CHAPTER SIX

Gangliosides in Cancer Cell Signaling

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Abstract

At the outer leaflet of the plasma membrane, gangliosides are found with other glycosphingolipids, phospholipids, and cholesterol in glycolipid-enriched microdomains, in which they interact with signaling molecules including receptor tyrosine kinases and signal transducers. The role of gangliosides in the regulation of signal transduction has been reported for many cases and in different cell types. The biosynthesis of gangliosides involves specific enzymes, mainly glycosyltransferases that control together with glycohydrolases, the steady state of gangliosides at the cell surface. Changes in ganglioside composition are therefore correlated with modifications of glycosyltransferases or glycohydrolases expression and result in the deregulation of cellular signals. In several types of cancers, the overexpression of

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disialogangliosides, such as GD3 or GD2 mainly results in the activation of cell signaling, increasing cell proliferation and migration, as well as tumor growth. In this chapter, we summarize our current knowledge of ganglioside biosynthesis, degradation, and of their role in cell signaling regulation in cancers.

ABBREVIATIONS

Cer	ceramide
CSC	cancer stem cells
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ER	endoplasmic reticulum
FAK	focal adhesion kinase
FGF	fibroblast growth factor
GalCer	galactosylceramide
GD3S	GD3 synthase
GEM	glycolipid-enriched microdomain
GlcCer	glucosylceramide
GM3S	GM3 synthase
GSL	glycosphingolipids
GT	glycosyltransferase
LacCer	lactosylceramide
PDGF	platelet-derived growth factor
RTK	receptor tyrosine kinase
TF	transcription factor
TGF	transforming growth factor
VEGF	vascular endothelial growth factor

1. INTRODUCTION

Gangliosides, the sialic acid-containing glycosphingolipids (GSL), are major components of the plasma membrane of eukaryotic cells. They are found in almost all tissues, especially in membranes of the central and peripheral nervous system where they make up to 6% of total lipids.[1] Localized at the outer leaflet of the plasma membrane, gangliosides interact with other GSLs, phospholipids, cholesterol, and transmembrane proteins, forming glycolipid-enriched microdomains (GEMs), also known as lipid rafts. Gangliosides are usually classified in four Series (0-, a-, b-, and c-Series) according to the number of sialic acid residues linked to the lactosylceramide (LacCer) (Fig. 1).[2] Normal extraneural-human tissues mainly express "simple" gangliosides from 0- and a-Series, whereas "complex"

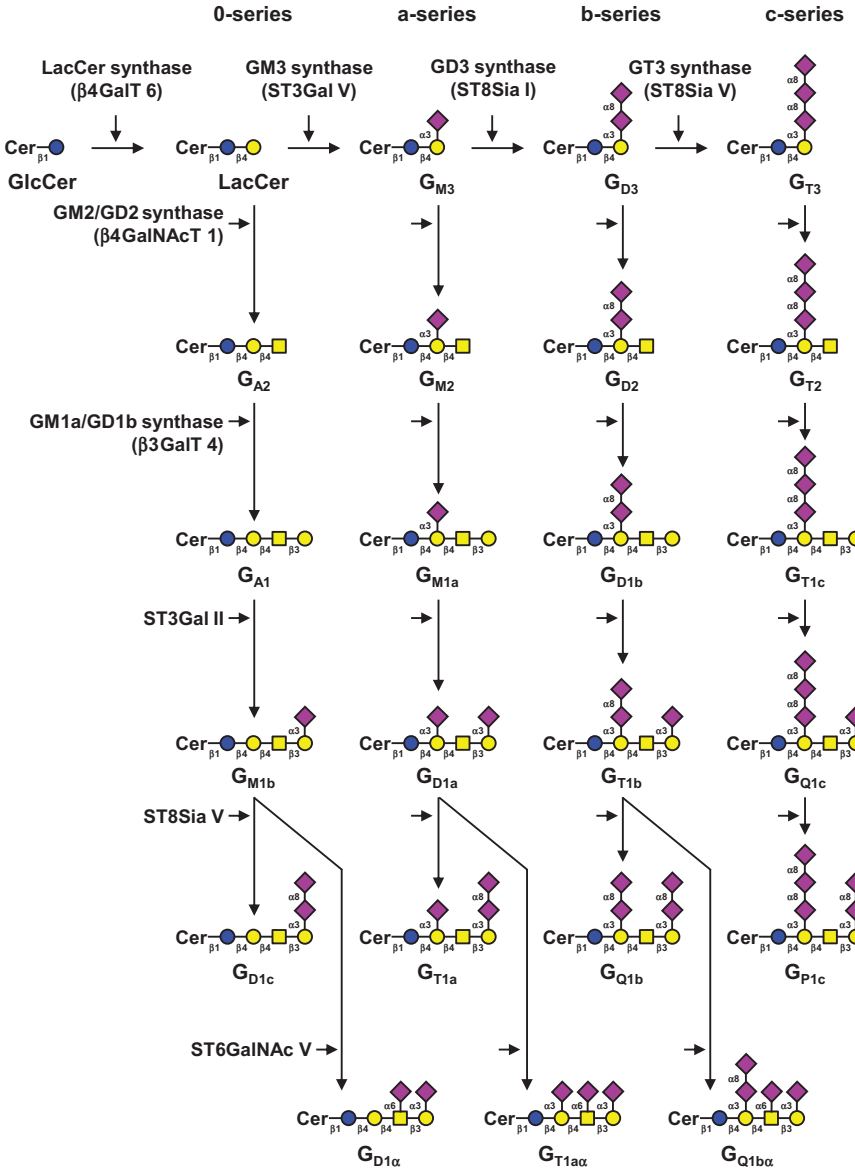

Fig. 1 Biosynthesis pathway for gangliosides. Gangliosides are synthesized by the stepwise addition of monosaccharides to ceramide (Cer). Cer is the acceptor for UDP-Glc: ceramide β-glucosyltransferase. Extension of GlcCer occurs by the action of UDP-Gal: GlcCer β1,4-galactosyltransferase (β4GalT 6) to make LacCer. The action of ST3 Gal V (GM3 synthase), ST8Sia I (GD3 synthase), and ST8Sia V (GT3 synthase) leads to the biosynthesis of the precursors of a-, b-, and c-Series gangliosides, respectively. The 0-Series gangliosides are directly synthesized from LacCer. Elongation is performed by the sequential action of *N*-acetyl-galactosaminyltransferase (β4GalNAcT 1), galactosyltransferase (β3GalT 4) and sialyltransferases (ST3 Gal II and ST8Sia V). α-Series gangliosides derive from the action of ST6GalNAc V on GM1b, GD1a, or GT1b. Gangliosides nomenclature is that of Svennerholm.[2] ●, Glc; ○, Gal; □, GalNAc; ◆, Neu5Ac.

