Studies in Glycolipids

Edited by Kenan Demir

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PREFACE

Aiming to fill the gap in its field, this book was prepared to be the first with its content. It was aimed to be beneficial not only to academicians and students working in the field of basic science, but also to all clinicians who research the relationship of glycolipids with cancer and immune system diseases. It is thought to be a comprehensive handbook in the fields of Medicine, Biology and Pharmacy, with its content that brings together physicians from different branches and covers up-to-date studies.

Gangliosides, which are the main glycolipids, Ceramides, Glycolipids and protein compounds, which are the main sources in the Glycolipid synthesis step, were selected as other basic topics in the book, which starts with the introduction of glycolipids. The in-vivo and in-vitro display of glycolipids is included in the book. The pharmacological and industrial uses of glycolipids, which have a wide range of uses, are discussed. The book has been developed on the topics of glycolipids and the immune system, sphingolipids and cancer, glycosphingolipid diseases, and glycolipids and infectious diseases to appeal to clinicians. Glycolipids and blood groups and glycolipid degradation products are presented as other study areas of glycolipids. This book will hopefully be useful to all concerned.

—The Editor

CHAPTER ONE

INTRODUCTION TO GLYCOLIPIDS

MUKADDER ERDEM

Introduction

All cells – prokaryote and eukaryote – are surrounded by a plasma membrane that determines the boundaries of the cell and separates its contents from the external environment. Lipids and proteins are the main components of the plasma membrane. The phospholipid bilayer forms the membrane structure by creating a steady barrier between two aqueous compartments, namely the inside and outside of the cell (Figure 1.1).

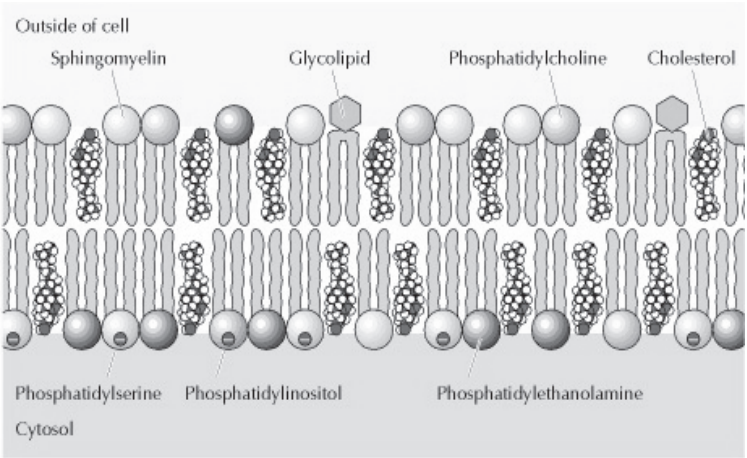

Figure 1.1: The plasma membrane lipid components (Cooper and Hausman 2007)

Mammalian plasma cells contain glycolipids and cholesterol in addition to phospholipids. Glycolipids and their carbohydrate portions are found on the outer surface of the cell membrane. Glycolipids constitute about 2% of membrane lipids (Cooper and Hausman 2007).

Glycolipids are found primarily on the plasma membrane and the body fluids of almost all vertebrate cells. Structural diversity is one of the important features of glycolipids and to date 172 neutral GSLs, 24 sulphated GSLs, and 188 gangliosides have been reported in vertebrates (Yu et al. 2007).

Almost all glycolipids are ceramide derivatives (Figure 1.2) as in sphingomyelin (ceramide: sphingosine + long chain fatty acid). So, referring to them as glycosphingolipids is more precise (Ferrier 2013).

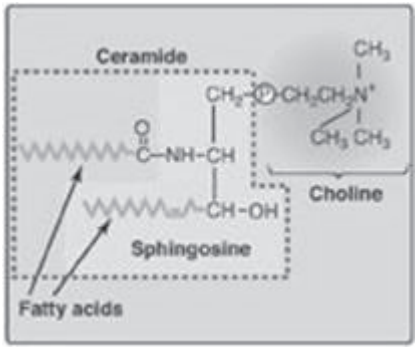

Figure 1.2: Sphingomyelin structure (Ferrier 2013)

Like phospholipids, glycosphingolipids are basic components of all cell membranes, but found in greater amounts in nerve tissue. They interact with the extracellular medium through their localization on the cell membrane. Therefore, they play a role in the regulating of cellular interactions (e.g. adhesion, recognition), growth and development (Ferrier 2013).

Membrane glycosphingolipids have a significant role in the regulating of signal transduction and membrane trafficking. These GSL- and cholesterol-rich laterally assembled micro domains (rafts) act as platforms for the attachment of lipid-modified proteins (e.g. glycosylphosphatidylinositol (GPI)-anchored proteins), so they function in regulating cellular processes.

Glycosphingolipids exhibit antigenic properties and are sources of blood group antigens such as A, B, O and some embryonic and tumor antigens. [The antigenic determinant part is the carbohydrate part of the GSL.] They also act as cell surface receptors for cholera, diphtheria toxins, and some viruses.

Deficient degradation of glycosphingolipids, seen in some genetic disorders, causes lysosomal accumulation of these compounds. Transformed cells with dysregulated growth show typical variances in the carbohydrate portion of the GSL (Ferrier 2013).

Glycolipid Structure

The basic structure of a glycolipid is formed by the attachment of a sphingolipid or a glycerol group to a mono- or oligosaccharide group. The SL or glycerol group can be acetylated or alkylated. So subclasses of glycosphingolipids and glycoglycerolipids are formed (Figure 1.3). Glycolipids interact and attach to the lipid bilayer surface with the hydrophobic structure of the lipid tail (Glycolipids-Physics Libre Texts 2019).

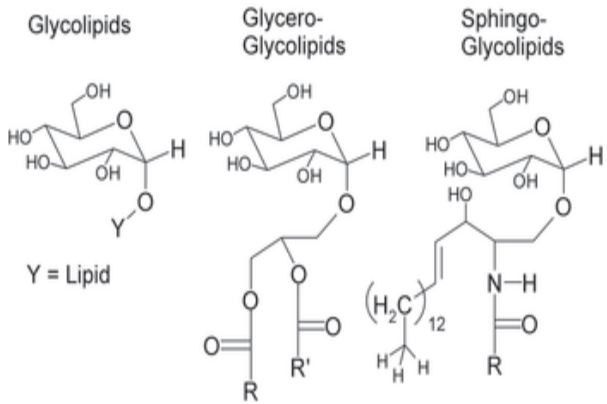

Figure 1.3: Glycolipid structure (Glycolipids-Physics Libre Texts, 2019)

A glycolipid structure consisting of two hydrocarbon chains attached to a polar head group containing carbohydrates is shown in Figure 1.4.

In order to fully understand the glycosphingolipids, we have to talk about sphingolipids. Sphingolipids are composed of a "sphingosine base" (serine + long chain fatty acyl-CoA) (Fahy et al. 2005; Hirabayash et al. 2006). The basic component of these lipids is called a sphingosine.

There are several classes of sphingolipids: the sphingoid base and simple derivatives, ceramides, and complex sphingolipids (Figure 1.5) (Sphingolipid-Physics Libre Texts, 2019).

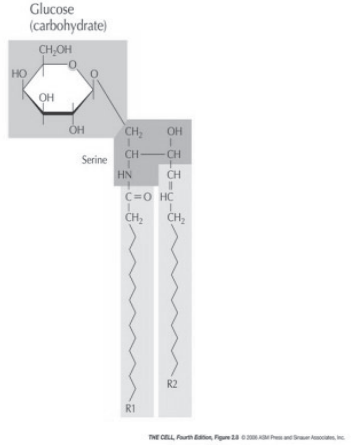

Figure 1.4: A glycolipid structure (two hydrocarbon chains + a polar head group (serine + carbohydrates e.g. glucose)) (Cooper and Hausman 2007)

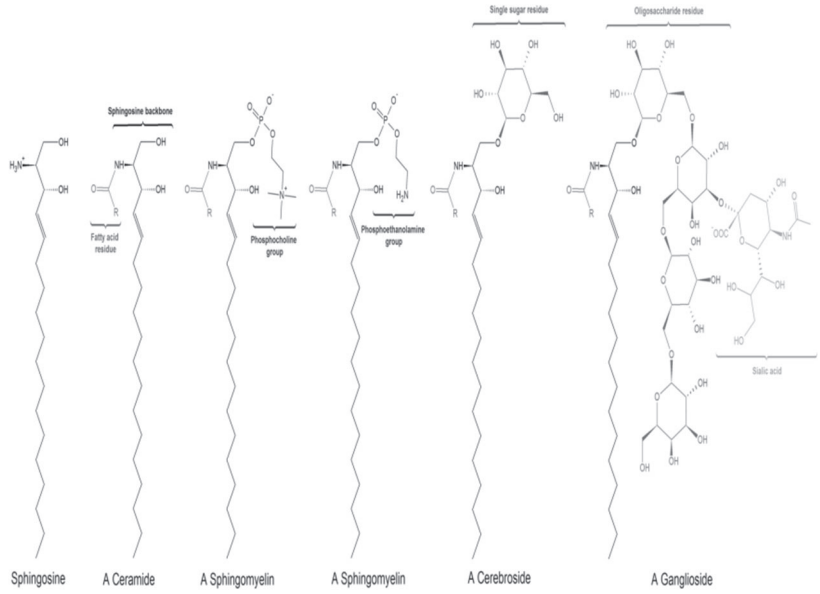

Figure 1.5: Sphingolipid's general structure (Sphingolipid-Physics Libre Texts, 2019)

Synthesis of Glycolipids

Glycolipid synthesis acts through a series of enzymes that sequentially add sugar to the lipid. Lactosylceramide is used to get glycosphingolipids via a series of reactions starting with the acylation and desaturation of D-erythro-sphinganine as a first step. Ceramide is extracellularly glycosylated then β-galactosylated in order to form lactosylceramide. Glycosyltransferases and sulfotransferases can provide further elongation. For example, galactosyltransferases transfer a galactosyl from UDP-Gal onto diacylglycerol for the synthesis of β-galactosyldiacylglycerol in plants. Then an additional transfer of a galactosyl from UDP-Gal causes further elongation (Yu et al. 2010).

Types of Glycolipids

Glycoglycerolipids

These are the glycolipids formed by the binding of diglyceride hydroxyl groups with mono-, di- or tri-saccharides by a glycosidic bond. Monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) are the major glycolipid components.

Monogalactosyldiacylglycerols and digalactosyldiacylglycerols are the main lipids that prevail in chloroplasts of photosynthetic organisms (e.g. plants, algae, bacteria) (Heinz 1996).

Glycosphingolipids

Glycosphingolipids consist of ceramide and one or more monosaccharide residues attached to it by beta glycoside bonds. The ceramide is formed by the binding of a long-chain fatty acid to the -NH2 group of sphingosines by amide bonds (ceramide: sphingosine + fatty acid). Glycosphingolipid subclasses:

Neutral glycosphingolipids; These do not carry any ionic charge. They consist of one or more sugar residues linked by an O-ester bond to the first carbon of ceramides (e.g. cerebrosides). Cerebrosides are ceramide monosaccharides, so they are the simplest neutral glycosphingolipids (e.g. galactosylceramide, glucosylceramide). Galactosylceramides are found in high concentrations in the myelin sheaths of all nerve tissues (central and peripheral). They can constitute 2% and 12% of the dry weight of gray and white matter, respectively (Figure 1.6) (Christie 2003). Glucosylceramide

(Glcß1-1'Cer) is the glycosphingolipid of non-nervous tissues. It is found in small amounts in the brain and some tissues (e.g. spleen, erythrocytes).

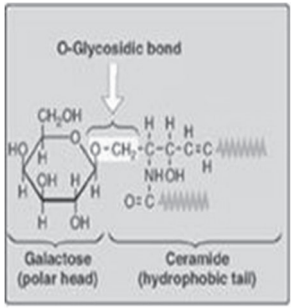

Figure 1.6: Structure of a neutral glycosphingolipid, galactocerebroside (Ferrier 2013)

Oligoglycosylceramides are neutral glycosphingolipids that contain two or more sugar units in their structure. They are found in high numbers in the cell membrane of most eukaryotic organisms (Christie 2003). The most important and abundant oligoglycosylceramide is β-D-galactosyl-(1-4)-β-D-glucosyl-(1-1')-ceramide, also called lactosylceramide (LacCer).

Acidic glycosphingolipids:

These are negatively charged at physiological pH. The negative charge comes from NANA (N-acetylneuraminic acid) in gangliosides (Figure 1.7), and sulphate groups in sulfatides (Ferrier 2013).

Subclasses are as follows:

*Sulfoglycosphingolipids***:** These are also sometimes called sulfatides or sulfatoglycosphingolipids. The 3rd carbon of the sugar residue attached to the ceramide is esterified with sulphate. The main sulfatides are 3-sulfate esters of galactosylcerebrosides. These are found in high amounts in nerve tissue, the myelin sheath and tissues with high sodium transportation such as the kidneys (Ishizuka 1997).

Gangliosides: These are complex glycosphingolipids derived from glucosylceramide containing one or more sialic acid molecules (as shown in Figure 1.7). They are found in high concentrations in the nervous tissue (e.g. up to 6% of the brain lipid weight). GM1 is one of the common

monosialo-gangliosides (G: ganglioside, M: monosialo, 1: migration rate on chromatography).

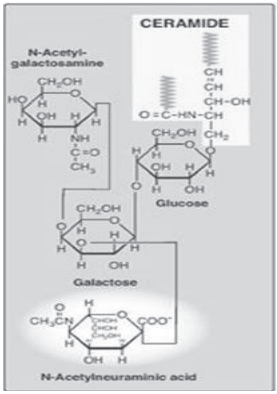

Figure 1.7: Ganglioside GM_2 structure (represents a hydrophobic hydrocarbon chain) (Ferrier 2013)

Glycolipid Distribution in the Cell

Glycolipids are commonly found in the membranes of cells and organelles. The proportion of glycolipids in the membranes of intracellular organelles is about two-thirds of the total cell glycolipid content (Gillard 1993). Glycolipid biosynthesis occurs in the Golgi apparatus. Sugar residues are added to the ceramide individually from the appropriate nucleotide sugar donors. While the first sugar transfer to ceramide occurs on the cytosolic surface of the Golgi complex, other sugar transfers take place on the lumen surface (Edidin 2003). The transportation of most glycolipids between the membranes occurs as small bilayer vesicles.

There is also the cytosolic distribution of some glycolipids. Glycolipids are found on the outer surface of the plasma membrane and on the lumen surface of organelles (Pike 2004). Glycosphingolipid distribution differs on the apical and basolateral sides in the cell, and they are commonly found on the apical side in polarized epithelial cells. Simons and Toomre (Simons and Toomre 2000) observed that cholesterol and glycosphingolipids form glycolipoprotein micro domains called rafts. The lipid content of the plasma membrane and the raft differs. While the raft cholesterol and sphingolipid ratio is 2-3 times higher than that of the cell membrane, the phospholipid content is relatively lower. A very large proportion of cell

glycolipids is packed in a raft. The intensive hydrophobic interactions of lipids in the rafts make them more saturated and denser regions than the other parts of the cell membrane.

Conclusion

Glycolipids undertake many functions in cell such as providing cell membrane stability, cell signal transmission, and intercellular interaction. The interaction of these cell surface markers plays an important role in the regulation of growth and development. Glycolipids are antigenic. The carbohydrate portions of certain glycolipids take part in the determination of human blood groups and safely determine which blood group will be given to which person in blood transfusion. They also act as cell surface receptors for cholera and diphtheria toxins as well as for some viruses. In this sense, more detailed research on glycolipids will be promising for the treatment and prevention of diseases.

Keywords: *Ceramide, ganglioside, glycolipid, glycoglycerolipid, glycosphingolipid, sphingosine*

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CHAPTER TWO

GANGLIOSIDES

GÖKÇE ATİKELER

Introduction

Gangliosides are glycosphingolipids containing sialic acid moieties in their lipophilic ceramide component and a carbohydrate chain. Ernst Klenk, a German scientist, was the first to isolate gangliosides from bovine brain tissue in 1942 (Fishman 1976). Later studies have shown that gangliosides are abundant in the nervous system of vertebrate animals, particularly in gray matter, and in other tissues and organs (liver, kidney) (Kolter 2012). Gangliosides are abundant in the nerve cell membranes of animals (Miller-Podraza et al. 1982).

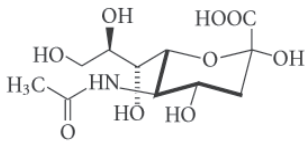
Gangliosides are involved in cell-cell interactions, the binding of bacterial toxins and viruses, and cell communication, which are particularly crucial for brain development and neural differentiation. The content and level of gangliosides change in chronic and neurodegenerative diseases, affecting both disease progression and treatment strategies.

Structure and Functions of Gangliosides

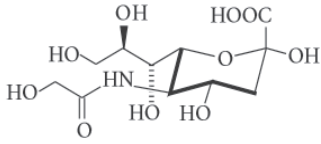
The lipid component of gangliosides, known as ceramide, is composed of long-chain fatty acids linked via an amide bond to amino alcohol (2-amino-1,3-dihydroxy-octadec-4-ene) sphingosine.

The oligosaccharide chain of gangliosides binds via a glycosidic bond to the first carbon atom of the sphingosine (Fishman and Brady 1976). The oligosaccharide component is a combination of glucose, galactose, and N-acetylgalactosamine. Oligosaccharide chains depend on the sugar structure, content, and linkages (Yu et al. 2011).

The negatively charged sialic acid residues separate gangliosides from neutral glycosphingolipids and sulfatides. There are generally 1-4 sialic acid residues, and sometimes seven. Sialic acid is another name for 5-amino-3,5-dideoxy-D-gylcero-D-galacto-non-2-ulopyrasonic acid or neuraminic acid derivatives (Sonnino et al. 2007). The three main sialic acids are 5-N-acetyl-, 5-N-acetyl-9-O-acetyl-, and 5-N-glycolyl (Figure 2.1), the first two of which are found in healthy individuals (Sonnino et al. 2007).



N-acetyl-α-neuraminic acid
(Neu5Ac)



N-glycolyl-α-neuraminic acid
(Neu5Gc)

Figure 2.1: Sialic Acids (Kolter 2012)

Svennerholm was the first to classify gangliosides in 1946. In the classification, the letter "G" indicates the members of the ganglion family, the letter "M" (mono) "D" (di), "T" (tri), or "Q" (tetra) indicates the number of sialic acid residues, and the number 1, 2, or 3 indicates the sequence of migration in thin-layer chromatography. Five subtracted from that number is the number of neutral carbohydrates in gangliosides (Kolter 2012). The galactose to which the sialic acid residues of gangliosides with 0, 1, 2, and 3 sialic acids bind, is referred to as the asialo (0-), a-, b-, and s-series, respectively (Figure 2.2). N-galactosamine to which sialic acid residues bind, is referred to as the α series.

For example, the ganglioside "GQ1b" is of the ganglio-series (G) with four sialic acid residues (Q), four neutral carbohydrate residues (5 – 1 = 4), and two sialic acids bound to the inner galactose (b) (Figure 2.3).

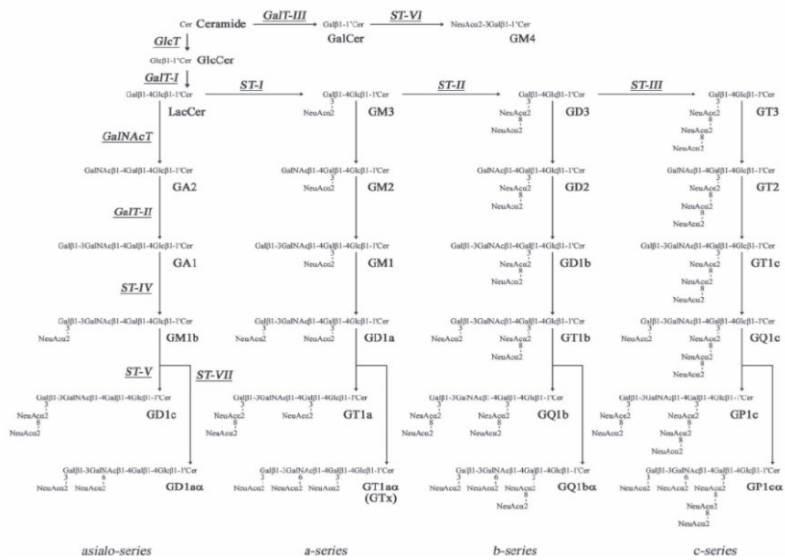


Figure 2.2: Structure and Biosynthetic Pathways of Gangliosides (Yu et al. 2011)

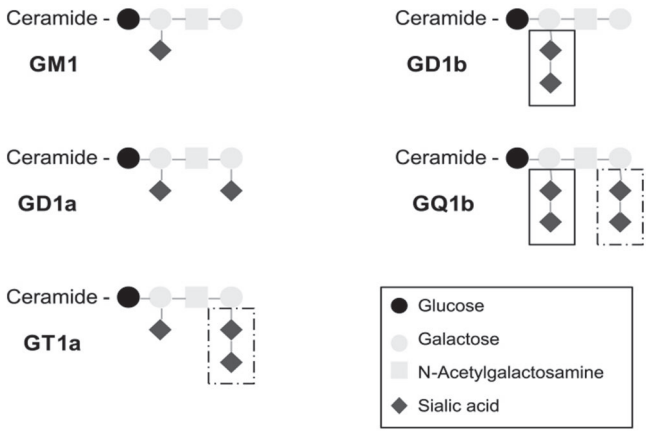


Figure 2.3: Structure of Gangliosides (Delmont and Willison 2015)

KDN (2-keto-3-deoxy-D-glycero-D-galacto-nononic acid) is a recently discovered member of the sialic acid family. The acyl group on the C-5 carbon is replaced by the hydroxyl group (Figure 2.4). KDN was first isolated from rainbow trout eggs (Nadano et al. 1986). Further studies

have found it to be widely distributed in nature, from bacteria to humans (Inoue S., Kitajima K., and Inoue Y. 1996). KDN is synthesized de novo from mannose (Angata et al. 1999). KDN synthesis requires CMP-KDN synthase and KDN-transferase enzymes. KDN expression is age-dependent. In rat liver, KDN decreases from birth to adulthood and then increases again with age (Campanero-Rhodes et al. 1999). The ratio of free KDN/free Neu5Ac in patients with ovarian cancer is positively correlated with the cancer stage, suggesting that KDN can be used as a biomarker for ovarian cancer (Inoue et al. 1998). High levels of KDN observed in ovarian cancer patients have facilitated research on other types of cancer.

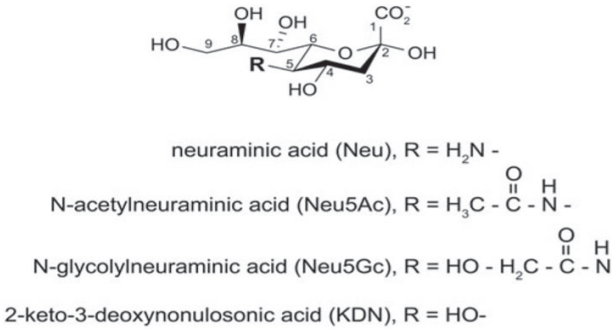


Figure 2.4: Chemical Structure of KDN (Wang et al. 2015)

Occurrence of Gangliosides

Most gangliosides in adult mammals belong to the ganglio, gala, and lacto series. Predominantly found in the brain, gangliosides are five times greater in gray matter than in white matter (Kolter 2012). Gangliosides are 6-10% of the lipid in the brain. Ganglioside production in the brain is proportional to neurogenesis, synaptogenesis, and cell proliferation (Rahmann 1995). The main gangliosides in the brain are GM1, GD1a, GD1b, and GQ1b. Simple gangliosides (GM3 and GD3) turn into complex gangliosides (GD1a and GT1b) during brain development (Yu et al. 2009). The content and structure of gangliosides in the brain change with age. Lipid-bound sialic acid concentrations decrease, while the number of gangliosides with complex carbohydrates increases.

Extraneuronal tissues (liver, bone marrow, kidney, and embryonic stem cells) have ganglio and lactosyl series. The structure and composition of gangliosides in the liver also change with age (Ozkok et al. 1999)

The serum also contains GM3, GD3, GD1a, GM2, GT1b, GD1b, and GQ1b, which are mainly transported by LDL (66%), HDL (25%), and VLDL (7%) (Senn et al. 1989).

Gangliosides are found in numerous vertebrate cells. At the cellular level, they are mostly found in the plasma membrane. The mitochondrial membrane has GD3 that regulates apoptosis (Garofalo et al. 2007). The nucleus membrane also has GD3 that helps to stabilize calcium (Ledeen and Wu 2011).

Gangliosides with O-Acetylated sialic acids are found mainly in growing cells and tissues. They are used as oncofetal markers in different tumors (Kohla et al. 2002) and may act as receptors for coronaviruses (Schwegmann and Herrler 2006).

Gangliosides are also found in different digestible foods (meat, milk, eggs, etc.). Milk contains GD3 and GM3 (McJarrow et al. 2009). Gangliosides in foods help to regulate intestinal microflora and prevent infections, especially in neonates. A considerable amount (80%) of gangliosides in foods is absorbed in the intestines, resulting in high levels of gangliosides in the serum, which is vital for brain development in neonates (McJarrow et al. 2009).

Biosynthesis of Gangliosides

The first stage of ganglioside synthesis is the formation of ceramide in the endoplasmic reticulum. L-serine and palmitoyl-CoA are catalyzed by the pyridoxal phosphate-dependent serine palmitoyltransferase, resulting in ceramides by the acylation of sphingosine by N-acyltransferases, a member of the LASS family of enzymes (Thomas Kolter 2012). Ceramides are transported to the Golgi apparatus via vesicle transport or ceramide transfer protein (CERT) (Yamaji and Hanada 2015) (Figure 2.5). UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, and CMP-N-acetylneuraminic acid are used as carbohydrate donors, which are added to ceramides by glycosyltransferase. The resulting glycosylceramides are then converted into LacCer by lactosylceramide synthase (Nishie et al. 2010). LacCer is the precursor of different glycosphingolipid series

(ganglio-, asialo ganglio-, globo-, and lacto-) (Sandhoff and Sandhoff 2018), which are generated by cell-specific glycosyltransferases.

While GM4 is derived from galactosylceramide (GalCer), many other gangliosides are synthesized from lactosylceramide (LacCer). First, GM3 (a simple ganglioside) is a result of sialic acid being added to LacCer by the LacCer α2-3 sialyltransferase (ST-I or GM3 synthase) enzyme. GD3 and GT3 are a result of sialic acid being added to GM3 and GD3, respectively, by the GM3 α2-8 sialyltransferase (STII or GD3 synthase) and GD3 α2-8 sialyltransferase (STIII or GT3 synthase) enzymes. GM3, GD3, and GT3 are the precursors of numerous a-b-c series gangliosides. Sialyltransferase enzymes result in more complex gangliosides (Figure 2.2). Asialo-series gangliosides are synthesized from LacCer by glycosyltransferases along a different pathway (Yu et al. 2011).

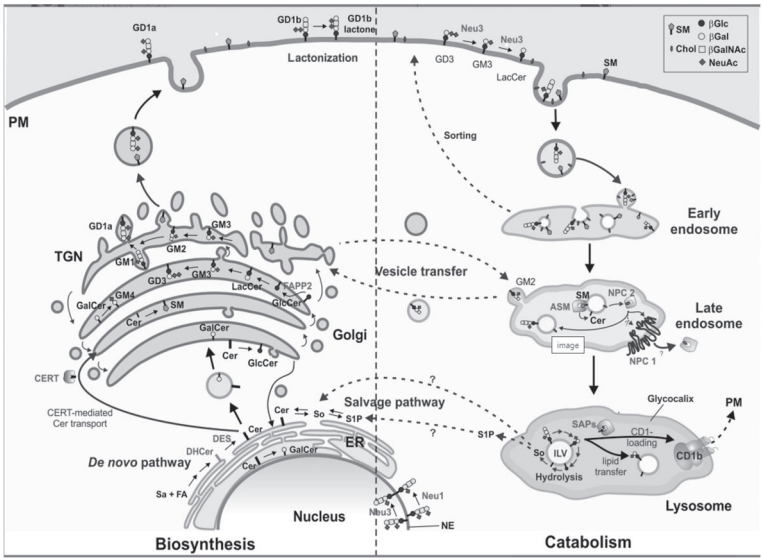


Figure 2.5: Ganglioside Biosynthesis and Catabolism (Sandhoff and Sandhoff 2018)

Degradation of Gangliosides

Gangliosides are degraded mainly in intraendolysosomal vesicles, endosomes, and lysosomes. However, they are also degraded by plasma membrane-associated sialidase (Neu3) (Kolter 2012). Luminal vesicles are the result of vesicle budding and fusion by endosomal complex proteins. Vesicles

are removed for lysosomal digestion through lipid sorting at the endosome stage (Wollert and Hurley 2010). Cholesterol is sorted out by two sterol binding proteins (NPC-1, NPC-2) (Abdul-Hammed et al. 2010) and degraded in sphingomyelin by sphingomyelinase. Anionic bis(monoacylglycero)phosphate (BMP) stimulates ganglioside degradation and is derived from phosphatidylglycerol in intraendosomal membranes (Gallala and Sandhoff 2011). Although the lysosomal perimeter membrane is resistant to lysosomal digestion, intra-endosomal membranes are degraded by sphingolipid activator protein (SAP) and hydrolytic enzymes (Sandhoff and Harzer 2013). The sialic acid and carbohydrate residues of gangliosides are removed by sialidase and exoglycohydrolase, respectively, while the resulting ceramides are hydrolyzed into fatty acids by ceramidases (Sandhoff and Sandhoff 2018). This degradation that occurs through the endocytosis-endosome-lysosome pathway requires acidic pH. On the other hand, sialidases and glycohydrolases require effector molecules, referred to as sphingolipid activator proteins (SAPs) (Kolter and Sandhoff 2005). Hereditary defects in prosaposin – the precursor of saposin A, B, C, and D – result in the deposition of glycosphingolipids and gangliosides (Sandhoff et al. 2018).

The first stage of the degradation of complex gangliosides in mammalian tissues is the removal of terminal sialic acid from oligosaccharide chains by neuraminidases and the formation of GM1 (Sandhoff and Sandhoff 2018). The degradation of GM1 proceeds with the formation of GM2 by the removal of galactose by the GM1-β-galactosidase enzyme (promoted by the GM2 activator protein or saposin B). Afterward, GM3 is formed by the removal of terminal N-acetylgalactosamine residues by the β-hexosaminidase A enzyme (promoted by the GM2 activator protein). GM3 is degraded to LacCer by α-sialidase and SAP B. LacCer is degraded to GlcCer by the β-galactosidase enzyme with the help of SAPs B and C. Glycosyl residues are also removed with the help of SAP B and C galactosidase. The resulting ceramide is degraded to sphingosine and free fatty acids, respectively, by ceramidase and SAP D (Figure 2.6). Hydrolytic stages in GM1 degradation are affected by pH, positively charged molecules, negatively charged molecules, and SAP. For example, GM2 is degraded by Hex A and GM2AP at $3.8<pH<4.5$ (Bierfreund et al. 1999).

Ganglioside degradation involves not only SAPs, but also anionic lipids, the absence of which makes the process difficult (BMP, phosphatidic acid, phosphatidylglycerol, and phosphatidylinositol) (Sandhoff R., Schulze H. and Sandhoff K. 2018). Anionic lipids stimulate, whereas cationic

lipids inhibit degradation (sphingosine bases). The terminal degradation product of lysosomal sphingolipids is sphingosines, which should be effectively removed from the lysosome for successful degradation. Ceramides stimulate glycosylceramide hydrolysis in the presence of fatty acids, monoacylglycerol, diacylglycerol, and anionic lipids, whereas sphingomyelin inhibits the hydrolysis of sphingosine and results in the deposition of glycosylceramide (Sandhoff et al. 2018).

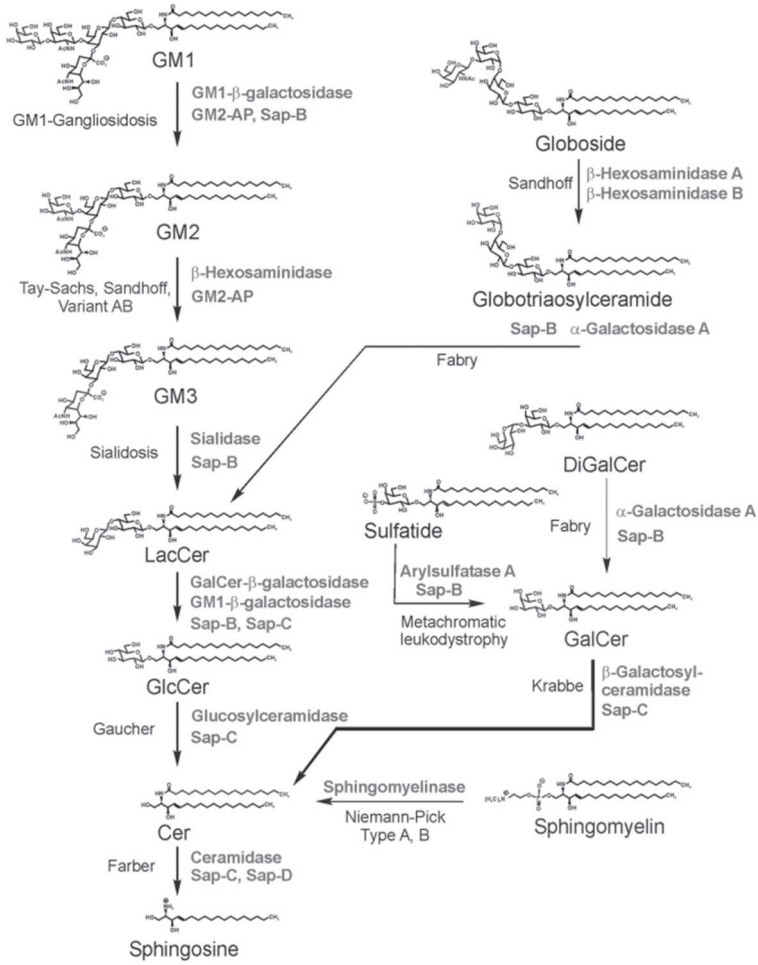


Figure 2.6: Ganglioside Degradation and Gangliosidoses (Sandhoff and Kolter 1995)

Gangliosidoses

GM1 and GM2 gangliosidoses are lysosomal storage diseases associated with ganglioside degradation defects. Gangliosides are found in the neuronal plasma membrane, and defects in ganglioside metabolism can cause fetal neurodegenerative diseases, such as GM1 and GM2 gangliosidoses due to defects in lysosomal ganglioside degradation (Sandhoff and Harzer 2013). All gangliosidoses are inherited autosomal recessive diseases with severe variable symptoms that can occur at any age as a result of genetic defects in ganglioside degradation.

GM1 gangliosidosis is a hereditary disease characterized by GM-1-β-galactosidase deficiency in lysosomes. GM1 gangliosidosis is caused by defects in the GLB1 gene and characterized by the deposition of GM1 and GA1 in neuronal cells (Brunetti-Pierri and Scaglia 2008). GM1 gangliosidosis presents in three clinical forms. Infantile (type 1) is characterized by the progressive deterioration of the nervous system. Its symptoms begin to appear in the neonatal period with a life expectancy of about two years. The other two types are juvenile (type 2) and adult/chronic (type 3) (Sperb et al. 2012). The severity and progression of the disease are proportional to the residual enzyme activity in cells. B-galactosidase is specific for oligosaccharide and keratan sulphate, and therefore, oligosaccharidosis and mucopolysaccharidosis findings can be regarded as extraneuronal clinical findings in the absence of B-galactosidase (Suzuki and Namba 2001).

