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Review

The Glycosphingolipidoses – from Disease to Basic Principles of Metabolism

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The glycosphingolipidoses are a set of diseases that are caused by defects in the lysosomal degradation of glycolipids derived from the plasma membrane. By investigating the molecular bases of the diseases, basic principles of storage disease pathology and of membrane digestion were discovered. The generation of mouse models has facilitated the development of new and promising therapeutic strategies for these diseases, most of which are not treatable at present. Lately, the discovery of the importance of glycosphingolipid metabolism for skin development has opened a new and interesting field.

Key words: Glycosphingolipid degradation / Glycosidase / Lysosomal storage disease / Skin / Sphingolipid activator protein / Therapy.

Introduction

Glycosphingolipids are complex lipids that form part of the outer layer of the eukaryotic plasma membrane. Here they form cell type-specific patterns that may change with differentiation, viral transformation or oncogenesis (Hakomori, 1984). Glycosphingolipids are known to participate in cell-cell recognition and are binding sites for several bacteria, viruses and toxins. Recently there has been evidence that they take part in the formation of rafts and caveolae and may therefore be essential for several signal transduction processes (Okamoto *et al*., 1998). Defects in their catabolism, which takes place in the lysosomes, lead to storage diseases, known as glycosphingolipidoses. These are rare but severe inherited diseases, such as Gaucher disease, metachromatic leukodystrophy, Krabbe disease and Tay-Sachs disease.

Glycosphingolipidoses have been known for quite a long time (for a review see Gravel *et al*., 1995). In 1881 Warren Tay, a British ophthalmologist, first described the clinical characteristics of 'infantile amaurotic idiocy', consisting of blindness ('cherry red spot'), slow growth, severe mental retardation, motor weakness and head enlargement, leading to death at an early age. In 1896 the American neurologist Bernard Sachs noted the distended cytoplasm of neurons in these patients. He also recognized its prevalence among Jews and coined the term 'amaurotic familiar idiocy' for this group of diseases, the prototype of which is now known as Tay-Sachs disease.

It was not until the 1930s that the storage material in the brains of Tay-Sachs patients was identified as a new group of acidic glycolipids. They were named 'gangliosides' by Ernst Klenk, who first analyzed them. In 1962 Svennerholm identified the major neuronal storage compound in Tay-Sachs disease as ganglioside GM2. Its structure elucidation by Makita and Yamakawa followed shortly after this and set the stage for the understanding of the underlying defect in this disease. It was obvious that the metabolism of GM2 was defective. Since GM2 and two other glycolipids (GA2 and globoside) that were shown to be stored to a minor extent in Tay-Sachs disease all contain a terminal N-acetylgalactosamine residue (Figure 1), hexosaminidase, the catabolic glycosidase that cleaves this residue was a natural suspect for the enzymatic defect. Surprisingly, hexosaminidase levels seemed to be normal or even slightly elevated in Tay-Sachs patients. In 1968 Robinson and Stirling found that human hexosaminidase could be separated into two forms, an acidic, heat labile form named hexosaminidase A (Hex A) and a basic, heat stable form named hexosaminidase B (Hex B). Shortly after this, in 1969, Sandhoff and O'Brien independently showed that the activity of hexosaminidase A, which in contrast to hexosaminidase B is able to process acidic substrates (Sandhoff, 1970), was absent or extremely reduced in Tay-Sachs patients. This discovery led to the development of a simple biochemical diagnosis for patients and carriers of the disease, which made it possible to screen risk-populations such as Ashkenasim Jews and give a prenatal diagnosis in risk-pregnancies. Simultaneously, the analysis of enzyme activity in a large amount of patients, carriers and normal controls led to the development of a new and exciting model for the clinical manifestation of metabolic storage diseases, which is described below.