gangliosides from b- and c-Series are essentially found in developing tissues, during embryogenesis, and mainly restricted to the nervous system of healthy adults.[3] The expression of complex gangliosides has been found to be increased in pathological conditions including several types of cancer, especially those from neuroectoderm origin, such as melanoma, neuroblastoma, glioblastoma, or breast cancer, but also lung cancer, in which they play a key role in invasion and metastasis,[4,5] making complex gangliosides attractive target molecules for cancer immunotherapy.[6–9] The biosynthesis of gangliosides is catalyzed by specific glycosyltransferases (GTs) that control, together with glycohydrolases, the cell surface expression of gangliosides. Changes in ganglioside composition are therefore usually correlated with modifications of the expression of GTs. For example, the increased GD3 synthase (GD3S) expression was reported for many types of cancer cells. Due to their extracellular orientation, gangliosides are involved in cell–cell and cell–matrix interactions. Within lipid rafts, gangliosides also interact with signaling molecules including receptor tyrosine kinases (RTKs) or signal transducers, controlling, and regulating different signaling processes potentially involved in cancer progression.[10,11] Although the role of gangliosides in the regulation of signal transduction has been reported in a variety of cancer cell lines, the molecular mechanisms sustaining these functions are not fully understood. Several examples have shown that direct carbohydrate-to-carbohydrate interaction through glycosynapses, interaction with extracellular matrix components, or crosstalk with integrins or other transmembrane proteins could be involved in the regulation of cell signaling by gangliosides.[12,13] Finally, within lipid rafts, gangliosides are also implicated in CD95 death receptor-mediated signaling and apoptosis, potentially providing new targets for cancer therapy.[14]

2. GANGLIOSIDE BIOSYNTHESIS AND DEGRADATION

The first step of ganglioside biosynthesis is ceramide (Cer) formation by endoplasmic reticulum (ER)-resident Cer synthases.[15] Cer is then transported to the Golgi apparatus by vesicular transport or by Cer transfer protein,[16] where it is used by specific GTs, which add monosaccharides in a stepwise manner to produce the glycosidic moiety of the ganglioside. The first glycosylation step corresponds to glucose or galactose residues transferred to the 1-hydroxy group of Cer, yielding the simple GSL glucosylceramide (GlcCer) or galactosylceramide (GalCer), respectively. Human

GalCer synthase is an ER-located transmembrane protein, with its catalytic site facing the lumen of the ER.[17] GalCer is a precursor for 3-sulfated-galactosylceramide (sulfatide) and GM4, both of which are synthesized in the Golgi apparatus, by the action of galactosylceramide 3-O-sulfotransferase or of α2,3-sialyltransferase ST3 Gal V, respectively.[18,19]

Human GlcCer synthase, encoded by *UGCG* gene, is a transmembrane protein localized on the cis-Golgi, with its catalytic site facing the cytosol.[20] Since higher GSL biosynthesis takes place in the lumen of the Golgi apparatus, GlcCer synthesized on the cytosolic side of the membrane has to translocate across the Golgi membrane to be used as a substrate by LacCer synthase (UDP-Gal: GlcCer β1,4-galactosyltransferase, β4GalT 6) and other GTs involved in ganglioside biosynthesis.[21] It was proposed by Halter and coworkers that the essential GlcCer flipping step is accomplished via a complex trafficking itinerary. Four-phosphate adaptor protein 2 (FAPP2), a cytoplasmic lipid transfer protein, transports GlcCer from the cytosolic side of the Golgi apparatus to the ER, then GlcCer has to flip across the ER membrane, which is facilitated by ATP-independent ER flippases, and be delivered to the lumen of the Golgi apparatus by vesicular traffic.[22,23] Eventually, GlcCer could be transported by FAPP2 directly from proximal to distal Golgi apparatus compartments.[24] Almost all complex GSLs are synthesized from GlcCer following additional reactions sequentially catalyzed by Golgi-localized GTs. LacCer synthase first transfers galactose from UDP-Gal to GlcCer to produce LacCer (Galβ1-4Glcβ1-Cer) in the Golgi apparatus. The transfer of the first sialic acid residue to LacCer is achieved by α2,3-sialyltransferase ST3 Gal V (GM3 synthase). GM3 synthase was recently found to be responsible for the synthesis of GM4 (sialylated GalCer), both in vitro and in vivo. The mechanism underlying GM4 expression remains unclear, and it seems that excess amounts of ST3 Gal V are necessary for GM4 synthesis in mammalian cells.[19]

Additional sialic acid residues can be added by sialyltransferases ST8Sia I (GD3S), and ST8Sia V (GT3 synthase), which synthesize GD3 and GT3, respectively. GD3S uses GM3 as a preferential substrate, but it is also able to sialylate GD3 to synthesize GT3,[25] whereas ST8Sia V shows a broader substrate specificity.[26] Although ganglioside biosynthesis pathways have been located to the Golgi apparatus, it was recently demonstrated that both ectopically and endogenously expressed GD3S were active at the plasma membrane, using GM3 and CMP-Neu5Ac as substrates, which could regulate local ganglioside composition.[27]

LacCer, GM3, GD3, and GT3 are the precursors for more complex gangliosides of the 0-, a-, b-, and c-Series, respectively, by sequential glycosylation steps catalyzed by β4GalNAcT 1, β3GalT 4, ST3 Gal II, and ST8Sia V (Fig. 1). In the central nervous system of mammals, a shift in the expression of gangliosides, from GD3 at early stages to GD1a at late developmental stages, is usually observed, which correlates with the transcriptional regulation of ST8Sia I and β4GalNAcT 1 observed during development.[28] Gangliosides GM1, GD1a, GD1b, and GT1b represent more than 90% of gangliosides in the brain of mammals and birds.