The infantile form usually occurs in the first six months of life. Individuals with the infantile form usually seem normal until symptoms appear, but their development slows down over time, and they begin to suffer from muscle atrophy. Individuals with the infantile form lose their skills over time and have loud and exaggerated startle reflexes. They show common symptoms of hepatosplenomegaly, skeletal abnormalities, seizures, reduced mental capacity, corneal clouding, a cherry-red spot in the macula of the eye, and cardiomyopathy due to gingival hypertrophy and heart muscle weakness.

The juvenile form (type 2) is an intermediate form. Individuals with the juvenile form have normal early development. They usually begin to show symptoms between the ages of 18 months and five years. It is characterized by developmental retardation but not by a typical facial appearance, a red spot in the macula of the eye, and organomegaly. The

juvenile form progresses more slowly than the infantile form, but the life expectancy is as short.

Individuals with the adult form (type 3) show symptoms in adulthood. The characteristic findings are dystonia and vertebral abnormalities. Life expectancy varies.

GM2 gangliosidosis is characterized by a GM2 ganglioside degradation defect. It manifests itself in three forms depending on hexosaminidase isoenzymes (Kolter 2012). Variant B, known as Tay-Sachs disease, affects hexosaminidase A and S but not hexosaminidase B. Variant O, known as Sandhoff disease, is characterized by a deficiency of beta-hexosaminidase A and B and a normal S activity. Variant AB is characterized by normal hexosaminidase A, B, and S activities, but a deficiency in GM2 activator protein due to mutations in the GM2 activator gene.

Tay-Sachs disease has three forms; infantile, juvenile, and adult/chronic. Individuals with the infantile form are normal at birth. However, the infantile form is characterized by progressive motor weakness, a cherry-red spot in the macula of the eye, increased startle responses at about 3 and 6 months, and progressive muscular atrophy, resulting in hypotonia and death within the first few years of life. The juvenile and adult forms are characterized by increased variant hexosaminidase A activity (Leinekugel et al. 1992) with very heterogeneous symptoms.

Sandhoff disease is characterized by GA2 accumulation in the brain and visceral organs as well as organomegaly and skeletal malformations, similar to the infantile form of Tay-Sachs disease. The juvenile form is also characterized by dementia and cerebellar ataxia with mental retardation.

Variant AB is characterized by GM2 and GA2 accumulation. It is similar to Tay-Sachs disease, but symptoms appear later (Kolter 2012).

As with sphingolipidosis, lysolipid (lysoGM2) increases, and can therefore, be used as a biomarker for Tay-Sachs and Sandhoff diseases (Kodama et al. 2011).

Analysis of Gangliosides

Chemical analysis was used to measure the ganglioside structure and levels, but today they can be measured in lipidomics in mass spectrometry (Farwanah and Kolter 2012). Mass spectrometry is the most widely used

method due to its sensitivity, accuracy, and high speed of analysis. Conventional methods involve a series of extraction and preparation steps (Merrill et al. 2005). Numerous methods can be used for purification (column chromatography and solid-phase extraction) (Muthing 2000). Gangliosides are extracted from tissue and body fluids using chloroform-methanol chemicals. Water in the extract solvent can help to improve the efficiency of extraction (Byrne et al. 1985).

Gangliosides can be classified according to their glycan level using thin-layer chromatography (TLC), HPLC, and mass spectrometry combined with other methods, facilitating the sorting of gangliosides by mass spectrometry (Sisu et al. 2011).

Various protocols have been developed for the mass spectrometry analysis of gangliosides. The ionization technique of biological material depends largely on electrospray ionization mass spectrometry (ESI-MS). However, matrix-assisted laser desorption/ionization (MALDI) is also used (Thomas Kolter 2012). ESI-MS technology can also be combined with liquid chromatography (LC/ESI-MS) (Spiro et al. 2020).

Functions of Gangliosides

Gangliosides perform numerous functions either by interacting with extracellular membrane-bound molecules (trans interactions) or by changing the properties of proteins in the same membrane (cis interactions) (Todeschini and Hakomori 2008). Trans interactions take place between the glycan part of gangliosides on the one side and lectins on the other side.

There are trans interactions between gangliosides and myelin-associated glycoprotein (MAG) in the nervous system. MAG recognizes NeuAcα2-3Galβ1-3GalNAc-termini (Schnaar 2010). It is necessary for miyelin stability and axon regeneration (Schnaar 2010). Cis interactions can be direct or indirect. Gangliosides can affect the activity of tyrosine kinase receptors in the plasma membrane. Thus, epidermal growth factor utilizing tyrosine kinase can also affect the functions of such molecules as insulin (Inokuchi and Kabayama 2007). GM3 binds to the extracellular domain of epidermal growth factor receptors and inhibits tyrosine kinase activity (Kim et al. 2020). GM3 inhibits EGFR activity in various cell cultures (Meuillet et al. 2000). It also inhibits insulin receptor signaling (Kim et al. 2020).

Cis and trans interactions of gangliosides are also crucial for immunity and infectious diseases (Hanada 2005). They can act as coreceptors for viruses, bacteria, and microbial toxins (Neu et al. 2011). The most obvious example is that GM1 acts as a receptor for cholera toxin (S'anchez and Holmgren 2011). Many pathogens use sialic acids in cell-surface glucoconjugates to gain access to the cell. Merkel cell polyomavirus, rotaviruses, and adenoviruses use GT1b (Erickson et al. 2009), sialic acid on GM1 (Haselhorst et al. 2009), and GD1a (Nilsson et al. 2011) as cell receptors, respectively. Due to the direct interactions between lipopolysaccharides and gangliosides, gram-negative bacteria use gangliosides to gain access to the cell (Day et al. 2015). The effects of gangliosides in the immune system (cell activation, signal transmission, cell interaction, etc.) vary from cell to cell. They are found in hematopoietic stem cells, mast cells, granulocytes (GM1), monocytes and macrophages (GM3), B lymphocytes (GM3), and T lymphocytes (GM1 and GM3) (Zhang et al. 2019). GM1 in T and B lymphocytes is particularly essential for the activation of these cells. Brain-derived gangliosides inhibit T cell proliferation (Chu and Sharom 1995) by binding to IL-2 receptors (Lu and Sharom 1995) or IL-4 and IL-5. Monocytes reduce the expression of MHC II antigens (Heitger and Ladisch 1996). NFkappaB inhibits the signal pathway (Caldwell et al. 2003). GM2 and GM3 inhibit NK cell activity (Grayson and Ladisch 1992).

Gangliosides are also involved in cell recognition, adhesion, and signal transmission on the cell surface (Yu et al. 2011). As stated earlier, gangliosides are found in the nuclear membrane as well as the plasma membrane and play a vital role in both cellular and nuclear calcium transport (Leeden and Wu 2008).

Gangliosides reduce the deposition of lipid peroxidation products in rat myocardiocytes (Maulik et al. 1993) and brain cells and increase the removal of free radicals (Avrova et al. 2002). Exogenous gangliosides affect cell functions and protect the cell against oxidative stress (Gong et al. 2018). The antioxidant effects of gangliosides can protect the sperm, oocyte, and embryo from reactive oxygen products (Kim et al. 2020). GT1b shows antioxidant effects by scavenging free radicals, while GM3 induces apoptosis in the early embryo period (Kim et al. 2020). Exogenous gangliosides promote oocyte maturity and pre-implantation embryonic development (Kim et al. 2020).

The biological importance and functions of gangliosides are observed in laboratory animals with ganglioside synthesis defects (Table 2.1). The selective degeneration of the organ of Corti results in hearing loss in ST-I knockout mice (Yoshikawa et al. 2009). Recent research has also reported attention-deficit hyperactivity disorder in ST-I knockout mice, suggesting that glycosphingolipids are involved in maintaining neuropsychological balance (Niimi et al. 2011).

Deficiency in -b and -c series gangliosides and impairment in the regeneration capacity of the damaged hypoglossal nerves together with intact neural tissue are observed in ST-II knockout mice (Okada et al. 2002).

Deficiency in the GalNacT gene results in the reduced GM1, GD1a, GD1b, and GT1b content in the brain. With time, animals exhibit marked impairment in motor coordination, axonal degeneration in sciatic nerves, and demyelination of optic nerves (Sugiura et al. 2005).

GalNacT and STII knockout mice exhibit GM3 ganglioside synthesis mainly in non-cerebral tissues, resulting in weight loss, progressive motor and sensory neuropathy, and impaired learning and memory over time (Tajima et al. 2009).

Ganglioside-deficient mice exhibit marked vacuolation in the cerebellum and white matter as well as cell apoptosis and axonal degeneration (Yamashita et al. 2005).

Gangliosides and Diseases

Gangliosides take place in the pathogenesis of some immune-mediated neurological diseases (e.g., Guillain-Barré syndrome). The pathophysiology of neurological symptoms in Guillain-Barré syndrome presents lipooligosaccharides in the cell membrane of Campylobacter jejuni, similar to gangliosides in nerves. Anti-ganglioside antibodies resulting from molecular similarity are the cause of neurological symptoms (acute polyradiculoneuropathy and acute quadriplegia) in Guillain-Barré syndrome (Kaida et al. 2009). GM3 is involved in the pathogenesis of insulin resistance and type 2 diabetes (Duncan et al. 2018). GM3 is synthesized by GM3 synthase. Diabetic mice exhibit high levels of GM3 and GM3 synthase in the kidneys, liver, fat, and muscle tissue (Tagami et al. 2002). Diabetic patients with microvascular complications also have high serum levels of gangliosides. GM3 synthase suppression in diabetic

rats results in an increase in insulin sensitivity and an improvement in hepatic steatosis (Dam and Paller 2018).

Although the pathogenesis of Alzheimer's disease is far from clear, lipid metabolism is believed to be involved in its pathology (Grimm et al. 2006). Aging and neurodegeneration change the physicochemical properties of membranes (Kalanj-Bognar 2006). For example, changes in the lipid content and distribution of membranes may contribute to the pathogenesis of Alzheimer's disease. Numerous studies show that individuals with Alzheimer's disease have low levels of gangliosides and high levels of β-amyloid, which are responsible for the pathogenesis of the disease (Mutoh et al. 2006). Research also shows that GM1 causes the deposition of amyloid β-protein and even binds to it and forms GA β complexes, which are involved in the pathogenesis of the disease (Yanagisawa et al. 1995). The deficiency of gangliosides, especially GM1, may also be involved in the pathogenesis of Parkinson's disease (Forsayeth and Hadaczek 2018).

Various types of cancer are marked by the overexpression of gangliosides on the surface of cancer cells due to modifications in glycosylation, and hence, increased levels of gangliosides. Central nervous system tumors (astrocytoma, neuroblastoma, meningioma, melanoma, and sarcomas) are marked by increased levels of gangliosides (GD3 and GD2), and GD3 facilitates the invasive potential and metastatic potential of cancer cells (Groux-Degroote et al. 2017). In recent years, there has been a growing body of research on the potential use of antibodies against gangliosides in immunotherapy for cancer (Krengel and Bousquet 2014).

Conclusion

Research has focused on the molecular structure of gangliosides since they were first identified in brain tissue. Advances in technology have allowed us to shed light on the functions and biochemical structures of gangliosides and their effects on cells. However, further investigation is required into how gangliosides are found and in which cells, how they recognize cells and affect their signaling pathways, which diseases they can be used to treat, and how antibodies against them affect cells.

Keywords: *Ganglioside, sialic acid, gangliosidosis, KDN*

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CHAPTER THREE

CERAMIDES

YASEMİN SAVRANLAR

Introduction

Lipids have many functions in the body; for example, energy storage, regulating hormones, maintenance of body temperature, signaling, storage of lipid soluble vitamins, membrane lipid layer formation (Taniguchi and Okazaki 2020), prostaglandin formation and a role in inflammation. There are different types of lipids. Signaling lipids: terpens and terpenoids, steroids, prostoglandins, fat soluble vitamins; Energy storage: triacylglycerols, free fatty acids and saponification; Structural lipids contain phospholipids, glycerophospholipids, sphingolipids and waxes.

Phospholipids are examined in two groups, phosphoglycerides (glycerophospholipids) and phosphosphingosides (sphingomyelins), according to the type of alcohol in their molecular structure (Figures 3.1, 3.2, 3.3). Membranes are made of phospholipids. Ceramides are the main structural and source elements of sphingolipids (Paciotti et al. 2020) and glycolipids (glycosphingolipids). The simplest sphingolipid is ceramide (Figure 3.4). Ceramide is formed by attaching a fatty acid to the amino group of sphingosine. A fatty acid binds with the second carbon of sphingosine's amino group. A ceramide is separated from another ceramide by the fatty acid it carries.

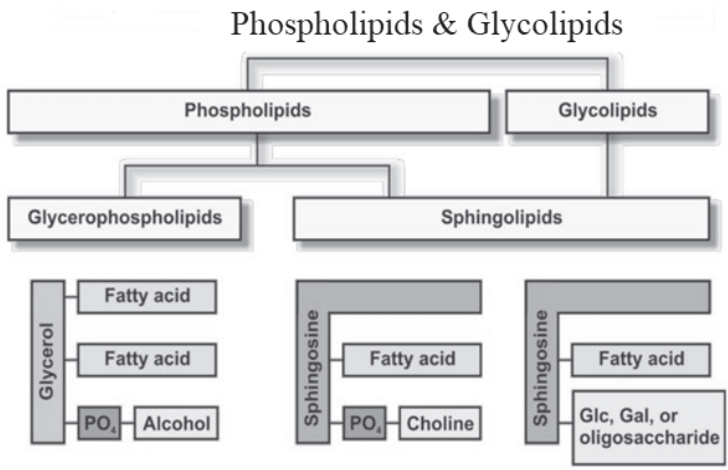

Figure 3.1: Structure of phospholipids and glycolipids (Source: https://www.slideserve.com/chesmu/phospholipids-2020)

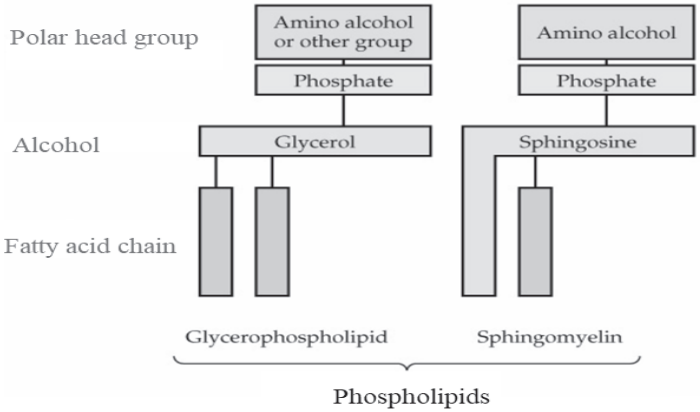

Figure 3.2: Structure of phospholipids (Source: https://docplayer.biz.tr/1985821-L-i-p-i-d-l-e-r-prof-dr-arif-altintas-altintas-veterinary-ankara-edu-tr.html-2020)

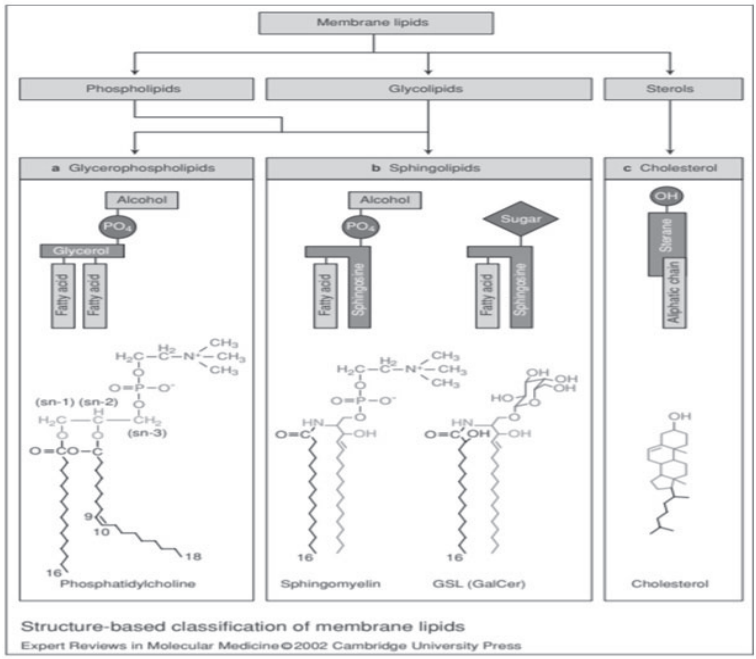

Figure 3.3: Lipids of the membrane. The plasma membrane contains sterols, glycolipids and phospholipids. These membrane lipids can be classified as sphingolipids, cholesterol and glycerophospholipids. The structure of syphingosine in membrane lipids combines with a fatty acid to form ceramide (Fantini et al. 2002).

Sphingolipids have a sphingosine or sphingoid backbone in contrast to the glycerol backbone of glycerophospholipids. They also have polar head groups and non-polar, long chain fatty acid tails. Many sphingolipids are also phospholipids as they contain a phosphodiester bond. However, other sphingolipids contain glycosidic linkages to sugars; if any lipid binds to a sugar it can be called a glycolipid. Sphingolipids are divided into four main subclasses according to their head group:

1. Sphingomyelins are the main class of sphingolipids, which are also phospholipids (sphingophospholipids). These molecules contain phosphatidylethanolamine or phosphatidylcholine as the head group and therefore contain a phosphodiester bond. The head groups of sphingomyelin have no net charge. Syphingomyelins are

the main components in plasma membranes of cells that provide myelination, such as Schwann cells and oligodendrocytes.

2. The second group is composed of the most complex sphingolipids. Gangliosides have one or more N-acetylneuraminic acid molecules (sialic acid) at the terminal (Figure 3.4). Gangliosides are glycolipids with polar head groups composed of negatively charged oligosaccharides. They play role in signal transduction, recognition, cell cell interaction.
3. Glycosphingolipids are sphingolipids and glycolipids with sugar head groups. These are not phospholipids since they do not contain phosphodiester bonds.
4. Glycosphingolipids are especially found on the outer surface of the cell membrane. Cerebrosides or globosides are glycosphingolipids (Figure 3.3). Globosides have two or more sugars, cerebrosides have a single sugar (Figures 3.4, 3.5). They also called neutral glycolipids because they don't have net charges (MCAT Biochemistry review 2019-2020).

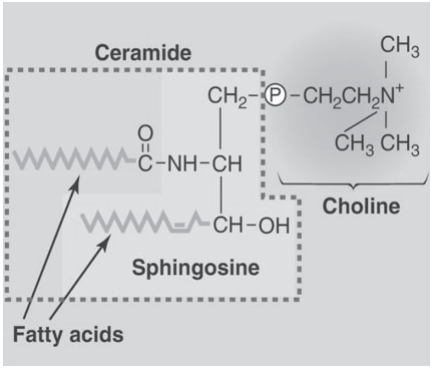

Figure 3.4: The structure of sphingomyelin, sphingosine and ceramide components (Lippincott Illustrated Reviews: Biochemistry 2017).

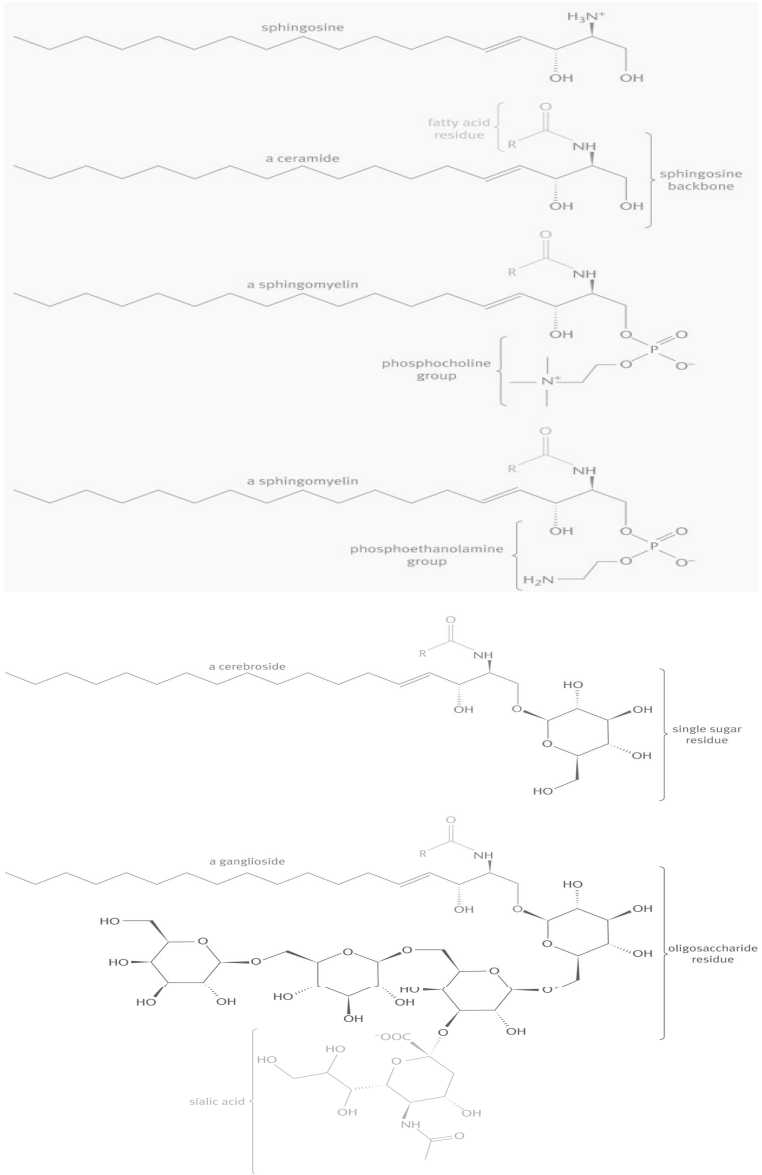

Figure 3.5: Types of sphingolipids. Sphingomyelins have phosphodiester bonds (phospholipids). Cerebrosides have a sugar. Gangliosides have oligosaccharides and terminal sialic acids (MCAT Biochemistry review 2019-2020)

Production and metabolism of Ceramide

Ceramide is N-acylated sphingosine with naturally occurring acyl chain lengths from 14 to 26 carbons. Ceramide is produced in three ways (Simon et al. 2019). The first is by de novo synthesis, which consists of several steps, starting with the merger of palmitoyl CoA and serine. The second is by the salvage pathway that recycles cellular sphingosine. The third is by hydrolysis from complex sphingolipids such as sphingomyelin and cerebrosides (galactosylceramide and glucosylceramide) (Simon et al. 2019). Ceramide diacylation by ceramidases generates sphingosine. The substrate of sphingosinekinases (Xia et al. 2020). Sphingosine1-phosphate (S1P) is a pleiotropic signal lipid that often opposes apoptosis and promotes angiogenesis and cell migration, justifying the inhibition of S1P formation.

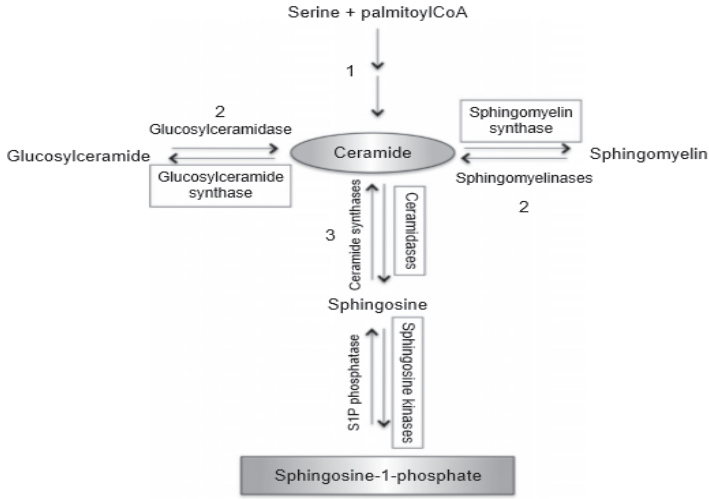

Figure 3.6: Ceramide is generated in three ways; (1) De novo biosynthesis, with palmitoyl CoA and serine; (2) Metabolism of complex sphingolipids (sphingomyelin and glucosylceramide, etc.); and (3) The salvage pathway (Gault et al. 2010; Amraoui et al. 2020).

Metabolism of sphingolipid explains the basic links of major sphingolipids in biosynthetic pathways and degradative pathways (Figures 3.6, 3.7). However, the reality is much more complex. For instance, predictions suggest that there are >28 different enzymes that use ceramide as a product or substrate. Therefore, ceramide is a "center" in the

metabolism of sphingolipid (Paciotti 2020). It serves as the precursor of sphingomyelin, ceramide phosphoethanolamine, ceramide phosphate and the overall glycosphingolipids. In addition, ceramide is the precursor to sphingosine in the degradation pathway, which is also the precursor to S1P (Hannun and Obeid 2011).

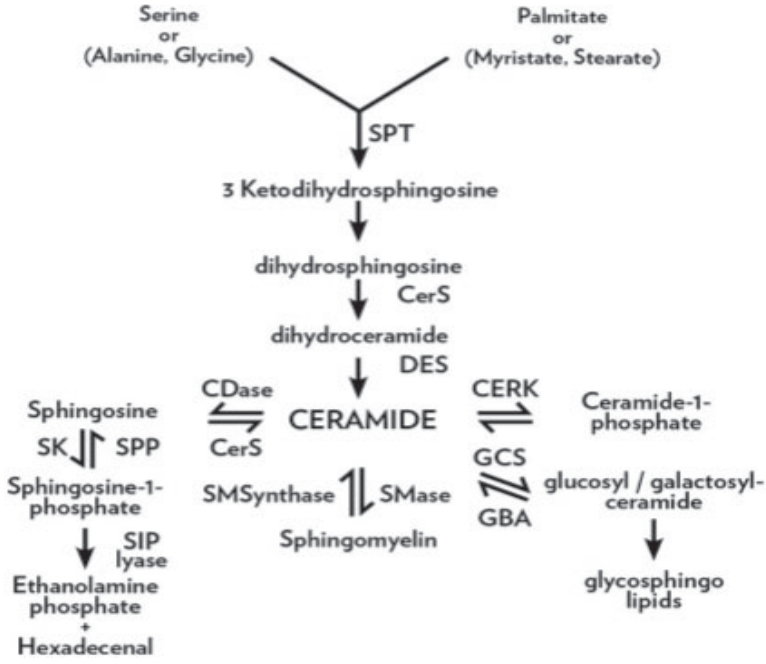

Figure 3.7: Metabolism of sphingolipid. GCS: glucosylceramide synthase; SPP: Sphingosine 1 Phosphatase; CERK: ceramide kinase; GBA: acid glucocerebrosidase; SMS: sphingomyelin synthase; SK: sphingosine kinase (Hannun and Obeid 2011; Park et al. 2020; Yura et al. 2020).

Ceramide is highly hydrophobic. It tends to remain in the membrane where it is produced unless it is moved. Ceramide metabolism is highly compartmentalized (Figure 3.8). Ceramide synthesized as de nova in the endoplasmic reticulum. Ceramide can even be produced by neutral glucocerebrosidase and sphingomyelinases in the plasma membrane. (Park et al. 2020). Enzymes in ceramide synthesis are also found in mitochondria and lysosome. this leads to the formation of ceramide specific to the area where it is located. Ceramide can be produced in the salvage path by a more complex mechanism. This rescue pathway

involves first cleavage of complex sphingolipids and sphingomyelin to ceramide and then to sphingosine in the endolysosomal system. The sphingosine which released can be recycled or recovered and then acylated to ceramide (Yura et al. 2020). It has also been reported that ceramide can be produced by the adverse effect of ceramides (Hannun and Obeid 2011).

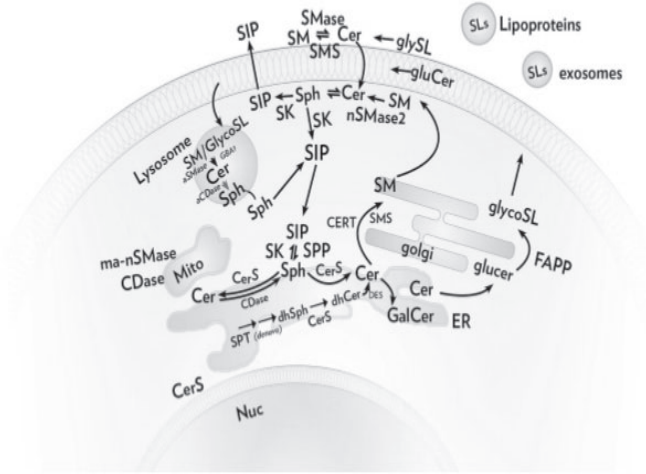

Figure 3.8: Sphingolipid metabolism in the cell. SMS: sphingomyelin synthase, ma-nSMase: mitochondrial associated SMase, glySL: glycosphingolipids, dhSph: dihydrosphingosine, aSMase: acid SMase, SK: sphingosine kinase, SLs: sphingolipids, dhCer: dihydroceramide, aCDase: acid ceramidase, CDase: ceramidase, Sph: sphingosine, Mito: mitochondria, Nuc: nucleus (Hannun and Obeid 2011)

Ceramides in the cell structure

Formation of the water barrier with keratinocytes:

The keratinocyte is the primary cell type of the epidermis. These cells are produced from the stratum basale layer of the epidermis. Cells separated from the basal layer have two functions:

1. They produce keratins, which are the basic structural proteins of the epidermis.
2. They participate in the formation of the epidermal water barrier (Figure 3.9).

Lamellar bodies allow the formation of the water barrier of the epidermis (Groen et al. 2011). The epithermal water barrier is required for the epithelium in mammals and is responsible for maintaining body homeostasis. The barrier consists mainly of two structures in end-differentiated keratinocytes: (1) lipid layer attached to the out of the plasma membrane, and (2) insoluble proteins accumulate inside the plasma membrane. When the keratinocytes in the stratum spinosum layer of the skin begin to produce keratohyalin granules, they also produce lamellar bodies.

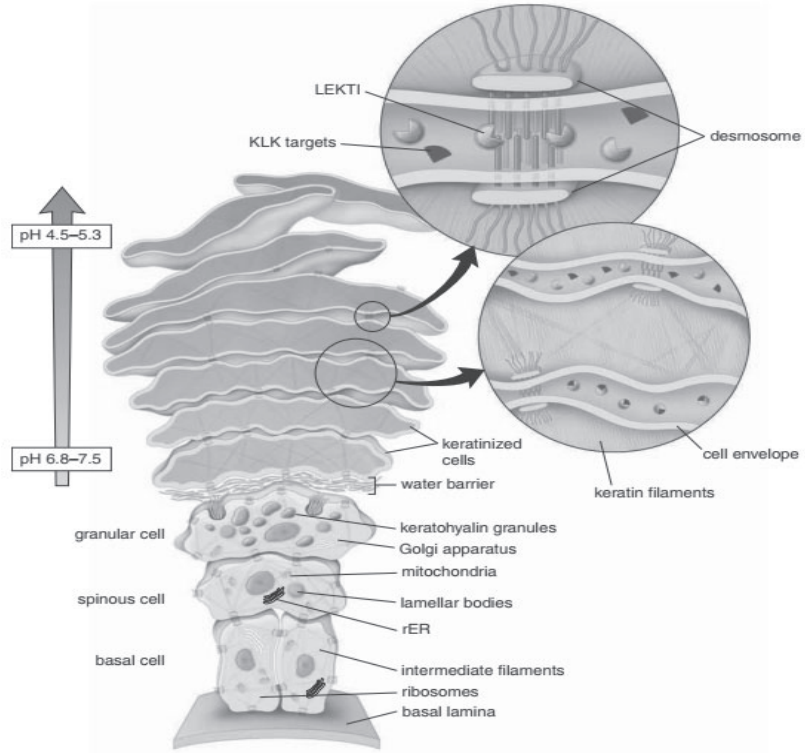

Figure 3.9: Keratinocytes in the epidermis. Keratinocytes project different stages in the life cycle of the cell, where cells pass from the basal sheet to the skin surface and desquamate from there. If we emphasize the lamellar layer in which ceramide plays a role, in the granular layer, a cell releases lamellar bodies outside the cell to form the water barrier of the epidermis (Histology: A Text and Atlas: with Correlated Cell and Molecular Biology 2016).

Structurally, lamellar bodies are tubular or oval membrane-bound organelles specific to the mammalian epidermis. In cells in the spinous and granular layer, lipids and enzymes such as ceramides, phospholipids, glycosphingolipids, secretory phospholipase A2 and acidic sphingomyelinase form the lamellar body in the Golgi apparatus (Borodzicz 2016). (Figure 3.10). In addition, lamellar bodies contain protease enzymes such as glycosidases, protease inhibitors, chymotriptic enzyme, cathepsin D, and acid phosphatase. The contents of the granules are excreted by exocytosis in the intercellular space among the stratum corneum and the stratum granulosum. The set-up of these lipid lamellae between cells is responsible for the forming of the epidermal water barrier (Figure 3.11). Lamellar bodies play roles in the desquamation of cornified cells, the formation of a cornified envelope and barrier homeostasis and the antimicrobial defense of the skin (Histology: a text and atlas: with correlated cell and molecular biology 2016).

The epidermal water barrier is composed of two parts:

1. The cell envelope is a structure consisting of the accumulation of insoluble proteins, 15 nm thick, inside the cell membrane and provides strong mechanical support. The cell envelope thickness increases in the epithelium subjected to considerable mechanical stress (soles, lips, palms, etc.). The cell envelope is formed by cross-linking larger structural proteins and proline-rich proteins. The structural proteins are envoplakin, loricrin, elafin, filaggrin, cystatin, desmosomal proteins (desmoplakin), keratin chains, and involucrin. Loricrin is the main structural protein and constitutes 80% of the cell envelope proteins. Loricrin, the insoluble protein, is the protein that has the highest amount of glycine known in the body.
2. The lipid envelope's thickness is 5 nm. The main lipid components of the lipid envelope are cholesterol, free fatty acids and ceramides, which belong to the sphingolipid class. Nevertheless, the most important component is the monomolecular acylglucosylceramide sheet that provides a coating like teflon on the cell surface. Ceramides also play an important role in cell communication and are not least for managing cell differentiation, the initiation of apoptosis, and the control of cell increase. So the cells move towards the free surface and the barrier is constantly supported by keratinocytes entering the terminal differentiation process (Histology: a text and atlas: with correlated cell and molecular biology 2016).

