Chapter 5

Prominent increase in plasma ganglioside GM3 is associated with clinical manifestations of type I Gaucher disease


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Abstract

Objectives and background
Patients with Gaucher disease show signs of insulin resistance. The ganglioside GM3 has recently shown to be a negative regulator of insulin sensitivity. In fibroblasts of Gaucher patients, deficient in degradation of glucosylceramide, an increased anabolism of this lipid to gangliosides occurs. The goal of the current study was to establish whether GM3 is elevated in plasma of type I Gaucher disease patients, and is related to disease manifestations.

Methods
Plasma GM3, glucosylceramide and ceramide were determined and compared to overall severity of disease, hepatomegaly, and plasma chitotriosidase activity.

Results
The ceramide concentration in plasma of untreated Gaucher patients was slightly but not significantly lower than in control subjects (median: 9.8 μmol/L, range: 5.7–14.7 μmol/L, (n=40) vs. median: 11.0 μmol/L, range: 5.1–18.0 μmol/L, (n=30)). Glucosylceramide was significantly (p=0.00) elevated. GM3 was also significantly (p=0.00) increased (median: 10.2 μmol/L, range: 4.3–19.1 μmol/L, (n=40) vs. median: 3.6 μmol/L, range: 2.7–5.4 μmol/L, (n=30)). Plasma GM3 concentrations correlated with plasma chitotriosidase activity (ρ=0.45, p=0.00), overall severity of disease (ρ=0.39, p=0.01) and hepatomegaly (ρ=0.49, p=0.00).

Conclusions
GM3 is elevated in plasma of most Gaucher patients. The increase is comparable to that of glucosylceramide, the primary storage lipid. The marked elevations in GM3 may play a role in the insulin resistance of Gaucher patients.
**Introduction**

Gaucher disease (OMIM 230800) is the most common lysosomal storage disorder\(^1\). The disease is due to a recessively inherited deficiency in lysosomal glucocerebrosidase (GBA1; EC:3.2.1.45), catalyzing the hydrolysis of the glycosphingolipid glucosylceramide to glucose and ceramide in lysosomes. Different phenotypes of Gaucher disease (types I, II, and III) are generally recognized, which are differentiated on the basis of the presence or absence of neurological symptoms. The most prevalent Gaucher phenotype is the non-neuronopathic type I variant. Although glucocerebrosidase is present in lysosomes of all cell types, type I Gaucher disease patients develop only pronounced lysosomal storage of glucosylceramide in macrophages. Recently, the protein (GBA2) responsible for the ubiquitous non-lysosomal glucocerebrosidase activity has been identified\(^2-4\). Most likely this enzyme largely protects most cell types of Gaucher patients from massive glucosylceramide accumulation. Lysosomal storage in macrophages of Gaucher patients can not be prevented due to the fact that large quantities of glycosphingolipids are directly introduced in their lysosomes by phagocytosis of senescent blood cells.

The glucosylceramide loaded macrophages of Gaucher patients show a characteristic morphology and are referred to as Gaucher cells. It has become clear that Gaucher cells are not inert containers of storage material but viable, chronically activated macrophages which contribute to the diverse clinical manifestations of Gaucher disease. In tissue lesions of Gaucher patients, mature storage cells, which are alternatively activated macrophages, are surrounded by newly formed, highly inflammatory cells\(^5-6\). Consistent with these observations, Gaucher patients show increased plasma levels of several pro-inflammatory and anti-inflammatory cytokines, chemokines, and hydrolases\(^7\). Factors released by Gaucher cells and surrounding macrophages are thought to play a crucial role in the development of common clinical abnormalities in Gaucher patients such as osteopenia, activation of coagulation, and gammopathies.

Metabolic abnormalities also occur in Gaucher patients. Patients show a markedly increased resting energy expenditure and in addition an increased hepatic glucose production pointing to insulin resistance\(^8,9\). Consistent with this, adiponectin levels are reduced in symptomatic Gaucher patients\(^10\). The precise cause for these metabolic abnormalities is presently unclear.
Recent reports implicate gangliosides like GM3 as important negative regulators of insulin sensitivity in liver and peripheral tissues. This is of particular interest since there are reports on abnormalities in GM3 in association with Gaucher disease. Firstly, cultured fibroblasts from Gaucher patients were found to show an increased synthesis of gangliosides, among them GM3. Apparently, the deficiency in lysosomal glucosylceramide catabolism in fibroblasts is partly compensated by increased anabolism of the lipid to gangliosides. Secondly, increased levels of the ganglioside GM3 were noted for spleen, liver, and brain from a relatively small number of Gaucher patients investigated, again suggesting a compensatory increase in ganglioside biosynthesis.