After synthesis, gangliosides are transported to the plasma membrane via vesicular transport where they are part of GEMs on the outer leaflet and key effectors in diverse cellular functions.[29–31] They can subsequently be released to the extracellular milieu or undergo endocytosis, and once internalized, they can be recycled back to the plasma membrane directly from endosomes, sorted from endosomes to the Golgi apparatus where they can be reglycosylated, or they can be degraded at the lysosomal level.[32,33]

Catabolism of gangliosides takes place mainly at the luminal surface of lysosomes by the action of glycohydrolases that sequentially cleave off the monosaccharide units from the nonreducing end of the ganglioside glycan chains.[34](Fig. 2). Adequate lysosomal ganglioside catabolism requires the presence of an acidic pH, glycohydrolases and in some cases also of lipid-transfer proteins for degradation of simpler gangliosides.[35] In this sense, it is known that GSLs with four carbohydrate residues or less require the presence of small lipid binding glycoproteins (GM2 activator protein or one of the four saposins A, B, C, and D), which extract glycolipids from membranes and present them to the soluble acid hydrolases.[34,36] Additionally, anionic lipids, such as bis (monoacylglycero)phosphate are key players in ganglioside degradation since they participate in the electrostatic attraction of glycosidases and lipid binding proteins to the ganglioside-containing luminal membrane surfaces. If a defect is inherited in the genes encoding glycohydrolases and lipid-transfer proteins, the substrate is accumulated in the organelle resulting in severe neurodegenerative pathologies called gangliosidosis.[35,37] Eventually, degradation of gangliosides can also occur at the plasma membrane by the action of sialidases (mainly Neu3), β-galactosidase, β-glucosidase, and β-hexosaminidase,[38–40] that can remodel cell surface glycans, and consequently cause rapid changes in critical signal transduction pathways. Interestingly, some of these enzymes are able to hydrolyze the ganglioside substrates present in both the plasma membrane from its own cell or when exposed at the cell surface of neighboring cells, evidencing new regulatory mechanisms of ganglioside expression.

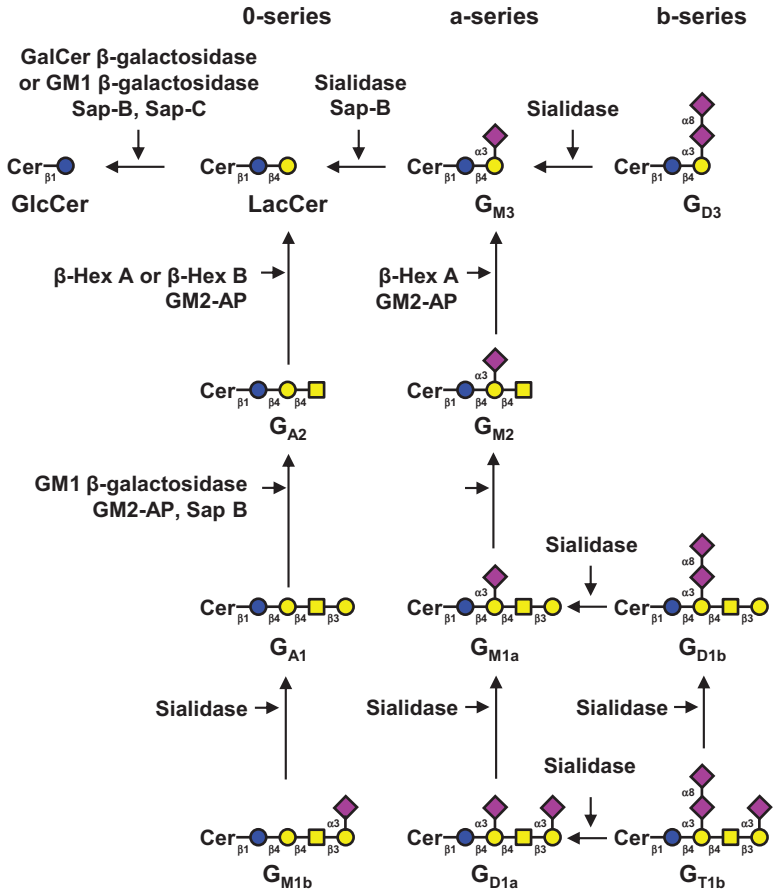

Fig. 2 Representative reactions of ganglioside catabolism. Enzymes and lipid-transfer proteins abbreviations are as follow: β-Hex A, β-hexosaminidase A; β-Hex B, β-hexosaminidase B; GM2-AP, GM2 activator protein; Sap-B, saposin B; Sap-C, saposin C. The sugar symbols are the same as in Fig. 1.

3. CELLULAR AND MOLECULAR BIOLOGY OF GTS AND SIALIDASES

3.1 Transcriptional and Cellular Regulation of GT Involved in Ganglioside Expression

The biosynthesis of glycan chains is mostly regulated at the transcriptional level. The tissue specificity of GT genes expression depends on the use of alternative promoters that control tissue specific transcripts, differing in their

5′ untranslated region (5′UTR), and on the involvement of specific transcription factors (TF).[41] However, the identity of TF controlling GT expression and regulation in normal and pathological conditions is largely unknown, and precise mechanisms involved in TF-mediated GT expression are available for only a limited number of genes. For example, the RelB subunit of NF-κB is implicated in the expression of α2,3-sialyltransferase genes *ST3GAL1*, *ST3GAL2*, and *ST3GAL6*, resulting in GD1a and sialyl-paragloboside overexpression on prostatic cancer cells.[42] Other examples are the well-described interactions of GATA-3 and T-bet TFs that regulate the expression of sialyl-LewisX homing receptors on Th1/Th2 lymphocytes[43] and the activation of the *B3galt4* promoter by the Ets-1 transcription factor on PC12 cells.[44]

Epigenetic modifications are also involved in the regulation of GT genes and several studies have shown a direct link between the methylation status of GT genes and their expression profile.[45] The epigenetic regulation of GT genes involved in ganglioside biosynthesis has recently been shown to be crucial for neural development and neuronal differentiation of neuronal stem cells. In mouse brain, histone acetylation of GT genes contributes to the modifications of ganglioside expression during development.[46] Acetylation of histones H3 and H4 on the GM2/GD2 synthase *B4GALNT1* gene promoter allows the recruitment of SP1 and AP-2, which activate *B4GALNT1* expression and neural differentiation in a primary neuroepithelium culture in response to exogenous GM1 addition.[47] In addition, GM1 itself directly binds to acetylated histones on *B4GALNT1* and neurogenic TF *NeuroD1* promoters in differentiated neurons, suggesting that GM1 is involved in the chromatin complex that promotes neuronal differentiation.[48]

The elevated expression of specific gangliosides described in many types of cancers and involved in tumor aggressiveness suggests that epigenetic regulation of ganglioside GT genes could underlie some pathogenic mechanisms, and future studies would be useful to develop new strategies and find new targets for disease treatment.