The Residual Enzyme Activity Model

Measurement of enzyme activities in a large number of individuals revealed that nature has supplied a generous safety buffer for normal life. Heterozygous carriers of Tay-Sachs disease have an enzymatic activity of 30 – 60% of

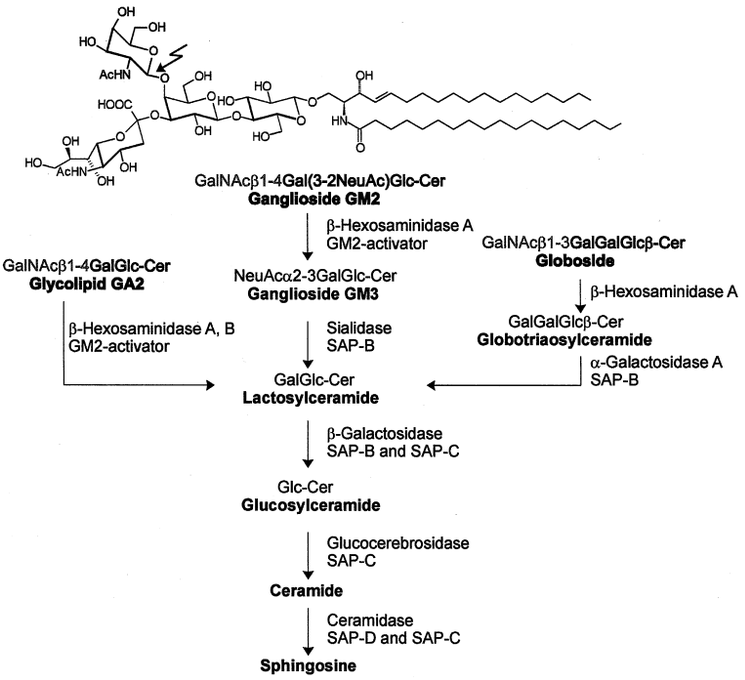

Fig. 1 Structure of Ganglioside GM2 and the Lysosomal Degradation Pathway for Selected GSLs.
The activator proteins that are necessary *in vivo* are indicated. The bond which is broken in GM2 is indicated by an arrow.

that found in normal individuals but do not show clinical symptoms. Even individuals with 10–15 % of normal activity lead a normal life. Only when the activity falls below that level, symptoms become apparent. These findings led to the development of a kinetic model relating the residual enzymatic activity to substrate turnover and to the development of clinical symptoms (Conzelmann and Sandhoff, 1983/84). According to this model, the critical threshold value at which clinical symptoms start to develop is reached, when υ_{max} of the residual catabolic activity equals the rate of influx of substrate to the lysosomes. When the activity is below this value, substrate is stored. The amount of substrate stored and therefore the timespan for substrate to accumulate to such an extent as to impair cellular function and lead to clinical symptoms depends on the amount of activity that is left. Also, the biosynthetic rate of the substrate and therefore the rate of influx differs between cell types. This means that a certain residual activity may be below the critical threshold for cells with high biosynthetic rates but above it for a different cell type.

This model explains the fact that there is a wide variety of clinical symptoms that are all caused by a defect in only one enzyme. This heterogeneity can be so extreme that two diseases that are caused by a defect in the same gene product are known by different medical names. A defect in α-iduronidase for example can lead either to Hurler disease or to Scheie disease, depending on the severity of the enzymatic defect (Neufeld and Muenzer, 1995).

In Tay-Sachs disease the phenotypes also differ extremely. While in classical Tay-Sachs disease the symptoms usually set in at about 3–5 months of age and progress rapidly from loss of motor skills to blindness and complete inertia, there are different cases where symptoms set in in juvenile or even adult patients. In these cases, the clinical phenotype is often quite different, involving psychosis, muscle wasting and weakness, but not blindness. Similarly, in classical Tay-Sachs disease there is storage of GM2 all over the brain, while in cases with adult onset the accumulation is restricted to certain areas of the brain, namely the brain stem and the ganglia. In complete agreement to the model, classic infantile onset Tay-Sachs disease is associated with no or very low residual ganglioside GM2 hydrolyzing activity, while patients with adult onset of the disease show a relatively high residual activity of 3–5% of normal controls. The activity found in patients with juvenile onset lies somewhere in between (Gravel *et al.*, 1995).

