinomas (20), and in melanomas from many species (21). The less common 7-O-Acetyl-GD₃ has been identified in hamster melanomas, and in human T cell lymphocytes (22, 23). In T lymphocytes, AcGD₃ species appear to be the epitopes for anti-CD60 antibodies (10), although a similar terminal structure on glycoprotein glycans also contributes (24). Antibodies against GD₃ and AcGD₃ have been used in melanoma immunotherapy, so far with limited success (25–28).

Address correspondence to Ajit Varki, CMM-E, Rm. 1065, Mail Code 0687, University of California, San Diego, La Jolla, CA 92093-0687. Phone: 858-534-3296; Fax: 858-534-5611; E-mail: avarki@ucsd.edu

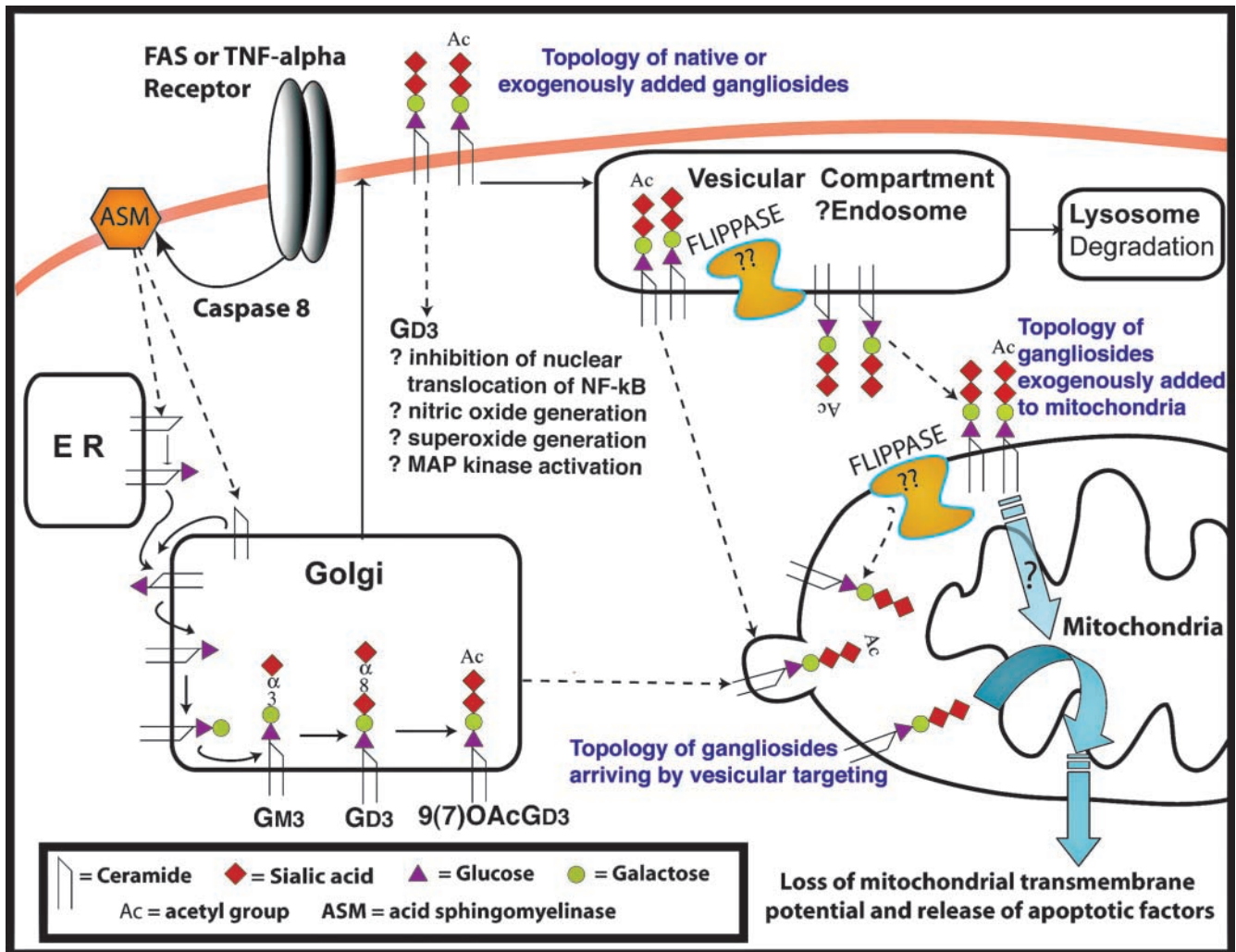


Figure 1. Subcellular compartments potentially involved in the biosynthesis, trafficking, and turnover of GD3 and AcGD3 are shown. Complexities involving 7- versus 9-AcGD3, and the possible action of O-acetylsterases are omitted. Various pathways for GD3-induced apoptosis are indicated, especially in relation to the proposed production and translocation of GD3 to mitochondrial membranes. Some of the unresolved topological issues are emphasized. Topological issues regarding ASM are not shown. See text for further discussion.

GD3-induced Apoptosis. GD3 synthesis is induced during Fas (APO-1/CD95)-mediated apoptosis, and is then thought to mediate the apoptotic effect by accumulating in mitochondria (an unconventional subcellular location for a ganglioside; reference 29). These phenomena are inhibited by blocking GD3 synthase expression, indicating that de novo synthesis of GD3 is required (30). In an apparently related process, apoptotic signaling is thought to occur through Fas-mediated activation of membrane-associated acidic sphingomyelinase (ASM), generating free ceramides (31, 32). It is proposed that these ceramides are converted to GD3 by returning to Golgi-like compartments (see Fig. 1). A similar ASM-GD3 pathway has been recently implicated in TNF- α -mediated apoptosis as well (33, 34). Independent work from others indicates that GD3 can be a component of the apoptotic response in cell types such as neurons (35), aortic smooth muscle cells (36), and keratinocytes (37). In keratinocytes, the structurally related gan-

glioside GT1b also has a proapoptotic effect, but unlike GD3, works in a fibronectin-dependent manner, apparently involving an integrin-linked kinase (37). In another study involving lymphoblastoid cells, GD3 association with the ezrin cytoskeleton protein was proposed to be involved in apoptosis (38). Also, low doses of GD3 stimulated superoxide generation and MAP kinase activation in human aortic smooth muscle cells (36).

