Defective galactosylation of serum transferrin in galactosemia

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The glycosylation of serum transferrin from galactosemic patients with a deficiency of galactose-1-phosphate uridyl transferase (EC 2.7.7 12) is abnormal but becomes normal after treatment with a galactose-free diet. To understand the structural and biochemical basis of the abnormal glycosylation, transferrin was purified from the serum of untreated and treated galactosemic patients and normal controls and the N-linked glycans analyzed by HPLC. The glycans from normal transferrin consisted predominantly (86%) of the disialylated biantennary complex type. The glycans from untreated galactosemic patients were more heterogeneous and contained four major truncated glycans in addition to a smaller amount (13%) of the disialylated biantennary complex type. The truncated glycans were deficient in galactose and sialic acid and their structures were consistent with a decrease in galactosyltransferase activity in hepatocytes, the probable cells of origin of the transferrin. This is postulated to be due to direct inhibition of the galactosyltransferase activity by the accumulated galactose-1-phosphate or to an effect on the formation of UDP-galactose, the donor substrate in the reaction. After treatment the proportion of the truncated glycans decreased and the proportion of the disialylated biantennary complex type increased, returning almost but never completely to normal, even after prolonged treatment in some cases. There was no clear relationship between the length of treatment and the normalization of glycosylation and the level of galactose-1-phosphate in red blood cells, the usual parameter for monitoring the treatment of galactosemics. It is suggested that the persistence of abnormally glycosylated proteins may contribute to the long-term complications in galactosemia.

Key words: galactosemia/glycosylation/transferrin

Introduction

Galactosemia is an autosomal recessive disorder caused by a deficiency of one of the enzymes involved in the utilization of dietary galactose; galactokinase (EC 2.7.1.6), galactose-1-phosphate uridyl transferase (EC 2.7.7.12), or uridine diphosphate galactose-4-epimerase (EC 5.1.3.2) (Segal and Berry, 1995). The most common form of galactosemia is due to a deficiency of galactose-1-phosphate uridyltransferase (GALT) (Isselbacher et al., 1956), which catalyzes the reaction between galactose-1-phosphate and UDP-glucose to form glucose-1-phosphate and UDP-galactose. The deficiency of GALT leads to the accumulation of galactose-1-phosphate and to the oxidation and reduction of galactose to galactonate and galactitol, respectively. The main clinical features of galactosemia are a failure to thrive, diarrhea and dehydration, vomiting, jaundice, hepatomegaly, hypoglycemia, and cataracts (Donnell et al., 1960). Treatment with a galactose-free diet prevents these acute severe symptoms, but there is increasing evidence that the long term prognosis for these patients is not as good as was first hoped. Long term complications include below-average IQ, ovarian dysfunction, speech difficulties, delayed growth, and impaired motor function and balance, suggesting persistent metabolic defects in the brain and ovarian tissues. The biochemical basis of both the acute and chronic symptoms is not well understood, and there is no correlation between outcome and genotype, residual GALT activity or the red blood cell level of galactose-1-phosphate, which is used to monitor treatment (Komrower, 1982; Waggoner et al., 1990; Schweitzer et al., 1993; Cleary et al., 1995).

The isoelectric focusing patterns of serum transferrin and the lysosomal enzymes, α-fucosidase and β-hexosaminidase, from untreated galactosemics are abnormal and similar to those seen in the carbohydrate-deficient glycoprotein syndrome (Jaeken et al., 1992). Following treatment, the less sialylated isoforms disappear and the isoelectric focusing patterns become normal (Winchester et al., 1995). It has been suggested that the decreased sialylation of serum glycoproteins in galactosemia is attributable either to a decrease in galactosylation resulting from decreased availability of UDP-galactose or to inhibition of galactosyltransferase by galactose or a metabolic derivative (Van Pelt et al., 1996). Other studies have shown that glycoproteins from cultured fibroblasts (Dobbie et al., 1990; Orinstein et al., 1992) and serum (Presott et al., 1997) from galactosemic patients have an increased capacity for galactosylation in vitro. In this article we have analyzed the N-linked glycans of serum transferrin from patients with galactosemia before treatment and shown that they are truncated and terminate predominantly in N-acetylgalactosamine. Furthermore, a small proportion of these abnormal glycoforms persist after treatment. We postulate that such aberrantly glycosylated proteins may contribute to the long term problems associated with treated galactosemic patients and that their detection may be a useful predictor of outcome of treatment.