This formerly confusing heterogeneity can now be explained by the described theory of residual enzymatic activity which has been shown to be valid for a large number of lysosomal metabolic diseases.

The Sphingolipid Activator Proteins

Linking the measurement of enzymatic activity to the clinical symptoms led to another important discovery. In some very rare cases, patients with typical Tay-Sachs symptoms showed a completely normal hexosaminidase A activity. It was found that by adding a homogenate of normal cells whose hexosaminidase A activity had been destroyed by a heat treatment to cell homogenates of these patients, the capacity to degrade GM2 was restored

(Conzelmann and Sandhoff, 1978). This demonstrated the existence of a heat stable factor which is necessary for GM2 degradation and which was absent in these patients. In 1979 this factor was purified in our laboratory and was named 'GM2 activator protein' (GM2AP) (Conzelmann and Sandhoff, 1979). Other proteins had already been found to activate glycosphingolipid degradation. Today we know that there are five such proteins, encoded by two genes. One gene on chromosome 5 encodes the GM2 activator protein, while another gene on chromosome 10 codes for a precursor protein, the sphingolipid activator protein precursor or SAP precursor, which is processed to four homologous proteins, the saposins or SAP-A to D (Sandhoff *et al*., 1995).

The function of these proteins seems to be another basic principle in the degradation of glycolipids. The degradation of glycosphingolipids with long sugar moieties extending far into the aqueous phase is independent of these activator proteins, while their presence is necessary for the degradation of lipids with short polar head groups. The problem that the activator proteins help to overcome is the interaction between a membrane-bound lipid substrate and a water-soluble enzyme. When the sugar chain is long enough, the terminal sugar is far enough from the lipid bilayer to be reached by the enzyme. But once the terminal sugar is too close to the membrane, the cleaving enzyme cannot reach it without assistance. In these cases the sphingolipid activator proteins are necessary. The mechanisms by which they help to overcome this hindrance seem to be different from protein to protein. While SAP-B stimulates the degradation of many lipids by several enzymes and seems to act like a physiological detergent, SAP-C is reported to be not only membrane perturbing but also to activate membrane-associated hydrolytic enzymes by forming complexes with the enzymes rather than with the substrates (Ho and O'Brien, 1971). The GM2 activator protein on the other hand is specific in activating the degradation of GM2 (Meier *et al*., 1991). It complexes the substrate and presents it to the degrading enzyme by either solubilizing or lifting it from the bilayer.

The discovery of the activator proteins also demonstrates another principle. While mutations in one gene can lead to very diverse clinical symptoms, as was discussed in the preceding paragraph, the opposite may also be true. Mutations in different genes, in the example described above in the GM2AP-gene and in the hexosaminidase α-chain gene, can lead to clinically indistiguishable symptoms. This principle is true for a large number of diseases and is not restricted to metabolic diseases.

The Topology of Intralysosomal Degradation

Components of the plasma membrane reach the lysosomes by the endocytotic pathway. It has been proposed that after successive steps of vesicle budding and fusion