The precise Golgi localization and supramolecular organization of glycosylation enzymes is also of critical importance. GTs involved in ganglioside biosynthesis are typical type II membrane-anchored proteins with a gradient distribution in the Golgi apparatus. The first glycosylation steps take place in the *cis*/medial-Golgi and the later in the *trans*-Golgi and *trans*-Golgi network. The relatively short-single transmembrane domain of GTs seems to be a critical signal for Golgi retention,[49] but the luminal and cytoplasmic domains also contain multiple signals involved in GT precise Golgi

localization.[50–52] Disulfide-bonded dimerization, glycosylation, and secretion of GTs can also play a role in the processing and function of the different enzymes in the Golgi apparatus.[52] Moreover, ganglioside GTs organize in distinct multienzyme complexes, and β4GalNAcT 1/β3GalT 4, β4GalT 6/ST3 Gal V/ST8Sia I, and ST8Sia I/β4GalNAcT 1 complexes have been characterized.[53–55] Evidence indicates that the β4GalNAcT 1/β3GalT 4 complex concentrates in a sub-Golgi compartment more distal than the one bearing the β4GalT 6/ST3 Gal V/ST8Sia I complex, suggesting that particular GTs organized in distinct complexes and concentrated in different Golgi subcompartments, are involved in the regulation of ganglioside biosynthesis. Regulation of ganglioside biosynthesis can also be linked to the synthesis of different GT isoforms exhibiting different properties or activities. As an example, mouse GM3 synthase (GM3S) has three isoforms (M1-GM3S, M2-GM3S, and M3-GM3S), with different N-terminal cytoplasmic tails. M1-GM3S is localized in the ER, as a result of retrograde transport signals, and has a low GM3 synthesis activity compared with the other isoforms. In contrast, both M2-GM3S and M3-GM3S are localized in the Golgi apparatus, but M2 isoform is rapidly degraded in lysosomes, whereas M3-GM3S is retained in the Golgi apparatus. The existence of different GM3S isoforms having such different properties, that it is probably important in the regulation of GM3 biosynthesis under various pathological and physiological conditions.[56]

3.2 Expression and Regulation of Ganglioside GTs in Cancer: The Key Role of GD3S

The expression and regulation of GTs involved in the synthesis of gangliosides can be altered in pathological conditions including cancer. In particular, overexpression of complex gangliosides is observed in malignant cells, such as melanoma, central nervous system tumors, lung and breast cancers due to the increased expression of GD3S gene *ST8SIA1*. The *ST8SIA1* gene consists of five coding exons spanning over 135 kpb,[57] and its 5′UTR have been described in melanoma, glioblastoma, neuroblastoma, and breast cancer cell lines, showing different transcription start sites located upstream the initiation codon on the first exon.[58–61] The analysis of *ST8SIA1* core promoter implicated Tumor Necrosis Factor in GD3S overexpression in estrogen receptor-negative breast cancer cells via the NF-κB pathway, as well as the repressive role of estradiol in estrogen receptor-positive breast cancer cells.[61] Indeed, estradiol inhibits the *ST8SIA1* core promoter activity by preventing NF-κB nuclear translocation in ER-positive MCF-7 breast cancer cell

line.[61] Other studies showed that Tumor Necrosis Factor modulates *B4GALNT1* expression in renal carcinoma,[62] and that NF-κB could play an essential role in the transcriptional regulation of the human GD3S expression in Fas-induced Jurkat-T cells.[63] Finally, testosterone was shown to activate ST3 Gal II sialyltransferase expression in prostate cancer cells through epigenetic regulation and NF-κB signaling.[64]

3.3 Regulation of Ganglioside Biosynthesis and Epithelial–Mesenchymal Transition

A growing body of evidence indicates that the epithelial–mesenchymal transition (EMT), which is undeniably implicated in cancer progression and metastasis, is likely associated with changes in ganglioside metabolism, and aberrant sialylation of gangliosides plays an important role in cell adhesion and motility in many cancers. Sarkar and coworkers revealed that GD3S could be involved in the EMT that bestows breast cancer cells with increased metastatic potential and cancer stem cell (CSC) properties.[65] Indeed, GD3S knockdown in the mesenchymal MDA-MB-231 cell line led to the expression of the epithelial cadherin and the downregulation of mesenchymal markers. GD3S silencing was also found to reduce the motility of breast cancer cells in vitro and metastasis in mice.[65] Surprisingly, normal restriction to the brain, the expression of ST6GalNAc V, a sialyltransferase implicated in the biosynthesis of α-Series gangliosides, decreases the interactions between breast cancer cells and the human blood–brain barrier.[66] If changes in GSL metabolism can modulate EMT, EMT can conversely affect the expression of genes encoding GTs. Thus, TGF-β-induced EMT is associated with a decreased expression of *B3galt4* gene in normal mouse mammary gland cells[67] and an increased expression of the GD3S in the MCF10A human epithelial breast cell line.[65] Moreover, many TFs involved in EMT have been identified to regulate the expression of genes encoding ganglioside-metabolizing enzymes. For instance, the Smad3/4 complex binds to the *B3galt4* promoter, leading to a decreased expression of the enzyme.[68] Zeb1 enhances the promoter activity of both GM3S and GD3S genes (*St3gal5* and *St8sia1*) by direct binding and furthermore represses the expression of a microRNA from the miR-200 family that targets the 3′UTR of the murine *St3gal5* gene (GM3S).[59,69] Finally, the overexpression of Twist/Snail enhances the expression of GD3S in transformed human mammary epithelial (HMLER) cells, as well as GD2 expression, suggesting a role of EMT in the origin of GD2-positive breast CSCs.[70] GD3S expression has also been shown to be triggered by NF-κB via FOXC2 transcription factor in human

triple-negative breast cancer cells.[65] Importantly, GD3S was shown to display higher expression in melanoma cell lines and in estrogen receptor-negative breast cancer tumors, which both have a mesenchymal-like gene signature.[71–73] High expression of GD3S was furthermore associated with poor histopathological grading and a decreased overall survival of patients, suggesting that GD3S overexpression contributes to increase the malignant phenotype of breast cancer cells.[74] In this context, it has been demonstrated that GD3S expression in an estrogen receptor-negative breast cancer cell line induced proliferative properties in the absence of serum or exogenous growth factors, and increased tumor growth.[75] Taken together, these findings show that GD3S plays a key role in the maintenance of a mesenchymal phenotype in cells that have undergone EMT, especially in response to Snail, Twist, and TGF-β1 in several biological systems.