Some of the above studies took advantage of the fact that when pure gangliosides (which are in the form of micelles in aqueous solution) are added to cells or organelles in vitro, they can become incorporated as integral components of the outer leaflet of their membranes (39). Addition of GD3 to intact cells induced apoptosis, and addition to isolated mitochondria gave a loss of mitochondrial transmembrane potential, along with release of apoptogenic factors such as cytochrome c and caspase-9. Gangliosides structurally related to GD3 did not exhibit these ef-

fects in most systems (29, 30, 40). Taken together, all these data constitute a strong case for a proapoptotic role for GD₃. However, the mechanisms of GD₃ action remain obscure, and there are puzzling topological issues (see below). Also, as GD₃ is only found in a minority of adult cell types, this may represent a specialized subset of apoptosis control pathways. Indeed, on initial study, GD₃ synthase null mice apparently did not exhibit gross developmental pathologies, nor show any differences from wild-type controls in Fas-mediated apoptotic reaction of thymocytes (41).

Is 9-O-acetyl GD₃ an Antiapoptotic Factor? If GD₃ is expressed in some normal as well as many cancer cells, how do they escape its apoptotic effects? Writing in this issue, Malisan et al. (6) offer an intriguing explanation. As many cells expressing GD₃ also have 9AcGD₃, the authors postulated that 9-O-acetylation could rescue the cell from GD₃-induced apoptosis. Indeed, in striking contrast to GD₃, 9AcGD₃ did not induce apoptosis when added to intact cells, nor did it affect transmembrane potential, or cause the release of apoptotic factors when added to isolated mitochondria. The effects were restored when 9AcGD₃ was chemically de-acetylated back to GD₃, showing that the findings were not due to an inhibitory contaminant. De-O-acetylation of endogenous 9AcGD₃ in intact cells was also achieved by transfecting cells with the viral 9-O-acetyl-esterase mentioned above. Cells that either express GD₃ synthase endogenously or by transfection became apoptotic when the viral esterase was also present, and this correlated with a reduction in 9AcGD₃. The authors conclude that by turning part of pro-apoptotic GD₃ into 'harmless' 9AcGD₃, 9-O-acetylation acts as an effective antiapoptotic mechanism. Together with the action of a putative endogenous 9-O-acetyl-esterase, an acetylation-deacetylation cycle is suggested as a subtle yet elegant means of regulating the apoptotic potential of GD₃. Consistent with this concept is our recent finding that the ganglioside 9-O-acetylation machinery is directly induced by the expression of GD₃ synthase (unpublished data). However, there are many unresolved issues.