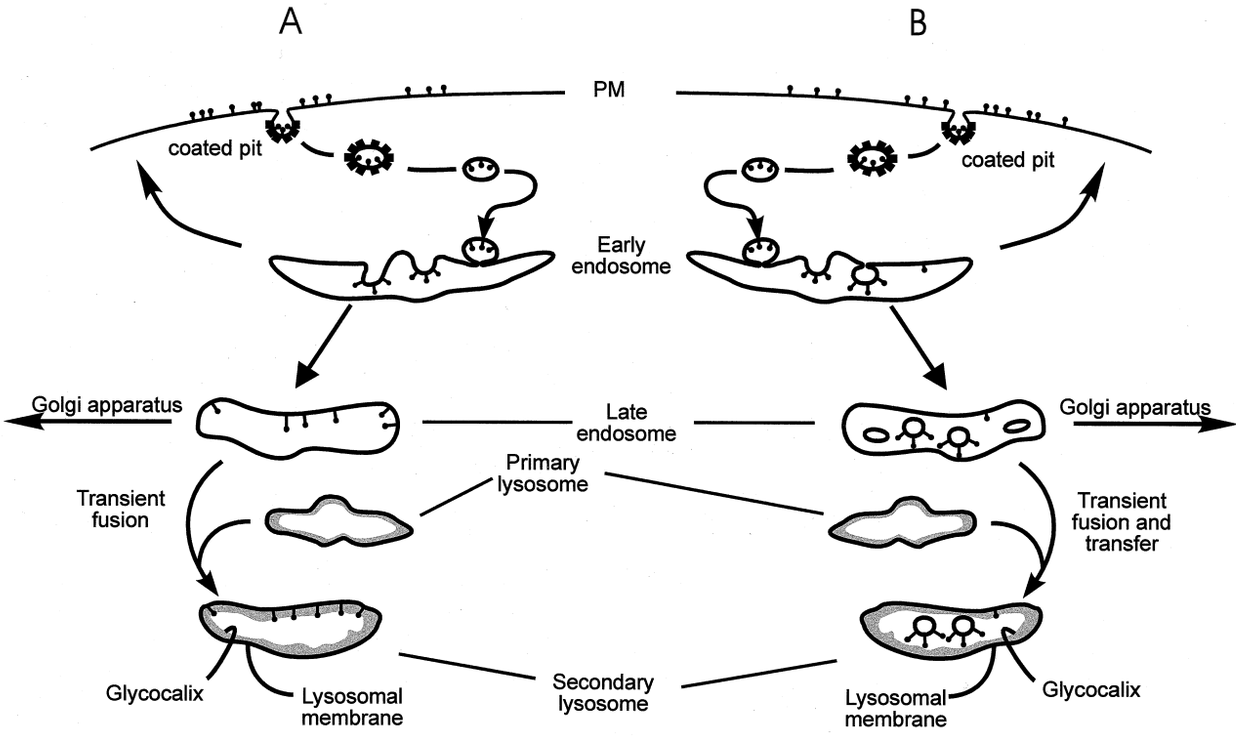

Fig. 2 Two Models for the Topology of Endocytosis and Lysosomal Digestion of Glycosphingolipids (GSLs) Derived from the Plasma Membrane.
Conventional model (A): degradation of GLSs derived from the plasma membrane occurs selectively within the limiting lysosomal membrane. Alternative model (B): during endocytosis glycolipids of the plasma membrane become incorporated into the membranes of intraendosomal vesicles (multivesicluar bodies). The vesicles are transferred into the lysosomal compartment when the late endosome fuses with primary lysosomes. PM: plasma membrane, •, glycosphingolipid.

they are integrated into the lysosomal membrane before they are catabolized. It is hard to conceive how the selective degradation of these components of the plasma membrane is supposed to take place without the destruction of the limiting lysosomal membrane.

An alternative hypothesis which has been proposed by our group (Fürst and Sandhoff, 1990) suggests that portions of the endosomal membrane that are enriched in components derived from the plasma membrane bud off into the endosomal lumen forming intraendosomal vesicles and other intraendosomal membrane structures. These vesicles are delivered to the lysosomal lumen by transient fusion of endosomes with lysosomes. Thus, components of the outer leaflet of the plasma membrane would face the lysosol on the outside of intralysosomal vesicles (Figure 2).

This hypothesis is supported by several observations: the fact that the inner surface of the limiting lysosomal membrane is protected by a glycocalix seems to argue against a degradation directly from this membrane. Furthermore, the massive accumulation of intralysosomal multivesicular storage bodies in the fibroblasts of patients with complete deficiency of the SAP precursor protein demonstrates the existence of such intralysosomal vesicles at least in cases of disrupted metabolism. Although they are less obvious, intralysosomal membrane structures have also been described for normal cells (Futter *et al*., 1996). In addition, in normal human fibroblasts the plasma membranes of which were loaded with biotin-labeled GM1, this ganglioside was traced to such intralysosomal structures by electron microscopy immunocytochemistry (Möbius *et al*., 1999).