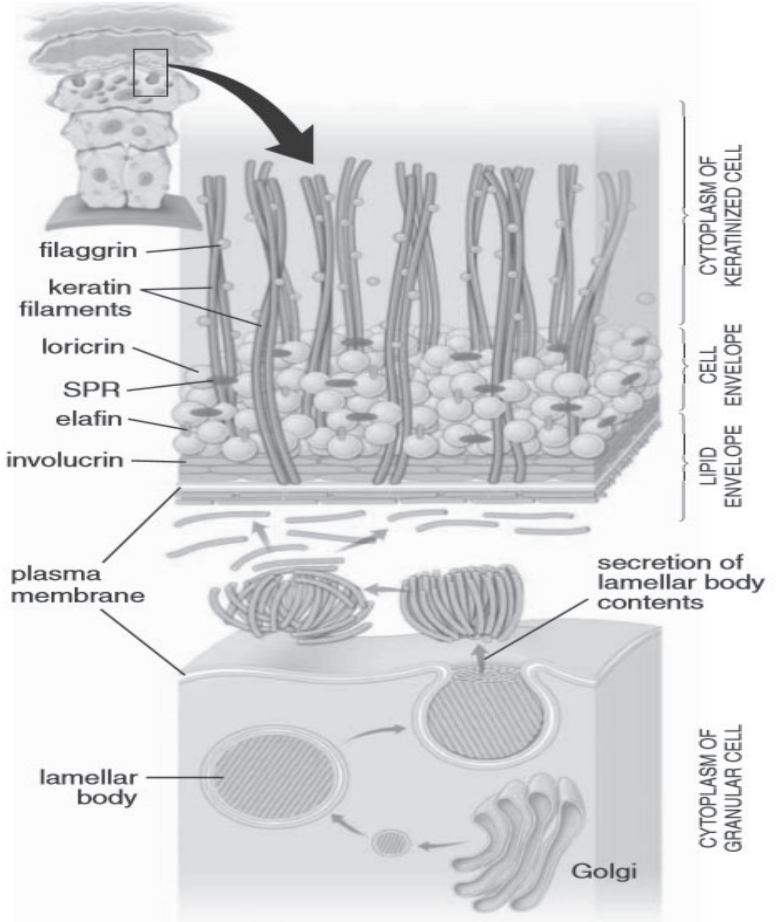

Figure 3.10: The epidermal water barrier. A mixture of ceramides, phospholipids and glycosphingolipids creates lamellae in lamellar bodies. Keratin filaments (tonofilaments) connected with filaggrin are attached to the cell envelope (Histology: A Text and Atlas: with Correlated Cell and Molecular Biology 2016).

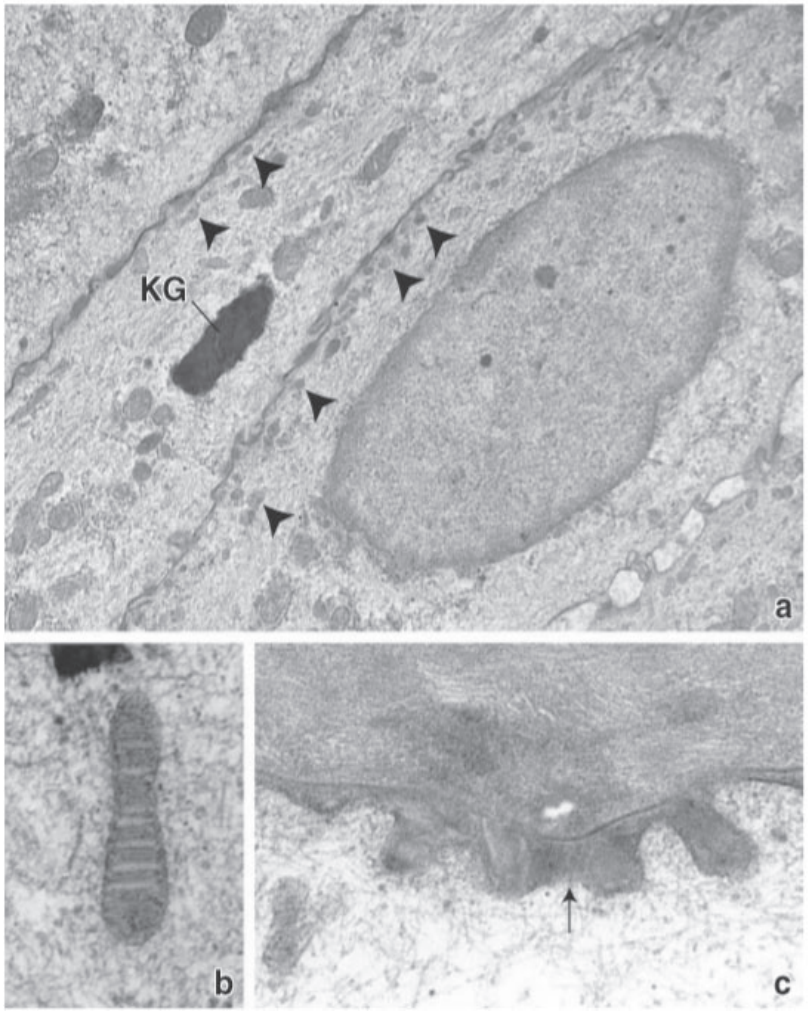

Figure 3.11: Keratinocyte-electron micrographs. a. A keratinocyte, most of the cytoplasm is filled with keratin flaments (tonofilaments). A keratohyalin granule (KG). Arrowheads point out lamellar bodies ×8,500. b. A lamellar body ×135,000. c. A keratinocyte under a keratinized cell. Lamellar bodies condense to form the lipid envelope between the cells ×90,000 (Histology: A Text and Atlas: with Correlated Cell and Molecular Biology 2016).

The plasma membrane

Lipids and proteins form the cell membrane. Phospholipid bilayer is the primary structure of the cell membrane (Taniguchi and Okazaki 2020). It creates a barrier between the extracellular and intracellular compartments. Proteins are embedded in the phospholipid bilayer and perform the functions of the plasma membrane, such as cell-cell recognition and the selective transport of various molecules.

Lipid rafts

A lipid raft is a region of the plasma membrane that has a wealth of cholesterol and sphingolipids (Handbook of Experimental Pharmacology 2013). Although there are few structural proteins in the lipid raft structure, other lipid raft structures are rich in certain proteins that regulate the function and content of this structure (Ketteler et al 2020).

Caveolin proteins are found in the lipid raft structure and are involved in the transport of vesicles and caveola structures (Figures 3.12, 3.13). The caveola structure is found in various cells such as epithelial cells, fat cells, endothelium, fibroblasts, type 1 alveolar cells, striated and smooth muscle cells (Histology and Cell Biology: An Introduction to Pathology 2016).

Caveolin 1, 2 and 3 from the caveolin protein family regulate the lipid raft structure and function (Ketteler 2020). Fotillins, glycosphingolipid-linked proteins, and Src tyrosine kinases are also regulative for the structure and function of lipid rafts.

Lipid rafts can participate in cell signaling by separating or concentrating specific membrane-associated proteins in geniune lipid areas.

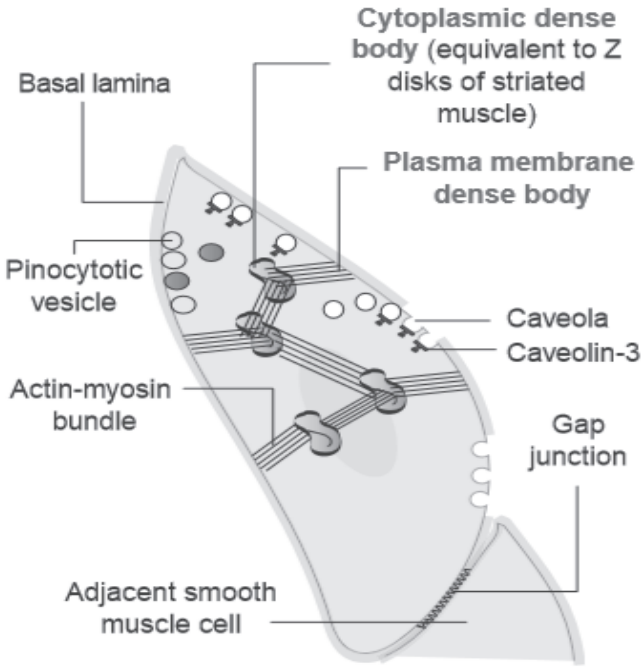

Figure 3.12: A smooth muscle cell (Histology and Cell Biology an Introduction to Pathology 2016)

Characteristics of smooth muscle

Smooth muscle is found in the ciliary body and iris (in eye), the wall of intestines and blood vessels, walls of tubular organs, and the arrector pili muscles in skin, among other sites etc. It consists of fusiform individual cells or fibers with a central nucleus. Smooth cells in the walls of large blood vessels produce elastin. Caveolae, depressions of the plasma membrane, are permanent structures involved in fluid and electrolyte transport (pinocytosis) (Ketteler et al. 2020). The caveolin-3 protein encoded by the Caveolin gene family is also associated with lipid rafts.

The caveolin-3 protein in the lipid raft forms a complex by binding with cholesterol and forms the caveola structure by invagination. The caveola structure separates from the plasma membrane to form pinocytic vesicles (Figure 3.12).

Invaginations of the plasma membrane, called caveolae, act as a primitive T tubule system, transmitting depolarization signals to the underdeveloped sarcoplasmic reticulum. The development of caveolae from lipid rafts and their diverse roles in several tissues are shown in Figure 3.13 (Histology and Cell Biology: An Introduction to Pathology 2016).

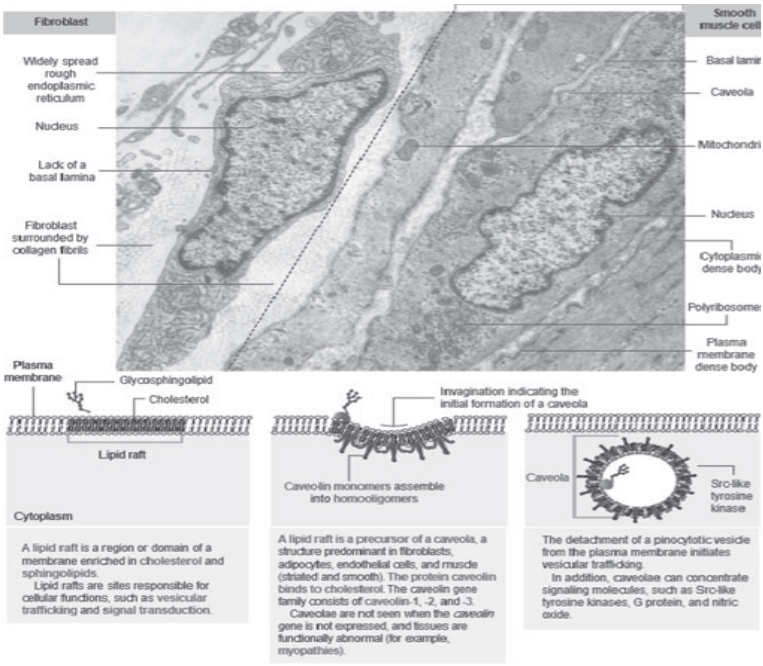

Figure 3.13: Formation of a caveola (Histology and Cell Biology: An Introduction to Pathology 2016)

The function of lipid rafts

Lipid rafts are involved in intracellular protein which can be bacterial toxins and signaling events and lipid movement. Lipid rafts also play a role in toxin/host-pathogen interactions (Figure 3.14) (Fantini 2002).

Lipid rafts also related to the formation of protein-associated diseases like Alzheimer's disease and prion diseases.

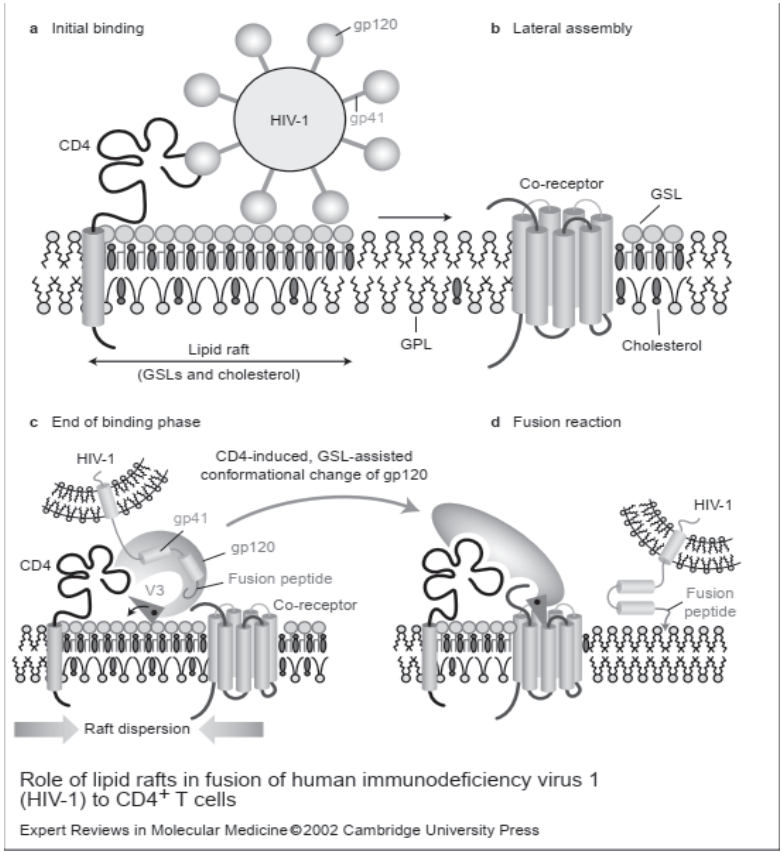

Figure 3.14: Human immunodeficiency virus 1 and $CD4^{+}$T cell fusion with lipid rafts. (a) Binding of $CD4^{+}$T cell and HIV-1 virus. There are micro-domains rich in glycosphingolipid and cholesterol on the surface of the CD4 cell (lipid rafts). HIV-1 surface glycoprotein gp120 binds to CD4. (b) Lateral assembly of the fusion complex of HIV-1. (c) End of the binding phase. The raft disperses and allows adjacent contact between the CD4–gp120 and the co-receptor complex. (d) The beginning of the fusion reaction (Fantini 2002)

Ceramides in the differentiation of keratinocytes

Keratinocytes in the stratum spinosum layer have an ovoid nucleus and a polygonal shape. Inside the cytoplasm there are small granules with a lamellar center called the membrane-covered granules or lamellar body.

Keratin intermediate filaments extend towards the spiny protrusions of cytoplasm and attach to the dense plate of the desmosome. The lamellar body first begins to appear in the stratum spinosum layer, and its amount increases in the stratum granulosum. The glycolipid and acylglucosylceramide which are products of lamellar bodies, are released into the intercellular space (Figure 3.15) (Histology and Cell Biology: An Introduction to Pathology 2016).

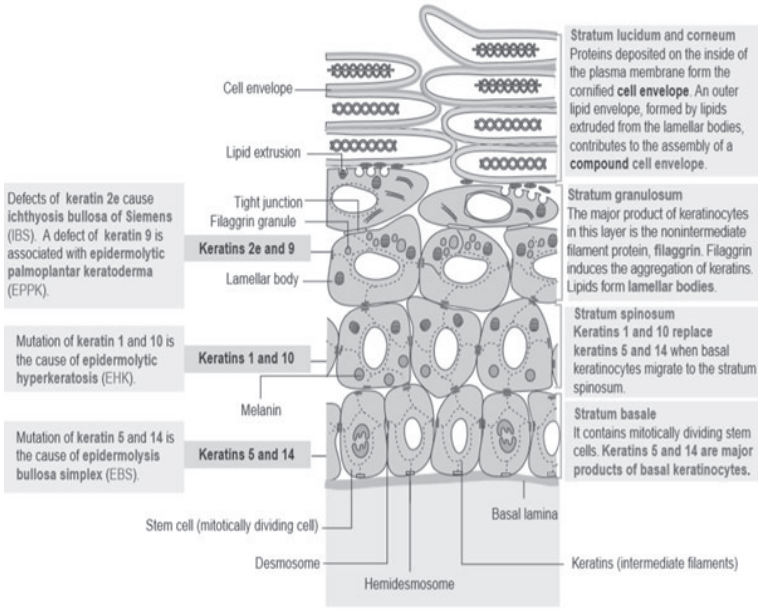

Figure 3.15: Differentiation of keratinocytes: Expression of keratins (Histology and Cell Biology: An Introduction to Pathology 2016)

In the space between cells, the lamellar lipid material makes a multilayer construction arranged in large layers that cover the surface of the keratinocytes of the superior layer in the stratum lucidum. The glycolipid layer forms the water barrier of the epidermis. The stratum lucidum is located as an intermediate layer between the stratum granulosum and the stratum corneum.

The stratum corneum and stratum lucidum layers consist of several layers of seedless keratinocytes. In the cytoplasm of these keratinocytes there are clusters of intermediate filaments of keratin-flaggrin (Figure

3.16). The keratin-flaggrin complex accumulates inside the plasma membrane to form a structure called the cornified cell envelope (Figure 3.16).

In addition, involucrin, small prolin rich protein, trichohyalin and loricrin are cross-linked by transglutaminase 1, 3 and 5, strengthening the plasma membrane near the desmosome. There is an insoluble lipid layer consisting of ceramide, fatty acid and cholesterol outside the cell. This lipid structure is delivered extracellularly by the lamellar body and is cross-linked to the cell envelope proteins forming the cornified cell envelope (Histology and Cell Biology: An Introduction to Pathology 2016).

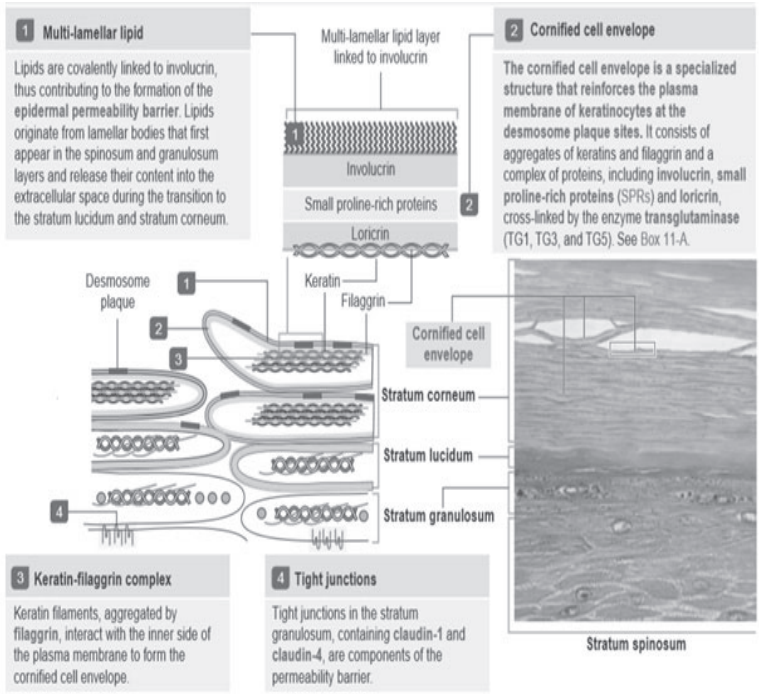

Figure 3.16: Components of the epidermal permeability barrier (Histology and Cell Biology: An Introduction to Pathology 2016)

Ceramides in apoptosis

Ceramide is the central molecule in sphingolipid metabolic pathways. Its generation and metabolism are key in understanding the advantageous and dysregulated sphingolipid responses to cancer therapy. Ceramide is best characterized as promoting apoptosis and cell senescence (Khiste et al. 2020). Among the major effectors of ceramide signaling are protein phosphatases PP2A and PP1, which are activated by ceramide. Through the activation of PP2A, ceramide promotes numerous signaling alterations including deactivation of Akt, PKC, and c-Jun; destabilization of c-Myc; and disruption of the Bax/Bcl-2 interaction. PP1 activation provokes dephosphorylation of SR proteins with a subsequent alternative subjoining of Bcl-X and Caspase 9 and the activation of retinoblastoma. Ceramide has a role in downstream PP2A and PP1 activation. Ceramide formed in the lysosome with acid sphingomyelinase has also been shown to directly bind and induce the autoproteolytic cleavage of cathepsin D, supporting the cleavage-induced activation of proapoptotic Bid (Beckham 2013). These functions of ceramide combine to cause cell cycle arrest, aging, apoptosis and cell death (Figures 3.17, 3.18) (Bhat et al. 2020). While the vast majority of the literature supports these anticancer effects of ceramide, it is worthwhile to acknowledge that antiapoptotic roles have been described for some specific ceramide species, highlighting the complexities of ceramide signaling that remain to be fully characterized (Beckham 2013).

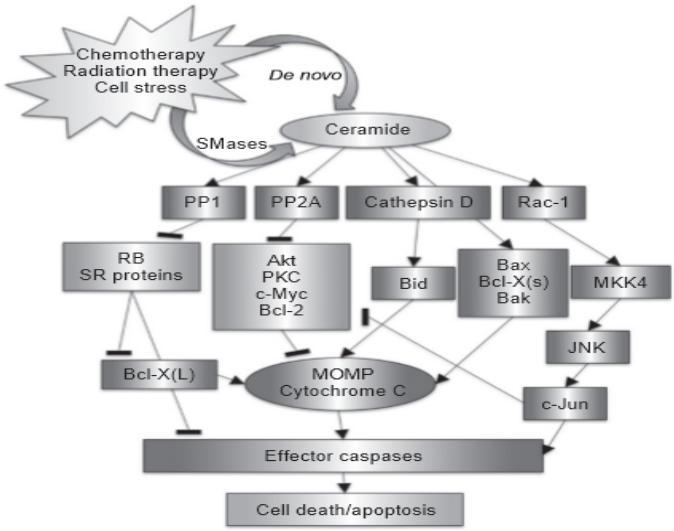

Figure 3.17: These downstream processes can lead to changes in apoptosis, senescence and growth arrest (Beckham 2013).

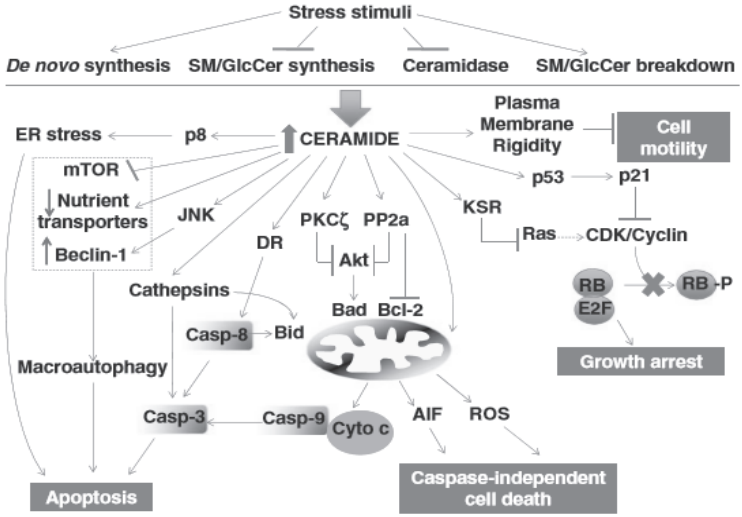

Figure 3.18: Signal cascades in apoptosis, growth arrest, cell motility and cell death regulated by ceramide (Bioactive Sphingolipids in Cancer Biology and Therapy 2015)

Conclusion

Ceramides are the main structural materials of sphingolipids which have important roles in cells. These roles are plasma membrane and organelle membrane formation, cell signaling, apoptosis, keratinocite differentiation, growth arrest, cell motility and cell death, formation of the epidermal water barrier, etc. Because ceramide is found in all cells, understanding the structure and function of ceramide will help us to maintain health and to struggle against diseases; Alzheimer's disease, prion disease, cancers, HIV, etc.

Keywords: *Ceramides, sphingosine, lamellar body, keratinocyte*

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CHAPTER FOUR

GLYCOLIPIDS AND PROTEIN COMPOUNDS

HATİCE SARAÇOĞLU

Introduction

Proteins that bind to glycolipids with high specificity may be divided into two groups. The first group includes activator proteins that play a role in the catabolism of glycolipids by lysosomal acid hydrolases, and the second group includes glycolipid transfer protein (GLTP). Activator proteins in the first group are glycoproteins localized in lysosomes, having a critical role in glycolipid binding and facilitating glycolipid transfer functions. GLTP facilitates the transport of different glyceroglycolipids and glycosphingolipids between membranes (Sasaki 1985).

Sphingolipid Activator Proteins

The catabolism of sphingolipids is carried out in digestive vacuoles called lysosomes by the effect of acid exohydrolases, starting at the hydrophilic tip of the molecule. For almost every degradation step, a hereditary enzyme deficiency causing sphingolipid storage diseases is known. Lysosomal hydrolases responsible for glycolipid catabolism have been purified and identified. Some of these are membrane-bound, while others are in soluble form. When the destruction of glycosphingolipids by soluble enzymes is examined in vitro, it has been observed that degradation rates are negligible. Because sphingolipids are amphiphilic molecules, they are distributed in water in the form of liposomes or micelles. Purified hydrolases may hardly affect these tightly packaged forms unless appropriate detergents such as bile salts are added. Detergents in appropriate concentrations form small micelles that may destroy oligosaccharide chains by hydrolases. However, the interaction between lysosomal hydrolases and glycolipid substrates occurs in another way, as lysosomes do not contain detergents in the in-vivo environment. Several non-enzymatic activator proteins have

been identified that occur with this function and accelerate the enzymatic catabolism of glycosphingolipids (Fürst et al. 1985).

Sphingolipid activator proteins (saposin, SAP), which are non-enzymatic small glycoprotein structures, are essential molecules for the breakdown of sphingolipids and membrane digestion. They bind hydrolases, which catabolize sphingolipids, and interact with intralisosomal membrane structures to make lipids accessible to their enzymes. As a result, saposins fill the physicochemical gap between lipids and hydrolases, and defects in their function may cause lipid accumulation in lysosomes. SAPs contain five molecules: SAP1, 2, 3, 4, and GM2AP (GM2 activator protein). Four of these (SAP1-4) produced by proteolysis from only one precursor protein named prosaposin (pSAP) are considerable homologous proteins. GM2AP, which plays a role in the destruction of GM1 and GM2 gangliosides, is inherited by a different gene and is unlike other SAP proteins (Schuette et al. 2001).

After the glycosphingolipids and gangliosides in the extracellular part of the cellular membrane are transported to the lysosome by endocytosis or phagocytosis, terminal sugar residues are removed by soluble lysosomal hydrolases before the ceramide backbone breaks down into free fatty acid and sphingosine. This process occurs at the water-lipid interface, as soluble enzymes move on membrane-bound substrates. Glycosphingolipids containing long carbohydrate residues, hydrolases are simply approachable because the terminal sugar is sufficiently far from the lipid bilayer. Conversely, activator proteins are required for the destruction of glycosphingolipids containing short carbohydrate residues (Schuetteet et al. 2001).

The Structure of Sphingolipid Activator Proteins

SAP1-4 produced by the proteolysis of pSAP are small proteins of nearly 80 amino acids. They contain six extremely preserved cysteines and an N-glycosylation site. The disulfide bonds found in SAP2, 3, and 4 are the same in all three. An intertwined annular structure is formed by disulfide bonds between the 1st and 6th, 2nd and 5th and 3rd and 4th cysteines in the sequence. These bonds, which probably provide high stability against acid, heat, and proteolytic enzymes, are also essential for functionality (Schuetteet et al. 2001).

Showing some structural differences, GM2AP is the fifth member of this group. This protein with a molecular weight of roughly 20 kDa, is the

largest SAP protein. It contains eight cysteines forming four disulfide bonds and one N-glycosylation site. Although similar to other saposins in that the first and last cysteine of the amino acid sequence form a disulfide linkage, the secondary structure of GM2AP is different. It contains a large amount of β-sheet and α-helix. There is an exclusive hydrophobic container that forms a large gap for ceramide and a possible recognition domain for the lipid-dependent carbohydrate chain next to this domain (Schuette et al. 2001).

The Functions of Sphingolipid Activator Proteins

It is known that pSAP may promote neurite growth and prevent the programmed cell death of neurons in vitro. It has been shown to prevent neurons from being influenced by ischemia and other damages in vivo and to have neurotrophic/neuroprotectant properties (Schuetteet al. 2001).

Specific defects in SAP2, 3, and GM2AP are known to result in disease. Genetic defects in SAP2 cause the metachromatic leukodystrophy variant form that begins in late infancy or childhood. The biological function of SAP3, which in its defect leads to juvenile variant Gaucher disease, is essentially the degradation of glucosylceramide. Heavy glucosylceramide accumulation has been detected in the liver of these patients. GM2AP deficiency leads to the GM2 gangliosidosis Aβ variant characterized by GM2 ganglioside and GA2 glycolipid storage (Schuette et al. 2001).

There are very few reported cases of SAP1 deficiency. SAP1 is a galactocerebrosidase activator and its deficiency causes a clinical picture similar to the early infantile type Krabbe disease caused by galactocerebrosidase deficiency (Calderwood et al. 2020).

Experimental studies show that SAP4 may be the activator protein of lysosomal acid ceramidase, and there are no reported cases of SAP4 deficiency so far. However, progressive damage of the Purkinje cells in the cerebellum and ataxia as well as progressive polyuria, renal tubular degeneration and hydronephrosis have been observed in SAP4 mutant mouse models (Matsuda et al. 2004).

A mutation in the pSAP gene that results in a total defect of SAP1-4 has been reported. In addition to the lipid storage in SAP2 and 3 deficiency, ceramide levels have increased in fibroblasts and various tissues in the patients (Schuette et al. 2001).

Although the amino acid sequences are very homologous and appear to have similar structures, experimental evidence suggests that the action mechanisms of the four SAPs are different. Soluble 1:1 complexes of SAP2 and sulfatide behave like a physiological detergent. However, no direct interaction of SAP2 with disruptive enzymes has been shown (Schuette et al. 2001).

The GM2AP and GM2 gangliosides have also been observed to form a soluble 1:1 complex. Its specificity to lipids is higher than that of SAP2, but besides GM2 it also attributes to other negatively charged lipids and encourages the destruction of GA2, GM1, and SM2. While hexosaminidase A and hexosaminidase B may degrade GM2 ganglioside when appropriate detergents are present in the environment, hexosaminidase A may degrade GM2 ganglioside in the presence of GM2AP without detergent. This suggests a possible specific interaction between the two proteins (Schuette et al. 2001).

The SAP3 effect mechanism is a protein-protein interaction. SAP3 does not form a complex with glycosylceramide, which is the storage compound it lacks, while it forms a 1:1 complex with the cleverer enzyme glucocerebrosidase and provides allosteric activation. It has also been shown that SAP3 acquires hydrophobic characteristics at acidic pH. In these situations, its affinity for membranes increases and promotes glucosylceramide degradation by facilitating the association of glucocerebrosidase with membranes (Schuette et al. 2001).

SAP1 has been shown to bind to GM1 and GM2 gangliosides, but little information has been provided about the mechanisms of function of SAP1 and SAP4. Also, at low pH, saposins are protonated and show disruptive effects on membranes as a result of increased affinity to anionic phospholipids. In liposomal leakage and fusion experiments, it has been shown that SAP3 and 4 strongly influence bilayer entirety, and SAP1 and SAP2 minimally (Schuette et al. 2001; Darmoise et al. 2010).

Glycolipid Transfer Protein

GLTP is a protein with the cytosolic placement that in vitro transfers glycolipids between two membranes, and the molecular weight is 24 kDa (Mattjus 2009). The first protein found to selectively transport glycosphingolipids between membranes has been identified in the spleen and called cerebroside transfer protein. Shortly thereafter, protein-

mediated glycolipid transfer activities were also identified in different tissues, including bovine and porcine brains (Brown and Mattjus 2007).

GLTP accelerates the transport of glycolipids to the ceramide or glycerolipid backbone only in the presence of β-linked sugar residue. Anionic and neutral glycolipids are transferred by GLTP, while sphingomyelin and phospholipids are not (Mattjus 2009).

GLTP is involved in the nonvesicular transport of glucosylceramide from the Golgi to the cellular membrane, a key intermediate substrate in the synthesis of advanced glycosphingolipids and cellular drug resistance (Zou et al. 2008). It assists the transport of different glycosphingolipids to liposomes. There is a reverse relationship between the length of the sugar chain of glycosphingolipids and their transfer rate. Glycosphingolipids are transferred at lower rates if they contain sialic acid or sulfate residue (Brown and Mattjus 2007).

GLTP also facilitates the transport of glucosyldiacylglycerol, galactosyldiacylglycerol and digalactosyldiacylglycerol from liposomes to mitochondria or liposomes, but not dimannosyldiacylglycerol. It also facilitates the transfer of galactosylceramide and derivatives of lactosylceramide which are oxidized by periodate and then reduced. Galactosylceramide derivatives are transferred lower than galactosylceramide, while lactosylceramide derivatives are transferred higher than lactosylceramide. GLTP does not facilitate the transfer of phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, and cholesterol. These results show that GLTP is specific for glycolipid transfer only (Sasaki 1985).

Although the biological functions of GLTP have not been clarified, recent studies have significantly improved knowledge of the important roles that GLTP plays in human cells.

Glycolipid transfer protein-containing domain 1 (GLTPD1), a gene predicted to be in the human genome, has been found to express a protein similar to GLTP, specifically transferring ceramide phosphate, rather than glycolipids. Therefore, the protein has been called ceramide phosphate transfer protein (CPTP). Depletion of CPTP triggers ceramide phosphate increase, stimulates arachidonic acid release with group IV cytosolic phospholipase A2α, and ultimately proinflammatory eicosanoids are synthesized. The data suggest that CPTP plays a crucial role in cellular homeostasis by protecting ceramide phosphate accumulation, and it is a

new regulator of proinflammatory eicosanoid production (Malinina et al. 2015).

Additionally, glycosphingolipids play significant roles in cell surface adhesion processes, neurodegeneration and cell death. Especially in colon cancer, onset and progression are heavily associated with the altered level of glycosphingolipids. GLTP seems to have a possible role in malignancies as it is the molecular carrier of glycosphingolipids to the cellular membrane (Samaha et al. 2019).

Conclusion

In addition to the important roles of glycolipids in the organism, SAPs and GLTP also play very important roles. Their pathologies result in diseases with high mortality and morbidity. These glycolipid-related proteins seem to have the potential to create new therapeutic targets in diseases as their functions become clear with further studies to be conducted in light of the data in the literature.

Keywords: *Sphingolipid activator protein, saposin, SAP, glycolipid transfer protein, GLTP*

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CHAPTER FIVE

GLYCOLIPIDS BEING VIEWED IN VIVO OR IN VITRO

FİLİZ YILMAZ

Introduction

Glycolipids, found mostly on nerve tissues, are the essential components of membranes. They are found on the cell surfaces of eukaryotic cells. While the lipophilic part is linked to the cell membrane, the carbohydrate part interacts with the outer environment. Cellular interactions figure in the formation of growth and development. The expression of glycolipids differs in terms of quantity and quality through different species, members of the same species, organs and even among the cells of an organ. They have an antigenic structure. Blood type antigens, various embryogenic antigens and tumor antigens are identified. They act as cell surface receptors for the diphtheria and cholera toxins and for some viruses.

Glycolipid metabolism disorders cause various fatal diseases. Any enzyme deficiency on any step during glycolipid synthesis or catabolism would cause an accumulation of glycolipid metabolites in organs and function loss. Therefore, the identification of glycolipid derivatives accumulating in organs, would provide the diagnosis, treatment and development for disease.

Until today, lots of different isolation and analysis methods have been used to understand the idiosyncrasy of glycolipids. With the methods described, the tissues of glycolipids have been purified, and different molecular types in their structure have been eluted and categorized through various analytic methods. The developed procedures can identify specific glycolipid derivatives; however, disorders may appear due to factors which cannot be kept out of the environment or the chemicals used during purification. The exposition of analytic data and structural

information decreases. The identification of different structural components is thus restrained. For this reason, the methods used for the identification of glycolipids need to be improved. In this section, we have compiled the methods used for analyzing glycolipids in the light of current essays found in the literature.

Chromatographic Methods

The chromatographic analysis method is one of the most preferred analysis methods used for the identification and determining of the quantity of components which make up the mixture. This method helps to resolve the materials purely, which is really difficult or even impossible to resolve when using other methods. Chromatographic methods are classified in five groups according to their effectuation style; paper chromatography, thin-layer chromatography (TYC), column chromatography (CC), gas chromatography (GC) and high performance liquid chromatography (HPLC).