This set of observations and the recent availability of a sensitive assay for plasma GM3 determination, prompted us to investigate the plasma concentration of this ganglioside in a large cohort of type I Gaucher disease patients. We here firstly report that plasma GM3 is strikingly increased in association with Gaucher disease manifestation. The potential physiological impact of GM3 elevation in Gaucher patients is discussed.

**Methods**

**Patients**

Plasma samples were collected of forty type I Gaucher disease patients who were referred to the Academic Medical Center in Amsterdam for assessment of the severity of their disease or the institution of therapy. The patients took part in an observational study for which approval for regular blood sampling was obtained by the institution’s ethical committee. Thirty healthy control subjects also consented to the use of their stored plasma samples for analysis of glycosphingolipids. A diagnosis of Gaucher disease was confirmed by demonstration of deficient activity of glucocerebrosidase in leucocytes and genotyping. The severity of the disease was classified using the modified severity scoring index (SSI). Thirteen of the patients had been splenectomized prior to the start of therapy. Liver volume was measured by spiral computed axial tomography. Excess liver volume was calculated as described before.
**Chitotriosidase activity assay**
The fluorogenic substrate 4MU-deoxychitobiose was synthesized as earlier described\(^2\). For the enzyme activity assay 25 μL plasma, diluted with BSA/PBS (bovine serum albumin/phosphate buffered saline, 1 g/L) and 100 μL substrate mixtures were incubated for 20 min at 37 °C. The substrate mixtures contained 0.11 mM 4MU-deoxychitobiose and 1 g/L BSA in McIlvain buffer, pH 5.2\(^2\). Reactions were stopped with 2.0 mL 0.3 M glycine NaOH buffer pH 10.6 and the formed 4MU was detected fluorometrically (excitation at 366 nm; emission at 445 nm). Only less than 10% difference in the duplicates was allowed. One unit (U) of activity is defined as 1 nmol of substrate hydrolyzed per hr. The chitotriosidase genotype of individuals was determined as described earlier\(^2\).

**Lipid measurements**
Plasma glucosylceramide, ceramide, and ceramidetrihexoside were determined exactly as previously described\(^2\). Ceramidetrihexoside was determined to establish alterations in the globoside anabolic pathway. All samples were run in duplicate and in every run 2 reference samples were included. Gangliosides, including GM3, were detected by analysis of the acidic glycolipid fraction obtained by Folch extraction. Gangliosides were desalted on a disposable SPE C18 column (Bakerbond, Mallinckrodt Baker Inc., Phillipsburg, NJ, USA) as described by Kundu\(^2\) and quantified following release of oligosaccharides from glycosphingolipids by ceramide glycanase (Recombinant endoglycoceramidase II, Takara Bio Inc., Otsu, Shiga, Japan) digestion. The enzyme was used according to the manufacturer’s instructions. Released oligosaccharides were labeled at their reducing end with the fluorescent compound anthranilic acid (2-aminobenzoic acid), prior to analysis using normal-phase high-performance liquid chromatography\(^2\). Throughout the procedure monosialoganglioside-GM1 (Sigma, St Louis, Mo, USA) was used as an internal standard. Similar procedures were used to measure the concentrations of ceramide, glucosylceramide, ceramidetrihexoside, and GM3 in homogenates of spleen specimens.

**Statistical analysis**
Results are given as median and [range]. To test differences between groups a Mann–Whitney U-test was used. Correlations were tested by the rank correlation test (Spearman coefficient, ρ). A p-value <0.05 was considered statistically significant.
Results

The median concentration of ceramide in plasma of Gaucher patients (9.8 [5.7–14.7 μmol/L]) was comparable to that of control subjects (11.0 μmol/L [5.1–18.0 μmol/L]) (p=0.19) (figure 1A). The glucosylceramide concentration was significantly higher in Gaucher patients (20.3 [7.2–54.2] μmol/L) compared to control subjects (5.7 [3.7–7.6] μmol/L)( p=0.00)(figure 1B). The ceramidetrihexoside concentration in plasma of Gaucher patients (1.7 [0.8–3.3] μmol/L]) was not significantly different from the concentration measured in control subjects (1.7 [1.2–2.6] μmol/L)( p=0.77) (figure 1C). GM3 concentrations were on average up to 3-fold elevated in plasma of Gaucher patients (10.2 [4.3–19.1] μmol/L) compared to control subjects (3.6 [2.7–5.4] μmol/L)( p=0.00) (figure 1D). Negroni previously reported values for total ganglioside concentration in human serum from control subjects between 4.0–8.9 μmol/L\textsuperscript{27}.