Further support for the hypothesis is derived from the biophysical properties of the sphingolipid activator proteins. It has been demonstrated that GM2AP can reversibly insert into lipid bilayers up to a lateral pressure of 25 mN/m (Giehl *et al*., 1999). With a lateral pressure of about 40 mN/m the limiting lysosomal membrane is therefore protected against the attack of GM2AP. The lateral pressure of intralysosomal vesicles on the other hand may be lower, due to their higher curvature. However, this factor alone is not sufficient to lower the pressure to such an extent that GM2AP can insert into these vesicles.

Another factor which may contribute to lowering the lateral pressure in these vesicles is their lipid composition. Since the lysosome is the site of lipid degradation, some lipids and products of their degradation are found either exclusively or in exceptionally high concentrations in these organelles. These lipids include bis(monacylglycero)phosphate (BMP), and possibly also phosphatidylinositol (PI), dolichol, dolicholphosphate and free fatty acids. To test whether these lipids have an influence on the degradation of glycosphingolipids with short sugar moieties, a detergent-free liposomal assay system for the degradation of glucosylceramide by glucocerebrosidase and SAP-C was established. By varying the curvature of the liposomes as well as their lipid composition it was demonstrated that degradation was faster when the substrate was incorporated in small liposomes with high curvature (as was expected) and that the presence of the anionic lipids BMP, dolicholphosphate and PI leads to a great increase in the speed of degradation of glucosylceramide (Wilkening *et al*., 1998). Similar results have been obtained for the degradation of GM2, sphingomyelin and ceramide in liposomes (unpublished). These findings also support our hypothesis that degradation of glycosphingolipids takes place on intralysosomal vesicles or other membrane structures and is facilitated by the presence of specific lysosomal lipids in these vesicles.

Therapeutic Efforts

When investigating metabolic diseases, one major objective is of course the development of therapies. The only glycosphingolipidosis for which a causal therapy exists today is the adult form of Gaucher disease which does not involve the central nervous system.

One favorable prerequisite for the development of therapies is the existence of suitable animal models for the disease. To provide these models, transgenic mice have been prepared. In the case of GM2 gangliosidoses, there are several different proteins involved in GM2 degradation. Hexosaminidase A consists of two subunits, α and β. Mutations in both subunits are known in humans. While mutations in the α subunit are associated with classical

Table 1 Mouse Models for GM2-Gangliosidoses and Corresponding Pathology in Mice.

Mouse model	Gene knock out	Pathology
B-variant (Tay-Sachs)	α-subunit Hex A ($\alpha\beta$) and S ($\alpha\alpha$) deficient	Minor storage of ganglioside GM2 in grey and white matter, restricted to certain regions of the brain, normal life span
0-variant (Sandhoff)	β-subunit Hex A ($\alpha\beta$) and B ($\beta\beta$) deficient	Storage of GM2 and GA2 throughout the CNS and of GM2, GA2 and globoside in visceral organs, reduced life span (5 months)
Double knock out	α- and β-subunit of the β-hexosaminidases Hex A ($\alpha\beta$), B ($\beta\beta$), and S ($\alpha\alpha$) deficient	Storage of GM2 and GA2 in grey and white matter; demyelination, mucopolysaccharidosis, life expectancy 6 to 12 weeks
AB-variant	GM2-activator	Comparable to B-variant mice defects in balance and coordination, normal life span

Tay-Sachs disease, mutations in the β subunit lead to Sandhoff disease which is characterized by an additional loss of hexosaminidase B activity (which consists of two β subunits) and additional storage of globoside in visceral organs but a phenotype almost identical to Tay-Sachs disease (Table 1). Both mouse models have been prepared, but interestingly the mouse with a disrupted Hex A gene, which should be a model for classic human Tay-Sachs disease, proved to be quite healthy due to an alternative metabolic pathway which exists in mice but not in humans. The specificity of mouse sialidase differs from its human counterpart in such a way that in mice it can transform GM2 to GA2, which can then be degraded further by Hex B (Sango *et al*., 1995). The mouse still accumulates GM2, but storage levels are so low that no symptoms develop. The Hex B knock out- or Sandhoff mouse on the other hand shows all the symptoms of the human disease and can therefore be used to test possible therapies (Sango *et al*., 1995).