Thin-Layer Chromatography (TLC)

TLC is used for qualitative and quantitative analyses. It is still one of the most preferred methods due to its easy usage when considered against other complex techniques, being cheap and providing important data (Khan 2020). For instance; the method used by Svennerholm to classify the ganglioside species is based on thin-layer chromatography. The letter G refers to ganglioside, the second letter refers to the sialylation degree (mono-, di and trisialic gangliosides) and the number refers to the migration line on the thin-layer chromatography (Svennerholm et al. 1964; Sántha et al. 2020).

In the literature, there are various examples of studies which analyze glycolipids through thin-layer chromatography. In 2019, thin-layer chromatography was preferred for evaluating the glycolipid profile during the pathogenisis research of the disease spinocerebellar ataxia type 2 (SCA-2). Thus, a decrease in GM1a of gangliosides in the cerebellums of SCA-2 patients was recorded and this contributed to the diagnosis of the disease profile (Sen et al. 2019). There are also some studies combining TLC analysis and immunostaining. The bands used in TLC analysis are visualized by immunostaining. In a study, three neutral glycosphingolipids in rodent brain tissue were examined, their location was evaluated through immunohistochemical staining and their quantity was evaluated through ELISA and immuno-TLC (Dasgupta et al. 2007). Serum is also an often-

preferred method for measuring glycolipid levels not only on tissue levels but also in body fluids. For instance, gangliosides were measured in the acid liquid of ovarian cancer patients by using the TLC method (Webb et al. 2012).

High Performance Liquid Chromatography (HPLC)

One of the chromatographic methods – high performance liquid chromatography (HPLC) – is a fast and cheap technique that provides the separation of complex compounds. Its main difference is that it separates through high pressure. The reduced size of HPLC columns and the formation of thin layers provide high performance while separating the complex compounds. Although HPLC does not provide an elaborate analysis of the idiosyncrasy of the compounds, it provides an understanding of what the compounds are (Sicard and Landgraf 2017).

In the literature, Sicard and Landgraf analyzed glucosylceramide and galactosylceramide – derivatives of glycolipid – with high performance thin-layer chromatography (HPTLC) (Sicard and Landgraf 2017). In another study, ganglioside derivative GM3 levels in diabetic rat muscle tissues were examined through HPTLC (Bozic et al. 2018). In order to show the lipid transfer in living cells, Backman et al. observed glycolipid metabolism in an in-vitro environment and measured the presence and level of glycolipids at the end of the experiment through immuno-HPTLC (Backman et al. 2019).

There are antibodies bound to gangliosides in the pathogenesis of some diseases. For instance; Campylobacteriosis jejunitis infection causes Guillain-Barré syndrome which is related to GM1 antibodies. Immuno-HPTLC (HPTLC-1) was admitted to be the gold standard so as to detect the antiglycolipid antibodies and confirm the autoreactivity results. Thanks to the identification of antiglycolipids, the diseases related to glycolipid metabolism will be diagnosed thoroughly (Lardone et al. 2019).

Mass Spectrometer Methods

Throughout the world, mass spectrometer methods have been frequently used due to their economy and speed compared to other trade practices. Mass spectrometry is commonly used for the quantitative assay of one or more compounds of complex organic (sometimes inorganic too) mixtures which are seen in petrol, the pharmaceutical industry and environmental researches.

Before mass spectrometer analysis, the resolution process is completed through chromatography or electrophoresis according to the features of the compound.

Liquid Chromatography-Mass Spectrometer (LC/MS)

Mass spectrometry is combined with liquid chromatography for the analysis of samples containing nonvolatile compounds. The material or the material mixture is replaced in the device after being dissolved by a proper solvent. The analysis is carried out as each material in the sample is resolved in liquid chromatography; again, each material's mass spectrum is measured, then they are taken to the mass spectrometer. There are computer-aided scanning libraries in most of the modern mass spectrophotometers. The spectrums loaded in computers, being compared to the sample's mass spectrum, can be used for diagnosis.

In the literature, in a research study on GM1 gangliosidosis disease, the accumulated GLB1 galactoside metabolites of brain and urine samples taken from gangliosidosis patients were analyzed by LC/MS (Lawrence et al. 2019). In another study, gangliosides and their roles in the human retina were examined using LC/MS. Moreover, ganglioside types in the retina and ocular structures were examined, and the brain and plasma in old people were compared to each other. While the retina has a multiple ganglioside profile, the brain has some ganglioside types (Sibille et al. 2016).

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Recent improvements and studies on the mass spectrometer have proved that complex lipids and lipid mixtures are essential for characterization (Akyar 2011; O'Brien et al. 2013). Tandem Mass Spectrometry (MS/SM), first of all, decomposes the biological mixtures chromatographically, then increases the specificity of the decomposed compounds via a two-stage MS analysis. During a single LC-MS/SM experiment, it is possible to measure more than one analyte. Especially during lipidomic analyses, hundreds of lipid types can be measured quantitatively (Murphy et al. 2011).

Fabry disease is a lysosomal storage disorder which causes glycosphingolipids to accumulate in body fluids and tissues. As the reagent of glycosphingolipids, globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3) are used for the diagnosis of the disease. However, for Fabry patients having residual enzyme activity,

these bio markers do not always increase. Because of this, Abaoui et al. started to research a new marker. GB3 isoforms were analyzed via the LC-MS/SM technique and thanks to this technique, GB3 derivatives which may be a marker, were defined (Abaoui et al. 2016). In another study, in a cerebrospinal fluid sample obtained at lower volume, more than one ganglioside was meant to be measured simultaneously. During the study, a new MS/SM technique for the GM1 and GM2 analyses was developed in the samples of cerebrospinal fluid taken from healthy individuals and patients with Tay-Sachs (Gu et al. 2008).

It is known that the GD2 (ganglioside) neural cristae based neuroblastoma which belongs to the glycolipid family is in the plasma membrane. Researchers studied whether GD2 can be used as a biomarker to make it possible for the patient to monitor the tumor burden or the response to the medical therapy. They developed HPLC-MS/SM which is a faster and more practical method instead of the often preferred TLC method, for the measurement of gangliosides. In the serums and plasmas of patients with the diagnosis of neuroblastoma GD2 measurements were carried out and their method was proven (Busch et al. 2018).

Electrospray Ionization-Mass Spectrometry (ESI/MS)

This method was first used in 1984 for the analysis of biomolecules such as proteins, polypeptides and oligonucleotides. It is a highly proper method to define the molecular ion peak for organic compounds and medicine molecules. ESI should be carried out at atmospheric pressure and ambient temperature. The advantage of this method is to define the molar mass of grand matter which can be easily split. ESI is a useful ionization technique especially for grand biological molecules which are hard to evaporate or ionize. The ESI method is used with liquid chromatography/mass spectrometry (LC/MS).

In the literature, the electrospray ionization/mass spectrometer technique was used besides liquid chromatography (LC-ESI/MS) so as to define the gangliosides in the rat brain tissue. It was emphasized that the ESI method is a basic, fast and effective method to measure the gangliosides in the tissues (Khoury et al. 2020). In the human body, renal cell carcinoma and the ganglioside measurements of the healthy tissue around it (ESI/MS) were compared to matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI-MS). Their data were compared and it was confirmed that the lipid measurements were similar to each other in both methods (Hayek et al. 2018).

Matrix-assisted Laser Desorption/Ionization/Mass Spectrometry Imaging (MALDI-MS)

The sample dissolves the beam from the short pulsed laser at a specific wave front in the absorption matrix, it is ionized and the mass analyzers are also extracted. It is used in combination with time path dependent mass spectroscopy (TOF, Time of Flight MS), but it is not suitable for use with liquid chromatography.

Since the molecule is examined to own its original form without a chemical process, advanced chemistry knowledge is not required for the use of MALDI-TOF or ESI/MS. In fact, as stated above, some applications do not require a separation step (e.g. with chromatography or electric freeze) and the sample can be put into MS and analyzed directly after a short process (Akyar 2011).

The distinction in mass analysis is generally made by the mass charge-ratio.

For those with a single charge, the simple spectrum is created for the MALDI-TOF device, but the ESI spectrum (reflecting a mixture of one, several, or many charged molecules) is more complex.

For this reason, MALDI-TOF has become more widely used in microbiology in terms of spectrum facility. However, the analysis of larger molecules can often be performed with ESI/MS (Akyar 2011). Lying et al. used flow cytometry and MALDI/MS to characterize glycosphingolipids in their stem cell studies (Breimer et al. 2017; Liang et al. 2011).

It has a feature that makes it superior to the known MS techniques; in addition to the definition of a large number of lipid molecules, it can display the spatial arrangement of lipids in the tissue.

This property allows researchers to define the physiological functions of membrane lipids. In a study investigating the lipid profile of a rat brain, glycolipids were shown using the MALDI-IMS technique. Thanks to this method, an evalution could be made about both the localization and the amount (Martinez-Gardezabal et al. 2017). In another study, Tay-Sachs and Sandhoff disease models consisting of mice were created and analyses were done with MALDI-MS. In the study, many different lipid and glycolipid types were identified and information about their localizations was obtained. In the study, the analysis of brain tissues, the electrospray

ionization-sequential mass spectrometry (ESI-MS/MS) method was also used to confirm the data obtained.

Spectroscopy

FTIR (Fourier-Transform Infrared Spectroscopy)

Spectroscopy is the measurement and interpretation of the electromagnetic radiation absorbed and emitted by an atom or molecule. Spectroscopic methods, the illumination of the molecular structure, elemental analysis and quantitative analysis are also used. IR spectroscopy is a branch of spectroscopy based on the material absorption of infrared rays.

Although the presence of Amyloid-p protein (Ap), which accumulates in Alzheimer's disease is known, the relationship between its involution mechanism and the Ap secondary structure and topography is still unclear. Matsubura et al. (2018) first verified the presence of Ap by atomic force microscopy and showed the structures of sphingomyelin, cholesterol and ganglioside (GM1). Using Fourier transform (FTIR) reflection-absorption spectroscopy, they demonstrated the construction of these lipid-associated fibrils, thereby determining the Ap secondary structure. A 20% mole content of GM1 (GM1 sphingomyelin, 20:40:40) was recorded. It has been shown that the fibrils are formed in turns and the parallel sheet layers within 48 hours.

Takahashi et al. investigated in detail the location of lipids in the hair structure, on the hair surface, the cuticle, and the cortex arsin and the distribution in the inner part of the hair (cortex, medulla, and melanin granules). They characterized the lipids and metabolites in the hair with infrared spectroscopy and a few mass spectrometry techniques (FTIR, TOF-SIMS, GCMS, and ESI-MS) (Takahashi and Yoshida 2014).

As a result, they showed that there are more unsaturated fatty acids in the cortex of the hair than on the hair surface.

Methods of Microscopic Evaluation

Although many studies have been done on the structure, biochemical importance and distribution of gangliosides or glycosphingolipids, there are limited studies on their localization and functions. Studies have also examined the cellular distribution of glycolipids by flow cytometry and immunohistochemistry. By combining data obtained by analytical methods,

the changing expressions of glycolipids and new glycosphingolipids could be identified.

Electron Microscopy Evaluation

In the medical field, electron microscopes are mostly used for structural examinations in cell and tissue research, and for further examinations in order to facilitate diagnosis in cases where light microscopy is insufficient for pathological biopsies. Especially, it is also necessary in the diagnosis of some congenital metabolic diseases. For example, in the electron microscopic examination of kidney biopsies in the diagnosis of Farby disease, Gb3 deposits, in the lysosomes of pedocytes are observed as "myelin figures" or electron-dense structures that form zebra bodies. Gb3 accumulation in pedocytes causes the deletion of the pedicel, thus affecting the structure of the cytoskeleton by changing its permeability, and may lead to protein loss. In addition, the accumulation of glycosphingolipid in the distal tubule cells is observed in electron microscopy. It is mentioned in the literature that it can be used for diagnosis in immunoelectric microscopy using anti-Gb3 antibodies.

Immunohistochemical Evaluation

Immunohistochemical analyses (IHC) in the medical field are performed to reach a safe diagnosis. The cytoskeleton and cell membrane receptor proteins of cells, tissue or body fluids are examined in IHC. These structures are accepted as antigens and the antigen–antibody complex is formed outside with specially produced antibodies, and this complex is made visible by staining with special dyes. The demonstration of the distribution of glycolipids in the body has been significantly advanced by the generation of specific antibodies against various glycolipid species. For example, the localizations of gangliosides (GM1, GD1a, GD1b, GT1b), which are densely packed in the nervous system, were determined by immunohistochemical staining (Santa et al. 2020). In 2007, they examined 3 neutral glycosphingolipids (GaLNa, GA1 and FMC-5) by immunohistochemical staining in their study on the brain tissue of rodents. Thanks to this method, it was shown that GA1 and FMC-5 were also contained in myelin sheaths, while GaLNa was found only in Purkinje cells in the cerebral cortex (Dasgupta et al. 2007). Similar to IHC, the immunofluorescent staining method gives important information about the localization and amount of glycolipids according to the staining intensity. For example, Kim et al. in their studies, determined the localization by the immunohistochemical staining of gangliosides and GT1 in embryo

development stages. Thus, they obtained important information about the localization and expression levels of gangliosides in embryos (Kim et al. 2008). Another alternative method for determining the localization of glycolipids has been used to visualize fluorescently labeled B subunit glycosphingolipids of bacterial toxins such as cholera toxin (Ctx B9 or Shiga toxin (Stx B9)). These microbial proteins selectively bind to specific glycolipids, detecting localization, but may also affect the multimeric structure and lipid organization due to binding. In the first studies in the literature on the localization of gangliosides in sensory ganglia, the cholera toxin B subunit (choleragenoid, CTB) was used by specific binding to GM1 ganglioside (Cuatrecas 1973).

The Cell Culture

Studies on the structure, biochemical importance and distribution of gangliosides or glycosphingolipids in in-vitro conditions have been carried out since the 1980s (Dasgupta et al. 2007).

Since in-vitro environments such as cell culture also contain a small amount of biological material, the isolation procedures should be chosen correctly and the analysis method should be limited (Breimer et al. 2017). Biswas et al. investigated the role of gangliosides in renal cell carcinoma (RCC), and the gangliosides in the RCC cell line were evaluated by immunohistochemical staining and the Elisa method (Biswas et al. 2009). Studies on Alzheimer's disease also examined the relationship between Ps and App deficiency with gangliosides in vitro and in vivo. Changes in ganglioside levels were determined by thin-layer chromatography and mass spectrometry (Grimm et al. 2014). In the literature, by using fluorescently labeled glycosphingolipids in vitro, cell membrane localization can be visualized under the confocal microscope. However, it should be kept in mind that the binding of the fluorophore to the glycan or lipid part of the glycosphingolipids alters the biophysical properties and may impair its biological function. Hence, researchers have evaluated the cell membrane's selective permeability in the living cell line by comparing different immunofluorescent probes (Dauner et al. 2016). In studies on glycolipids, normal or pathological metabolic pathways can be identified by using radiolabeled gangliosides in the culture medium (Schwarzmann et al. 2018). Nadia et al., while investigating the interaction between neighboring cells in their study of the most enzymatic effects in the plasma membrane, used radioactively labeled gangliosides (GD1 a). At the end of

the experiment, the levels of iron ganglioside in the cultured cells were evaluated by the HPLC-ESI/MS method (Papini et al. 2004).

Positron Emission Tomography

The PET imaging method is frequently used in the diagnosis and treatment follow-up of cancer patients. In the literature, the glycolipid derivate ganglioside was visualized in vivo with the PET device. In their studies in 2019, Butch et al. learned about the primary focus and metastasis of cancer using the radioactive marking of osteosarcoma marker GD2. The study was performed on experimental animals and is a valuable study in terms of providing information on in-vivo conditions. This study inspires us in the diagnosis and treatment follow-up of metabolic diseases (Butch et al. 2019).

Conclusion

In this chapter, we have compiled the methods used for analyzing glycolipids in light of current essays found in the literature. The methods for identifying glycolipids need to be increased. Thus, glycolipids can be named and the definition of metabolic diseases will be easier.

Keywords: *Chromatography, mass spectrometer, spectroscopy, glycolipids*

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CHAPTER SIX

THE PHARMACOLOGICAL AND INDUSTRIAL USE OF GLYCOLIPIDS

AYKUT ÖZTÜRK

Introduction

Glycolipids were first studied by J. H. Law in 1960 and entered our lives in this process (Law 1960). Glycolipids have a structure formed by the covalent bonding of a lipid and a carbohydrate. Glycans are a rich network of glycolipids and glycoproteins on the plasma membrane. This network structure is also called the glycocalyx. This layer almost completely covers the outside of the cell and plays a role in communication between cells. The carbohydrate portions of glycolipids are located closer to the extracellular surface. Glycolipids take part in various tasks that are vital for cells such as the adhesion of cells to each other and the exchange of substances between cells. In addition to their basic functions, they also have very different roles such as creating special dice subunits. For this reason, glycolipids are also referred to as glycosphingolipids. The forename sphinx has been added to draw attention to the uniqueness of the Sphinx. It has also been used to reflect the unknown tasks of glycolipids. The nomenclature of glycosphingolipids can often be confusing. In the naming of glycosphingolipids, galactosphingolipids are formed by attaching galactose to this structure instead of glucose.

The International Union of Basic and Applied Chemistry (IUPAC), the main descriptor of this subject in the world, defines glycolipids as one or more saccharides linked by a glycoside linkage to a hydrophobic unit such as an acylglycerol, a ceramide (N-acylsphingoid), or a spinhoids. Glycolipids are part of a larger group known collectively as glycoconjugates, which include glycoproteins, proteoglycans, glycopeptides, peptidoglycans and lipopolysaccharides (Chester 1997).

Cell membranes have a rich structure of glycoconjugates. Although glycolipids are an important component of the human cell membrane, they are not specific to the human cell and can be found in many living cell membranes, from bacteria to fungi, plants and animals. There are various differences between the glycolipids in the cell membrane among these organisms, especially in the configuration of glucose and galactose. They can be easily produced by transferring sugar residues with glycosyltransferases to sterols, ceramides and diacylglycerols (Warnecke and Heinz 2010).

Glycolipids acquire a stable conformation in biological membranes even under the influence of weak bonds such as van der Waal interactions and hydrogen bondings. In physiological and pathological processes, this stable structure in the cell membrane has undertaken various tasks such as recognizing other cells, adhering to other cells, ensuring communication between cells, receiving signals from the extracellular environment and transmitting these into the cell. It also has many more effects such as antimicrobial and antiviral activity, macrophage activation, cell differentiation or a fibrinolytic effect, antioxidant (Rodrigues et al. 2006).

Glycolipids have a wide range of uses today. It is especially thanks to the rapid development of new biological surfactants that glycolipids have taken their place in many areas of our lives, from pharmaceuticals and cosmetics to cleaning materials, from cleaning soil pollution and extending the shelf life of foods to paints and coating materials.

Glycolipids in Pharmacology

The main task of glycolipids is to enable the main carbohydrate groups to recognize cells and activate the antibody response mechanism when necessary. They interact with highly specific saccharide receptors and initiate biological adhesion. It is thought that by taking advantage of these properties, glycolipids and other glycoconjugates can be used for drug delivery to targeted cells. Pioneering studies have focused on glycolipids increasing the mucoadhesion of orally administered drugs in the gastrointestinal tract, resulting in the increased bioavailability of drugs and a decrease in their side effects. Subsequent research has related to the specific binding formed by glycolipids with lectins (Bies et al. 2004; Ponchel and Irache 1998).

Lectins are non-immune proteins that can be found in nature in many sources, from viruses to bacteria and from plants to animals, which specifically bind certain monosaccharides and oligosaccharides. Lectins

have a role in the recognition mechanism of cells at the cellular and molecular levels. This binding and adhesion occurs via glycolipids and glycoproteins. The concept of lectin-mediated adhesion can be applied to the gastrointestinal tract, as well as to other biological barriers such as the nasal mucosa, eye, buccal space and blood-brain barrier. In addition to using glycolipids to target endogenous lectins, drugs linked to lectins in reverse logic have also been considered to bind specific glycolipids (Lehr & Gabor 2004; Minko 2004). Although there are thousands of animal lectins, the group that includes structurally important types is "type C". This is called type C because they are dependent on calcium. The most important members of the C type lectin family are asialoglycoproteins, collectins, selectins and phycolinas.

The first lectin family member receptor group to be identified in animals is the asialoglycoproteins (Hudgin et al. 1974). These receptors, which provide clearance of deacialylated proteins by lysosomal and endocytosis, are mostly expressed by the hepatocytes on the surface (Bies et al. 2004). Collectins bind to the microbial cell wall, particularly mannose proteins. In binding to the surface carbohydrate of pathogens, they lead to complement system activation and cytokine production or phagocytosis. Selectins bind to sialylated carbohydrate moieties and exist in the cell as transmembrane or soluble proteins. They have been identified as platelets (P-selectin), endothelium (E-selectin) or lymphocytes (L-selectin), depending on the cells in which they are located. These glycoproteins have many important functions such as physiological roles, the attachment and rolling of neutrophils and monocytes on the endothelium, and the formation of acute inflammation. Galectins are sulfhydryl-dependent (S-type) lectins that mainly recognize-galactose and have many functions such as inflammation, allergic reactions, regulation of cell growth (Ghazarian et al. 2011). Phycoholins can bind to surfaces that are "fibrinogen-like" and contain N-acetylglucosamine independently of calcium, and by binding to microorganisms in plasma, they increase the phagocytosis of neutrophils and monocytes, and can also activate the complement (Lu et al. 2002).

Lectins are altered in many pathological conditions and are overexpressed in some diseases, a condition making them a target for new drug research. For example, in many patients with epithelial tumor such as colon, thyroid and breast carcinomas, high levels of galectin-1 have been detected and this increase has been found to be positively correlated with the metastatic phenotype (Faivre and Rosilio 2010). Increased galectin-3 expression is associated with neoplastic progression, particularly malignancies of the

neck, head, stomach, thyroid, and central nervous system (Francavilla et al. 2009). However, the increase in serum levels of selectins is not specific to the tumor and they increase in various infections such as HIV, acute ischemic stroke and plasmodium falciparum.

The idea of a glycosylated carrier is not actually a very new field of study. This idea was put forward in the 1980s and various studies have been done since. The rapid development in nanotechnology has triggered the development of drugs for the physiologically impaired body parts and even the cell-specific gene. Many nano-vectors have been found for this purpose. This field has been paved with the special bonding of glycoproteins with lectins and the classification of lectins. Although there are many receptor options to target liver parenchymal cells, Asialoglycoprotein receptors (ASGP-R) are at the top of these receptors. Since the liver is the primary metastasis organ for many tumors, it has been the main target organ in antineoplastic drug studies. The scientific results obtained so far give the promise of using a variety of new DNA-based pharmaceuticals for the treatment of liver diseases. Liposomes smaller than 100 nm in diameter can reach transendothelially implanted hepatocytes without using any targeting ligands. However, application of cell-specific targeting technology to liposomes will reduce drugs adverse effects while increasing gene delivery efficiency. For example, the high sensitivity of liver cells to galactose has made galactolipids the most important target in the development of liver gene therapy liposomes (Pathak et al. 2008). Many studies, both in vitro and in vivo, have shown that asialoglycoprotein receptors are the main group of receptors that allow galactosylated liposomes to adhere to liver cells (Nishikawa et al. 2003; Shimada et al. 1997). They found that an increase of DNA replication for ASGP receptors in hepatocyte cells, and liposomes composed of Gal-C4-Chol, 3p [N'N'N'dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioleoylphosphatidylethanolamine (DOPE) had higher transfection activity. Liposome / DNA complexes prepared by using galactosylated cholesterol derivatives were efficiently recognized by ASGP receptors and as a result showed that they lead to gene expression (Pathak et al. 2008).

As mentioned earlier, lectin-like receptors that recognize sugar conjugated ligands are found in large numbers in the cells of the mononuclear phagocyte system (MPS).

It can benefit from this property to increase the effectiveness of treatments for diseases that use MPS cells as the main host, such as HIV. Stavudine, which is frequently used in the treatment of AIDS and has an

average half-life of one hour, maintained its level in liver, spleen and lung tissues for up to 12 hours when intravenously administered to rats as a mannocylated liposome, and it also reduced the side effects of anemia and leukocytopenia (Garg et al. 2006). Similarly, scintigraphic imaging and body distribution of the drug and liposomes labeled with galactosylated liposomes and 99mTc of the stavudine active ingredient showed that liposomal formulations were better absorbed by the liver and spleen, and less retained in bone tissue (Garg et al. 2008). In tissues rich in galactose-specific receptors, a significant level of stavudine was maintained, the half-life of the drug was prolonged, hepatic cellular uptake was increased and hematological toxicity decreased. Similarly, it was observed that mannosylated liposomes loaded with zidovudine, another active ingredient frequently used in the treatment of AIDS, accumulate more in the lymphatic system (Kaur et al. 2008). An antibiotic isolated from Penicillium nigricans strains against Leishmania donovani, MT81, was tested in hamsters in the form of liposomes. Compared to the free drug, mannosylated liposomes caused low toxicity on kidney and liver functions and were able to eliminate active intracellular amastigotes in spleen macrophages (Mitra et al. 2005). In order to prevent stenosis, which is a serious complication that may occur after angiography, sialyl lewis x-coupled doxorubusin liposome was applied and it was shown to accumulate more in the wounded vessel walls and thus significantly reduce post-angioplasty stenosis (Tsuruta et al. 2009).

Glycolipids are also an important receptor component for pathogenic microorganisms, making glycolipids an important pathogenicity pathway (Imberty and Varrot 2008). Escherichia coli normally lives in the intestinal flora without causing any disease. However, the same bacteria can cause diarrhea, urinary tract infections and even sepsis in humans and is responsible for some of the neonatal meningitis. One of the important reasons for this is that E. coli strains allow fimbriae with their own glycoprotein structure to specifically attach to cells. For example, type 1 fimbria lectins of uropathogenic E. coli strains allow it to bind to the upper urinary tract and bladder epithelium (Imberty et al. 2005). Helicobacter pylori is a bacterium that causes gastric ulcers and cancers and has a high prevalence in society. One of the important factors in the pathogenicity of H. pylori is the presence of specific adhesion receptors for Helicobacter pylori in the gastric mucosa. The sialyl-dimeric-Lewis x glycosphingolipid is identified as the H. pylori receptor and contributes to the ability of many H. pylori strains to adhere to sialylated glycoconjugates and resulting chronicity (Mahdavi et al. 2002). Similar expressions can be used not only for bacteria but also for fungi, for example Candida galabrata has the cell

wall protein containing asialo-lactosil which is responsible for adhesion to glycolipids in human epithelial cells, and pathogenicity also plays a prominent role (Nimrichter et al. 2005). Therefore, drug delivery vector therapy is used in the treatment of various infections. For this purpose, sialo-mannan and mannosylated liposomes are especially preferred. For example, one of the treatment ideas to reduce H. pylori adhesion is cholesteryl oligoethyleneglycol glycosides embedded in liposomes (Bardonnet et al. 2005). H. pylori is actually sensitive to many antibiotics, but the deterioration of drugs in the acidic environment remains a major obstacle. Glycosylated liposomes are minimally affected by this pH change and have the potential to increase the success rate of antibiotic therapy (Bardonnet et al. 2009).

The large number of lectins in pathogenic organisms is an important obstacle that makes their detection difficult. However, bacterial and fungal lectins will be detected thanks to the genome projects that have been and will be done. More specific treatments will be developed based on the glycolipids to which these detected lectins bind.

Biosurfactants in Pharmacology

Glycolipids have been used industrially as surfactants for many years. Surfactants that can be synthesized biologically from various living things are called biosurfactants. The use of biosurfactants as carrier vectors in drug delivery first came to the agenda in 1988. Glycolipids can spontaneously change form in complex structures as a result of their amphiphilic structure depending on both solvent and temperature (Faivre et al. 2009). In addition, they can increase oral drug absorption in a non-specific way (Falconer and Toth 2007). Based on these properties, synthetic glycolipids, as an innovative approach, have the potential to be used for the distribution of drugs assembled to a designated area in the body. The basic materials that can be used to bind specific cells and tissues are used as liposomes, niosomes or lipid nanoparticles.

Alkylglycosides and alkylpolyglycosides are the main groups of these new surfactants (neosurfactans) that do not precipitate resistant to high temperature (106 Centigrade) and seawater salinity (Zulkifli et al. 2019). The most basic forms of glycolipids are alkyl αD-glucosides, alkyl αD-lactosides, alkyl a-D-maltosides and alkyl aD-melibiosides (Milkereit et al. 2007). The most fundamental point in creating a new glycolipid-based surfactant is the carbon number, carbon chain length and chemical structure in the structure. For example, long-chain and basic structures

may be better detergents. The main neosurfactant glycolipid structures used in pharmaceutical and cosmetic fields are mannosylerythritols, lipids, sophorolipids, rhamnolipids and trehalose-conjugated lipids (Lourith and Kanlayavattanakul 2009).

Mannosylerythritol lipids; while decreasing bcl-2 expression, they increase caspase 3 and 12 (Fan et al. 2016). Mannosylerythritol lipids are mostly used for gene delivery. They can the separation of the therapeutic gene from the complexes by a destabilizing effect on endosome membranes (fusion) in the endocytotic pathway or accelerate the fusion between DNA-liposome complexes and the plasma membrane (Ueno et al. 2007). As liposomes, they increase the efficiency of gene transfer in mammalian culture cells by 5-7 times (Naughton et al. 2019). Nanoparticles produced with zinc and silver for the treatment of in-vitro hepatocellular carcinoma and diabetes have cytotoxic effects on cancer cells (Bakur et al. 2019). Besides these and similar oncological studies, mannosylerythritol has been reported to increase the viability of papilla cells by 150% (Varvaresou and Iakovou 2015). Papilla cells are an important factor in regulating the growth and development of hair follicles; they have the potential to be used to regain damaged hair that has lost elasticity.

Sophorolipids; these alone have antibacterial, antiviral, and antifungal activity (Roelants et al. 2019). Sophorolipids have been shown to inhibit protein kinase C activity in cancer treatment studies on human promyelocytic leukemia cell line HL60 (Chen et al. 2006). They have also been shown to be effective against human pancreatic cancer cells (Fu et al. 2008). They have anticancer activity against human cervical cancer using HeLa and CaSki cells, leading to the activation of caspase-3, caspase-8 and caspase-9. They have been shown to induce apoptosis with increased intracellular calcium levels (Li et al. 2017; Nawale et al. 2017). They are also used as a drug delivery vector; for example, gellan gum-gold nanoparticle conjugates and their doxorubicin-loaded derivatives have been shown to be cytotoxic against human glioma and human glioma stem cell lines (Dhar et al. 2011).

Ramnolipids; these are widely used in many areas such as environmental cleaning, oil recovery, food production, cosmetics and pharmacy (Amani 2015). They are used in tomato cultivation against zoospores of Pythium and Phytophthora fungi (Sharma et al. 2007). They also have antiviral activity and have been reported as an inhibitor of Herpesvirus (Remichkova et al. 2008). They have also been used successfully in the control of Nicotiana glutinosa leaves infected with tobacco mosaic virus

(TMV) and Potato virus X disease (Haferburg et al. 1987). They have cytotoxic effects on human breast adenocarcinoma (MCF-7) cells in the form of silver nanoparticles (Dwivedi et al. 2015). They are especially used as a pharmaceutical agent for periodontal regeneration and re-epithelization of mucous membrane tissues in the treatment of gum diseases (Inès and Dhouha 2015). As a drug delivery vector, they have the potential to be useful tools for dermal drug delivery because human skin has a high tolerance to rhamnolipids (Müller et al. 2017). In an in-vivo mouse experiment performed as a nanoparticle, SCC7 reduced the growth of tumor cells along with photodynamic therapy (Yi et al. 2019).

Trehalose-conjugated lipids; these are used to stabilize a wide variety of biomaterials, cells, tissues and including proteins for numerous preservation applications in the food, pharmaceutical, and cosmetic industries (O'Neill et al. 2017). Trehalose lipids are known to have antiviral and antimicrobial properties. They provided higher resistance to intranasal infection with influenza virus in mice (Azuma et al. 1987). The human promyelocytic leukemia cell line induces cell differentiation into monocytes rather than cell proliferation in HL60 (Isoda et al. 1997). Trehalose liposomes (DMTre) have inhibitory effects on the growth of lymphoblastic leukemia (MOLT-4) cells in vitro and in-vivo therapeutic effects in xenograft mice (Matsumoto et al. 2016).

Industrial Use of Glycolipids

Glycolipids have been a research subject that has increased in popularity in recent years. One of the main reasons for this is the increasing demand for new products produced from natural resources and the cosmetics industry's focus on sustainable and renewable products. For example, ethylene oxide is one of the most common nonionic surfactants (surfactants) found in many different products such as detergents and personal care products. Ethylene oxide is a toxic substance and carries the risk of developing breast cancer and lymphoma in humans (Jinot et al. 2018). Therefore, replacing nonionic surfactants (surfactants) with degradable carbohydrate main groups has become an important research area.

Surfactants are compounds that reduce the tension or interfacial tension between two surfaces. Surfactants function as detergents, moisturizing agents, emulsifiers, foaming agents and such. Sugar-based surfactants with the most industrial use; sorbitan esters, alkyl polyglycosides, fatty acid N-methyl glucamides, and sucrose.

Alkyl polyglycosides are obtained by the reaction of alcohols with 8 to 16 chain carbon atoms and glucose (Von Rybinski and Hill 1998). Fatty acid glucamides are prepared by the reductive alkylation of glucose followed by acylation with fatty acids. The longer the alkyl chain length of fatty acid glucamides prepared by reductive alkylation of glucose and acylation with fatty acids, the greater the surface tension reducing effect (Eastoe et al., 1996). Fatty acid N-methyl glucamides are frequently used in detergents (Lichtenthaler 2006). While sorbitan esters are mainly derived from sorbitol, many sorbitan esters are available depending on the type and length of the fatty acid chains. The biggest advantage compared to other surfactants mentioned is the low cost of high purity sucrose (b-D-fructofuranosyl a-D-glucopyranoside). Sucrose fatty acid esters, also called sucroesters, are effective emulsifiers used in the food and cosmetics industries (Queneau et al. 2008).

The biodegradability and aquatic toxicity of the new glycolipids being marketed are looked at on the basis of environmental thinking. These biologically- and sugar-based surfactants have generally proved to biodegrade rapidly in both aerobic and anaerobic conditions and have low water toxicity compared to conventional polyoxyethylene-based nonionic compounds (Kronberg and Lindman 2003; Femina Carolin et al. 2020). The 12 principles of green chemistry (Kargozar et al. 2019) are based on the synthesis of less harmful and safer chemicals, the use of safer solvents, the planning of products that degrade after use, and the production of environmentally-friendly chemicals based on the principles of new glycolipid-based surfactants (Grüninger et al. 2019).

Glycolipid surfactants have applications in almost every chemical industry such as household and industrial cleaning, paper, agrochemicals, and personal care or pharmacy (Le Guenic et al., 2019). Alkyl glycosides are widely used in the cosmetics industry as surfactants, foamers or viscosity enhancers and for detergent effects. Sorbitan esters are often found as lipophilic nonionic emulsifiers in creams and emulsions for topical application. Although a mild skin irritant in acute and long-term studies, they are relatively non-toxic (Lanigan et al. 2002). Polyethylene glycols with molecular weights ranging from 200-10000, frequently used in cosmetic products, are also considered safe for human health (Fruijtier-Pölloth 2005).

Biosurfactants in Industry

Biosurfactants are amphiphilic surfactants synthesized by living things, that is, they have both hydrophobic and hydrophilic properties. Synthesis by living things provides biodiversity and they can be biodegraded compared to chemically synthesized surfactants, thus they are less toxic. The main neosurfactant glycolipid structures used in pharmaceutical and cosmetics fields are mannosylerythritol lipids, sophorolipids, rhamnolipids and trehalose-conjugated lipids (Lourith and Kanlayavattanakul 2009).