Gaucher cells laden with cerebrosides mainly glucosylceramide, can be found predominantly in spleen and liver causing gross enlargement of these organs. Nilsson et al.\textsuperscript{17} reported of a 200–500 times higher glucosylceramide concentration and a two- to six-fold elevation in GM3 concentration in spleen and liver of types I, II, and III Gaucher patients. They did not find major differences between the three types with respect to elevations in glucosylceramide and GM3 concentrations. To confirm their results and to see if the glycosphingolipid composition in spleen and plasma is comparable, we analyzed spleens from four Gaucher type I patients and two control subjects on ceramide, glucosylceramide, ceramidetrihexoside, and GM3 content (table 1). The ceramide concentration in spleens of Gaucher patients was on average three times higher than in control spleens. Glucosylceramide was almost thousand-fold elevated and GM3 was five times higher in Gaucher spleen versus control spleen.

It is known that splenectomized Gaucher patients have higher concentrations of glucosylceramide in the blood compared to Gaucher patients who did not have a splenectomy \textsuperscript{28}. In our study thirteen out of 40 Gaucher patients were splenectomized and glucosylceramide levels were significantly higher (p=0.024) in these patients, consistent with was found earlier. GM3 concentrations were also significantly higher in the splenectomized patients (11.6 μmol/L [7.3–19.1] μmol/L]) compared to the non-splenectomized patients (9.5 μmol/L [4.3–13.6] μmol/L),(p=0.010).
Figure 1  Plasma concentrations of ceramide (Cer) (A), glucosylceramide (GlcCer) (B), globoside ceramidetrihexoside (CTH) (C) and GM3 (D) were determined as described in Methods and are expressed as μmol/L. Displayed data are collected from 40 untreated symptomatic Gaucher patients and 30 control subjects. In all dot plots the bar represents the median.

In Gaucher patients GM3 and glucosylceramide concentrations are significantly correlated (ρ=0.64, p=0.00)(figure 2a). Glucosylceramide is known to correlate with important parameters of Gaucher disease, like chitotriosidase, overall disease severity and hepatomegaly. In our study we investigated the correlation of GM3 with these disease parameters. GM3 concentration is significantly correlated to chitotriosidase activity (ρ=0.45, p=0.00)(figure 2b), SSI (ρ=0.39, p=0.012)(figure 2c) and excess liver volume (ρ=0.49, p=0.00)(figure 2d).
Table 1  Concentrations of ceramide, glucosylceramide, ceramidetrihexoside and GM3 in spleen specimens of Gaucher patients and controls. Concentrations of ceramide (Cer), glucosylceramide (GlcCer), ceramidetrihexoside (CTH), and GM3 were determined in spleen specimens of type I symptomatic Gaucher patients (n=4) vs. that of control subjects (n=2) as described in Methods. Concentrations are expressed as mmol/kg wet weight.

<table>
<thead>
<tr>
<th></th>
<th>Cer (mmol/kg wet weight)</th>
<th>GlcCer (mmol/kg wet weight)</th>
<th>CTH (mmol/kg wet weight)</th>
<th>GM3 (mmol/kg wet weight)</th>
</tr>
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<tbody>
<tr>
<td>Patient 1</td>
<td>0.32</td>
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<tr>
<td>Patient 2</td>
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<td>28.1</td>
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<td>0.14</td>
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<td>Patient 3</td>
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<td>38.1</td>
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<tr>
<td>Patient 4</td>
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<td>Mean ± SD</td>
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<td>29.7±9.5</td>
<td>0.02±0.001</td>
<td>0.15±0.04</td>
</tr>
<tr>
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<td>0.02</td>
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<td>0.02</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>0.09±0.001</td>
<td>0.03±0.01</td>
<td>0.01±0.002</td>
<td>0.03±0.01</td>
</tr>
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Figure 2  Correlation of plasma GM3 with plasma glucosylceramide (A), chitotriosidase activity (B), SSI (C), and excess liver volume (D). Plasma chitotriosidase activity in the case of carriers for 24-bp duplication in chitotriosidase gene (open symbols) was multiplied by two.
Discussion