Results

Analysis of isoforms of serum transferrin

Human serum transferrin has two N-glycosylation sites, which are normally both fully occupied by disialylated biantennary glycans to generate tetrasialotransferrin (Spik et al., 1975). This was the predominant isoform in the transferrin purified from normal human serum (Figure 1a). Less acidic forms, i.e., less sialylated forms were present in the serum of all the untreated galactosemic patients (Figure 1b). These forms were either absent or present as...
a much lower proportion of the total transferrin in the serum of patients who were on a galactose-free diet (Figure 1c).

Analysis of the N-linked glycans on serum transferrin

N-Linked glycans were released from the serum transferrin purified from normal controls, untreated galactosemic patients and patients who had been on treatment for various lengths of time, and, analyzed by HPLC (Figure 2, Table I). It can be seen (Figure 2a) that the N-linked glycans from the normal transferrin consisted predominantly of the disialylated biantennary type (86%), as has been reported by us (Iourin et al., 1996; Charlwood et al., 1997) and others previously (Spik et al., 1975). The identity of this glycan was established by its retention time and cochromatography with an authentic standard. In contrast, the glycans released from the transferrin from an untreated galactosemic patient (Figure 2b) were more heterogeneous and contained four major truncated glycans in addition to a smaller amount (13%) of the normal disialylated biantennary type. After treatment the proportion of the truncated glycans fell and the proportion of the disialylated biantennary glycan increased, reflecting the normalization of the glycosylation seen in the intact transferrin (Table I). In one patient who had been on the galactose-free diet for 9 months, the proportion of the disialylated biantennary glycan on the transferrin had increased to 82% with a concomitant decrease in the truncated glycans (Figure 2c). However, in other patients who had been on the diet for a comparable period the proportion of normal glycans was much lower.

Table I. Relationship between treatment and level of galactose-1-phosphate in red blood cells and proportion of disialylated biantennary glycans on serum transferrin in patients with galactosemia

<table>
<thead>
<tr>
<th>Weeks of treatment</th>
<th>Galactose-1-phosphate (µmol/g)</th>
<th>% Disialylated biantennary glycans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0*</td>
<td>2.8</td>
<td>13</td>
</tr>
<tr>
<td>0</td>
<td>4.0</td>
<td>13</td>
</tr>
<tr>
<td>0</td>
<td>8.75</td>
<td>13</td>
</tr>
<tr>
<td>Treated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.34</td>
<td>21</td>
</tr>
<tr>
<td>24</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>38</td>
<td>0.3</td>
<td>82</td>
</tr>
<tr>
<td>42</td>
<td>0.22</td>
<td>58</td>
</tr>
<tr>
<td>50</td>
<td>0.15</td>
<td>28</td>
</tr>
</tbody>
</table>

* and # indicate measurements on same patients.

Structures of the truncated N-linked glycans on serum transferrin in galactosemia

The structures of the truncated glycans were either established by comparison of retention times and cochromatography with authentic samples or deduced from the calibration of the column with authentic standards (Figure 3). Component 1 (rt, 24.8 min) was shown to be the core pentasaccharide, Manα1→3Man[Manα1→6]β1→4GlcNAcβ1→4GlcNAc, by cochromatography and calibration.

The structure of component 2 (rt, 29.1 min) was deduced to be the core pentasaccharide with an additional N-acetylglucosamine attached to one of the peripheral mannose residues, i.e., (GlcNAcβ1→2)Manα1→3Man[Manα1→6]β1→4GlcNAcβ1→4GlcNAc. It had a retention time most consistent with the addition of an N-acetylglucosamine residue in a terminal position to compound 1 or the loss of an N-acetylglucosamine residue in a terminal position from GlcNAcβ1→2Manα1→3Man[Manα1→6]β1→4GlcNAcβ1→4GlcNAc. The retention time of an authentic sample of the latter was 31.7 min. The possibility of α1→6 fucosylation of the N-acetylglucosamine at the reducing end of the core pentasaccharide cannot be completely excluded but the data obtained under our chromatography conditions were more compatible...
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Fig. 2. HPLC of fluorescently labeled glycans released from transferrin. (a) Normal control; (b) untreated galactosemic patient; (c) patient on galactose-free diet for 38 weeks. The structures of the truncated glycans, 1–4, are shown in Figure 3.

with the addition of an N-acetylglucosamine residue at a nonreducing terminal of the core pentasaccharide rather than core fucosylation.