Mannosylerythritol lipids are mainly derived from Pseudozyma spp. (P. antartica, P. aphidis) and a small number of Ustialgo spp. (Arutchelvi et al. 2008). The moisturizing properties of mannosylerythritol lipids are greater than those of soybean oil and polysorbate, so they are preferred in the cosmetics industry (Arutchelvi and Doble 2011). They exhibit antioxidant and protective effects on skin cells even under oxidative damage, and are included in anti-aging cream formulations for skin care (Morita et al. 2013). This surfactant is used in detergent formulations as it enhances the emulsification of hydrocarbon in water, and it is antitumor and antioxidant. It can also be used for medical purposes that show activity (Silva 2017).

Sophorolipids are obtained from Candida apicola, bombicola and batistae, Wickerhamiella domericquae or Cryptococcus curvatus (Van Bogaert et al. 2007). Sophorolipids are very good solubilizers, have hygroscopic properties (the ability to reduce water molecules in the environment of any substance by diffusion or condensation on the wall) and are applied as moisturizers or softeners in cosmetics. They have the potential to be used as a biological pesticide in the future, as a flavoring in food, and as a shelf-life extender of fruits and vegetables (Roelants et al. 2019).

The class of rhamnolipids includes dirhamnolipids and monorhamnolipids. The lipophilic part of these glycolipids is rich in b-hydroxydecanoic acid and consists of 8 to 14 carbons. The main natural producers of ramnolipids are Pseudomonas spp., Especially P. aeruginosa. Rhamnolipids are the best known and most used surfactants due to their low production costs on an industrial scale (Rahman and Gakpe 2008). They are widely used in many areas such as oil recovery, food production, cosmetics and pharmacy. All this aside, rhamnolipids are used in the reclamation of organically contaminated soil and soil contaminated with heavy metals, putting them in an important place within glycolipids (Liu et al. 2018). In

addition to mannosylerythriol lipids, rhamnolipids are used in various formulations such as antacids, acne pads, anti-dandruff products, insect repellents, contact lens solutions, nail care products, deodorants and kinds of toothpaste (Klekner and Kosaric 1993). Thanks to the extremely low possibility of skin irritation, cosmetics containing rhamnolipids have been patented and included in the formulation of anti-wrinkle and anti-aging products (Rikalović et al. 2015).

Lipids conjugated with trehalose are produced by Rhodoccus spp., especially R. erythropolis, R. opacus, and R. ruber (Franzetti et al. 2010). Their area of use is wide; they are a good fat retaining and emulsifying agent like other glycolipids. They are used to prevent soil pollution, just like rhamnolipids. They have various biological properties such as antimicrobial, antiviral, anti-adhesive, anticancer or immunomodulatory properties. They are also used in the biomedical field (Mnif et al. 2018).

Conclusion

Glycolipids are a basic substance existing in almost all living things, but with structural differences. They participate in molecular recognition mechanisms on the surface of cells in the human cell membrane. This feature is used for drug targeting. They can be synthesized de novo or produced by biotechnology. Over the last 60 years, glycolipids with many different structures have been discovered and characterized them isolated from the producer strain. Microbial glycolipids have many more than expected properties, such as the ability to reduce surface stress, emulsification capacity, foaming strength, dissolution and mobilization capabilities. Glycolipids offer a wide range of applications in agriculture, pharmaceuticals, cosmetics, soil and water cleaning.

Surfactants such as textile, plastic, paper, food, cosmetics and pharmacy are needed in industrial life and they are used frequently. Annual surfactant production worldwide was as high as 13 million tons in 2008, and total demand is expected to exceed 24 million tons in 2020 (Zoller 2008). Both the increase in current surfactant demand, and the market and consumer orientation to green products, have been the driving force for the development of new biological surfactants in the last few years. They will also be more preferred in the near future, as the biosurfactant derivatives of glycolipids reduce environmental pollution. While bio-surfactants have enormous potential, the major barriers to their use are their higher cost compared to synthetic surfactants and the complexity of protein-carbohydrate interactions.

Taking advantage of glycolipid-lectin interactions, glycolipids are also used as drug delivery vectors. They are particularly promising in treating resistant infections and increasing the effectiveness of cancer drugs. They can be used in a wide variety of forms such as liposomes, niosomes, and microemulsion, both as a drug dispenser and alone.

Glycolipid-derived biosurfactants produced by microorganisms that fully comply with green chemistry appear to be very promising molecules. Glycolipids will be more preferred in the near future as they reduce environmental pollution and are successful drug delivery vectors.

Keywords: *Glycolipids, pharmacology, industry, biosurfactants, liposomes*

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CHAPTER SEVEN

GLYCOLIPIDS AND IMMUNITY

FATMA NUR KARAKUŞ

Introduction

Glycolipids, proteoglycans, and glycoproteins comprise the structure, referred to as the glycocalyx, covering the plasma membrane. The glycocalyx is responsible for the antigenic characterization of the cell and also plays a crucial role in cell-cell interaction, cell adhesion-migration, signal transduction, immune responses, and many others.

Glycosylation is a major post-translational modification that gives new physical, chemical, and biological characteristics to lipids and proteins. Cells, tissues and organs have specific glycoconjugate structures. Glycolipids and glycoproteins have a broad spectrum of oligosaccharide side chains, suggesting that they play an unlimited structural and functional role in metabolic processes, including immune responses.

The word "immunity" is derived from the Latin word *immunitas*, meaning a tax exemption and legal immunity granted to Roman senators. Immunity has been historically defined as the resistance of an organism to infectious diseases. Today, however, all branches of medicine focus on immunology for the treatment of cancer, immunodeficiency, and autoimmune and allergic disorders.

While other branches of medicine deal with organ systems, immunology focuses on cells, and molecules and lymphoid tissues synthesized and secreted by cells and their interaction with other tissues (Hekim and Alkan 2017). Cell-cell interactions, signaling mechanisms, and cell migration, in which glycoconjugates play a major role, are the key to immunity.

There are large varieties of glycolipids involved in numerous biological activities. Immunity is a dynamic system with complex mechanisms. This chapter addresses the interactions between glycolipids and immunity and the diseases caused by them and treatments for those diseases.

Glycolipids and Innate Immunity

The term "innate immunity" refers to the first defense mechanisms that are always ready to fight microbes and other dangerous situations. Innate immunity not only eliminates numerous pathogens without involving adaptive immune mechanisms but also plays a key role in many stages of adaptive immune response mechanisms.

Innate immune responses involve physical barriers (epithelial tissues), antimicrobial factors (sweat, tears, etc.), tissue-resident cells (macrophages, and mast and dendritic cells, etc.), leukocytes (neutrophils, monocytes, and NK cells), plasma proteins (complements and kinins), and cytokines.

Inflammation and antiviral responses are the primary defense mechanisms of the innate immune system (Abbas et al. 2017; Hekim and Alkan 2017).

Inflammation is the accumulation of leukocyte and plasma proteins in infected or damaged extravascular tissues (Abbas et al. 2017). Leukocytes circulating in the bloodstream should migrate to extravascular tissues to perform effector functions, such as fighting off infections and wound recovery. Selectins, integrins, chemokines and their ligands, and cytokines are involved in that migration.

Selectin (cluster of differentiation/CD62) is a family of cell adhesion molecules. Selectins and selectin ligands are found on both endothelial cells and leukocytes. There are three members of the selectin family; P(latelet)-selectin (endothelia activated by CD62P-Histamine or thrombin), E(ndothelial)-selectin (endothelium activated by cytokines, such as CD62E-TNF and IL-1), and L(eukocyte)-selectin (CD62L-neutrophils, monocytes, T lymphocytes, and B lymphocytes). They play a significant role in the low-affinity adhesion of leukocytes to postcapillary venule endothelial cells (Murphy and Weaver 2017).

Although the major ligands of selectins are known to be sugar residues in various glycoproteins, recent research has shown that glycolipids can

also be functional selectin ligands. Enzymatic studies suggest that E-selectin ligands on the surface of neutrophils may be glycolipids because they are protease-resistant and sialidase and β-galactosidase sensitive (Bochner et al., 1994). Further research has shown that myeloglycans (glycosphingolipids containing fucosyl and sialyl oligosaccharides) in human neutrophils are mainly endogenous selectin ligands (Schnaar 2004; Sperandio et al. 2009).

Selectin-mediated cellular traffic may affect the metastasis of tumor cells and leukocyte infiltration in autoimmune diseases, and therefore, inhibition of selectin adhesion or synthesis of selectin ligands may be a treatment option (Chen and Fukuda 2006; Woollard and Chin-Dusting 2007). In some cases, the induction of selectin ligands may also be beneficial. Sackstein et al. (2008) reported an increase in bone tissue formation due to an increase in the tropism of mesenchymal stem cells – the membrane glycans of which are reformed – toward post-transplant bone marrow.

Further studies are required on the roles of glycolipids, which can be selectin ligands, in immune responses and disease mechanisms, and their use in therapy.

Innate immunity can respond to only a few types of microbial molecules (~1000) because it recognizes common molecular patterns in many microbes. Receptors that detect the few types of microbial molecules are also few. Unlike adaptive immune receptors, which are generated by the somatic recombination of gene segments, innate immune receptors are encoded in the germline. Molecules recognized by receptors, known as *pattern recognition receptors* (PRRs), are classified as *pathogen-associated molecular patterns* (PAMPs), *microbe-associated molecular patterns* (MAMPs), *damage-associated molecular patterns* (DAMPs), and *self-associated molecular patterns* (SAMPs).

C-type lectin receptors (CLRs) are an essential group of PRRs involved in the recognition of glycolipid antigens. C-type lectin receptors are the *macrophage-inducible C-type lectin* (Mincle) and the *macrophage C-type lectin* (MCL). Mincle and MCL are expressed on dendritic cells, neutrophils, monocytes, macrophages, and some subtypes of T and B lymphocytes (Kawata et al. 2012; Smith and Williams 2016; Lu et al. 2018). These are type-II transmembrane proteins containing an extracellular C-type lectin domain, a transmembrane region, and a short cytoplasmic tail.

Mincle associates with the Fc receptor γ-chain (FcRγ) via the arginine residue in the transmembrane domain. The phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in FcRγ results in adhesion sites for the *spleen tyrosine kinase* (Syk), which provides CARD9-BCL10-MALT1 mediated NF-κB signaling pathway activation and proinflammatory cytokine expression. The transmembrane domain of MCL does not have positively charged residues to associate with the negatively charged residues of FcRγ. MCL and Mincle form heterodimers (Figure 7.1) (Lu et al. 2018; Smith and Williams 2016).

Mincle recognizes *Mycobacteria* (trehalose dimycolate [TDM], glycerol monomycolate [GroMM], glucose monomycolate [GMM]), *Malassezia* (glyceroglycolipids, mannosyl fatty acids linked to mannitol), *Nonomuraea sp.* (brartemisin), and *Streptococcus pneumoniae* (α-glucosyl diacylglycerol) glycolipid antigens. Mincle also recognizes the PAMPs of several microorganisms. The Mincle ligands of those microorganisms are not yet defined (Lu et al. 2018).

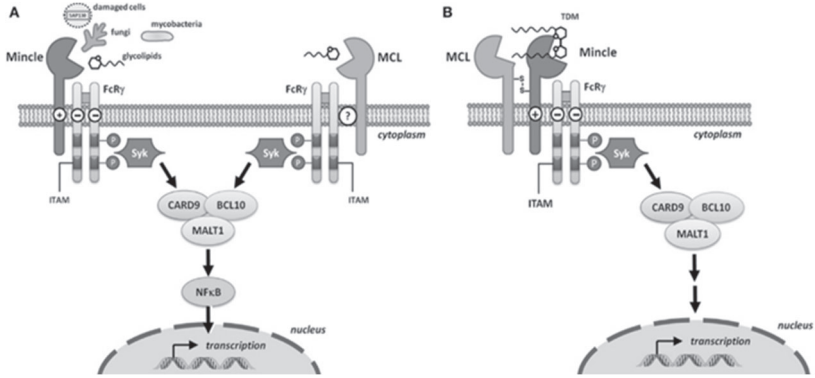

Figure 7.1: Left) The binding of glycolipids to Mincle results in phosphorylation of ITAM in FcRγ and activation of NFκB. NFκB is the transcription factor responsible for inducing the synthesis of cytokines that promote the activation of naive T cells. Right) MCL and Mincle form heterodimers and activate the FcRγ-mediated signaling pathway (Smith and Williams 2016)

Apart from fighting infections, Mincle is also actively involved in immune homeostasis because it recognizes DAMPs as ligands. β-glucosylceramide (β-GlcCer) released by damaged cells is the first identified endogenous glycolipid ligand recognized by Mincle (Nagata et al. 2017). Homozygous mutations in β-glucocerebrosidase (GBA1) which plays a role in β-GlcCer degradation causes Gaucher disease, which is

characterized by systemic inflammation (Grabowski et al. 1990; Tsuji et al. 1987).

Mincle also recognizes the cyclopropane fatty acid α-glucosyl diglyceride in *Lactobacillus (L.) plantarum*, which is a commensal probiotic, as ligands (Shah et al. 2016). This indicates that Mincle is also involved in microbiota-mediated immunomodulation.

Mincle is also effective in adaptive immune responses. Mincle activation results in the development of naive T cells into Th1 and Th17 subgroups. Mincle serves as an adjuvant receptor and stimulates antibody production (Lu et al. 2018; Smith and Williams 2016), and promotes autoimmunity, tumor progression, and sterile inflammation (Patin et al. 2017).

All these effects on the immune system make Mincle a promising therapeutic target.

Glycolipids and Adaptive Immunity

The adaptive immune system is composed of cellular immune responses mediated by T cells and humoral immune responses mediated by B cells. Adaptive immunity is stimulated by the recognition of antigens through antigen receptors on lymphocytes.

Adaptive immunity is specific. Innate immunity responds to similar molecular patterns of different microbes, while adaptive immunity responds to specific antigens on microbial and non-microbial agents. Another distinct feature of adaptive immunity is its diversity, for antigen-specific receptors are encoded by genes generated by the somatic recombination of gene segments. This diversity is also referred to as the "lymphocyte repertoire," which is estimated to be capable of recognizing 10^7-10^9 antigens. Adaptive immunity has a memory that allows it to respond faster and more efficiently the next time the body is exposed to the same antigen. Adaptive immunity can distinguish between self and non-self-antigens (self-tolerance). The loss of this ability can result in autoimmune diseases (Abbas et al. 2017; Hekim and Alkan 2017).

While most T lymphocytes only recognize peptides, B lymphocytes recognize small chemicals, lipids, carbohydrates, nucleic acids, proteins, as well as peptides. Dendritic cells, macrophages, and B lymphocytes (antigen-presenting cells [APCs]) present antigens to T lymphocytes. This

process is achieved by the interaction of major histocompatibility complex (MHC) molecules on the surface of APCs with T cell receptors. The contact interface (also known as the supramolecular activation cluster – SMAC) is called the immunological synapse, which has a bullseye-like configuration (Figure 7.2). The central supramolecular activation cluster (cSMAC) contains the T-cell receptor (TCR) complex, CD4 or CD8 coreceptors, costimulatory receptors, enzymes (e.g., PKC-θ), and adapter proteins. The peripheral supramolecular activation cluster (pSMAC) is the domain that contains integrins that stabilize binding.

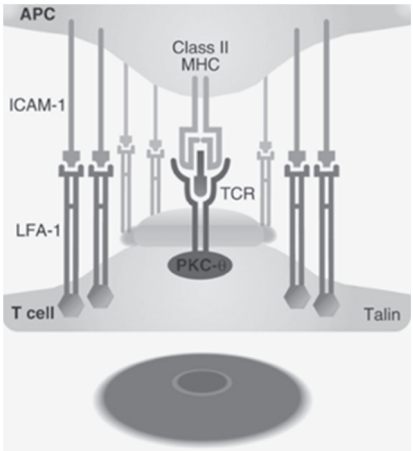

Figure 7.2: Immunological Synapse. Talin and LFA-1 p-SMAC (Green). PKC-θ and TCR c-SMAC (Red) (Abbas et al. 2017)

As a result of the TCR and costimulatory receptor signaling, rearrangements in the cytoskeleton allow lipid rafts to coalesce. The immunological synapse is formed in coalescent lipid rafts and makes T-cell responses longer and more efficient (Abbas et al. 2017).

Lipid rafts are small glycolipid-rich domains of the cell membrane (Figure 7.3).

Glycolipids make up only five per cent of lipids in the extracellular layer of the cell membrane. However, their location (lipid rafts) allows them to play a pivotal role in cell-cell interaction, cell migration, signaling, and immune responses (Alberts et al. 2008).

Lipid rafts also have a high content of glycosylphosphatidylinositol (GPI)-anchored proteins (Alberts et al. 2008). GPIs are a complex family of glycolipids common in eukaryotes. GPIs are either attached to the C-termini of proteins during post-translational modification or are freely present on the extracellular surface of the cell membrane. Mammals have approximately 150 GPI-anchored proteins involved in numerous activities, including neurogenesis, embryonic development, and immune responses (Malik 2018).

Immunity-associated GPI-anchored proteins are CD24 (in B lymphocytes and granulocytes), CD52 (Campath-1 antigen, modulation of T lymphocyte responses), CD55 (Decay-accelerating factor [DAF]), CD58 (Lymphocyte function-associated antigen [LFA-3], leukocyte adhesion), CD59, CD90 (Thy-1, in thymocytes, $CD34^{+}$ hematopoietic progenitor cells, and neurons), CD 177 (NB-1, neutrophil-specific antigens), and Ly6C (classical monocyte marker in mice) (Abbas et al. 2017; Malik 2018; Murphy and Weaver 2017; Zhao et al. 2017).

CD55 (DAF) and CD59 are two of the regulatory proteins of the complement system, which is a major effector mechanism of humoral immunity. The PIGA gene encodes one of the enzymes involved in the synthesis of GPIs on the X chromosome. The somatic mutation of the PIGA gene in the hematopoietic cell clone affects the functions of CD59 and DAF proteins, resulting in paroxysmal nocturnal hemoglobinuria characterized by intravascular hemolysis, chronic hemolytic anemia, and venous thrombosis (Abbas et al. 2017; Murphy and Weaver 2017).

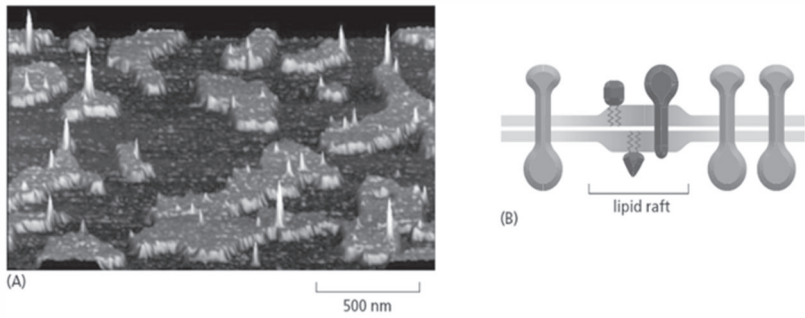

Figure 7.3: A) Surface contours of a synthetic bilayer membrane containing lipid rafts based on atomic force microscopic analysis. Lipid rafts are domains that are thicker than the rest of the lipid bilayer (orange). The yellow spikes are GPI-anchored proteins. B) Increased thickness in lipid rafts and differences in lipid

content result in specific membrane proteins (dark green) concentrating in these regions (Alberts et al. 2008)

Glycolipid Antigen Presentation

Classical T cells are known to use their TCRs to recognize MHC I/II-peptide complexes displayed on the membranes of APCs. A non-classical T lymphocyte group, which makes up a significant portion (7% $CD4^+$, 0.2% $CD4^-$ $CD8^-$) of $TCR\alpha\beta^+$ T lymphocytes in the human circulation, can recognize lipid antigens (de Lalla et al. 2011). CD1, which is an MHC I-like molecule, is involved in antigen presentation to lipid-specific T cells.

The human CD1 gene cluster, discovered in 1986, is classified into two groups based on sequence homology. CD1a, CD1b, CD1c, and CD1e are Group 1 CD1 molecules, while Group 2 CD1 contains only CD1d (Shamshiev et al., 2002). CD1 molecule expression was observed in all mammals analyzed. In humans, CD1 molecules in both groups are expressed, whereas mice lack group 1 CD1 isoforms.

CD1 molecules consist of an α chain (α1, α2, α3) non-covalently linked to β2-microglobulin (β2m) and are structurally similar to MHC class I molecules (Figure 7.4) (Porcelli 2005).

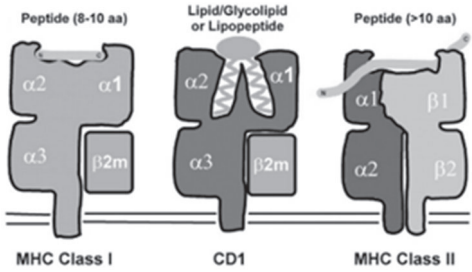

Figure 7.4: Schematic representation of three antigen presenting families of molecules (Porcelli 2005).

Some characteristics differentiate CD1 molecules from MHC molecules. Human CD1 genes are mapped to chromosome 1, while MHC locus is located on chromosome 6 (Reinink and Van Rhijn 2016). CD1 molecules display less polymorphism than MHCs. MHC molecules have six pockets in their antigen-binding groove (A-F), whereas CD1 molecules only have two (A and F). The CD1 groove is narrower and deeper than the MHC

groove. The CD1 groove is also rich in hydrophobic residues that allow for the stable binding of lipids to it. In general, the alkyl chains of lipid antigens are embedded in the CD1 groove, while their polar parts extend outward to interact with TCRs (Cotton et al. 2016). All CD1 molecules except CD1e present antigens. CD1e is indirectly involved in antigen presentation by transferring lipid antigens to other CD1 molecules (De La Salle et al. 2005).

CD1a-c molecules are expressed in $CD4^+$ $CD8^+$ thymocytes and professional APCs, whereas CD1d molecules are expressed in numerous cells, including non-hematopoietic cells in the skin, liver, and colon (Dougan et al. 2007; Van Kaer et al. 2016). These different expression patterns of CD1 molecules suggest that local T cell responses can be shaped by these molecules (Ryu et al. 2018).

CD1 molecules are loaded with lipid antigens as soon as they are synthesized in the endoplasmic reticulum (ER). This process requires the assistance of various lipid transfer proteins (LTPs). Some of the LTPs in the ER are microsomal triglyceride transfer protein (MTP), saposins (also known as sphingolipid activator proteins [SAPs]), Gm2A (lysosomal LTP, that enzymatically degrades ganglioside GM2, which is also involved in lipid antigen transfer to CD1d), Niemann-Pick Type C1 and C2 (NPC1 and NPC2, lipid transfer to CD1d) and CD1e (Schrantz et al. 2007; Cantu et al. 2004).

Peptide antigens bound to MHC molecules should possess adhesion motifs, while CD1 molecules are, in theory, capable of binding almost all phospholipids and glycolipids. However, some chemical properties, such as the length and saturation of acyl chains, limit the lipidome that can be presented by CD1 (Vartabedian et al. 2016). The subcellular location (endosome/lysosome network) of CD1 molecules also affects the type of lipid it will carry.

The endogenous lipids known to be presented by CD1 molecules are the squalene in the skin, gangliosides (e.g., GM2), sulfatides in the central nervous system, phospholipids (e.g., phosphatidylethanolamine and phosphatidylglycerol), and α-monoglucosylceramides (De Jong et al. 2014).

CD1 molecules also present lipid antigens of numerous pathogens and microorganisms in the microbiota. *Mycobacterium leprae* and *Mycobacterium tuberculosis* (lipoarabinomannan [LAM], phosphatidylinositol mannoside [PIM]), *Ehrlichia muris* and *Sphingomonas spp.* (α-glucuronyl

[GSL-1] and α- galacturonyl [GSL-2] ceramides), *Aspergillus fumigatus* (Asperamide B), *Bacteroides fragilis* (Bf α-galactoceleceramide [Bf-αGalCer]), *Borrelia burgdorferi* (α-galactosyl diacylglycerols [BbGL-II]), *Streptococcus pneumoniae* (α-glucosyl diacylglycerol), *Helicobacter pylori* (cholesteryl α glycosides modified by lipids [α-CAGs]), *Entamoeba histolytica* (EhPIb), and *Listeria monocytogenes* (anteiso C-15 and anteiso C-17 phosphatidylglycerol) are sources of exogenous glycolipid antigens presented by CD1 molecules (Figure 7.5) (Smith and Williams 2016).

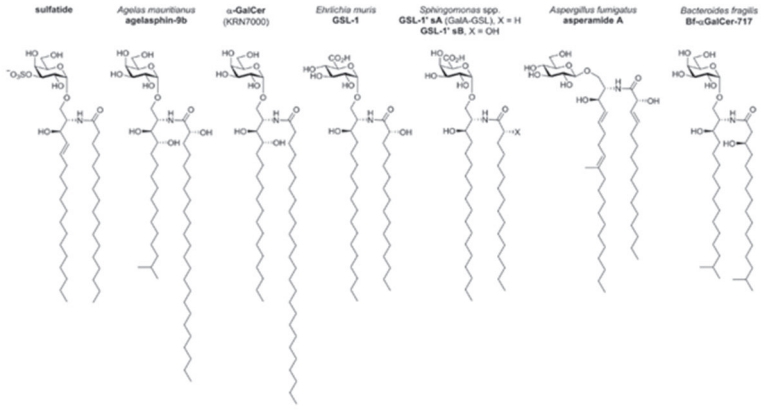

Figure 7.5: Glycosphingolipid antigens presented by CD1d (Smith and Williams 2016)

CD1-Restricted T Lymphocytes

CD1-restricted T lymphocytes remain an understudied topic because Group 1 CD1 molecules are not expressed in mice. Much of that learned so far has been derived from in-vitro studies on human cells.

CD1a (2%), CD1b (1%), and CD1c (7%)-restricted T cells are considerably higher than CD1d (0.1%)-restricted cells in the human bloodstream. Further research is warranted on T cells involved in lipid antigen responses via Group 1 CD1 molecules (de Lalla et al. 2011; Ryu et al. 2018).

T cells responding to glycolipid antigens via CD1d molecules are referred to as natural killer T (NKT) cells. As the name implies, NKT cells have the properties of both natural killer (NK) cells involved in innate immunity and T cells involved in adaptive immunity. NKT cells have both NK cell markers (CD161 in humans and NKG2D and NK1.1 in mice) and

T lymphocyte markers (TCR, CD4, CD8, CD25, CD69, and CD122) (Van Kaer et al. 2011). Innate and adaptive immune responses are not actually two separate systems but intertwined mechanisms. NKT cells act as a bridge since they have the properties of both types of immunity.

NKT cells are classified based on surface expression, gene expression, and the TCR diversity. Based on surface expression, NKT cells are classified into $CD4^+$ and $CD4^-$ $CD8^-$ (double negative-DN) populations in mice and $CD4^+$, $CD4^-$ $CD8^-$ (double negative-DN), and $CD8^+$ in humans (Heller, Berga-Bolanos, Naler, & Sen 2018). Based on gene expression, NKT cells are classified into three subsets; (1) NKT1 expressing T-bet, (2) NKT2 expressing GATA-3, and (3) NKT17 expressing RORγt (Lee, Holzapfel, Zhu, Jameson, & Hogquist 2013). In recent years, two more subsets have been identified: (1) follicular helper NKT cells (NKTfh) expressing Bcl-6, and (2) NKT10 expressing Nfil3 (Chang et al. 2012; Lynch et al. 2015; Sag et al. 2014). PLZF (promyelocytic leukemia zinc finger) transcription factor is a key regulator of NKT cell differentiation. NKT1, NKT17, and NKT2 express PLZF at low, medium, and high levels, respectively (Kovalovsky et al. 2008; Savage et al. 2008).

NKT cells have a quite limited TCR diversity compared to classical T cells. Based on the TCR, NKT cells are classified into two subsets: (1) Type I NKT (invariant NKT-iNKT), and (2) Type II NKT (diverse NKT-dNKT). Type I NKT cells have semi-invariant αβ TCRs; which are composed of an invariant α chain (Vα24-Jα18 in humans and Vα14-Jα18 in mice) and β chains with limited heterogeneity due to the tendency to use specific genes. αβ TCRs in dNKTs also have limited diversity but do not contain invariant chains.

iNKT cells respond strongly to α-galactoceleceramide (α-GalCer), a synthetic isoform of the marine sponge glycolipid. iNKT cells are also sensitive to numerous microbial and endogenous glycolipid antigens (e.g., Isoglobotrihexosylceramide [iGb3]) presented via CD1 (Figure 7.6) (Lalazar et al. 2006; Joyce et al. 1998; Kawano et al. 1997; Mattner et al. 2004). Antigenic targets of dNKT cells are glycolipids and phospholipids, such as sulfatide, lysophosphotidylcholine, β-glucosylceramide, Liso-GL-1 and phosphatidylglycerol. α-GalCer and even many other α anomeric glycolipids are not antigenic stimuli for dNKT (Dhodapkar and Kumar 2017).

NKT cells manifest their effects on immunity by both secreting cytokines and directly participating in cell-cell interactions. NKT cells

have immunoregulatory properties due to their proinflammatory and anti-inflammatory effects. They can produce high levels of cytokines (IL-4, IL-10, IFN-γ, TNF) even hours after activation, which takes days and even weeks for classic T lymphocyte responses. The ability of NKT cells to produce both Th1- and Th2-associated cytokines is a sign of their regulatory functions. NKT cells can promote immune responses to infections and tumors and suppress autoimmune diseases and allograft rejection. They are also involved in the pathogenesis of and protection against some diseases.

Activation of iNKT cells in mice plays a role in the pathogenesis of Concanavalin A-mediated immune-mediated hepatitis (Lalazar et al. 2006). NKT2 cells localized on mucosal surfaces in the lungs and intestines contribute to Th2-mediated airway hyperreactivity (Stock, Lombardi, Kohlrautz, and Akbari 2009). NKT2 and NKT17 are associated with impaired mucosal homeostasis in the intestine. In the collagen-induced arthritis model, CD1d ligands have a protective effect. NKT cells can interact with immune cells, as well as non-immune cells (e.g., epithelial cells, adipocytes, and keratinocytes). Adipose tissue-resident NKT cells suppress tissue inflammation and protect against type-2 diabetes. Impairment in adipose tissue-NKT cell interactions makes mice more prone to obesity. Studies also show that NKT cells in keratinocytes may play a role in the pathogenesis of allergic contact dermatitis and systemic lupus erythematosus (SLE)-associated skin pathologies (Heller et al. 2018).

Circulating NKT cell numbers decreased in diabetes mellitus, multiple sclerosis, scleroderma, SLE, and rheumatoid arthritis diseases (Kojo 2001; Hammond and Godfrey 2002; Araki et al. 2003;Miyake and Yamamura 2007). Adoptive transfer of NKT cells has a therapeutic effect in many immune mediated animal models, including graft versus host disease, experimental autoimmune encephalomyelitis (EAE) and immune mediated colitis. The amount of NKT cells was observed to increase in the circulation of patients with Gaucher disease (Lalazar et al. 2006). People with HIV had fewer NKT cells in their peripheral blood circulation than healthy individuals (Unutmaz 2003). Inappropriate activation of NKT cells is associated with psoriasis and atherosclerosis (Bobryshev and Lord 2005; Nickoloff et al. 1999)

All of these results make NKT cells a target for potential immunotherapies. α-GalCer, which is a potent activator of NKT cells, has a protective effect against *Pseudomonas aeruginosa*, *Streptococcus*

pneumoniae, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Novosphingobium capsulata*, *Staphylococcus aureus*, *Plasmodium falciparum*, *Trypanosoma cruzi*, influenza, respiratory syncytial virus, cytomegalovirus, diabetogenic encephalomyocarditis virus, Japanese encephalitis virus, and hepatitis B virus (Van Kaer et al. 2011).

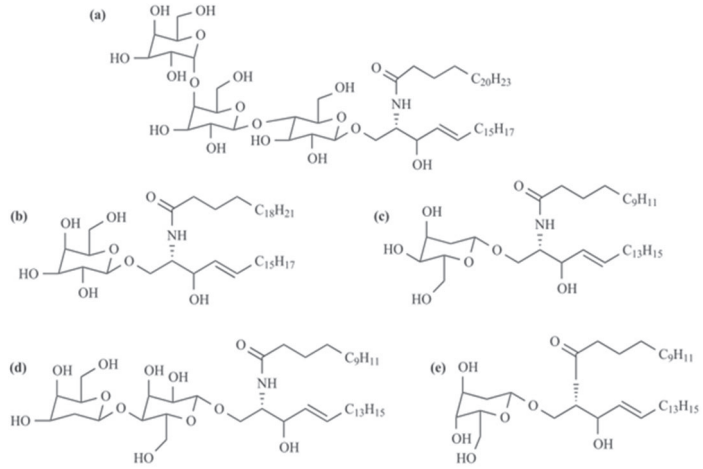

Figure 7.6: Some α and β glycolipids that are an antigenic target for NKT cells. (a) Isoglobotrihexosylceramide (iGb3). (b) α-galactosylceramide (α-GalCer). (c) β-glucosylceramide (GC). (d) β-Lactosylceramide (LC). (e) β Galactosylceramide (GLC) (Lalazar et al. 2006)

iNKT cell responses were manipulated using α-GalCer analogs. Some of these analogs acted as anticancer and anti-autoimmune disease agents, and vaccine adjuvants against cancers and infections (Hung et al. 2017).

NKT cells promote antitumor immunity through various mechanisms. Both in-vivo and in-vitro studies show that 7DW8-5, an analog of α-GalCer, can be used as an anticancer agent against human breast cancer (Seki et al. 2019). Recent preclinical studies in the field of chimeric antigen receptor (CAR) cell immunotherapy have focused on NKT cells and reported promising results for neuroblastomas and B-cell lymphomas. CAR-NKT cells were effectively localized in tumor tissues, and no graft versus host disease induced by classical CAR-T cells was observed (Heczey et al. 2014; Tian et al. 2016).

NKT cells have positive or negative effects on infectious, autoimmunity, and inflammatory diseases, hypersensitivity reactions, and cancers. Once we better understand the related mechanisms, the parameters affecting treatment efficacy, and the similarities and differences in NKT cells between humans and mice, we can use the immunomodulatory effects of NKT cells more effectively for therapeutic purposes.

Conclusion

Glycosylation results in a vast repertoire of glycans defined as glycoms. Almost all molecules involved in immunity are glycosylated (Quick 2009). Therefore, glycans play a significant role in numerous immunological mechanisms, such as intracellular signaling, cell-cell interaction, cell migration, and antigen recognition.

Glycolipids are important members of the glycom. Numerous microbial or endogenous molecules have a glycolipid structure. These molecules and related receptors and cells are used to better understand the immunological mechanisms and pathogenesis of diseases and to develop immunotherapies.

The endogenous glycolipid antigens that first come to mind are blood group antigens (see chapter 11) and tumoral antigens (see chapter 8). Many types of cancer lead to changes in the cell membrane glycolipid structure:

1) The synthesis of sugar chains is discontinued by the inhibition of glucosyltransferase enzyme, resulting in a simpler glycolipid profile.
2) Neosynthesis leads to the display of new glycolipids in tumor cells.
3) Tumor cell membrane glycolipids are rearranged (abnormal fucosylation, abnormal sialylation, etc.), and those cells go through changes in antigenic expression (Durrant et al. 2012; Dyatlovitskaya and Bergelson 1987).

Unlike healthy cells, membrane glycosphingolipids accumulate in tumor cells. The release of those glycosphingolipids into the blood changes the glycolipid profile of the serum of patients with cancer. Tumor-related gangliosides reduce antitumor immunity by suppressing immune-competent cells (Wiederschain 2017).