3.4 Expression and Regulation of Sialidases

As stated, gangliosides are mono- or multisialylated GSLs, which are synthesized, among other GTs, by a set of specific sialyltransferases in a cell type-dependent manner. The opposite reaction, desialylation, is catalyzed by a family of sialidases that operate at different cellular levels. Sialidases hydrolyze the terminal nonreducing sialic acid linkage in glycolipids and glycoproteins. To date four types of mammalian sialidases have been identified and characterized, designated Neu1, Neu2, Neu3, and Neu4, with each one having different subcellular localization and substrate specificity.[76,77]

Human Neu2 is the only member of the mammalian sialidases that has been crystallized[78] and it shows the typical β-propeller fold already described for bacterial and viral sialidases,[79,80] indicating that despite the low-sequence identity between viral, bacterial, and mammalian sialidases, they have a common folding topology, with active site residues highly conserved among species. Homology models of the remaining three members of the family have been developed based on the crystal structure of Neu2. All of them are assumed to share the typical β-propeller structure organized in six blades, each composed of four antiparallel β-sheets.[81] However, several structural differences in the active sites of these enzymes have been found, and this provides better insight into the differential substrate recognition and activity of each member.

Neu1 is a lysosomal sialidase that hydrolyzes preferentially glycoproteins and oligosaccharides. It is associated with carboxypeptidase protective protein/cathepsin A and β-galactosidase as a complex, with dissociation of the complex leading to sialidase inactivation.[82] Besides its well-known lysosomal

catabolic function, Neu1 can be also targeted to the cell surface and assume the previously unrecognized role as a functional modulator of cellular receptors.[83] Neu2 and Neu4 have broad substrate specificity and can act on glycoproteins and oligosaccharides, as well as on glycolipids. Neu2 is a cytosolic sialidase while Neu4 has been found to be present in lysosomes, mitochondria, and the ER.[76]

Among human sialidases, the plasma membrane–associated sialidase Neu3 is by far the most studied. Neu3 is a key glycosidase for ganglioside degradation that preferentially hydrolyzes α2–3 and α2–8 terminal sialyl linkages, but is ineffective on an α2,3 inner sialic acid branch. Consequently, gangliosides GM3, GD3, GD1a, GD1b, and GT1b, but not GM1 and GM2, are substrates for the enzyme.[84] Neu3 has been characterized at the molecular level in various animal species and found to be ubiquitously expressed. However, a remarkable upregulation of Neu3 has been observed in various human cancer cells. Neu3 modifies the cell surface ganglioside composition by shifting polysialylated species to the monosialo derivatives and GM3 to LacCer. Besides, sialidase Neu3 is able to hydrolyze gangliosides present in the cell surface of neighboring cells, supporting its transcatalytic activity[85] and interestingly, it was recently demonstrated that Neu3 can also desialylate *N*-glycans present in glycoproteins.[86] The major subcellular localization of Neu3 sialidase is the plasma membrane, and it is also expressed in other cellular membrane components, such as the endosomal compartments.[87,88] Given that Neu3 is tightly associated to the plasma membrane,[88,89] it is thought to be an important molecule for various cell surface events. Indeed, many of the stimuli triggered by the sialidase involves modulation of transmembrane signaling not only through the modulation of ganglioside expression, but also by direct interaction with signaling molecules, as will be discussed in the following sections. Recently, the participation of Neu3 in endocytosis was also reported, regulating specifically the molecular machinery involved in clathrin-mediated internalization.[87]

4. REGULATION OF CELL SIGNALING BY GANGLIOSIDES IN CANCER CELLS

The role of gangliosides as regulators of signal transduction was first established by supplementation with exogenous gangliosides directly added in the culture medium of cancer cells.[90] With the identification and the molecular cloning of ganglioside biosynthetic enzymes, numbers of papers

reported ectopic expression or antisense inhibition strategies targeting specific GTs to analyze the role of gangliosides in the regulation of signal transduction. All these approaches have clearly demonstrated that gangliosides are fine regulators of RTKs signaling[91] and that changes in cell membrane ganglioside composition that occur in cancer cells result in different cellular responses.[92,93] RTKs activate various intracellular signaling pathways and regulate cell survival, proliferation, differentiation, migration, and invasion. RTKs are usually activated by the binding of the ligand, inducing receptor dimerization and autophosphorylation of the kinase domain. In cancer cells, RTKs signaling is often overactivated through mutation or chromosomal translocation, leading to constitutive kinase domain activation and upregulation of downstream signaling.[94] At the plasma membrane, RTKs are localized in GEMs with other lipid raft-associated proteins including integrins and tetraspanins. Changes in ganglioside expression modify GEMs molecular composition and structure, leading to the reorganization and/or the exclusion of RTKs from GEMs.[95–97] Several RTKs including receptors for epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor, platelet-derived growth factor (PDGF) or nerve growth factor were demonstrated to be positively or negatively regulated by gangliosides in cancer cells.[11,93] These different observations show contrasting effects of ganglioside expression on cell signaling, monosialogangliosides mostly downregulating RTKs activation whereas disialogangliosides acting as activators of RTKs signaling pathways (Fig. 3).[13]

4.1 Monosialogangliosides Negatively Regulate Cell Signaling

Converging evidence demonstrates that monosialogangliosides, especially GM3, negatively regulate the activity of RTKs in cancer cells.[98] The negative effect of GM3 was first demonstrated for EGF receptor (EGFR) signaling in a variety of cell lines including hepatoma, hepatocellular carcinoma, and neuroblastoma cells.[99–102] In murine hepatoma cells Hca-F25, GM3 expression suppressed cell motility and migration via the inhibition of EGFR phosphorylation and the PI3K/Akt signaling pathway.[99] GM3 inhibits the dimerization of EGFR by preventing the autophosphorylation of the intracellular kinase domain in response to ligand binding.[101] It was shown that GM3 directly interacts with EGFR on a site distinct from the EGF-binding site through direct carbohydrate–carbohydrate interactions between GM3 and a GlcNAc-terminated *N*-glycan on EGFR.[103] It was also shown that the inhibition of the sialidase Neu3 suppresses cancer-cell growth by attenuation of EGFR signaling through GM3 accumulation.[104] In human