There is compelling evidence for cellular adaptations in glycosphingolipid metabolism in the case of a lysosomal glucocerebrosidase deficiency. Firstly, Saito and Rosenberg demonstrated that cultured fibroblasts from Gaucher patients show increased conversion of glucosylceramide to gangliosides\textsuperscript{15}. Secondly, the accumulation of glucosylceramide in fibroblasts of Gaucher patients, or even in cells treated with Conditurol-B-epoxide, the potent covalent inhibitor of glucocerebrosidase, is surprisingly small. In our experience about 5 nmol glucosylceramide/mg protein accumulate in completely glucocerebrosidase-deficient fibroblasts. This amount is about doubled, to 9 nmol glucosylceramide/mg protein, when concomitantly the non-lysosomal glucocerebrosidase (GBA2) is inhibited with a hydrophobic iminosugar\textsuperscript{30}. This sharply contrasts to the accumulation of 26 nmol ceramidetrihexoside/mg protein in fibroblasts of Fabry patients compared to control fibroblasts. These latter cells are completely deficient in lysosomal alfa-galactosidase A activity generating glucosylceramide via the intermediate actosylceramide. Clearly, a large quantity of lysosomal glucosylceramide does not inevitably end up as inert storage material during glucocerebrosidase deficiency but is re-used in synthesis to gangliosides. Direct experimental evidence of such type of pathway was earlier elegantly generated by Trinchera et al. by studying the fate of labeled exogenous glycosphingolipid in rat liver\textsuperscript{31}. Consistent with this, our study indicates that in glucocerebrosidase deficient Gaucher patients, as compensatory mechanism GM3 is increased. Our findings with plasma specimens are consistent with very early observations by Nilsson and coworkers who reported increased levels of GM3 in spleen, liver, and brain of investigated Gaucher patients\textsuperscript{16,17}. It is of interest to note that the globoside ceramidetrihexoside is not abnormally high in plasma of Gaucher patients. Apparently, increased anabolism of glucosylceramide is restricted to the gangliosides and involves not globosides. A chronically increased production of gangliosides like GM3 may not be without consequence. We and others have recently demonstrated that elevated GM3 in tissues is associated with insulin resistance and abnormal glucose homeostasis. Obesity, an established risk factor for insulin resistance, is associated with elevations in glycosphingolipids. Importantly, it was demonstrated in two independent studies employing different classes of inhibitors of glycosphingolipid synthesis that lowering of the gangliosides leads to improved glucose homeostasis by increased insulin sensitivity in liver and peripheral tissues\textsuperscript{13,14}. In view of this
it is not surprising that an increased hepatic gluconeogenesis, a characteristic sign of insulin resistance of the liver, has been described for Gaucher patients. Furthermore, the observed reduction in plasma adiponectin in Gaucher patients is usually observed in association to insulin resistance. Hyperinsulinemic euglycaemic clamp studies are presently being conducted in the Academic Medical Center to monitor more closely insulin sensitivity in Gaucher patients during follow-up of enzyme replacement therapy. It will be of interest to correlate the insulin sensitivity of individual Gaucher patients with plasma GM3 levels to test the possibility of a causal relationship between the ganglioside and regulation of glucose homeostasis.

Compared to the increased glucosylceramide, the extent of elevation of GM3 in plasma of symptomatic Gaucher patients is remarkable. It should be noted that the GM3 elevation is almost just as pronounced as that of glucosylceramide, the primary storage lipid. Like plasma glucosylceramide, increased plasma GM3 correlates with the body burden of Gaucher cells, overall severity of disease, and a clinical sign like hepatomegaly. It appears that increases in GM3 may be considered as a hallmark of Gaucher disease manifestation and reflecting the cellular adaptations to impaired lysosomal catabolism of glucosylceramide. Attention is warranted to the occurrence and consequences of secondary abnormalities in monogenetic disorders like Gaucher disease.

References


Aguilera B, Ghauiharali-van der Vught K, Helmond MT, et al. Transglycosidase activity of chitotriosidase: im-