Numerous immune mechanisms are tailored to recognize exogenous glycolipid antigens. Although they are defense mechanisms, they are sometimes the cause of pathogenicity. For example, cells with ganglioside GM1 in their membranes (e.g., intestinal epithelial cells) become the target of cholera toxin. Cholera toxin entering cells leads to a prolonged increase in the intracellular cyclic AMP (cAMP) concentration, resulting in Na^+ and water efflux into the intestinal lumen, and hence, diarrhea (Alberts et al. 2008). The same glycolipid with a lower specificity is also a target of *Escherichia* coli toxin. Some gangliosides containing additional neuraminic acid residues (GD1b, GT1, and GT1b) also act as receptors for tetanus and botulinum toxins (Wiederschain 2017).

Mincle is a PRR that recognizes PAMP, DAMP, MAMP, and SAMP in glycolipids. CD1 molecules are MHC class I-like molecules that present glycolipid antigens to NKT cells. Both Mincle and NKT cells have immunoregulatory effects. Synthetic glycolipid analogs are developed for Mincle and NKT to generate either proinflammatory or immunomodulatory responses. In this way, effective treatments are developed for infections, cancers, and autoimmune and inflammatory diseases. Some of the glycolipid antigens (TDM and α-GalCer) recognized by Mincle and NKT have adjuvant properties, opening up new possibilities for vaccine research and development. The dose, duration, and administration (e.g., intramuscular injection) of glycolipid antigens, and toxicity, and even the length of carbohydrate chains (Goff et al. 2004) affect the research outcomes.

Studies on the glycolipid antigens that have been identified and related immune mechanisms have allowed us to better understand the pathogenesis of diseases and to develop immunotherapies. There are, however, numerous glycolipid antigens and immunological mechanisms that have not yet been identified. The promising research results suggest the need for further research.

Keywords: *Glycolipid antigens, Mincle, CD1, NKT cells*

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CHAPTER EIGHT

SPHINGOLIPIDS AND CANCER

GÖKSENİN ÜNLÜGÜZEL ÜSTÜN

Introduction

Sphingolipids are structural components of the cell membrane and play a vital role in providing the barrier function and fluidity of the membrane (Hannun and Obeid 2008). Sphingolipid metabolism products (e.g. ceramide-1-phosphate (C1P), sphingosine-1-phosphate (S1P) and ceramide), undertake primary tasks in many cellular functions such as growth, survival, angiogenesis, migration, inflammation, immune cell trafficking, cancer. They control signal transduction functions within the cell, and so regulate biologic cancer courses like proliferation, growth, invasion, migration, and/or metastasis (Schneider et al. 1987; Dressler et al. 1992; Spiegel and Milstien 2003; Kunkel et al. 2013; Bujko et al. 2017). Ceramide and S1P have opposite effects on cell survival, one pro-apoptotic and the other pro-survival, respectively. Ceramide and S1P determine the fate of the cell by affecting various features of cancer biology, and radio/chemoresistance (Oskouian and Saba 2010). Radiation, chemotherapy, and/or oxidative stress induce ceramide and sphingosine formation, and the increased amount of sphingosine and ceramide causes senescence, cell death, and/or cell cycle arrest (Ogretmen and Hannun 2004). Metabolic ceramide conversion leading Sphingomyelin, Sphingosine-1-phosphate (S1P), or glucosylceramide formation have pro-survival and anti-apoptotic effects in the cell (Cuvillier et al. 1996; Lee et al. 1998). Nowadays, almost all of the main enzymes involved in sphingolipid metabolism have been determined. Thus, allowing us to test whether we can use new approaches in signalling regulation and cancer treatment.

Metabolism

Ceramide consists of sphingosine and a fatty acyl chain, ranging in length from C14 to C26, linked to it by an amide bond. Ceramide is the metabolic and structural precursor for complex sphingolipids (e.g. sphingomyelin, ceramide 1-phosphate and GlcCer) (Gouaze-Andersson and Cabot 2006). Ceramide, sphingolipid metabolism mid product, exhibits pro-apoptotic and antiproliferative effects (Ogretmen et al. 2004; Ponnusamy Meyers-Needham et al. 2010). Intracellular ceramide levels are arranged by complex and combined metabolic pathways, each containing a specific enzyme series (Futerman and Hannun 2004; Futerman and Riezman 2005). Intracellular ceramide production can be eventuated by sphingomyelinase (SMase) from sphingomyelin (SM) (Tani et al. 2003; Carré Graf et al. 2004), cerebrosidase (CRS) from glycosphingolipids, ceramide 1-phosphate phosphatase (C1PP) from S1P and via the de novo pathway (Michel and Van Echten-Deckert 1997; Gouaze-Andersson et al. 2006). The synthetic pathway of sphingolipids is summarized in Figure 8.1 below.

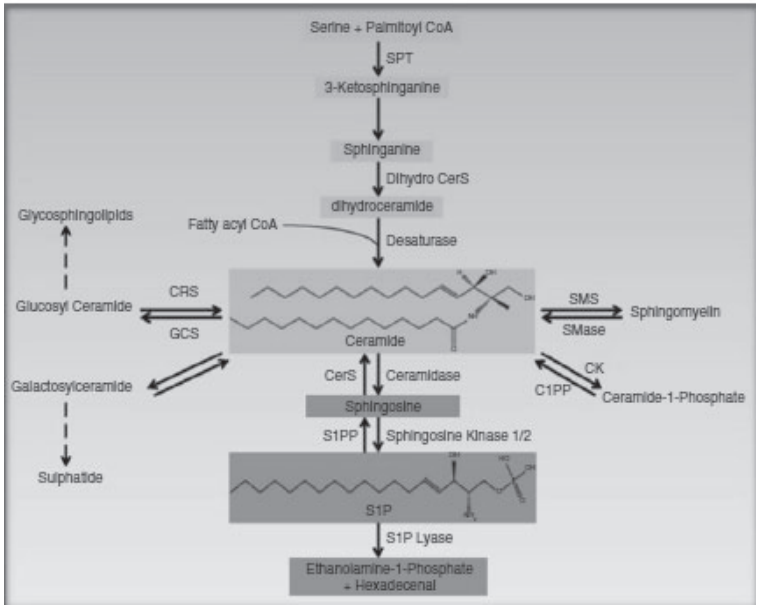

Figure 8.1: The synthetic pathway of sphingolipids (Selvam and Ogretmen 2013)

The de Novo Synthetic Pathway

The formation of 3-ketosphinganine from serine and palmitoyl CoA by serine palmitoyl transferase is the starting point in sphingolipid synthesis. Then 3-ketosphinganine is reduced by 3-ketosphinganine reductase to form sphinganine (Hanada 2003). Next, sphinganine is acylated with dihydroceramide synthase (sphinganine N-acyl transferase) to form dihydroceramide (Pewzner-Jung et al. 2006). Afterwards, dihydroceramide desaturase/reductase converts dihydroceramide to ceramide. The cytosolic leaflet of the ER is the place for all of the above reactions (Mandon et al. 1992; Hirschberg et al. 1993; Michel et al. 1997).

Ceramide is the precursor of five different products.

1. Sphingosine: This is obtained from ceramide by ceramidase (CDase). Then it can be metabolized to S1P by sphingosine kinase 1 (SPHK1) and sphingosine kinase 2 (SPHK2). S1P is either dephosphorylated by S1P phosphatases (S1PP) to regain sphingosine or irreversibly hydrolyzed by S1P lyase to ethanolamine 1-phosphate and hexadecenal (C18 fatty aldehyde) to exit the sphingolipid metabolic cycle (Becker et al. 2005; Kraveka et al. 2007).
2. Galactosylceramide (GalCer): This is formed by glycosylation via galactose transfer to ceramide in the lumen of the endoplasmic reticulum (ER) (Sprong et al. 1998).
3. Glycosylceramide (GlcCer): GlcCer synthase (GCS) glycosylates the ceramide into GlcCer. It is the precursor of complex glycosphingolipids (Futerman and Hannun 2004; Futerman and Riezman 2005).
4. Sphingomyelin (SM): This is formed by phosphorylcholine transfer from phosphotidylcholine (PC) to ceramide by sphingomyelin synthase (SMS) in the Golgi apparatus and diacylglycerol (DAG) is formed as a by-product (Pagano 1988; Futerman et al. 1990; Jeckel et al. 1990).
5. Ceramide 1-phosphate (C1P): Ceramide kinase (CERK/CK) phosphorylates the ceramide to form C1P (Sandhoff 2013; Selvam and Ogretmen 2013).

Enzymes

The functions of enzyme series involved in sphingolipid synthesis and their known relevance to cancer types are the subject of this topic.

1. SPT (serine palmitoyl transferase): This takes part in the first step of de novo sphingolipid synthesis and is localized in the ER. The enzyme consists of two large subunits and one small subunit. There are 3 types of large subunit – SPTLC1, SPTLC2, SPTLC3 – and 2 types of small subunit – SPTSSA or SPTSSB. It can cause paclitaxel-induced neurotoxicity, which can be seen in cancer treatment. In breast cancer, its activity increases in response to chemo- and radiotherapy and provides tumor suppression (Han et al. 2009; Kramer et al. 2015; Bode et al. 2016).
2. 3-ketosphinganine reductase: This is involved in de novo sphingolipid synthesis and is localized in the ER.
3. CERS1-6 (dihhydoCerS): The CERS gene family, also known as the Lass (longevity assurance) gene, has been identified in mammals. The proteins encoded by this gene family are integral membrane proteins, each member showing a unique tissue distribution (Pewzner-Jung et al. 2006). CERS has six different types (CERS1-6):
    - CERS1: This is responsible for the synthesis of C18 (dihydro) ceramides. It induces mitophagy in leukaemia cells and cancers of head and neck providing tumor suppression (Koybasi et al. 2004; Meyers-Needham et al. 2012; Thomas et al. 2017).
    - CERS6: C16 (dihydro) is responsible for the synthesis of ceramides. It stimulates cell death by inducing caspase activation in lung cancer cells. It maintains ER and Golgi integrity in head and neck cancer cells. Its amount increases in breast cancer tissues. Taking all these studies together, CERS6 plays a role in providing tumor suppression (Schiffmann et al. 2009; Suzuki et al. 2009; Senkal et al. 2010; Jensen et al. 2011; Senkal et al. 2011; Fekry et al. 2016; Sofi et al. 2017).
4. DES (dihydroceramide desaturase): This takes part in ceramide synthesis and is localized in the ER. It leads to tumor suppression by inducing cell cycle arrest in neuroblastoma cells (Rahmaniyan et al. 2011).
5. CERK/CK (ceramide kinase): C1P (ceramide-1-phosphate) is formed by its activity. Its cellular localization is not clear. There are studies showing that it is localized in the plasma membrane (Ponnusamy et al. 2010), Golgi apparatus (Futerman et al. 2005), or cytoplasm (Futerman et al. 2004). It induces cell survival in breast and lung cancer cells (Pastukhov et al. 2014; Payne et al. 2014).
6. C1PP (ceramide 1-phosphate phosphatase): This dephosphorylates C1P to ceramide.

7. CDase (ceramidases): These take part in the deacylation of ceramide to sphingosine. According to the pH of the environment in which they function, they are divided into three different forms: neutral, acidic and alkaline. They are found in various intracellular localizations, there are even studies in which the mitochondrial localization is defined (Tani et al. 2003).
    - NCDase (Nötral CDase): This is localized in the plasma membrane (Carré et al. 2004).
    - Alkaline CDase: This is localized in the ER/Golgi complex. There are 3 types called ACER1, ACER2 and ACER3 (alkaline ceramidase 1-3). Systemic inflammation may increase with ACER3 and cause colitis and colorectal cancers (Becker et al. 2005).
    - AC (acidic CDase): This is lysosomally localized (Kraveka et al. 2007). There are studies showing that radiotherapy-induced AC expression may cause radiotherapy resistance, leading to relapse in prostate cancers (Tirodkar et al. 2015). It is responsible for resistance to cell death in prostate cancer. It shows a pro-survival cancer signaling effect by providing metabolic communication between ceramide catabolism and S1P synthesis (Eliyahu et al. 2007; Beckham et al. 2013; Cheng et al. 2013).
8. CerS (ceramide synthase): This metabolises sphingosine to ceramide.
9. SMS (sphingomyelin synthases): This is responsible for the conversion of ceramide to sphingomyelin. Two types have been described.
    - SM1: This is localized in the Golgi apparatus (Pagano 1988; Sandhoff 2013).
    - SM2: This is localized in the plasma membrane (Han et al. 2009).

CERT (ceramide transport protein)➔ Ceramide synthesized in the ER must be transported to the Golgi apparatus for conversion into sphingomyelin. At first, it was thought that the transfer was through vesicular transport. However, with the discovery of CERT, it was understood that the transfer of ceramide (C16-20) to the Golgi for the SM1 function was through the CERT-dependent non-vesicular route (Meyers-Needham et al. 2012; Kramer et al. 2015). Sphingomyelin synthesis regulation occurs by CERT-dependent ceramide transport (Heering et al. 2012; Degagné et al. 2014). CERT enhances pro-survival signalisation by

inhibiting pro-apoptotic ceramide signaling in breast, ovarian, and colorectal cancer cells. It has been reported that the decrease in CERT expression leads to changes in plasma membrane sphingomyelin levels and supports the EGFR (epidermal growth factor receptor) signaling pathway in some types of breast cancer (Lee et al. 2012). Pharmacological inhibition of CERT by CHC (3-chloro-8β-hydroxycarapin-3,8-hemiacetal) can effectively induce ceramide-dependent HeLa cell death (Wijesinghe et al. 2014). In light of the available data, it seems that by targeting CERT-dependent ceramide transport which is inhibiting the ceramide metabolism and consequently ensuring cell death, we can provide new approaches in cancer treatment and overcoming drug resistance (Hullin-Matsuda et al. 2012).

10. SMases (Sphingomyelinase): This has three types, acid, neutral and alkaline, depending on the pH of the environment in which it is active (Sofi et al. 2017). It takes part in providing cancer cell death, growth arrest and tumor suppression by hydrolysing SM to ceramide.
    - ASMase: The production of ceramide by this type induces apoptosis in lymphoblasts (Santana et al. 1996; Carpinteiro et al. 2015; Goreliket al. 2016).
    - NSMase: The production of ceramide by this type mediates cell cycle arrest in breast cancer cells (Trajkovic et al. 2008; Shamseddine et al. 2015; Airola et al. 2017).
    - Alk-Smase: Its deficiency may cause tumor size growth in colon cancers.
11. GCS (glucosyl ceramide synthase): This is involved in the glycosylation of ceramide to glucosylceramide (GlcCer) and is localized in the Golgi apparatus. Although both SM synthesis and GlcCer synthesis occur in the Golgi apparatus, they are different from each other in terms of enzyme localization and the transport of ceramide from the ER to the Golgi. While SM synthesis takes place in the luminal leaflet of the Golgi, GlcCer synthesis takes place in the cytosolic leaflet (Koybasi et al. 2004; Meyers-Needham et al. 2012). In addition, SM synthesis requires CERT-dependent non-vesicular ceramide transport, while in GlcCer synthesis ceramide is transported by vesicular transport (Thomas et al. 2017). GCS mediates drug resistance in breast and oral cancers and has a pro-survival effect (Roh et al. 2015; Kim et al. 2016). Pharmacological GCS inhibition has been tried for use in overcoming chemotherapy resistance in head, neck and hepatocellular

cancers (Liu et al. 2011; Stefanovic et al. 2016). Both GCS and CERK can turn cell resistance into apoptosis. Therefore, it would be wise to select these two for a treatment approach targeting ceramide-mediated cancer cell death.

12. CRS (cerebrosidase).
13. SPHK (sphingosine kinase): This phosphorylates the ceramide-derived sphingosine to S1P. It has two isoforms, SPHK1 and SPHK2.
    - SPHK1: This is located in the cytosol (Fekry et al. 2016). It can be secreted from the cell, but the relation of this secreted SPHK1 with physiological events is not yet clear (Lee et al. 2011; Jensen et al. 2014). In the cell, it mediates pro-survival signaling and in addition causes metastasis in the bladder, lung cancer and melanoma (Kawamori et al. 2009; Wang 2013; Zhang et al. 2014; Postepska-Igielska et al. 2015).
    - SPHK2: This is located in both the cytosol and the nucleus (White-Gilbertson et al. 2009) and provides the production of nuclear S1P. It causes HDAC1 and HDAC2 inhibition in breast cancer cells and increased telomerase stability in lung cancer cells. As a result, it shows a pro-survival effect in the cell (Hait et al. 2009; Selvam et al. 2015).
14. S1PP (S1P phosphatase).
15. SPL (S1P lyase): This breaks down S1P into ceramide. By inducing ceramide accumulation, it leads to cell death and tumor suppression in colon and gastric cancers (Trajkovic et al. 2008; Degagné et al. 2014).

Sphingolipids in Cell Physiology

Sphingolipids have a wide variety of functions. One of them is to be a structural part of cell membranes, and the other is to take a crucial part in intracellular signalling. Among the simple sphingolipids, ceramide, ceramide-1-phosphate and sphingosine are the most well-known being involved in intracellular signaling pathways. Growth, apoptosis, differentiation, proliferation, senescence, and motility can be given as examples of these functions. S1P and ceramide oppositely affect all these processes. The path the cell turns in is determined by the balance between S1P and ceramide, which are metabolically linked. Complex glycosphingolipids show a wide variety of influences on cell physiology, by acting like microbial toxin binding agent, signal transduction modulator, growth factor, cell adhesion mediator, and antigen. A cell's

choice of life or death depends on the balance of the intracellular levels of these sphingolipids, which can be metabolically transformed into each other. This balance is controlled by special enzyme series via destruction or construction. SPHK1 seems to play the most vital role among these enzymes as it increases pro-survival S1P with its activity, while lowering pro-apoptotic ceramide and sphingosine levels. SPHK1 overexpression in cell cause higher cellular growth rate and resistance to apoptosis induced by TNF-a or exogenous ceramide (Xia et al. 2000; Edsall et al. 2001). Ceramide-1-phosphate also acts as an inhibitor on S1PP, reducing ceramide recycling and preventing ceramide-induced apoptosis, thus it is anti-apoptotic (Chalfant et al. 2002; Chalfant and Spiegel 2005). The main factors determining the equilibrium between cell death and survival are the S1P and ceramide molecules and the SPHK1 and ceramide kinase enzymes.

Cellular processes in which sphingolipid species are involved:

1. S1P: Processes regulated and/or mediated by S1P are;
   - Inflammation
   - Cell survival
   - Cell migration and invasion
   - Autophagy
   - Angiogenesis and vascular maturation
   - Cytoskeleton rearrangement
   - Embryonical heart development
   - Maturation of vascular system
   - Calcium homeostasis (Spiegel et al. 2003)
   - Immunity and lymphocyte circulation
   - Tumorogenesis (Sabbadini 2006)
   - Cell growth (Spiegel et al. 2003)
   - riggering of signal transduction autocrinally (Payne et al. 2002)
   - Suppression of apoptosis (Spiegel et al. 2003)
   - Induction of cell differentiation and proliferation. Cytokines, vitamin D3, GPCR agonists, growth factors, phorbol esters and antigens cause SPHK activation. This causes the S1P level to increase. (Payne et al. 2002; Pyne and Pyne 2002). On the contrary, IP3-independent calcium mobilization and DNA synthesis are downstream effectors of S1P in intracellular pathways (Young and Nahorski 2002; Spiegel et al. 2003).

2. C1P (ceramide-1-phosphate): This shares similar functions with S1P (Chalfant et al. 2005):
   - Cell migration
   - Cell proliferation
   - Inhibition of apoptosis
   - Regulation of inflammation.
3. Ceramide: This mediates and/or regulates processes like:
   - Cell death
   - Senescence
   - Cell cycle arrest
   - Necroptosis
   - Cell differentiation
   - Autophagy
   - Mitophagy
   - Cytoskeleton rearrangement (Hannun and Obeid 2018).
4. Sphingosine: This acts similarly to ceramide (Chalfant et al. 2005). It mediates and/or regulates processes such as:
   - Apoptosis
   - Cell cycle arrest
   - Cell differentiation.
5. Dihydroceramide: This mediates and/or regulates processes such as:
   - Cell cycle arrest
   - Apoptosis
   - Inhibition of cell growth
   - Autophagy (Hannun and Obeid 2018).
6. Sphingomyelin: This mediates and/or regulates processes such as:
   - Cell growth
   - Cell adhesion (Hannun and Obeid 2018).
7. Galactosylceramide: This mediates and/or regulates processes such as:
   - Inflammation
   - HIV-1 infection (by the binding of HIV-1 gp120 to GalCer on the cell membrane).
8. Glucosylceramide: This mediates and/or regulates processes such as:
   - Multidrug resistance in cancer cells
   - Inflammation
   - Cell adhesion
   - Cell differentiation (Hannun and Obeid 2018).

9. Lactosylceramide: This mediates and/or regulates processes such as:
    - Cell proliferation
    - Cell adhesion
    - Angiogenesis
    - Generation of reactive oxygen species
    - Inflammation (Hannun and Obeid 2018).

Ceramide in Cancer Cell Death

Apoptosis

Apoptosis in the cell can be achieved in two ways: the extrinsic or intrinsic route. While the extrinsic route occurs through the activation of cell surface receptors such as TNFαR1 and Fas, the intrinsic route begins with the permeabilization of the mitochondrial outer membrane of the pro-apoptotic Bcl-2 protein family members. When surface receptors are activated in the extrinsic route, they interact with the adapter protein called FADD and caspase 8 is activated. This activates the Bcl-2 family member Bid. The activated Bid translocates to the outer membrane of the mitochondria and initiates the intrinsic route. Consequently, both apoptosis pathways converge on caspases. Bid can also be activated by cathepsin D, the lysosomal aspartate protease, and ASMase activity is required for the activation of cathepsin D by TNFα (Heinrich et al. 1999; Heinrich et al. 2004). Due to ceramide's effects on Bcl-2 family proteins, it is predicted that it causes apoptosis through the mitochondrial (intrinsic) route (Kim et al. 2001; Chalfant et al. 2002; Ruvolo et al. 2002; Von Haefen et al. 2002; Kolesnick and Fuks 2003; Scorrano et al. 2003; Stoica et al. 2003; Kashkar et al. 2005). Ceramide binds directly to cathepsin D and converts the enzyme to its active isoform, which causes Bid to be activated. All of these findings show that ceramide has important effects in the regulation of Bid processes. In addition, ceramide is thought to affect apoptosis with its biophysical properties and effects on membrane organization. Endogenous ceramide can accumulate in lipid rafts. These ceramide-rich membrane regions facilitate the functioning of the receptor-mediated extrinsic pathway by providing localization for Fas receptors, etc. (Grassme et al. 2001; Kilkus et al. 2003). All of these findings show us that ceramide is directly linked to apoptosis. As a result, ceramide is very important in terms of how various cancer cells respond to stimuli for apoptosis induction.

Ceramide in Tumor Suppression and Cell Death

Cellular stress increases ceramide production through the activity of CERS1-6 and SMases, which results in the death of cancer cells and tumor suppression. In radiation-induced apoptosis, ceramide accumulates in the outer membrane of mitochondria and induces BAX uptake into mitochondria. As a result, the outer membrane permeability of mitochondria increases, caspases are activated and cell death occurs (Siskind et al. 2008; Chang et al. 2015; Chipuk et al. 2012). In chemotherapy, lysosomal related transmembrane protein 4B (LAPTM4B) stabilizes the lysosome membrane by regulating ceramide transport from late endosomal organelles, and apoptosis occurs through ceramide-mediated caspase 3 activation (Blom et al. 2015). To give an example for this, the binding of phosphatase 2A inhibitor (I2PP2A) with ceramide or FTY720 (a sphingosine analog drug) in lung cancer leads to the activation of serine/threonine-protein phosphatase 2A (PP2A), allowing cancer cells to undergo receptor-dependent necroptosis (Mukhopadhyay et al. 2009; Saddoughi et al. 2013). In addition, cellular stress caused by chemotherapy causes C18 ceramide to accumulate on the outer membrane of the mitochondria. These C18 ceramides bind to the lipid form of microtubule-associated protein 1 light chain 3β (LC3B-II), leading to the aggregation of autophagosomes. As a result, this leads to lethal mitophagy through the activation of dynamin-associated protein 1 (DRP1) and mitochondrial fission (Sentelle et al. 2012; Dany et al. 2016). Tetrahydrocannabinol can induce autophagy in glioma cells by triggering the ceramide and/or dihydro-ceramide cumulation in the Endoplasmic Reticulum. Lysosomal and autophagosomal membrane permeability increase with this cumulation process, leading to apoptotic cell death subsequent cathepsin release (Saddoughi and Ogretmen 2013; Jiang and Ogretmen 2014; Hernández-Tiedra et al. 2016). Providing elongation and maturation of the early autophagosomal membranes through autophagy-related protein 9A (ATG9A) cumulation is another function of ceramide yielded from SM by ASMase activity (Corcelle-Termeau et al. 2016).

S1P in Cancer Growth and Metastasis

S1P is considered as a pro-survival lipid (Maceyka et al. 2002; Hla, 2004; Taha et al. 2006). Five different G protein-coupled S1P receptors (S1PR1-5) have been identified in mammalian cell membranes. SPHK1 and SPHK2 convert ceramide to S1P, leading to cancer growth and metastasis in two different ways: S1PR dependent and S1PR independent.

S1PR-dependent oncogenic S1P signaling

S1P produced after SPHK1 activation in the plasma membrane combines with S1PR1-5 to provide oncogenic signaling (Pitson et al. 2003). S1P is secreted from breast cancer cells with the effect of estradiol by the help of ATP-binding cassette sub-family C member 1 (ABCC1), ATP-binding cassette sub-family G member 2 (ABCG2) (Takabe et al. 2010) or protein spinster homologue 2 (SPNS2) (Hisano et al. 2012). Thus begins autocrine and paracrine oncogenic signaling. S1PR signaling drives inhibition of PP2A and BAX caspase 3 signal and induction of survival autophagy. In other words, it functions as apoptosis inhibition, migration induction and cell proliferation induction, which causes drug resistance seen in chemotherapy. (Hisano et al. 2012; Takabe et al. 2010). S1PR-dependent oncogenic signaling and its results are shown in Figure 8.2.

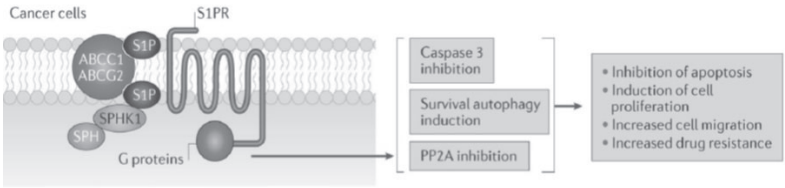

Figure 8.2: S1PR-dependent S1P signaling (Ogretmen 2018)

S1P signalisation through S1PR1 has been reported in B cell-like diffuse large B cells and T lymphoblastic lymphoma cells. S1PR2 is associated with S1P signaling in AML, melanoma and bladder or cervical cancer cells. Activation of S1PR3 leads to lung cancer progression and metastasis in mouse models. Increased S1PR4 expression causes shorter disease-free survival in estrogen receptor-negative breast cancer, while S1PR5 signaling causes mitogenic progression in HeLa cells (Ogretmen 2018).

Secreted S1P increases tumor metastasis. SPNS2 selectively induces S1P secretion from endothelial cells. This endothelial SPNS2-dependent S1P release affects S1PR functions in cancer and immune cells, leading to a weakening of cytotoxic T-cell function, thus facilitating tumor metastasis. S1P release from lymphoid and vascular endothelial cells activates S1PR signaling in cancer cells and increases the likelihood of metastasis by inhibiting the expression of breast cancer metastasis-suppressor 1 (BRMS1). All these results show that secreted S1P enables cancer and host immune cells to communicate with each other and increases tumor metastasis. In the light of these data, it is predicted that the

use of sonepcizumab (systemic S1P inhibitor), JTE013 (cancer cell S1PR2 inhibitor), and FTY720 (cancer cell S1PR1 inhibitor) will decrease metastasis (Visentin et al. 2006; Ponnusamy et al. 2012; Liang et al. 2013; Brizuela et al. 2014; Van der Weyden et al. 2017). Lymphoid and endothelial S1P release and oncogenic effects are shown in Figure 8.3.

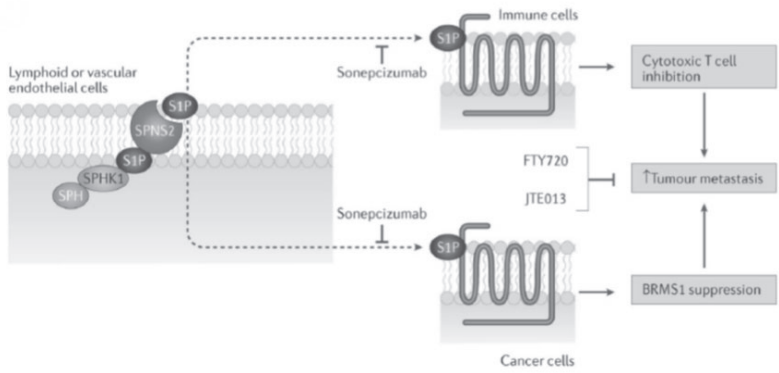

Figure 8.3: Lymphoid and endothelial cell S1P secretion (Ogretmen 2018)

S1PR-Independent Oncogenic S1P Signaling

S1PR-independent S1P shows different oncogenic signaling functions in the cytoplasm, mitochondria, and nucleus within the cell.

In the cytoplasm, S1P from SPHK1 causes induction of polyubiquitylation of receptor-interacting serine/threonine-protein kinase 1 (RIPK1) by binding directly to TNF receptor-associated factor 2 (TRAF2). Subsequently, it indirectly leads to the phosphorylation of the inhibitor of NF-κB kinase (IKK) and the degradation of NF-κB inhibitor (IκB) and enables the activation of nuclear factor-κB (NF-κB) (Alvarez et al. 2010). Again, it binds directly with peroxisome proliferator-activated receptor-γ (PPARγ) in the cytoplasm and induces tumor angiogenesis and/or metastasis by increasing PPARγ-dependent gene expression (Xiong et al. 2013; Etemadi et al. 2015; Parham et al. 2015).

S1P from mitochondrial SPHK2 binds to mitochondrial membrane prohibitin 2 (PHB2) and induces mitochondrial respiration through cytochrome oxidase (complex IV) activity. (Strub et al. 2011).

S1P originating from the nuclear membrane-located SPHK2 binds to histone deacetylase 1-HDAC1 and HDAC2 and inhibits their enzymatic effect. Thus, it prevents deacetylation of histone H3. And it induces the expression of CDKN1A encoding p21 and FOS encoding the proto-oncogene FOS (Hait et al. 2009; Selvam et al. 2015). In addition, SPHK2-derived S1P binds to telomerase reverse transcriptase (TERT), which is the catalytic subunit of telomerase in the nuclear membrane, preventing the degradation of TERT and thus inhibits telomere damage and senescence (Selvam et al. 2015). Rapid TERT degradation caused by the inhibition of SPHK2 (either by genetic loss or pharmacological inhibition) can provide tumor suppression by causing severe telomere damage and rapid senescence (Selvam et al. 2015). S1P oncogenic signaling pathways independent of intracellular S1PR are summarized in Figure 8.4.

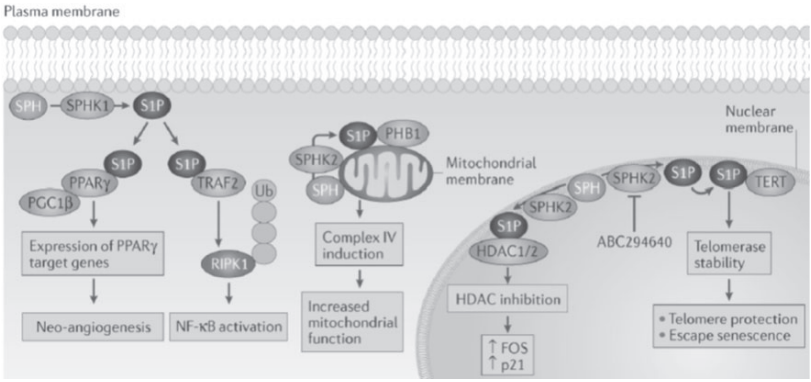

Figure 8.4: S1PR-independent oncogenic S1P signaling (Ogretmen 2018)

Sphingolipid Usage in Cancer Therapy

Chemo- and radiotherapy induce ceramide production by causing cellular stress, and they show some tumor suppression effects through the subsequent pro-cell death mechanisms. Conversely, the increased conversion from ceramide to S1P within the cell leads to chemo- and radiotherapy resistance. Therefore, understanding the intrinsic mechanisms of ceramide and S1P will provide us with new tools in fighting cancer. It is a fact that ceramide induces apoptosis and S1P leads the cell to cancer pathogenesis. Therefore, providing high ceramide levels in cancer cells and preventing S1P formation and accumulation are a desired tumor suppressor treatment approach in cancer treatment.

There are various studies showing the effects of sphingolipids on cell response to chemo- and radiotherapy. For chemotherapy, for example, daunorubicin provides apoptosis in human p388 leukemia and U937 histiocytic lymphoma cells via de-novo ceramide synthesis (Bose et al. 1995). In another phase 2 study, it was shown that the effects of gemcitabine plus doxorubicin combination therapy were stronger when intracellular CERS1-generated C18 ceramide levels were increased in recurrent head and neck cancers (Deng et al. 2008; Saddoughi et al. 2011; Senkal et al. 2007). In order to achieve apoptosis with radiotherapy, there should be signalisation caused by mitochondrial ceramide biogenesis originating from CERS activation. Radiation-induced ceramide not only causes cancer cell apoptosis, but also leads to gastrointestinal toxicity by generating gastrointestinal stem cell death (Rotolo et al. 2012).

One of the biggest problems in cancer treatment is drug resistance against chemotherapeutic agents during treatment. The relationships between changes in sphingolipid metabolism and the development of chemotherapeutic resistance have been documented in several studies. For example, conversion of ceramide to glucosylceramide by GSC leads to drug resistance in many cancer types (Liu et al. 2008). SPHK1/S1PR signaling protects cancer cells from chemotherapy-induced apoptosis. SPHK1 overexpression causes the development of resistance against cetuximab in colorectal carcinoma cells (Rosa et al. 2013). Similarly, in chronic myeloid leukemia cells, SPHK1 overexpression causes the development of resistance against imatinib by inhibiting PP2A through S1PR2 activation (Salas et al. 2011).

Therefore, it seems like a smart approach to adjust sphingolipid metabolism in order to overcome chemotherapeutic resistance. Increased gluCer and/or S1P levels lead to the development of resistance to cancer therapy. Therefore, GSC and SPHK1/2 can be targeted to overcome drug resistance and the levels of gluCer and S1P, the final products of these enzymes, can be used as markers in determining chemotherapy resistance.

Sphingolipids, besides mediating the effects of chemo- and radiotherapy, also regulate antitumor functions in many immune cell types (Fang et al. 2017). For example, STAT3-induced S1PR1 signaling increases malignant progression in both cancer and immune cells (Lee et al. 2010). S1P released from dying cells acts as a beacon that leads to the recruitment of macrophages and increases the phagocytosis of apoptotic cells (Luo et al. 2016). Therefore, it seems that new antitumor immunotherapy approaches can be developed by targeting S1P signaling.