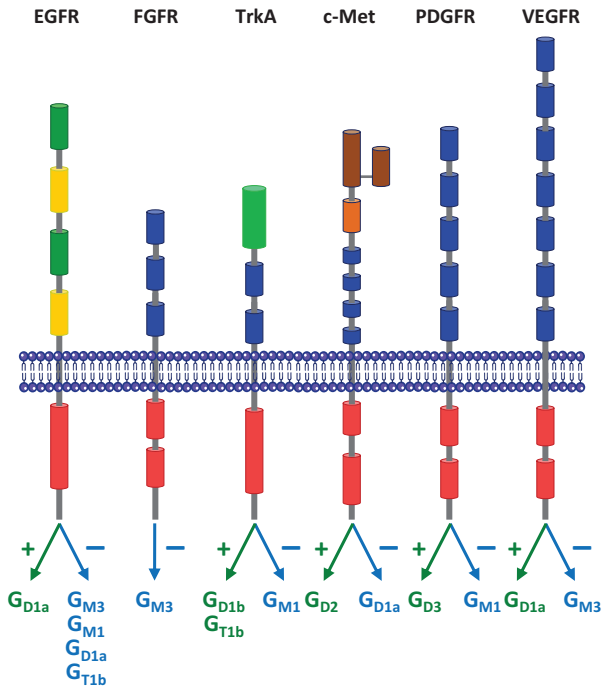

Fig. 3 Main effects of gangliosides on RTKs signaling. Gangliosides can either inhibit (*red arrows*) or activate (*green arrows*) RTK signaling. FGFR, FGF receptor; PDGFR, PDGF receptor; VEGFR, VEGF receptor. *Adapted from Julien S, Bobowski M, Steenackers A, Le Bourhis X, Delannoy P. How do gangliosides regulate RTKs signaling? Cells. 2013;2:751–767.*[11]

neuroblastoma cells, proliferation and EGFR phosphorylation is not only inhibited by GM3 but also by GM1, GD1a, or GT1b. [102] GM3 is also implicated in the decrease of vascular endothelial growth factor (VEGF) receptor 2 phosphorylation and subsequent inhibition of Akt signaling pathway in human umbilical vein endothelial cells.[105] GM3 decreases VEGF receptor 2 activation by blocking receptor dimerization and VEGF binding through a GM3-specific interaction with the extracellular domain of VEGF receptor 2.[106] In cultured retinal glial cells, GM3 depletion by GlcCer synthase inhibition enhances tyrosine phosphorylation of the FGF receptor, activates PI3K/Akt pathway and increases the interactions of the FGF receptor with integrins.[107,108]

The monosialoganglioside GM1 also negatively regulates RTK signaling. For example, in human glioma cells, GM1 expression by transfection with GM2/GD2 synthase and GM1/GD1b synthase cDNAs, resulted in reduced

PDGF receptor phosphorylation and signaling, due to the exclusion of the receptor from GEMs.[109] The Csk binding protein PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains) regulates PDGF receptor partitioning in caveolae and its association with Src family protein tyrosine kinases by controlling GM1 levels at the plasma membrane.[110] In rat pheochromocytoma cell line PC12, the expression of GM1 by transfection of β3GalT 4 cDNA that converts GM2 into GM1, inhibited nerve growth factor-induced TrkA dimerization and phosphorylation, as well as the downstream pathways.[111]

4.2 Tandem Disialogangliosides as Activators of RTKs

Contrasting with the negative effect of monosialogangliosides, more complex gangliosides showing Neu5Acα2–8Neu5Acα2-3 disaccharide motif linked to LacCer such as GD2 or GD3, are considered as positive regulators of RTKs signaling. In PC12 cells, the introduction of the GD3S (*ST8SIA1*) cDNA resulted in the overexpression of GD1b and GT1b. These gangliosides triggered a conformational change of TrkA that formed constitutively active dimers, activating its downstream signal pathways including Erk1/2 and PI3K/Akt, and leading to the enhancement of cell proliferation.[112] Furukawa and coworkers analyzed in depth the implication of GD3 in the malignant properties and cell signaling of human melanoma cells. By stable transfection of the GD3S cDNA into GD3-negative SK-MEL-28 mutant, they showed the enhancement of p130Cas, paxillin, and focal adhesion kinase (FAK) phosphorylation in GD3-positive cells.[113] The adhesion signals via integrins were also enhanced due to the clustering of integrins into GEMs associated with GD3 expression.[114] They also found higher amounts of constitutively activated Yes kinase in GEMs in GD3-positive cells.[115] They suggested that GD3 ganglioside plays a crucial role in the convergence of adhesion and RTKs signals, leading to the synergistic effects of those signals on malignant properties of melanomas.[116] Similarly, in glioma cells and tissues, GD3 ganglioside, PDGF receptor α, and activated kinase Yes colocalized in GEMs, promoting proliferation, invasion and a malignant phenotype.[117] In MDA-MB-231 breast cancer cells, the expression of GD3S induced the accumulation of b- and c-Series gangliosides including GD3, GD2, and GT3.[118,119] Among these complex gangliosides, GD2 was found to be involved in the constitutive activation of c-Met, and the subsequent activation of MEK/Erk and PI3K/Akt signaling pathways, leading to enhanced cell migration, proliferation and tumor growth in severe combined immunodeficiency (SCID) mice. This was shown by competition assays

using anti-GD2 mAb that inhibited c-Met phosphorylation, demonstrating the role of the GD2 glycan moiety in c-Met activation.[119] Moreover, silencing of the GM2/GD2 synthase efficiently reduced both GD2 expression and c-Met phosphorylation. Of importance, the GD2-dependent activation of c-Met occurred in the absence of hepatocyte growth factor.[75] Several papers have recently underlined the role of GD2 ganglioside and GD3S in renewal and properties of cancer stem cells. GD2 was identified as a new specific cell surface marker of $CD44^{hi}CD24^{lo}$ breast CSC from human breast cancer cell lines and patient samples, capable of forming mammospheres and initiating tumors.[70] Gene expression analysis revealed that several GT genes involved in GD2 biosynthesis (*ST3GAL5*, *B4GALNT1*, and *ST8SIA1*) are highly expressed in CSC.[70,120] The reduction of GD2 expression by *ST8SIA1* knockdown reduced mammosphere formation and cell motility and completely abrogated tumor formation in vivo, changing the phenotype from CSC to non-CSC.[70,120] Similarly, GD3 and GD3S are also key drivers for glioblastoma stem cells maintenance and tumorigenicity.[121] Finally, it was also demonstrated that the interaction of GD3 with EGFR is responsible for sustaining its expression and downstream signaling to maintain the self-renewal of mouse neural stem cells in vitro.[122]