What About the GD₃:9AcGD₃ Ratio? A substantial amount of GD₃ continues to be present alongside 9AcGD₃ in all the situations examined. Also, in our own work using CHO cells (42), expression of GD₃ synthase was accompanied by 9-O-acetylation of only a minor fraction of the GD₃, and yet no obvious apoptosis was observed. A dominant effect of 9AcGD₃ over GD₃ could explain such findings. However, mixing experiments by Malisan et al. appear to rule this out (6). It is possible that the ratios of GD₃ and 9AcGD₃ in whole cell extracts are misleading, and that 9AcGD₃ is selectively enriched in a critical cellular sub-compartment involved in mediating in proapoptotic effects of GD₃. This could be checked by immunoelectron microscopy using the available antibodies. However, our own work with melanoma cells indicated a similar distribution for GD₃ and 9AcGD₃ in melanoma cells, with a novel intracellular distribution only for another Sia variation called de-N-acetyl GD₃ (9).

Other Unresolved Subcellular and Topological Issues. How does the newly synthesized GD₃, which is normally found in the ER-Golgi-plasmalemma pathway reach the mitochondria? Perhaps this occurs via vesicular trafficking associated with the cytoskeleton (33, 38), or through the proposed mitochondria-associated membranes bridging to ER/Golgi elements (43). Regardless, as GD₃ is synthesized within the lumen of the Golgi and is 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 (see Fig. 1). In contrast, the experiments with isolated mitochondria involve added GD₃, which should be incorporated with its polar glycan head-group facing outward, in the cytosol-facing leaflet of the outer mitochondrial membrane (see Fig. 1). It is hard to imagine that these two topologically unique forms of GD₃ mediate the same biological actions. Thus, one must postulate a "flippase" for gangliosides in the mitochondria and/or in the ER-Golgi-plasmalemma pathway that could transfer the polar headgroup between the two leaflets.

A Receptor for GD₃? A more basic question is why GD₃ is active in this pathway, but not other gangliosides with closely related structures, including 9AcGD₃. This implies the existence of a receptor that specifically recognizes the structure of GD₃, including the outer Sia residue that becomes O-acetylated in 9AcGD₃. Perhaps this proposed receptor is the same as the putative "flippase" protein? In this regard, it is interesting that there are studies describing "glycolipid transfer proteins" in the cytosol (44, 45). Another potential candidate is Bid, a proapoptotic cytosolic factor of the Bcl-2 family, which is cleaved by FAS-induced caspase 8 (46). Truncated Bid associates with lipid membranes, has an affinity toward acidic phospholipids, and is thought to be involved in membrane lipid transfer to mitochondria (47). It is thought that Bid affects the structural state of multidomain antiapoptotic Bcl-2 proteins in the outer membrane by changing the lipid environment in mitochondria. Perhaps Bid or another Bid-like proapoptotic factor could be the putative GD₃ flippase as well? This would fit with the earlier observation that enforced expression of Bcl-2 attenuated GD₃-induced apoptosis. Yet another possibility is that a transient de-N-acetylation of GD₃ could cause it to associate strongly with phospholipids (48). Such complexes might then be flipped over by the previously well-known phospholipid transfer proteins.

Other Missing Pieces of the Puzzle. In addition to the mechanism of "flipping," the immediate downstream effectors of GD₃ that induce the mitochondrial changes need to be elucidated. Cloning of the putative GD₃:9(7)O-acetyl transferase(s) and esterase(s) would also help in understanding the regulation of 9(7)O-acetylation in relation to GD₃ expression in different cell types and within different subcellular compartments. Finally, what about the effects of the less common intermediate form 7AcGD₃, which cannot be deacetylated by any known O-acetyl-esterase? Until a clearer picture emerges regarding all these issues, the biological significance and precise mechanisms of GD₃-induced

apoptosis remains somewhat of a mystery, and O-acetylation of GD3 remains an enigmatic modification in continued search of definitive functions.

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