There are several approaches for the usage of sphingolipids in cancer therapy. One of these approaches is to use exogenous ceramide analogs while another is to use ceramide metabolism inhibitors. Enzymes involved in sphingolipid metabolism such as SM synthases, CDases, CK and GSC also appear as possible therapeutic targets. In many different studies, it has been shown that apoptosis induction and the following tumor suppression can be achieved by down-regulation or inhibition of the activity of acidic-CDase and CK (Samsel et al. 2004; Mitra et al. 2007; Morales et al. 2007; Holman et al. 2008). One of the main goals is to find more selective and more active ceramide analogs or sphingolipid metabolism inhibitors and then make their usage available in the clinic for cancer treatment. Some ceramide analogs and sphingolipid metabolism inhibitors, developed based on this approach follow, although most of them are still preclinical and phase 1-2 studies. As an example of ceramide analogs; Pyridinium ceramide, which targets mitochondria, is tried in head and neck squamous cell carcinoma (HNSCC), lung, colon and breast cancers, 4,6-diene-ceramide in breast cancer and C16-serinol in neuroblastoma. Among the sphingolipid metabolism inhibitors being tested; A-CDase inhibitor B13 in the colon and HNSCC, neutral/alkaline ceramidase inhibitor D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol (D-MAPP) in squamous cell carcinoma, GCS inhibitor PPMP/PPPP (1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) in solid tumors, a sphingosine kinase inhibitor dimethylsphingosine in leukemia, colon, and breast cancers and Safingol (Lt-dihydro-sphingosine) in solid tumors can be given as examples (Ogretmen 2018).

All of these studies show that the induction of ceramide production or the inhibition of S1P metabolism or signalisation pathways is an innovative cancer therapy approach.

Conclusion

Comprehending S1P and ceramide's intrinsic mechanisms will provide us with new tools in fighting cancer. It is a fact that ceramide induces apoptosis and S1P leads the cell to cancer pathogenesis. Therefore, providing high ceramide levels in cancer cells and preventing S1P formation and accumulation are a desired tumor suppressor treatment approach in cancer treatment. One of the main goals is to find more selective and more active ceramide analogs or sphingolipid metabolism inhibitors and then make their usage available in the clinic for cancer treatment. A variety of genetic models focusing on sphingolipid

metabolism's main enzymes can be constructed nowadays. Thus, the roles of these intermetabolites and enzymes in cancer biology and treatment can be examined. This has opened new doors for us to develop innovative and structure-function-based anticancer drugs.

Keywords: *Sphingolipid, apoptosis, ceramide, S1P, oncogenic signaling*

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CHAPTER NINE

GLYCOLIPID DISORDERS

ÖZLEM SEZER

Introduction

Sphingolipidoses are inherited disorders with several mutations. Mutations seen in these disorders, impair the functions of enzymes involved in lysosomal sphingolipid degradation. The accumulation of non-degradable lipids in lysosomes results in fatal pathological phenotypes.

Glycosphingolipidoses and Sphingolipidoses

Sphingolipidoses are a class of lysosomal storage disorders with genetic heterogeneity. Sphingolipid degradation causes sphingolipid storage disorders (Wasserstein and McGovern 2008) (Figure 9.1). The underlying genetic mutations cause insufficient activity in specific enzymes, and so complex lipid substrates are accumulated in tissues (Table 9.1). The very severe forms of sphingolipidoses cause neuropathy, resulting in death in early infancy. The milder forms do not exhibit neuropathy or appear at later ages with a longer life expectancy. The intermediate forms usually have a mild and non-neuronopathic phenotype, but they develop into more severe phenotypes, including neuropathy. Sphingolipidoses are classified as rare disorders, but the actual numbers of cases are probably higher than the official figures because the right diagnosis often requires enzyme assays and genetic tests (Arenz 2017).

Sphingolipids are present in all cells, but not all cells are equally affected by genetic defects that cause sphingolipidoses (Arenz 2017). Enzyme activities vary significantly in healthy individuals. Enzyme activity of 20-30% generally does not cause sphingolipidoses, but if it falls below a certain threshold, the related sphingolipid begins to accumulate. The rate of sphingolipid accumulation in a cell depends on its synthesis

and degradation rates (Arenz 2017). Most patients exhibit clinical manifestations of a neuronopathic form in the absence or deficiency of the enzyme. However, they develop a nonneuronal phenotype at later ages with milder clinical findings and a 5-10% higher residual enzyme activity than in healthy individuals (Arenz 2017).

Except for the adult forms of Fabry and Gaucher diseases, enzyme deficiency results in death in early infancy. Except for the X-linked Fabry disease, all of them are autosomal recessive disorders (Schulze and Sandhoff 2011).

Table 9.1: Sphingolipid Storage Disorders

DISORDER	ENZYME/ SUBSTRATE	AGE OF ONSET / CLINICAL CHARACTERISTICS	GENOTYPE
GM1 Gangliosidosis-Generalized Gangliosidosis			
Infantile	β-galactosidase/ GM1 gangliosidosis	Infantile form/ Hepatosplenomegaly (HSM) Progressive psychomotor retardation Tonic-clonic seizure A cherry-red spot in the macula Dysostosis	GLB1 Mutations specific to some families
Juvenile	β-galactosidase/ GM1 gangliosidosis	Late infantile or juvenile form/ Progressive psychomotor retardation	
Adult	β-galactosidase/ GM1 gangliosidosis	Late-onset, adult, chronic form/ Slowly progressing dementia	
GM2 Gangliosidosis			
Tay-Sachs (Ganglioside lipidosis)	Hexosaminidase A/ GM2 gangliosidosis	Infantile form/ Progressive neurodegeneration A cherry-red spot in the macula Excessive startle reflex Blindness	HEXA >90 Acute infantile phenotype 35% enzymatic, In non-Jewish populations R247W and R249W "pseudodeficiency allele"

			95% of all mutant alleles in Ashkenazi Jewish populations (G269S, 1277insTATC and 1421+1G>C)
Late-onset Tay–Sachs	Hexosaminidase A/ GM2 gangliosidosis	Childhood-Young adult/ Dysarthria, dementia, ataxia	HEXA
Sandhoff	Hexosaminidase A/B GM2 gangliosidosis	Infantile/ Progressive neurodegeneration Hepatosplenomegaly Similar to Tay-Sachs, but progresses much faster	HEXA/B
Krabbe disease	Galactocerebrosidase Galactocerebroside	Infantile/ Loss of myelin Mental retardation Accumulation of globoid cells in the white matter Irritability Seizure, 2y exitus	GALC/*PSAP* 809G> A mutation causes late-onset disease
Fabry disease (Glycolipid lipidosis)	α Galactosidase A/ Trihexosylceramide	Early childhood, Adult/ Angiokeratoma Renal failure Limb pain	GLA
Gaucher disease (Cerebroside lipidosis)			
Type 1	Glucocerebrosidase/ Glucocerebroside	Childhood, Adult/ Hepatosplenomegaly Erosion of long bones Foam cell formation	GBA/*PSAP* 90-95% in Ashkenazi Jewish populations (N370S, L444P, 84insG and IVS2+1)
Type 2	Glucocerebrosidase/ Glucocerebroside	Infantile/ HSM Erosion of long bones Foam cell formation	
Type 3	Glucocerebrosidase/ Glucocerebroside	Infantile/ Progressive neurodegeneration	
Niemann-Pick (Sphingomyelin lipidosis)			
Type A	Sphingomyelinase/ Sphingomyelin	Infantile/ HSM Mental retardation	SMPD1
Type B	Sphingomyelinase/ Sphingomyelin	Early childhood, Adult/ Progressive HSM Infiltrative lung disease	

Type C	Sphingomyelinase/ Sphingomyelin	Juvenile or adult	NPC1–NPC2
Farber's	Ceramidase/ Ceramide	Infantile/ Progressive pain in joints Hoarse voice Nodules under the skin Mental retardation Exitus in a few years	ASAH
Metachromatic leukodystrophy-MLD (Sulfatide lipidosis)			
Infantile	Arylsulfatase A/ Sulfatide	Infantile/ Mental retardation No deep tendon reflexes (DTR) Demyelination Exitus in the first decade	ARSA/*PSAP* two pseudodeficiency alleles
Juvenile	Arylsulfatase A/ Sulfatide	Childhood, Young adult/ Gait abnormalities, Mental deterioration, and emotional disturbances	
Adult	Arylsulfatase A/ Sulfatide	After the second decade/ Emotional disorders Psychosis	

Source: Wasserstein and McGovern (2008)

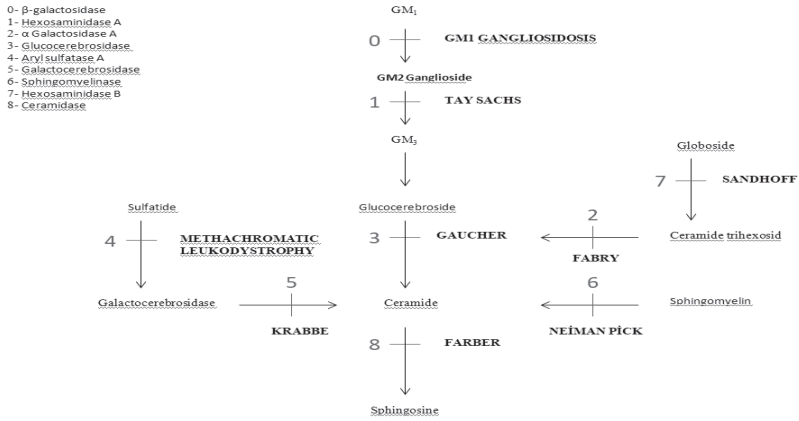

Figure 9.1: Defective activities causing sphingolipid storage disorders (Schulze and Sandhoff 2011)

GM_1 Gangliosidosis (Generalized Gangliosidosis)

GM1 gangliosidosis (generalized gangliosidosis) is caused by mutations in the GLB1 gene located at 3p21.33 of chromosome 3, which encodes the beta-galactosidase enzyme. Low enzyme activity leads to the accumulation of toxic gangliosides in body tissues, especially in the central nervous system. GM1 gangliosidosis is a rare panethnic lysosomal storage disorder, which is clinically characterized by a wide range of variable neurovisceral, ophthalmological, and dysmorphic features. The incidence of GM1 gangliosidosis is about 1 in 100.000-200.000 live births (NINDS; Orphanet; Schulze and Sandhoff 2011; Tonin 2019).

There are three types of GM1 gangliosidosis, depending on the age of onset.

Type 1 (infantile form) is the most severe and rapidly progressing form of GM1 gangliosidosis, appearing in the first 6 months of life. It is characterized by neurodegeneration, seizures, hepatosplenomegaly, coarse facial features, skeletal abnormalities, eburnation, muscle atrophy, exaggerated startle response, and gait abnormalities. About half of the cases have a cherry-red spot in the macula. The infantile form may cause deafness and blindness until the age of one year and results in death from cardiac complications or pneumonia by the age of three years (NINDS; Orphanet; Schulze and Sandhoff 2011; Tonin 2019).

Type 2 (late infantile/juvenile form) GM1 gangliosidosis appears between 7 months and 3 years of age. It is characterized by delayed motor and cognitive development, ataxia, seizures, dementia, and speech disturbances (NINDS; Orphanet; Schulze and Sandhoff 2011; Tonin 2019).

Type 3 (late-onset/adult/chronic form) GM1 gangliosidosis starts between 3 and 30 years of age. It is characterized by dystonia. The severity of Type 3 depends on the level of beta-galactosidase activity. Muscle atrophy and neurological complications are less prevalent and progress more slowly in Type 3 than in Types 1 and 2. Some cases of Type 3 show symptoms of corneal clouding and muscle contraction. Angiokeratomas may occur in the lower part of the body. The size of the liver and spleen is within normal limits (NINDS; Orphanet; Schulze and Sandhoff 2011; Tonin 2019).

GM1 gangliosidosis can be hard to diagnose because it has a broad clinical spectrum. Clinical suspicion is based on the symptoms of coarse

facial features, hypertrophy, a cherry-red spot in the macula, visceromegaly, dysostosis, and psychomotor retardation. A peripheral smear test and oligosaccharide screen are the first tests to be performed. The bone marrow examination shows Gaucher-like foam cells. The definitive way of diagnosing GM1 gangliosidosis is by performing β-galactosidase enzyme assays or GLB1 molecular genetic testing (NINDS; Orphanet; Schulze and Sandhoff 2011; Tonin 2019).

Prenatal and preimplantation genetic diagnosis is possible in the case of a family history of GM1 gangliosidosis. Beta-galactosidase activity in chorionic villus (CVS) cells or GLB1 molecular activity in amniotic fluid cells may also be analyzed for diagnosis.

GM1 gangliosidosis is an autosomal recessive disorder, and therefore, family members afflicted with any form of it should seek genetic counseling (NINDS; Orphanet; Schulze and Sandhoff 2011; Tonin 2019).

The prognosis of GM1 gangliosidosis depends on the type. While Type 1 results in death within the first year of life, Type 3 has a variable prognosis (NINDS; Orphanet; Schulze and Sandhoff 2011; Tonin 2019).

Tay-Sachs Disease (Ganglioside Lipidosis)

Tay-Sachs disease is caused by mutations encoding the alpha subunit in the HEXA gene located on chromosome 15q23-24, which encodes the hexosaminidase A enzyme. It is characterized by the accumulation of GM2 ganglioside due to enzyme deficiency. Its incidence is 1 in every 320.000 live births (Cachon-Gonzalez et al. 2018).

There are three variants of Tay-Sachs disease, depending on the age of onset (Cachon-Gonzalez et al. 2018).

In the infant form (Type 1), the age of onset is between 3 and 6 months. Usually, the first symptom of Type 1 is exaggerated reactions to loud sounds. Psychomotor retardation, which manifests itself with hypotonia, amorosis and megalencephaly, develops after 8 months of age. There may be a cherry-red spot in the macula; however, it is not specific. Muscular weakness progresses, resulting in paralysis. Type 1 leads to death by causing a decerebrative status in childhood. Leucocytes and fibroblast cultures (skin biopsy) have either very low or no hexosaminidase A enzymatic activity. Type 1 resembles atypical Friedreich's disease, with

the exception of heart symptoms and bone symptoms such as scoliosis (Cachon-Gonzalez et al. 2018).

The juvenile form (Type 2) starts between 2 and 6 years of age. The characteristic symptoms are behavioral disorders, locomotor ataxia and progressive mental function impairment. It turns into a state of decerebration, resulting in death at around 15 years of age. It shows more pronounced hexosaminidase A enzymatic activity than Type 1. The juvenile spinal amyotrophy is similar to Kugelberg-Welander syndrome. Type 2 may or may not affect mental functions and behaviors (Cachon-Gonzalez et al. 2018).

The adult/chronic form (Type 3) may begin at about ten years of age. However, it often goes undiagnosed until adulthood. It has two different clinical forms (Cachon-Gonzalez et al. 2018).

In populations with high incidence, heterozygous individuals should be screened for Tay-Sachs. Prenatal and preimplantation diagnosis is available in and recommended for such populations. Tay-Sachs disease is autosomal recessive.

Fabry Disease (Glycolipid Lipidosis)

Fabry disease is a glycosphingolipid metabolism disorder with mutations in the GLA gene (Xq21.3-q22) in which the deficiency or complete absence of lysosomal alpha-galactosidase A activity is seen. Cellular lysosomal accumulation of globotriaosylceramide (Gb3) is seen due to low enzyme activity (Arends 2017).

Fabry disease (FD) is an inherited lysosomal storage disorder presenting itself progressively and multi-systematically with the incidence of 1 in every 80.000 live births. Specific neurological, cutaneous, cardiovascular, renal, cochleovestibular, and cerebrovascular signs exist in the disease (Arends 2017).

Fabry disease manifests itself in a broad spectrum from mild to severe. Its clinical signs are mild in heterozygous females whereas they are severe in hemizygous males having no residual alpha-galactosidase A activity. The characteristic signs seen in Fabry disease are pain, angiokeratoma, proteinuria, renal failure, cardiomyopathy, arrhythmia, loss of hearing, transient ischemic attacks and strokes. Also, female patients may exert mild to severe symptoms depending on the type of mutations. The initial

symptom of FD is pain, presenting itself as chronic or episodic. Burning and tingling sensations are seen in chronic pain whereas only a burning sensation is seen in episodic pain. Pain may go away in adulthood. Individuals with Fabry disease may develop anhidrosis or hypohidrosis, which causes heat and exercise intolerance, as well as organ failure with age. Multisystemic organ involvement complications of FD cause untreated men and women to have a 10- and 20-year lower life expectancy than the general population, respectively (Arends 2017).

An accurate diagnosis requires the demonstration of enzyme deficiency in hemizygous males. However, molecular testing is recommended for females due to random X-chromosomal inactivation. Fabry disease is an X-linked disorder. Genetic counseling is very difficult in atypical, late-onset variants. Enzyme assays and DNA tests in chorionic villi or cultured amniotic cells (prenatal diagnosis) can only be conducted on male fetuses. Preimplantation diagnosis is possible (Arends 2017).

Sandhoff Disease

The hexosaminidase A beta subunit and hexosaminidase B deficiency cause Sandhoff disease. The enzymatic defect results in GM2 ganglioside accumulation in neuronal and peripheral tissues. HEXB gene localization is defined on chromosome 5q13. Sandhoff disease is a lysosomal storage disorder with a prevalence of about 1 in 130.000. The disease progresses with central nervous system degeneration (Tavasoli et al. 2018).

The symptoms in Sandhoff disease, as in Tay-Sachs disease, are an increment in head circumference and the startle response, vision loss, progressive motor and mental deficiency, and a macular cherry-red spot. Individuals with Sandhoff disease may have a doll-like facial appearance. Sandhoff disease is asymptomatic in the first 3-6 months of life, but then appears and progresses rapidly with hepatosplenomegaly, and recurring respiratory infections. Spinocerebellar ataxia or dystonia may occur in later-onset or adult cases. The disease may or may not affect intellectual functions (Tavasoli et al. 2018).

The disease is an autosomal recessive inherited trait with poor prognosis and death by 4 years of age (Tavasoli et al. 2018).

Gaucher Disease (Cerebroside Lipidosis)

Mutations in the GBA gene (1q21) and/or PSAP gene respectively, coding glucocerebrosidase and saposin C, cause Gaucher disease (GD) (Stirnemann et al. 2017).

Gaucher disease is one of the lysosomal storage diseases. It has three main forms – Types 1, 2, 3, a fetal form and a variant form, also called Gaucher-like disorder in which cardiac involvement is seen. Gaucher disease prevalence and annual incidence are 1 in 100.000 and 1 in 60.000, respectively. However, it can be as high as 1 in 1.000 in Ashkenazi Jewish populations (Stirnemann et al. 2017).

Splenomegaly, hepatomegaly, osseous pain, osteonecrosis, pathological bone fractures and cytopenia are seen in Type 1 (the chronic and non-neurological form). 90% of Gaucher cases present as Type 1 (Stirnemann et al. 2017).

The acute and neurological form called Type 2 is associated with organomegaly. It has an early onset and develops rapidly progressive brainstem dysfunction, resulting in death before 2 years of age (Stirnemann et al. 2017).

The subacute neurological form called Type 3 manifests itself in childhood or adolescence. Systemic signs in Type 1 together with progressive encephalopathy are characteristic features of Type 3 (Stirnemann et al. 2017).

Fetal movements are decreased or absent in the fetal form.

Progressive cardiovascular calcifications such as on the aorta and/or mitral valves are seen in Gaucher-like disease – ophthalmoplegia.

Glucosylceramide accumulates in reticuloendothelial system cells, such as liver, spleen, and bone marrow (Gaucher cells) cells in glucocerebrosidase deficiency (Stirnemann et al. 2017).

Gaucher disease is diagnosed based on leukocyte glucocerebrosidase levels in the circulation. Then diagnosis is confirmed with genotyping. The disease is an autosomal recessive inherited trait.

Type 1 has a good prognosis. Type 2 results in death before the age of 2. Type 3 results in death within a few years with no specific treatment (Stirnemann et al. 2017).

Niemann-Pick Disease (Sphingomyelin Lipidosis)

Niemann-Pick disease (NPD) is an inherited metabolic disorder also known as lipid storage disease. It is characterized by excessive lipid accumulation in the lung, liver, spleen, brain and bone. NPD's neurological symptoms are ataxia, muscle atrophy, brain degeneration, allodynia, spasticity, and speech impediment, as well as dysphagia, eye palsy, learning problems, hepatosplenomegaly, corneal clouding, and a cherry red halo around the retina. Three types of NPD are defined – Types A, B, and C (Santos-Lozano et al. 2015). SMPD1 gene mutations are the cause of Type A and Type B while NPC1 and NPC2 gene mutations are the cause of Type C1 and C2 respectively.

Type A (classical infantile form) is the most severe form of NPD that begins in early infancy with a high prevalence in Jewish populations. Its symptoms are weakness, hepatosplenomegaly, lymphadenopathy, and brain injury at the age of six months. Individuals with type A seldom survive beyond 18 months of age.

Type B (visceral form) begins in the juvenile period. Liver, lung and spleen involvement without brain involvement is seen in Type B.

In Types A and B, toxic quantities of sphingomyelin accumulate in each cell due to insufficient sphingomyelinase activity. The histochemical property of Types A and B is that they contain foam cells. Individuals with Type A have 5%, and those with Type B have 5-10% lower ASM activity than the general population. Individuals with Types A and B have the same quantities of SM in their organs, whereas those with Type B have very little or no lipid accumulation in their MSS (Santos-Lozano et al. 2015).

Type B, caused by a deficiency of NPC1 or NPC2 proteins, can occur in the early stages of life and develop in young people or adults. Individuals with Type B exert extensive brain damage that results in a lack of up and down eye movements, progressive vision and hearing loss, moderate hepatosplenomegaly, ataxia and dysphagia.

Brain and visceral organ involvement can be seen in Type C1 that may be developed at any age. Type C2 usually shows pulmonary involvement; although similar to type C, it is more severe (Santos-Lozano et al. 2015).

Farber Disease

Mutations in the ASAH1 gene (8p22), encoding acid ceramidase (ACDase) cause Farber disease. ACDase is localized in the lysosome and catalyses the reaction of ceramide to sphingosine. ACDase inactivity causes ceramide to accumulate in many tissues (Ehlert et al. 2007).

Farber disease is a subcutaneous tissue disease characterized by the painful and progressive deformation of joints, subcutaneous nodules, and progressive hoarseness due to laryngeal involvement, and different phenotypes with respiratory and neurological involvement, and clinical signs (Ehlert et al. 2007).

The clinical manifestation of Farber disease varies from patient to patient.

Clinical manifestations of the classical phenotype (at around 3-6 months of age) are pain and swelling in the hands and feet, pronounced subcutaneous nodules on stiff joints and pressure points, and progressive hoarseness, ending up with aphonia due to vocal cord infiltration, as well as, cardiac, pulmonary, and neurological defects, progressive neurological dysfunction, seizures, paraparesis, and developmental delay. The neonatal type, the most severe form of Farber disease, is characterized by hydrops fetalis, hypotonia, and developmental failure, as well as rapid neurological deterioration, and granulomatous infiltrations and megaly in the liver, spleen, and lungs. The classical phenotype also has milder forms that do not present neurological defects and have a longer life expectancy (Ehlert et al. 2007).

For diagnosis, clinical findings with laboratory findings are necessary. Peripheral leukocytes and cultured lymphoid cells or skin fibroblast ACDase activity as well as cultured cell ceramide concentration are used as laboratory findings. Molecular genetic tests for the identification of mutations in the ASAH1 gene help to improve diagnostic accuracy (Ehlert et al. 2007).

Only those families presenting known disease mutations are candidates for a prenatal diagnosis that can be done by DNA testing or alternatively,

by the determination of ACDase activity in amniotic fluid cell cultures or chorionic villi (Ehlert et al. 2007).

Krabbe Disease

GALC gene (14q31) mutations cause Krabbe disease. The GALC gene encodes galactocerebrosidase, an enzyme localized in the lysosome. This enzyme catalyzes the hydrolysis of galactosylcerebroside and galactosylsphingosine to galactose. Accumulation results in oligodendrocytic apoptosis and central/peripheral nervous system demyelination. Krabbe disease at the end leads to deterioration in the white matter of both the peripheral and central nervous system. It has three forms: infantile, late infantile/juvenile, and adult (Graziano and Cardile 2015). Except for GALC gene mutations, prosaposin (PSAP) gene (10q21-q22) mutations may cause the infantile form, though rarely. This gene encodes saposin A (sphingolipid activator protein) which is required for GALC activity (Graziano and Cardile 2015).

The prevalence of Krabbe disease in Northern Europe is 1 in 100.000, while its worldwide incidence is 1 in every 100.000 live births. 85-90% of cases from Europe are the infantile form (the most common form) (Graziano and Cardile 2015).

The onset age of the infantile form is 2 to 6 months and it develops in three stages.

- The first stage is characterized by irritability, muscle stiffness, poor head control, eating difficulties, fever attacks and developmental delay.
- The second stage is characterized by opisthotonos, myoclonic seizures, developmental regression, vision deficits, and hypotonic episodes.
- The third stage is characterized by hypotonia, blindness, and deafness. Individuals in the third stage fall into a vegetative state and generally die before 2-3 years of age.

The late infantile/juvenile and adult forms are seen between 1 and 8 years and after 8 years of age, respectively. They present a variety of symptoms and disease progression which are mostly slower in older age. The late infantile/juvenile form has symptoms similar to those of the infantile form. However, the adult form presents itself with weakness, gait abnormalities, paresthesia, hemiplegia, and/or vision loss. The late

infantile/juvenile form has varying cognitive regression, but the adult form generally does not.

Abnormalities in nerve conduction velocities (slower), in electroencephalogram and in white matter as well as an enzyme assay-GALC deficiency in leukocytes or cultured fibroblasts are used for diagnosis. In the brain, MRI demyelination, gliosis, late-stage cerebral atrophy and cerebral calcifications can be seen. Histologically, the white matter contains characteristic globoid cells. Mutation analysis confirms the diagnosis (Graziano and Cardile 2015).

Enzyme assay or mutation analysis can be used for prenatal diagnosis in families at risk. Knowing the family-specific disease-causing mutations allows for preimplantation genetic diagnosis.

Krabbe disease is autosomal recessive. At-risk couples should be informed that the chances of them having a child with the disorder is 25%.

The infantile form (<2-3 years of age) mostly results in neurodegeneration and early death. The late infantile/juvenile form becomes fatal 2-7 years after the symptoms appear. Individuals with the adult form can survive many years following the onset of symptoms.

Metachromatic Leukodystrophy (Sulfatide Lipidosis)

Metachromatic leukodystrophy (MLD) is a rare autosomal recessive inherited lysosomal storage disorder, that causes the gradual deterioration of motor and neurocognitive functions. It is characterized by the accumulation of intralysosomal sulfatide in various tissues. It is caused by deficiency of the saposin B and arylsulfatase A enzymes. It is an autosomal recessive disorder with a prevalence of 1 in 1.000.000 (Jabbehdari et al. 2015).

Most individuals with MLD have mutations in the ARSA gene, which encodes the arylsulfatase A enzyme. Arylsulfatase A helps with the degradation of sulfatides in lysosomes. Individuals with MLD have mutations in the PSAP gene as well. The mutations in the ARSA or PSAP genes reduce the sulfatide degradation capacity, resulting in their accumulation in the cell. Excess sulfatides are harmful to the nervous system. The accumulation of sulfatides destroys the myelin-producing cells, resulting in nervous system dysfunctions. Some cases of MLD with very low arylsulfatase A activity do not show signs of metachromatic

leukodystrophy, which is called pseudo-arylsulfatase A deficiency (Jabbehdari et al. 2015).

Metachromatic leukodystrophy has three forms: late infantile, juvenile, and adult (Jabbehdari et al. 2015).

The late infantile form generally appears 12-20 months after birth and results in death by five years of age. After the age of one, it is noteworthy that infants develop gait abnormalities and a tendency to fall. Intermittent pains in the extremities are remarkable in the late infantile form. Other striking features are progressive mental deterioration, loss of vision and skills, dysphagia, convulsions, and dementia before two years of age. Individuals with the late infantile form also develop muscle atrophy and eventual loss of ability to walk.

The juvenile form appears between the ages of 3 and 10 years and results in death 10 to 20 years following the onset of the symptoms of progressive mental deterioration, reduced academic achievement, ataxia, seizures, and dementia.

The adult form appears after 16 years of age. Its symptoms are ataxia, seizures, tremor, reduced concentration, mental problems, and dementia. It generally results in death within 6 to 14 years of the onset of symptoms.

Metachromatic leukodystrophy appears at an early age and there is rapid deterioration. It causes motor disturbances and cognitive impairments and damages the white matter significantly. MRI of the brain can show signal hyperintensities with white and gray matter involvement. Sulfatide accumulation in the kidneys, gall bladder and central and peripheral nervous system can cause organ dysfunction, which is an important aspect of MLD (Jabbehdari et al. 2015).

Current Treatments for Sphingolipidoses

There is a growing body of research on treatments addressing the underlying metabolic defects of sphingolipidoses. Some of these treatments are bone marrow transplantation (BMT), hematopoietic stem cell transplantation, enzyme replacement therapy (ERT), substrate reduction therapy (SRT), enzyme stabilization, pharmacological chaperones (PC) and gene therapy (Platt and Lachmann 2009; Santos and Amaral 2019).

Enzyme replacement therapy (ERT) is the most common treatment. However, one of the major challenges of ERT is that enzymes cannot

cross the blood-brain barrier. Besides, all types of ERT are temporary and require the periodic administration of therapeutic agents. The immune system may also perceive replacement enzymes as foreign. In substrate reduction therapy (SRT), glycosphingolipid synthesis is blocked by using inhibitors. However, these inhibitors may also affect other sphingolipids with undesirable off-target effects. It may cause the partial depletion of glycosphingolipids in the plasma membrane and severe side effects due to off-target interactions by inhibitors. Unlike most ERT, some molecules used in SRT can cross the blood-brain barrier. Pharmacological chaperones (PC) assist in protein folding, and therefore, can be used to treat variants of disorders caused by enzyme misfolding. However, it also has a high tendency to be off-target. Some of these limitations can be mitigated by CRISPR-Cas9 genome editing. However, harmful off-target effects resulting from CRISPR-Cas9 editing should not be overlooked. As in other techniques, a careful assessment of results requires several checkpoints at the gene and protein levels (Santos and Amaral 2019).

Allogeneic bone marrow transplantation (BMT) is used for the treatment of numerous sphingolipid storage disorders. In BMT, stem cells derived from donor bone marrow are used to reproduce the microglial cells in the brain that produce the missing enzyme (Schulze and Sandhoff 2011).

Conclusion

All sphingolipidoses, apart from the X-linked Fabry disease, are autosomal recessive lipid storage disorders. Sphingolipidoses are a class of complex lipid storage disorders rarely seen in communities with a high rate of consanguineous marriage. Using genetic assays and prenatal and preimplantation genetic methods for the diagnosis of sphingolipidoses and considering treatment options in patients with this group of genetic diseases can help to protect future generations.

Keywords: *Disorder, glycolipid, glycosphingolipidoses, sphingolipidoses*

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CHAPTER TEN

GLYCOLIPIDS AND INFECTIOUS AGENTS

SELİM GÖRGÜN

Introduction to Glycolipids

Sphingolipids are a class of lipids that serve as cell signal modulators and structural components of skin, membranes, lipoproteins, and other biomaterials (Merrill et al. 1997). Sphingolipids contain ceramide (a fatty acid amide derivative of sphingosine), sphingomyelin (a combination of ceramide with phosphorus and choline), cerebrosides (a combination of ceramide with sugars, such as glucose and galactose), ceramide oligosaccharides (linkage of a glycosidic hetero oligosaccharide to ceramide), and gangliosides (a carbohydrate and at least one sialic acid linked to ceramide). Glycosphingolipids (cerebrosides) have glucose and galactose, but not phosphoric acid. Cerebrosides are also known as membrane lipids. Glucose and galactose cerebrosides are the most common types of cerebrosides (Merrill et al. 1997).

Johann Ludwig Wilhelm Thudichum, a German scientist, was the first to discover cerebrosides towards the end of the nineteenth century (Merrill and Stevens 1989). Cerebrosides are mostly found in white and gray matter, and in nerve myelin sheaths (Merrill and Stevens 1989). Symptoms of plague, which caused the death of tens of thousands of people, are a result of the degradation of the galactocerebroside structure (Perry and Fetherston 1997). Research also shows that structural changes in cerebrosides affect human organs, resulting in diseases and infections (Perry and Fetherston 1997).

Bacterial Lipopolysaccharides

Bacterial lipopolysaccharides (LPSs) are a member of the glycolipid family, which is the highly conserved lipid part, known as lipid A. Bacterial

lipopolysaccharides are involved in the stability of the outer membrane's permeability barrier and host–pathogen interactions. LPSs are produced by numerous gram-negative bacteria (Whitfield and Trent 2014) and are found on the outer membrane of gram-negative bacteria. LPSs cover approximately 75% of the cell surface in *Escherichia coli* and *Salmonella typhimurium*. LPSs are divided into two types; smooth (S-LPSs) and rough (R-LPSs). S-LPSs are further subdivided into three parts; (1) a glycolipid anchor, called Lipid A, (2) a linker oligosaccharide (core), and (3) an O-specific polysaccharide (O-chain). On the other hand, R-LPSs (lipooligosaccharides, also referred to as LOSs) lack O-specific polysaccharide chains due to a genetic mutation or the nature of the bacteria (Casillo et al. 2019).

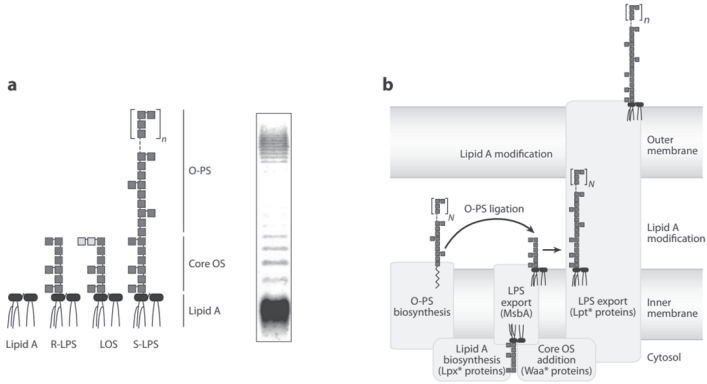

Figure 10.1: Lipopolysaccharide (LPS): a. different forms of LPS; b. biosynthesis of LPS (Whitfield and Trent 2014)

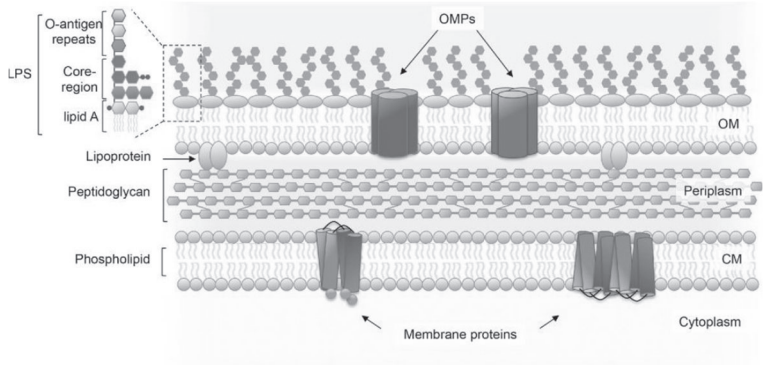

Figure 10.2: Cell wall structure of gram-negative bacteria (Maldonado et al. 2016)

Lipid A, which is the amphipathic glycolipid part of an LPS, stimulates the immune system by binding to toll-like receptor 4 (TLR-4) and also activates intracellular caspase-4 and -5 (Xiao et al. 2017). Lipid A, which is produced by different gram-negative bacteria, has various structures that cause different immune activities (Xiao et al. 2017).