In inherited metabolic diseases the therapeutic efforts classically aim at restoring the activity that is missing in the patients. This can be done either by 'enzyme replacement', that is administering the missing enzyme as it is done in the adult form of Gaucher disease, by gene therapy, which means introducing a functional gene by a suitable vector, or by bone marrow transplantation. In this case healthy hematopoetic cells which produce the missing protein are used to deliver this protein to the affected tissues.

One major problem in the therapy of most glycophingolipidoses is the involvement of the central nervous system (CNS). The blood-brain barrier constitutes a severe hindrance for the delivery of any therapeutic agent to the brain. Since proteins do not pass through this barrier, enzyme replacement is ruled out as an easy therapy for any disease with CNS involvement. Similar problems apply for gene therapy, since most vectors do not pass the blood-brain-barrier. An additional problem is the infection of non-dividing neuronal cells by the vectors commonly applied in gene therapy. Nevertheless, some investigations into this therapeutic possibility are still ongoing (Akli *et al*., 1996).

Bone marrow transplantation (BMT) should be one possibility for these diseases since macrophages derived from the bone marrow can pass the blood-brain-barrier to some extent to become microglia. These cells could serve as sources of enzyme in the brain.

Sandhoff mice which were subjected to BMT showed a reduced lipid storage in peripheral tissue. Their life expectancy was doubled, but they still died prematurely and neuronal storage was not diminished to a significant extent (Norflus *et al*., 1998). Similar results were obtained with the twitcher mouse which is a model for Krabbe disease (Hoogerbrugge *et al*., 1988), which shows that BMT is not an effective therapy at least for the severe infantile variants of these diseases.

A different approach to the therapy of storage diseases is called substrate deprivation or 'trickle down approach'. Here, therapy is not directed at replacing the lacking enzymatic activity but rather at reducing the influx of the stored substrate. One prerequisite for this approach is the presence of a residual catabolic enzymatic activity. This means that it will be most useful for the juvenile or adult forms of the diseases, but may fail in the infantile forms with no or very low residual activity.

To test the suitability of such an approach in the GSL storage diseases, the effect of an inhibition of the GSL biosynthesis had to be explored. To this end a knock out mouse was prepared which is defective in GalNAcT (β1,4-N-acetylgalactosaminyltransferase), the enzyme that catalyzes the third step in the biosynthesis of complex gangliosides (Takamiya *et al*., 1996, Liu *et al*., 1999). Although this mouse did not synthesize any complex GSL it had an almost normal life, although it was infertile and did show some demyelination at an advanced age. This result shows that this approach is possible, since lack of complex gangliosides does not lead to severe clinical manifestations.

To test the effect on a disease-model, a double knock out mouse was prepared which was defective in the Hex B gene (which results in severe symptoms, see above) and the GalNAcT gene simultaneously. This mouse had a significantly increased lifespan when compared to the Hex B knock out mouse. It did not store complex GSL of the ganglio-series in any tissue and performed almost normally in the behavioral tests. However, after about seven months of age, this double knock out mouse started to display phenotypic abnormalities. It was shown that this pathology was caused by accumulation of N-linked oligosaccharides, a secondary substrate for hexosaminidase (Liu *et al*., 1999). Although this form of secondary storage disease may prove to be a limitation for substrate deprivation therapy in some cases of GSL storage diseases like Sandhoff disease, the absence of GSL storage and increased lifespan make this form of therapy look very promising, at least for patients with high residual enzymatic activities.

For a therapy that is based on these findings, an inhibitor of GSL biosynthesis is necessary. N-butyldeoxynojirimycin (NB-DNJ) was found to be an inhibitor of the glucosyl-transferase which catalyzes the biosynthesis of glucosylceramide (Platt *et al*., 1994). By inhibition of this step, the biosynthesis of all GSL derived from glucosylceramide is inhibited. Also, this compound is able to pass the blood-brain barrier.