Disialogangliosides that belong to the a-Series, such as GD1a, were found to have different effects on cell signaling. In mouse osteosarcoma cell variant FBJ-LL cells, GD1a inhibits the hepatocyte growth factor-induced motility and scattering through the suppression of phosphorylation of c-Met.[123,124] On the contrary, in normal human dermal fibroblasts, GD1a promotes ligand-independent EGFR dimerization, enhances EGFR-mediated activation of the MAPK signaling pathway and EGFR phosphorylation is significantly reduced with the knockdown of ST3 Gal II, an enzyme that converts GM1 into GD1a.[125,126]

5. GANGLIOSIDES AND APOPTOSIS SIGNALING

Gangliosides, especially the disialoganglioside GD3, are involved in mitochondrial damage and apoptosis. GD3 was first implicated in apoptosis in lymphoid and myeloid tumor cells where the activation of the apoptosis-inducing CD95 death receptor (Fas) induced GD3 synthesis and accumulation in the membrane of mitochondria, leading to disruption of mitochondrial transmembrane potential and apoptosis in a caspase-independent fashion.[127] Apoptosis can be induced by direct exposure to GD3

exogenously added in the medium, or by overexpression of GD3S. GD3 accumulates within mitochondria of cells undergoing apoptosis and inhibition of GD3S expression prevents GD3-induced mitochondrial changes, caspase 9 activation, and apoptosis.[128] Although apoptosis could be induced by GM1 in thymocytes and by GD1b in MCF-7 breast cancer cells, most data available show that GD3 is the major player in programmed cell death.[129,130] Importantly, the association of specific gangliosides, mainly GD3 with cytoskeletal elements, such as the cytoplasmic peripheral membrane protein Ezrin, is required to trigger CD95/Fas-mediated apoptosis.[131] Efficient apoptotic signaling mediated by the CD95 death receptor required its translocation in GEMs. Thus, the structure and composition of GEMs play a major role in the promotion of either survival or cell death.[14] It has been shown that the proapoptotic activity of GD3 is suppressed by sialic acid O-acetylation. In childhood acute lymphoblastic leukemia, sialic acid O-acetylation on GD3 prevents its apoptotic effect and promotes survival of lymphoblasts.[132] Gangliosides bearing 9-O-acetylated sialic acid are expressed at high levels in tumors of neuroectodermal origin, such as melanoma, where they play roles in the growth and migration.[133] In tumor cells, 9-O-acetyl-GD3 could therefore modify the proapoptotic activity of GD3 either by affecting its transport to the mitochondria, or its interactions with mitochondrial components.

The targeting of tumor-associated gangliosides with antibodies also affects signaling pathways and leads to cell death including apoptosis.[134,135] As an example, the antiproliferative and proapoptotic activity of GD2-specific mAb 3F8 has been reported in human melanoma cells.[136] In human neuroblastoma cell lines, treatment with the anti-GD2 mAb 14G2a downregulated the PI3K/Akt/mTOR signaling network, decreasing cell viability.[137] In 2015, Dinutuximab (a chimeric monoclonal antibody to GD2 ganglioside) was approved by the US Food and Drug Administration (FDA) and is currently used in a combination immunotherapeutic regimen for the treatment of children with high-risk neuroblastoma.[138]

6. BIOLOGICAL ROLES AND RELEVANCE OF SIALIDASES IN CANCER

During the past few years, experimental evidence pointed out the potential involvement of mammalian sialidases in the occurrence of various kinds of tumors. Changes in sialidase expression levels have been observed in

primary tumor samples, as well as in cancer cell lines, with different sialidases having been reported to either promote or revert malignant phenotypes. In fact, it seems like three of the sialidases, Neu1, Neu2, and Neu4 showed a tendency for downregulation, while Neu3 showed marked upregulation during carcinogenesis.[139]

Multiple observations suggest that, in addition to lysosomal catabolism, Neu1 modulates transmembrane signaling through desialylation of surface molecules, presumably after mobilization to the cell surface. In this sense, overexpression of Neu1 leads to suppression of experimental pulmonary metastasis and reversion of malignant phenotype in murine B16 melanoma cells.[140] This was consistent with pioneering observations that metastatic potential is inversely correlated with lysosomal-type sialidase activity in transformed rat 3Y1 fibroblasts.[141] It was proposed that a low level of Neu1 in tumors causes hypersialylation of the carbohydrate portion of glycoproteins, in line with the observation that malignant cells are often hypersialylated. To elucidate the mechanism underlying the modulation of malignant properties by Neu1, the human sialidase gene was overexpressed or silenced in colon cancer HT-29 cells. When Neu1-overexpressing cells were injected transsplenically into mice, in vivo liver metastasis was significantly reduced. Neu1 overexpression suppressed cell migration, invasion and adhesion in vitro, whereas its silencing resulted in the opposite. One of the target molecules of Neu1 was found to be integrin β4, which undergoes desialylation and decreased phosphorylation followed by attenuation of focal adhesion kinase and ERK1/2 downstream pathway.[142] Furthermore, it has been demonstrated that cell surface-residing Neu1 downregulates the proliferation of human aortic smooth-muscle cells and fibroblasts by desialylation of other cell surface receptors that directly propagate mitogenic signals, such as PDGF and insulin-like growth factor-2 receptors. Moreover, fibroblasts derived from patients with sialidosis bearing exclusive deficiency in Neu1 demonstrate higher basal proliferation rates and greater responsiveness to PDGF and insulin-like growth factor-2 than normal fibroblasts.[143] Although, several cancers show a tendency for decreased Neu1 expression, recent reports suggest that this is not a general property of cancer cells. For example, in ovarian cancer tissues of patients, Neu1 was expressed at a higher level than that in adjacent normal tissues, and in OVCAR3 and SKOV3 ovarian carcinoma cells, Neu1 knockdown inhibited proliferation, invasion, arrested cells cycle at G0/G1 phase, and induced apoptosis in vitro.[144] In line with these results, it has been proposed that Neu1 hydrolyzes α-2,3-sialic acid residues of glycosylated receptors, such as EGFR and TrkA on the cell

surface to remove steric hindrance and to facilitate growth factor association, subsequent receptor activation, and downstream signaling.[145,146]