Lipid A may trigger systemic inflammation resulting in tissue damage and even death. To avoid these adverse effects, some gram-negative bacteria (e.g. Yersinia Pestis) alter the Lipid-A structure (Xiao et al. 2017).

Bacteria are exposed to an environment dominated by some host or exogenous factors such as inflammatory cells and antibiotics during chronic infections. Bacterial modulation of LPS synthesis and structure, are independent of the type, bacteria, and site of infection. In *Pseudomonas aeruginosa*, alterations in LPS synthesis due to mutations also contribute to bacterial resistance during chronic infections (Maldonado et al. 2016).

Glycolipids and Viruses

Glucosylceramidase regulates the entry of influenza and other endocytosed viruses into cells. Influenza viruses are trafficked to late endosomes for fusion, and glucosylceramide (GlcCer) is involved in lipid transport along the endocytic pathway. GlcCer regulates epidermal growth factor receptor traffic along the endocytic pathway. It is mediated by the glycoproteins of enveloped viruses that enter cells through endosomes (Drews et al. 2019).

Symptomatic Zika virus (ZIKV) infection was observed about six days before the onset of neurological symptoms in most patients with Guillain-Barré syndrome (GBS) (88%). Anti-glycolipid antibodies, especially against GA1, were found in half the cases (Cao-Lormeau et al. 2016). It is also believed that anti-galactocerebroside antibodies (10%; ELISA technique) in patients with Guillain-Barré syndrome were involved in demyelination by cross-reacting with *Mycoplasma pneumoniae* bacteria molecules (Ang et al. 2002).

Numerous mammalian viruses can recognize cell-surface glycoproteins or glycolipid glycan receptors. B19 virus in humans binds globoside glycolipids of the P blood-group antigen series, especially globotetraose (Gb4), on erythrocytes and hematopoietic progenitors. Gb4 interaction induces some structural changes in the B19V capsid and allows binding to a co-receptor for viral entry. Most Coronaviruses that cause respiratory

and gastrointestinal infections also use glycans as receptors (Thompson et al. 2019).

Pharmacological up-regulation of ceramide inhibits human immunodeficiency virus (HIV) infection in TZM-b1, a derivative of the HeLa cell. This antiviral effect leads to a reduction in HIV entry and deterioration of the host membrane structure, resulting in the production of non-infective viral strains. Myr treatment also successfully suppresses hepatitis C virus (HCV) replication. In adenoviruses, it uses de novo ceramide biosynthesis to regulate host SR proteins required for pathogenesis. Both play different roles for de novo ceramide in the life cycles of flaviviruses that induce ceramide biosynthesis. While ceramide has an antiviral function against Dengue Virus, it plays a role in the production and replication of West Nile virus. (Soudani et al. 2019).

Glycolipids and Fungi

Although there are several ways to explain the functions of glycolipids in yeast, it is not fully elucidated. Thanks to their amphiphilic nature, glycolipids promote the attachment and subsequent transport of water-insoluble substrates to cells. Biosurfactants help microorganisms to adapt to the environment. Reduced surface tension properties allow glycolipids to be involved in osmotic pressure relief and microbial mobility, which helps them find a better environment for growth, reproduction, and colonization. Glycolipids are also extracellular carbon stock for fungi. The conversion of carbon to glycolipids makes it less available to other microorganisms. The antimicrobial activity allows glycolipids to launch a biological war against their competitors in the immediate environment. Therefore, glycolipids serve as secondary metabolites, although they are natural and low-molecular-weight molecules that are not involved in the central metabolism of living cells (Jezierska et al. 2018).

Yeasts produce different types of glycolipids. Of those glycolipids, sophorolipids are composed of two glucose molecules and one fatty acid. Mannosylerythritol lipids (MELs) are another glycolipid class produced in large quantities. Cellobiose lipids, as the name implies, are composed of a cellobiose residue and a fatty acid chain (Jezierska et al. 2018).

Table 10.1: Yeasts producing glycolipids (Jezierska et al. 2018, 1313)

Glycolipid	Yeast
Cellobiose lipids	*Ustilago maydis*
	Pseudozyma flocculosa
Mannosylerythritol lipids	*Pseudozyma antarctica*
	Pseudozyma aphidis
	Ustilago maydis
Sophorolipids	*Starmerella bombicola*
	Rhodotorula bogoriensis
	Candida apicola
Trehalose lipids	*Rhodococcus erythropolis*

The Effect of Glycolipids on Bacterial Infections

The immune system recognizes its antigens, and antigenic variation affects the immune regulation and autoimmune status of the individual. The memory of the immune system is a result of previous infections or an intrinsic property. In some cases, bacterial infections stimulate reactive T cell activation by glycosphingolipids, either contributing to the immune response or increasing susceptibility to autoimmune diseases (De Libero et al. 2005).

Numerous pulmonary and other pathogens bind specifically to the carbohydrate backbone in some glycolipids. For example, uropathogenic *E. coli* is actively involved in urinary system infections by binding to glycosphingolipids in urinary system epithelial cells. Infections often recur, if left untreated. It has long been known that some bacteria (*Actinomyces naeslundii, Propionibacterium granulosum*, and *Pseudomonas aeruginosa*) are more isolated from the cultures of patients with respiratory tract infections and prolonged hospital stays (Krivan et al. 1988). *Acinetobacter baumannii* is a common nosocomial infectious agent that causes multidrug-resistant infections (World Health Organization 2017). These infections are treated with colistin, which is a last-resort antibiotic that generally causes colistin-resistant strains due to high antibiotic resistance caused by modifications of the terminal phosphate parts of lipopolysaccharide derivative Lipid A, which reduces membrane electronegativity. Colistin resistance can be quickly identified using manual microtube dilution, standard MIC, and mass spectrometry. Glycolipid phenotyping (mass spectrometry) plays a crucial role in diagnosis and characterization. Recent research has reported a correlation

and specificity of more than 90% between colistin sensitivity (the standard MIC test) and glycolipid fractions (Leung et al. 2019), which results in a reduced incidence of resistant cases, length of hospital stay, and mortality.

Mycobacterium species are bacterial agents that still maintain their pathogenicity. *M. leprae* causes common demyelinating neuropathy, resulting in hand and foot injury, paralysis, and even blindness. Phenolic glycolipids (FGL) produced by *M. leprae* play an essential role in disease pathogenicity. *M. leprae* is phagocytosed by monocytes and then causes the release of monocyte chemoattractant CCL2. FGL-1 from *Mycobacterium leprae* stimulates the production of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) from infected macrophages, causing damage to mitochondria and demyelination in neurons (Dunlap and Khader 2017).

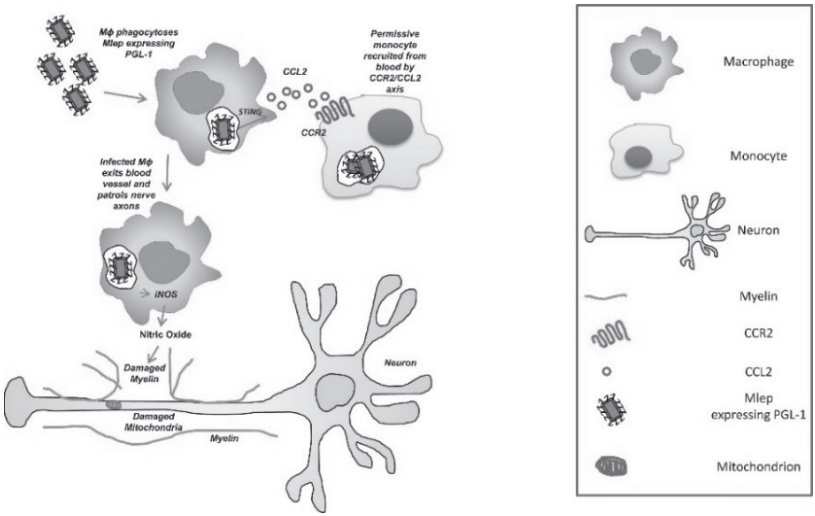

Figure 10.3: *M. leprae*- and macrophage-associated nerve demyelination (Dunlap and Khader 2017)

The MHC-associated CD1 molecules presenting lipid antigens to T lymphocytes are transmembrane glycoproteins involved in T-cell activation with glycolipid antigens from CD1 a to d. The alpha galactosylceramide and mammalian self-glycolipids, which serve as signal molecules, have a similar structure. This leads to T-cell stimulation and autoimmune reactions with foreign or modified mammalian core glycolipids (Moody and Besra 2001). In bacterial infections (*E. coli,*

Bacillus subtilis, S. aureus, and *M. bovis*), glycoprotein-specific T cells are stimulated by the infection itself or its components (liposaccharides, lipoteichoic acid, lipopeptides, etc.). Cytokines released by activated T cells contribute to the infection response or autoimmune reactions (De Libero et al. 2005). In infections, endogenous and exogenous glycolipids also activate natural killer T cells and are involved in the recognition of the bacterial cell wall (Mattner et al. 2005).

Cytotoxic T cells linked to glycolipid-specific CD1s (from a to c) are involved in the host immune response against mycobacterium in tuberculosis (Schaible and Kaufmann 2000). Macrophages are the first to meet *Mycobacterium tuberculosis* in the lung alveoli. Phenolic glycolipids stimulate macrophages and the release of CCL2, which enables the migration of monocytes. Mycobacteria survive by transferring themselves from macrophages to monocytes by fusion reaction in the cell. Mycobacteria with an insufficient number of phenolic glycolipids have low pathogenicity, and therefore, cannot survive the phagocytosis (Cambier et al. 2017).

Human immunodeficiency virus 1 (HIV1) and *Mycobacterium tuberculosis* co-infection cause immediate immune suppression, which resulted in the death of about 400,000 people in 2015, according to the World Health Organization (WHO). Mycobacterial glycolipids affect HIV1 infection and replication and increase mortality (Pouget 2018).

Glycosphingolipids are binding receptors for bacteria, viruses, and toxins on the host cell surface and are molecules that assist the life cycle of the HIV1 virus. Asialo GM1 ganglioside binds to *Pseudomonas aeruginosa*, *Bifidobacterium bifidum*, and *Lactobacillus*, while GSL binds to *E. coli*, *Propiobacterium*, and *Candida albicans*. *E. coli* and *Shigella* toxin bind to the GO3 receptor, cholera toxin, heat-sensitive *E. coli* enterotoxin binds to the ganglioside GM1 receptor, and *C. botulinum neurotoxin* A, B, and tetanus toxin bind to gangliosides. Gangliosides in breast milk reduce binding to cholera and *E. coli* toxins, and thus, show protective effects against those diseases (Aerts et al. 2019).

Conclusion

In addition to being structural elements of microorganisms, glycolipids are involved in the binding of different bacterial toxins to host cells, and in the host's immune system response. Nowadays, drug resistance is increasing

in the fight against infectious agents and research on glycolipids and its components is gaining importance.

Keywords: *Glycolipids, bacteria, virus, fungus*

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CHAPTER ELEVEN

BLOOD GROUPS AND GLYCOLIPIDS

CANAN ÜNAL

Introduction

A blood group is a classification of blood based on the type of oligosaccharidic antigens on the surface of red blood cells (erythrocytes = red blood cell = RBC), which constitute approximately 40-45% of the total amount and 99% of the formed elements of blood. Karl Landsteiner was the first to classify human blood into A, B, O, and AB groups (Rafael 1995). His classification refers to A and B antigens expressed on RBCs and serum anti-A and anti-B antibodies against antigens not present on RBCs. People with type A blood have A antigens, those with type B blood have B antigens, those with AB blood have both A and B antigens, and those with type O blood have neither A nor B antigens. The synthesis of the ABO antigens is catalyzed by gene-encoded glycosyltransferase enzymes. Blood group A consists of two phenotype subgroups A1 (80%) and A2 phenotypes (20%) (Laura 2005). A neonate may have ten possible genotypes and six phenotypes of the ABO blood group, depending on the parents' genes (Winifred 1980) (Table 11.1).

Table 11.1: ABO blood group system and glycosyltransferase enzymes

RBC		SERUM	
GENOTYPE	PHENOTYPE	ANTIBODY	GLYCOSYLTRANSFERASE
A^1A^1 A^1A^2 A^1O	A_1	Anti-B	α-3-N-Acetyl-D-galactosamine
A^2A^2 A^2O	A_2	Anti-B[b]	α-3-N-Acetyl-D-galactosamine
BB BB	B	Anti-A	α-3-D-Galactocele
A^1B A^2B	A_1B A_2B	- -[b]	α-3-N-Acetyl-D-galactosamine and α-3-D-Galactocele
OO	O	Anti-A and Anti-B	-

[b] Anti-A_1 may sometimes be present in the serum of A_2 and A_2B blood groups.

The incidence of diseases depends on the blood group phenotype. For example, gastric and duodenal ulcer is common in individuals with blood group O. In the relationship between blood groups and cancer development, the risk of gastric cancer is higher in individuals with blood group A (Johnson 1964; Gary 1971).

Erythrocyte Membrane Antigens

The erythrocyte membrane is a flexible and dynamic structure of carbohydrates, lipids, and proteins. Adults and neonates have the same number of glycolipids in their erythrocyte membranes. However, the number of long-chain neutral glycolipids and gangliosides depends on enzymatic activities during erythropoiesis and increases in the branching of carbohydrate chains during the transition from fetal to adult

erythrocytes. Neonates have fewer long-chain neutral glycolipids and more gangliosides in their erythrocyte membranes than adults (Michiko 1983).

Like other cells, erythrocytes are covered by a layer of glycocalyx composed of glycolipids, glycoproteins, and proteoglycans (Michael 1992). The negatively-charged glycocalyx (10-15 nm in thickness) prevents RBC aggregation and their adhesion to the endothelium and protects against microbial invasion (Marion 2004).

The carbohydrate and protein structures on the outer surface of the RBC membrane form blood group antigens. The International Society of Blood Transfusion (ISBT) has identified more than 300 antigens and 33 blood group systems (ABO, Rh, Kell, Kidd, Duffy, Lewis, etc.) (Storry 2014). Blood group antigens are RBC membrane antigens. However, they are also known as histo-blood group antigens (HBGAs) because they are expressed on epithelial and endothelial cells, and the skin, hair, and exocrine glands (saliva and seminal fluid) (Fumiichiro 2012).

Biochemical Basis of Histo-Blood Group Antigens

Histo-blood group antigens are expressed differently on cells and tissues. A, B, and H determinants are present as glycoproteins in glands and as free oligosaccharides in milk and urine (Winifred 1980). Blood group antigens, which play a clinically significant role in blood and tissue transfusions, are of a glycosphingolipid structure. Johann Ludwig Wilhelm Thudichum originally discovered glycosphingolipids in brain tissue in 1874 and named them “cerebrosides”. Glycosphingolipids are abundant in brain and nerve tissues. Glycosphingolipids in the membrane serve as modulators in signal transduction and as receptors for antigens/toxins (Wedeking 2007; Hakomori 1986). A glycosphingolipid consists of a hydrophobic ceramide moiety and a hydrophilic carbohydrate chain (galactose and glucose).

Glycosphingolipids are classified into four series based on their core carbohydrate structure; ganglio-series, globo-series (Gb), (neo)lacto series type 1, and lacto series type 2 (Senitiroh 2003). The content of RBC membrane glycosphingolipids varies from species to species. In human RBCs, carbohydrates bind to lacto series type 2 glycosphingolipids as well as globo-series structures (Gb3-Gb4). Gb4Cer, which is a globo-series, constitutes only 58% of the membrane glycolipids (Masao 2009).

The glycosyltransferase enzymes involved in the synthesis of ABO, H, secretory, and Lewis histo-blood group carbohydrates are encoded by the ABO, FUT1, FUT2, and FUT3 genes, respectively (Henry 2000). The H antigen is the precursor in the synthesis pathway of the A and B antigens. The α1,2-fucosyltransferase enzyme, which synthesizes the H antigen in RBCs, is encoded at the H locus located on chromosome 19. Glycosyltransferases, which are protein products of the A and B alleles, are encoded at the ABO gene locus located on chromosome 9. AB antigens are not produced, and the precursor H antigen remains the same in individuals with blood type O because transferase enzymes are not encoded in allele O (Schenkel-Brunner 2000).

The α1,2-fucosyltransferase enzyme expressed on the epithelium of secretory organs (salivary glands, and gastrointestinal and respiratory tracts) is encoded at the Se locus located in the FUT2 gene. The α1,2-fucosyltransferase enzyme catalyzes the production of the H antigen in bodily secretions. Secretory genes with Se/Se or Se/se genotypes secrete the H antigen with or without A and/or B antigens depending on the AB0 genotype (Laura 2005) The Lewis gene encodes α1,3-fucosyltransferase for phenotypes Le^x and Le^y (Vibeke 2000).

ABO, H, secretory, and Lewis histo-blood group carbohydrates have six types of monosaccharides; β-D-glucose (Glc), β-D-N-acetylglucosamine (GlcNAc), β-D-galactose (Gal), β-D-N-acetylgalactosamine (GalNAc), α-fucose (Fuc), and D-mannose (Man) (Schenkel-Brunner 2000).

In a nutshell, the glycosyltransferase (UDP-GalNAc-specific GalNAc transferase) that produces the A antigen is encoded by the A allele, where the immunodominant sugar is N-acetyl-galactosamine (GalNAc). The glycosyltransferase (UDP-Gal-specific Gal transferase), which produces the B antigen, is encoded by the B allele, where the immunodominant sugar is D-galactose (Gal) (Peter 2015) (Figure 11.1). The AB blood group has both enzymes, and thus, both GalNAc and Gal oligosaccharide chains (Laura 2005).

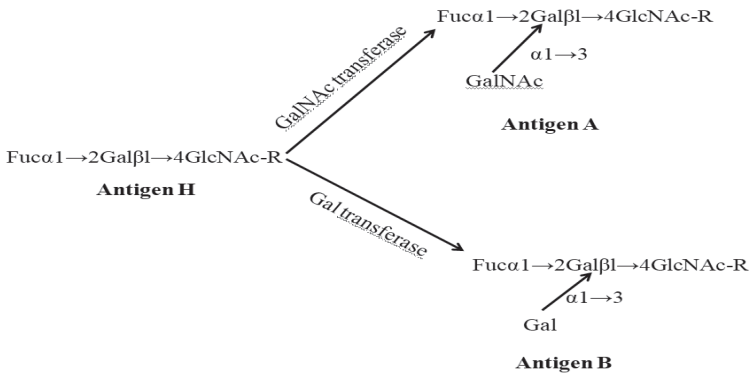

Figure 11.1: Diagram of ABH antigens (Peter 2015).

After the H antigen is formed, the N-acetylgalactosamine and galactose are further added in the α1,3 linkage, respectively, where GalNAc and Gal transferase enzymes, which are encoded, respectively, by the A and B alleles, serve as catalysts (Hakomori 1999).

ABH and Lewis antigens consist of precursors by the sequential addition of monosaccharides catalyzed by a series of glycosyltransferase enzymes (Jacques 2001). There are six types of precursor oligosaccharide chains identified for histo-blood group antigens (Rafael 1995) (Table 11.2).

Table 11.2: Biochemical structures of the six types of precursor oligosaccharide chains (Rafael 1995).

TYPE	TERMINAL PRECURSORS
1	Galβ1→ 3GlcNAcβ1-R
2	Galβ1 → 4GlcNAcβ1-R
3	Galβ1 → 3GalNAcα1-R
4	Galβ1 → 3GalNAcβ1-R
5	Galβ1 → 3Galβ1-R
6	Galβ1 → 4Glcβ1-R

Type 1 and Type 2 precursors can be part of O- or N-glycoproteins and lacto series glycolipids. Type 1 is present in the exocrine glands and the epithelium of the respiratory, gastrointestinal, and genitourinary tracts,

while Type 2 is predominantly found in the vascular endothelium and hematopoietic tissues. Brunner's glands express the Type 2 antigen chain under the control of the H gene, while the crypts of Lieberkuhn and the acini of the salivary glands express Type 1 and Type 2 antigens under the control of the Se gene. In short, the Se gene is involved in the expression of Type 1 and Type 2 antigens, whereas the H gene can only express the Type 2 antigen. Type 2 oligosaccharide chains are the most prevalent in RBC membrane antigens. Type 3 is an antigen present as an extension of Type 2 carbohydrate with the addition of galactose to the terminal N-acetylgalactosamine. Type 3 antigen, which can also be present linked the musins, is expres in the salivary glands and kidneys.

Another antigen found in the salivary glands and kidneys is Type 4 present in glycolipids of the globo and gaglio series. Type 5 is a synthetic antigen and has not been isolated from human tissues. Type 6 is present in human intestinal cells, the longest part of the digestive system (Rafael 1986; Clausen 1989; Schenkel-Brunner 2000; Angstrom 2004; Craig 2012; Williams 2016).

Conclusion

The ABO blood group antigens on the surface of RBCs have a glycosphingolipid structure. The synthesis of ABO antigens is catalyzed by gene-encoded glycosyltransferase enzymes. The function of blood group antigens is not yet well understood by the scientific community. However, they are known to play a crucial role in blood and cell/tissue/organ transfusion, and the forensic evaluation of biospecimens (Fumiichiro 2012; Henrik 1989). For example, exposure to foreign antigens as a result of a small amount of fetal blood entering the maternal circulation during a blood transfusion, pregnancy, or labor induces the immune system. Some diseases and microbial infections may also cause changes in blood group antigens and increases in the amount of blood group antibodies. Hematological cancers may cause modifications in oligosaccharide chains carrying ABO blood group antigens. For example, thalassemia may increase the body's demand for RBCs and reduce the expression of ABO blood group antigens (Laura 2005; Marion 1990). It is also argued that blood group antigens are involved in the differentiation of endothelial cells and the adhesion of metastatic tumor cells (Vibeke 2000).

Keywords: *ABO blood group, antigen, glycosphingolipid, glycosyltransferase*

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CHAPTER TWELVE

GLYCOLIPID DEGRADATION PRODUCTS

RÜMEYSA GÖÇ

Introduction

Glycolipids (GL) are derivatives of lipid sphingosine. Cerebrosides and gangliosides are two of those sphingolipids (SL). Glycosphingolipids (GSL) gain access to the cell by endocytosis. They are trafficked to lysosomes, where glycosphingolipids and hydrolytic and non-recyclable bonds are degraded by enzymes.

Lysosomal enzymes degrade proteoglycans, glycoproteins, and glycolipids that gain access to the cell by endocytosis. Lysosomes combine with endocytic vesicles, and lysosomal proteases digest the protein component. Carbohydrates are degraded by lysosomal glycosidases. Lysosomes contain both endoglycosidases and exoglycosidases. Endoglycosidases break the chains into shorter oligosaccharides, and then, exoglycosidases, for each type of linkage, remove sugar residues one at a time from non-reducing ends. Deficiency of lysosomal glucosidase causes the accumulation of carbohydrates partially degraded by proteoglycans, glycoproteins, and glycolipids in membrane-covered vesicles inside the cell. Those residues can cause dysfunction and organ enlargement (Goni and Alonso 2002).

Cellular Ingestion

Sphingolipids of plasma membrane are continuously taken up into the cell through the endocytotic membrane flow. Moreover, cytosolic surface lipids may be translocated to alternative membranes via monomeric transportation. When the cell is in a resting state, most sphingolipids on the exoplasmic side of the cell membrane lack access to the cytosolic side with the exception of "sphingosine". Sphingosine, either exogenously

added or lysomally produced, is *per se* translocated to the cytosolic leaflet and comes into balance with intracellular membranes.

GSL and gangliosides not only have a relationship with sphingolipid-rich plasma membrane areas (lipid rafts) but also laterally affect and modulate the activity of membrane-related proteins, particularly tyrosine kinase receptors, in the nervous system (Rosen et al. 2005). The clustering of a protein in SL-enriched membrane areas promotes the interaction between rafts and lipid components. High enrichment of protein kinases, either receptor or non-receptor and other signaling molecules, in lipid rafts suggests new models for interpreting ganglioside-mediated signal transduction.

First, sphingosine, sphingosine 1-phosphate (S1P), and ceramide regulate the expression of lipid homeostasis genes more or less directly (Worgall, 2007). Another view is that inflammatory cytokines as a non-lipid signal, which can be produced as a result of changes in SL metabolism, function in the regulation of some SL metabolizing enzymes (Mechtcheriakova et al. 2007). Second, SL metabolizing enzyme expression depends on the cell type and developmental phase. For example, fundamental changes are seen in brain ganglioside patterns throughout development (Ngamukote et al. 2007).

Lysosomal sphingosine 1-phosphate, which is present in a concentration of approximately 0.5 μM extracellularly, in plasma, shows its effects mostly through cell surface G-protein coupled receptors (Rosen et al. 2005; Spiegel and Milstien 2003). The role of S1P in the cell has been comprehensively understood by the modulation of genes that control S1P degradation, transport, sphingoid base phosphorylation, and high affinity binding in mice models (Olivera et al. 2007).

S1P lyase is the only enzyme that catalyzes the non-recyclable degradation of S1P. Deterioration of this enzyme results in the cumulation of phospo-sphingoid bases, leading to multiple defects and postnatal death (Meyer zu Heringdorf et al. 2002).

In some studies, it has been suggested that while the cell is in a stimulated state, SM is transported to the cell surface by scramblase protein and then converted to ceramide with N-SMase (Goni and Alonso 2002; Spiegel and Milstien 2003). However, it is not entirely clear how this ceramide, which can be used for SM and GlcCer synthesis, reached these synthesis localizations. Although the mode of stimulation is not

exactly known, a Golgi transfer protein carries SM with high specificity and strongly stimulates SM re-synthesis. Ceramide is produced in the lysosome's inner membrane, where it is unable to leave, and in the end cannot exit the lysosome lumen (Goni and Alonso 2002). Exogenously added S1P binds to its own cell surface receptors and migrates to the cytosolic surface via CFTR. CFTR is an ABC carrier, which is a cystic fibrosis conductivity regulator (Mao et al. 2000). Moreover, when galactosylsphingosine and glucosylsphingosine are added to the cell, they are translocated towards the cytosolic surface and then acylated. After translocation, lysosphingolipids move freely inside the cell because they are removed from the membrane, whereas the GalCer and GlcCer perform their cytosolic surface functions. Only one research study reported that an exogenously added GlcCer plasma membrane rotated towards the cytosolic surface (Huitema et al. 2004; Worgall 2007). It is not yet fully known whether complex glycosphingolipids reach the cytosolic surface of the plasma membrane. It was, however, reported that there were specific interactions between glycosphingolipids and cytosolic proteins (e.g. calmodulin) (Yamaoka et al. 2004). What is more, gangliosides must have reached a cytosolic surface before reaching mitochondria during signaling events (Riboni et al. 1996).

Endocytosis

Sphingolipids, and other lipids, follow the mass membrane flow along endocytotic and exocytotic vesicular transport pathways. Inner membranes of late endosomes (Yamaoka et al. 2004; Hannun 1994) have a large number of complex glycosphingolipids. Most of the glycosphingolipids are recycled to the plasma membrane through early, late and recycling endosomes. What is more, some of the complex sphingolipids, especially GlcCer, reach the Golgi complex (Huitema et al. 2004). This is also true for GL binding toxins such as E. coli verotoxin, cholera and shiga toxin. Toxin-GL complexes travel retrogradely from the Golgi to the endoplasmic reticulum, then the active subunit is transported across the membrane to the cytosol here. However, only a fraction of the complex GL reaches the ER in the absence of toxins (Koch et al. 1996).

The plasma membrane GSL degrades in lysosomes. Along the pathway to lysosomes, the GSL, which was initially in the plasma membrane, can be transported to intracellular regions, where it is glycosylated by more complex products that reach the plasma membrane again. Lysomally produced sphingosine and ceramide (simple SLs) can also avoid further

decomposition and be reused for complex plasma membrane and signaling SL re-synthesis (Riboni et al. 1996). The plasma membrane is a region where complex SL is concentrated to show its biological functions and it is also active in the regulation of SL metabolism. Bioactive ceramide production depends on SM hydrolysis (Goni and Alonso 2002) by sphingomyelinases. These SMases are found in the cell membrane or transported from intracellular regions as a stimulant (Hannun 1994). Recently, it has been shown that sphingomyelin synthase (SMS) found in the Golgi, and SMS found in the plasma membrane, called SMS2, are encoded by different genes (Huitema et al. 2004).

In some instances, SL-protein interactions are specific and medium-affinity interactions between the GSL oligosaccharide chain and amino acid residues of the extracellular loops of the protein, a glycosylated protein. Also SL-protein interactions are between the hydrophilic portion of a glycosylphosphatidylinositol (GPI) anchor event of GPI-linked proteins and a portion of the protein represented by sugar residues in glycans of a glycosylated protein.

The linkage of a protein with a rigid membrane area might, however, lead to conformational changes in the polypeptide chain that affect, independent of high-affinity with other lipid raft components.

Catabolic fragments originating from plasma membrane SL through hydrolytic enzyme activity represent or are converted to lipid mediators (ceramide, sphingosine, and S1P) that affect specific signaling cascades, and thus, modulate cell proliferation, differentiation, motility, or apoptotic cell death.

The main pathway of sphingolipid degradation starts with headgroup removal and continues with ceramide hydrolization into free fatty acids and sphingoid bases. Subsequently, the degradation end products are further metabolized or reused. GL cleavage takes place in the lysosome, via complex glycosidases and activator proteins or saposins. Defects in any step may cause lysosomal storage diseases (Tessitore et al. 2004). Only Glc-Cer synthesis occurs in the cytosolic leaflet of the Golgi. An acid sphingomyelinase (SMase) degrades SM in the lumen of the lysosome (Yamaoka et al. 2004). Alternatively, there are acid and neutral sphingomyelinases on the cytosolic surface of cellular membranes acting as ceramide-producing enzymes, working in signal transduction (Fensome et al. 2000). Yeast also involves SMase activity necessitating the gene ISC1, the product of which is structurally related to neutral sphingomyelinases

(Sawai et al. 2000). Ceramides are degraded by ceramidases which are of three types: acidic, neutral and alkaline, determined by the environmental pH. Acidic ceramidase, whose gene has been cloned from humans, is in lysosomes (Koch et al. 1996). Two ER-associated alkaline ceramidases are found in yeast (Mao et al. 2000). Cells incorporate most of the sphingoid bases and ceramides into SLs. A small amount is found in the cell as free or phosphorylated derivatives. These are either synthesized or derived from SL degradation and are again used in the SL synthesis pathways in the ER and the Golgi. S1P and sphinganine-1-phosphate are the final substrates in sphingolipid hydrolysis and a lyase converts them into ethanolamine phosphate and a C16 aldehyde (Zhou and Saba 1998; Tani et al. 2005). Sphingoid long-chain base-1-phosphates are also important intra- and inter-cellular second messengers, whose concentration is regulated by the linkage of kinases (Olivera et al. 2007), the lyase, and a phosphatase (Mao et al. 2000; Tani et al. 2005; Mechtcheriakova et al. 2007).

Ceramide

Ceramide is transported via non-vesicular transportation mediated by CERT (ceramide transfer protein) to the Golgi luminal side; in other words, to the main center of conversion to SM by the SMS1 enzyme (Yamaoka et al. 2004), which favors C16–C20 fatty acid ceramides. Galacto-GSL's precursor, GalCer is formed at the ER luminal side. All other GSL series are derived from ceramide which is translocated to the Golgi via vesicular transport. Then ceramide is glycosylated by membrane-bound glycosyltransferases. GlcCer is the main glycosylated precursor of many GSL series (e.g. globo-, isoglobo-, ganglio-, lacto- and neolacto-) (Yamaji et al. 2008). Finally, newly synthesized GalCer and GlcCer are delivered to the luminal surface of the Golgi containing all transferases (e.g. Gal transferases, GalNAc transferases and GalCer sulfotransferase) which sequentially add sugar residues to the growing oligosaccharide chain to synthesize more complex GSLs. These transferases can also reach the plasma membrane (Warnock et al. 1994). Neosynthesized GSL is relocated from the Golgi to the plasma membrane through the mainstream exocytotic vesicular transport.

SM and ceramide levels in plasma membranes are mutually regulated by two different enzyme activities in response to changes in cell physiology. The plasma membrane-bound ceramides and SPHK (sphingosine kinases) responsible for the formation of sphingosine and/or

S1P have been identified at the cell side (Slife et al. 1989; Tani et al. 2005). Both a sialidase (Schengrund and Rosenberg 1970) and a sialyltransferase (Preti et al. 1980) are active in synaptosomal membranes. Metabolic research on chicken embryos (Matsui et al. 1986) and rat brain (Durrie et al. 1989; Ngamukote et al. 2007) has verified of a synaptosomal membrane sialyltransferase in the brain (Tessitore et al. 2004). Therefore, GSL sialylation may take place outside the Golgi compartment and play a critical role in modulating plasma membrane GSL patterns. Cultured rat cerebellar granule and human neuroblastoma cells can desialylate exogenously added gangliosides under experimental conditions blocked by a cell-impermeable sialidase inhibitor (Riboni et al. 1996; Deng et al. 2000). Lastly, (glyco) sphingolipids are released – as shedding vesicles (e.g. monomers or aggregates) – from the cell surface to the extracellular environment and then taken up by neighboring cells (Tessitore et al. 2004).

GSL can modulate plasma membrane activity through direct SL–protein or indirect (via lipid rafts) interactions (cis). This capacity accounts for the roles played by GSL in regulating cellular activity to ensure the improvement and homeostasis of the nervous system (Rajendran and Simon 2005).

Functions of Galactosylceramide and Sulfatide

Failure of GalCer and sulfatide lysosomal degradation, results in the accumulation of these two sphingolipids in myelin, leading to neural diseases with progressive demyelination (Eckhardt et al. 2007).

GlcCer is affected by three known hydrolases, one lysosomal and the other two non-lysosomal. GBA2 and GBA3 genes encode these non-lysosomal hydrolases (Tessitore et al. 2004). Lactosylceramide (LacCer) is a metabolic intermediate for GSLs with all GlcCer. The simultaneous defect of β-galactosidase and β-galactosylceramidase, results in LacCer accumulation, stunningly leading to a milder phenotype in mice (Tohyama et al. 2000).

Gangliosides, which are abundant in neurons, are also found on the surface of numerous types of cells. Therefore, deterioration in ganglioside metabolism causes neurological impairment. GM1 and GM2 gangliosidoses are hereditary diseases, caused by a lysosomal degradation defect, leading to lysosomal ganglioside accumulation. Such metabolic disorders result from inadequate hydrolase activity (β-hexosaminidase or GM1-β-

galactosidase) that normally degrades the oligosaccharide chain of gangliosides, and from defects in the activators (GM2-activators and saposins) of such enzymes. It is still a moot point whether sialidase (which removes sialic acid from gangliosides) defects are caused by primary sialidase deficiency or by cathepsin deficiency. Ultimately these defects lead to ganglioside cumulation (Tessitore et al. 2004).

Conclusion

Although there is a growing body of evidence that supports lipid classification based on area in various endocytotic organelles, most of that proof has been derived from lipid precursors, toxins, antibodies, and glycosphingolipid-bound viruses, and GPI-proteins as raft markers. Precursors of LacCer and globoside are endocytosed by a clathrin-independent subgroup of vesicles taking up SM, suggesting that classification can be performed at the plasma membrane (Reddy et al. 2001). Both of these pathways result in different classes of early endosomes associated with the Golgi. Glycolipid-bound toxins follow both clathrin-dependent and -independent pathways (Rajendran and Simons 2005; Reddy et al. 2001). Despite progress in recent years, more research is warranted to determine the quantitative lipids and the size of pathways.

Keywords: *Glycolipid, sphingolipid, endocytosis, lysosome*

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