NB-DNJ has previously been evaluated as a possible antiviral agent in a clinical trail and did not show severe side effects on humans (Fischl *et al*., 1994). Oral administration of this compound to healthy mice resulted in a depletion of GSLs in multiple organs without causing any overt pathology, not even the effects observed in the knock out mice (see above) since an inhibitor will never lead to a complete block of a metabolic step. To test the effect of NB-DNJ on GSL storage in mice with an enzymatic defect, a study was performed in Tay-Sachs mice. These mice store GM2 slowly without developing clinical symptoms (see above), which makes it possible to monitor the effect of a treatment over a longer period of time. It was

demonstrated that administration of NB-DNJ considerably reduced the amount of GM2 stored in the brains of Tay-Sachs mice (Platt *et al*., 1997), a result which makes this compound a very promising candidate for a drug treatment of GSL storage diseases. Its potential will have to be evaluated further by testing it on symptomatic mouse models like the Sandhoff mouse and finally in humans. These studies are currently in progess.

Although it may be necessary to eventually develop a mix of inhibitors to eliminate the pathology caused by secondary substrates in some special cases, this approach seems to be the most promising route to the development of a therapy for GSL storage diseases known to date. In combination with BMT it may even be a possible therapy for the infantile forms of the diseases.

Sphingolipids in Epidermal Barrier Formation

Another area in which sphingolipids play an important role is the formation of the water permeability barrier of the skin. The barrier is essential for mammalian terrestrial life, since it restricts excess transepidermal water loss and therefore protects the organism from desiccation. It is localized in the outermost stratum corneum layer of the epidermis where corneocytes are embedded in a matrix of extracellular lipid membranes mainly consisting of long-chain ceramides, free fatty acids and cholesterol. Some of these ceramides possess an ω-hydroxyl group on their long-chain N-acyl moiety and are covalently attached to proteins on the surface of corneocytes, probably forming the hydrophobic scaffold (lipid-bound envelope, LBE) on which the extracellular lipid membranes are initially deposited (Figure 3).

To date, the formation of the epidermal barrier to water loss is not completely understood. It is known that glucosylceramides together with other polar lipids are secreted into the extracellular space of the stratum corneum by lamellar bodies and are subsequently processed by a set of co-localized lysosomal lipid hydrolases into a more hydrophobic mixture, enriched in ceramides.

While most human patients with a partial defect in β-glucocerebrosidase do not display any visible skin phenotype, there are some reports of so-called 'collodion babies', infants whose skin feels sticky and who die shortly after birth. In several cases this phenotype has been