As mentioned before, upregulation of Neu3 is observed in various types of tumors including colon,[147] renal,[148] ovarian,[149] prostate,[150,151] and head and neck cancers.[152] In general, its upregulation has been associated with apoptosis suppression and increased cell proliferation, invasion, and migration. In colon cancer HCT-116 cells, Neu3 overexpression increases the level of antiapoptotic molecules, such as Bcl-2, and decreases the amount of proapoptotic molecules, such as caspase-3.[147] In colon cancer DLD-1 cells, this sialidase also regulates cell adhesion by enhancing tyrosine phosphorylation of integrin β4 and promoting adhesion to laminins.[153] In human prostate cancer–cell lines PC-3 and LNCaP, upregulation of Neu3 causes androgen-independent proliferation by enhancing expression of androgen receptor signaling-related molecules.[150] This was consistent with another report in which mice injected with PC-3 M prostate carcinoma cells carrying the Neu3 siRNA developed fewer bone metastases than control mice.[151] In squamous carcinoma HSC-2 and SAS cells, Neu3 promoted cell motility and invasion, accompanied by the increased expression of matrix metallopeptidase 9.[152] Furthermore, it has been recently demonstrated that Neu3 influences the expression of stem cell pluripotency marker genes and Wnt/β-catenin signaling related genes in HT-29 and HCT116 colon cancer cells, suggesting that this sialidase might contribute to maintenance of an undifferentiated and tumorigenic state of cancer cells.[154] On the other hand, silencing of the Neu3 gene in different cancer cell lines significantly inhibited cell growth, decreasing cell proliferation and increasing the propensity to undergo apoptosis.[150,155,156]

The role of Neu3 in tumorigenesis in vivo has been studied in animal models. Neu3 transgenic mice showed increased azoxymethane-induced aberrant crypt foci formation in the colonic mucosa due to suppression of apoptosis,[157] whereas Neu3 knock-out mice were less susceptible than wild-type mice to the colitis-associated colon carcinogenesis induced by azoxymethane and dextran sodium sulfate.[158]

One of the possible molecular mechanisms that explains the changes caused by Neu3 upregulation is that this sialidase stimulates EGFR phosphorylation in response to EGF with a consequent activation of its downstream molecules, ERK1/2 and Akt.[86,156,157,159] As already mentioned, ligand-dependent activation of EGFR is known to be inhibited by ganglioside GM3 through carbohydrate–carbohydrate interactions between GM3 and *N*-glycans in the ectodomain of EGFR.[160] Analysis of glycolipid content

in cells overexpressing Neu3 indicated a significant decrease in GM3 substrate and a marked increase in LacCer, suggesting that modulation of gangliosides as a consequence of Neu3 catalytic activity could remove the inhibitory effect exerted by GM3 on EGFR, promoting its activation. Interestingly, it was also proposed that Neu3 might activate EGFR through direct desialylation of the receptor, suggesting that glycoproteins may be also among the physiological substrates of Neu3.[86] Therefore, Neu3 may protect against cell death both by promoting EGFR activation through its association and eventual desialylation, as well as through gangliosides pattern modulation. In addition, Neu3 might exert an influence on the cell surface by interacting directly with other signaling molecules including integrin β4,[153] low-density lipoprotein receptor-related protein 6,[154] Rac-1,[161] Cav-1,[162] Src,[159] and Grb-2.[163] In conclusion, the net effect of Neu3 upregulation in cancer cells seems to be the activation of prosurvival pathways that are used as an advantage for tumor progression.

Much less is known about the involvement of the other two members of the sialidase family, Neu2 and Neu4, in carcinogenesis. In clear contrast to Neu3, some studies suggest that downregulation of Neu2 may contribute to the invasive properties and protection against programmed cell death of tumors. For example, ectopic expression of Neu2 in B16 melanoma cells led to marked suppression of lung metastasis accompanied by decrease in invasiveness and cell motility.[164] Similar results were obtained when the cytosolic sialidase was overexpressed in highly metastatic mouse colon adenocarcinoma NL17 cells.[165] In the same sense, Neu2 expression in myeloid leukemia K562 cells remarkably weakened the antiapoptotic axis Bcr-Abl, leading to an increased susceptibility to apoptosis and a marked reduction in proliferation rate. The molecular link between Neu2 activity and Bcr-Abl signaling pathway is not completely understood, but may rely on the desialylation of some cytosolic glycoconjugates.[166]

Regarding Neu4, the level of this sialidase was found to be markedly decreased in human colon cancers as compared with the noncancerous mucosa.[139,167] Besides, transfection of Neu4 in cultured human colon cancer cells resulted in acceleration of apoptosis and in decreased invasion and motility. The siRNA-mediated Neu4 targeting, on the other hand, caused a significant inhibition of apoptosis and promotion of invasion and motility.[167] It has been proposed that Neu4 plays a physiological role in controlling sialyl-Lewis antigens through desialylation of O-glycans, therefore contributing to invasive properties of cancer.[168] In clear contrast,

Neu4 is upregulated in glioblastoma stem cells and promotes β-catenin signaling, enhancing stem-like malignant cell growth.[169,170]

Overall, it is clear that sialidases are likely to be involved not only in catabolism of glycoconjugates but also in functional modulation of transmembrane signaling through protein interactions with signaling molecules. Moreover, the effects of sialidase expression on malignant phenotype may be due to different mechanisms depending on the cell type and cellular conditions.

7. CONCLUSIONS

The last 3 decades have been very fruitful in increasing our knowledge about the metabolism of gangliosides and related lipids, their expression in different physiological and pathological conditions, and very importantly, the cellular signal mechanisms regulated by these sialoglycolipids. It is now clear that gangliosides regulate RTK signaling in cancer cells either by inhibiting or activating receptor dimerization and autophosphorylation. From a general point of view, monosialogangliosides, such as GM3 or GM1 can be considered as negative regulators of RTKs signaling whereas disialogangliosides including GD2, mostly activate RTKs-mediated signal transduction involved in cell proliferation and migration, angiogenesis, and tumor metastasis, making both gangliosides and related glycolipids important predictive biomarkers for clinical outcome of different tumors.[171]

Undoubtedly, gangliosides have reemerged as promising targets for developing anticancer therapies, which includes new and better anticancer antibodies; the development of immunogene therapies, such as antiganglioside-specific chimeric antigen receptor (CAR) to redirect T cells to particular tumor cells;[172] and the design of novel and specific inhibitors of glycolipid synthesis. In this context, the understanding of the mechanisms by which gangliosides regulate cell signaling is of primary importance to identify new targets in cancer therapy.

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