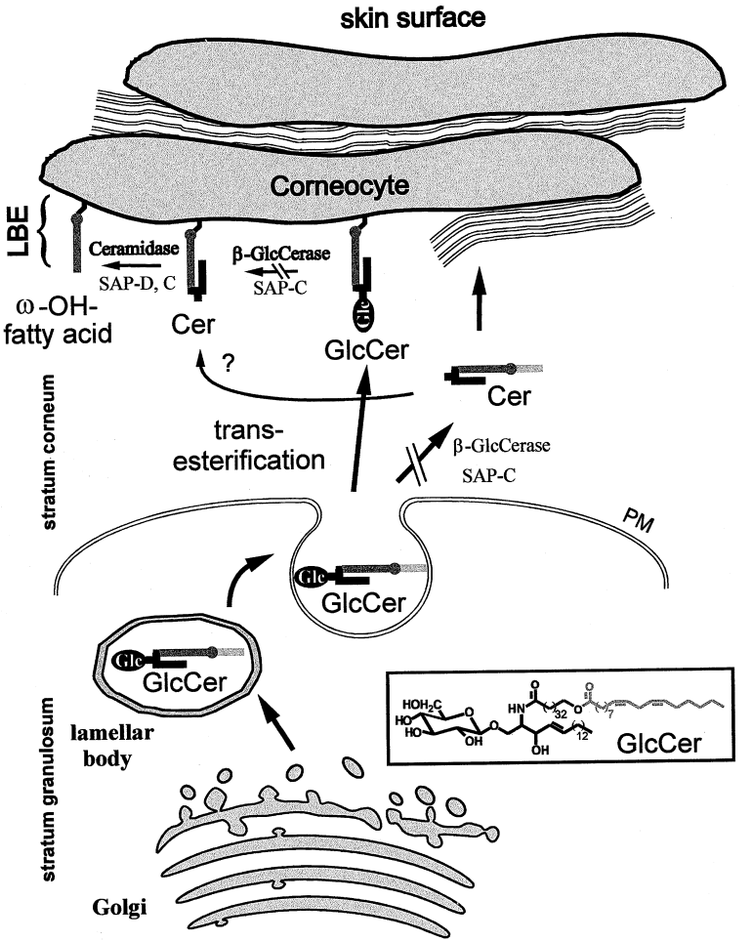

Fig. 3 Extracellular Metabolism of GlcCer and Formation of the Lipid-Bound Envelope (LBE).
PM: plasma membrane, Cer: ceramide, consisting of sphingosine (black) and long-chain ω-hydroxyl fatty acid (dark grey). Linoic acid (light grey) is found esterified to the ω-hydroxyl moiety. Catabolic blocks in β-glucocerebrosidase- and SAP-C deficiency are indicated by —//↦.

shown to be associated with a severe infantile form of Gaucher disease, characterized by a complete defect of β-glucocerebrosidase (Liu *et al*., 1988).

These findings are confirmed by a mouse model for this disease which has been created by introducing point mutations into the murine gene encoding β-glucocerebrosidase. Due to an almost complete loss of β-glucocerebrosidase activity, these mice show a severely abnormal skin and die 24 h after birth. When analyzing the epidermal lipid composition of these mice, it was found that the amount of glucosylceramides was increased while the amount of ceramides was reduced (Doering *et al*., 1999a). These observations demonstrate that the processing of glucosylceramide to ceramide is essential for barrier competence but that only a nearly complete loss of enzymatic activity leads to epidermal pathology in humans.

Lipid analysis of the β-glucocerebrosidase deficient mice and of mice with a complete deficiency of the SAP precursor protein which are therefore also deficient in SAP-C, a cofactor of β-glucocerebrosidase, revealed the existence of a new glycoconjugate: it was demonstrated by our group that ω-hydroxylated glucosylceramides are covalently attached to corneocytes (Doering *et al*., 1999a and b, Figure 3). In the SAP precursor protein- and β-glucocerebrosidase deficient epidermis this novel glycoconjugate accounted for 30 and 70% of the total protein-bound lipid, respectively. Concomitantly, levels of related protein-bound ceramides and fatty acids were decreased in the epidermis of these mice, all of which showed an abnormal epidermal permeability barrier.

These data support the concept that free ω-hydroxylated glucosylceramides are transferred to carboxylated side chains on surface proteins of the corneocytes and are subsequently processed by the sequential action of β-glucocerebrosidase and acid ceramidase which need sphingolipid activator proteins (SAPs C and D) to enhance their activity towards these rather hydrophobic substrates.

The results obtained demonstrate how the study of metabolic diseases can lead to insights into related metabolic pathways, in this case the formation of the epidermal barrier. Further investigations in this area may give rise to a better understanding of skin malfunctions as they are observed in diseases like psoriasis or atopic dermatitis.

Concluding Remarks

The analysis of sphingolipid storage diseases led to the understanding of underlying metabolic principles and to a new topological model of lysosomal digestion. The discovery of the sphingolipid activator proteins was an important step in this process. However, their role in the selective degradation of membrane components within the lysosome is still far from clear. Morphological studies as well as the development of appropriate biochemical approaches for the investigation and reconstitution of intralysosomal membrane structures will hopefully help to achieve a better understanding of this process.

As a result of the knowledge obtained about the GSL metabolism, a new approach towards a therapy of GSL-storage diseases has been found. Here, further research is required to overcome the problems on the way from the concept towards a workable therapy. A new and just emerging area of research is the contribution of glycolipid metabolism to the formation of the water permeability barrier of the skin. The clinical relevance of this field can only be estimated at this point but it seems to be profound.

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