Component 3 had a retention time of 33.6 min, slightly less than that of the authentic sample of the truncated triantennary structure with N-acetylglucosamine at the end of each branch (rt, 34.5 min). The retention time of glycans 3 was consistent with the addition of a bisecting N-acetylglucosamine to GlcNAcβ1→2Manβ1→3Man[GlcNAcβ1→2Manα1→6]β1→4GlcNAcβ1→4GlcNAc rather than of an α1→6 linked fucose residue to the reducing terminal N-acetylglucosamine residue or a galactose residue at the nonreducing end. Additional-

ly, any exposed galactose residues would be expected to be capped rapidly with sialic acid under conditions in which there is a general decrease in galactosylation.

Component 4 (rt, 39.2 min) cochromatographed with an authentic sample of the truncated tetra-antennary oligosaccharide with each branch terminating in an N-acetylglucosamine residue. Thus each of the truncated oligosaccharides is deficient in galactose and sialic acid and there is an increase in glycans (2, 3, and 4) with N-acetylglucosamine at the nonreducing ends. This increase in exposed N-acetylglucosamine residues would explain the increase in galactose acceptor capacity of glycoproteins from the serum of patients with galactosemia (Prestoz et al., 1997).

Relationship between level of galactose-1-phosphate in red blood cells and glycosylation of serum transferrin

The effectiveness of treating patients with galactosemia with a galactose-free diet is usually monitored by measuring the level of galactose-1-phosphate in red blood cells (Gitzelman, 1995). As treatment also leads to the formation of normally glycosylated serum transferrin, the correlation between the red blood cell galactose-1-phosphate level and the percentage of disialylated biantennary glycans present in serum transferrin was investigated (Table 1). The levels of galactose-1-phosphate in the red blood cells of untreated galactosemics were greater than 1.0 µmol/g and the proportion of serum transferrin with normal disialylated biantennary glycans was 13%. Although the level of galactose-1-phosphate in the red blood cells fell on treatment the proportion of disialylated biantennary glycans ranged between 18 and 82%. There was not a clear correlation between the level of galactose-1-phosphate and the length of treatment or state of glycosylation.

Discussion

The structures of the truncated glycans found on serum transferrin from untreated galactosemic patients, are consistent with a decreased capacity to galactosylate glycoproteins. This defect in galactosyltransferase activity could be due to inhibition of the galactosyltransferase enzyme itself or to decreased availability of the substrate, UDP-galactose. There is some evidence that galactose-1-phosphate inhibits galactosyltransferase when at high concentrations in cells (Roth et al., 1971), and it has been shown to inhibit milk UDP-galactosyltransferase activity (Segal, 1995). It is also a competitive inhibitor and substrate for UDP-glucose pyrophosphorylase (EC 2.7.7.9 ), which also catalyzes the formation of UDP-galactose from galactose-1-phosphate and UTP (Oliver, 1961). Galactose, galactose-1-phosphate or other galactose derivatives may inhibit other enzymes involved in the synthesis of UDP-galactose or its transport into the Golgi, thereby lowering the effective substrate concentration. The question of whether a deficiency of UDP-galactose is responsible for under-galactosylation has been discussed for many years because of the technical difficulty of measuring UDP-galactose accurately. Ng et al. (1989) showed that there were decreased amounts of UDP-galactose in red cells, fibroblasts, and liver, but other groups have failed to confirm this finding (Berry et al., 1992; Kirkman, 1992; Gibson et al., 1993, 1994).

The defect in galactosyltransferase activity in galactosemia provides a genetic, albeit secondary, model for studying the factors that regulate the pattern of glycosylation in human hepatocytes, in which transferrin is synthesized (Figure 4). The first potential substrate for galactosyltransferase in the glycopro-
tein processing pathway is the N-linked glycan, GlcNAcβ1→2 Manα1→3Man|GlcNAcβ1→2Manα1→6β1→4GlcNAc- β1→4GlcNAc. However, this structure was not found on transferrin from patients with galactosemia. The probable reason for this is that it is also a substrate for at least two other enzymes, N-acetylglucosaminyltransferase III and N-acetyl glucosaminyltransferase IV. Two of the abnormal glycans that are found on transferrin from patients with galactosemia, 3 and 4, are the expected end products for the processing routes initiated by these enzymes. This indicates that in the absence of galactosyltransferase, partially processed N-linked glycans are diverted to alternative pathways that give rise to bisected or more highly branched glycans. The most abundant truncated glycan, 2, is the immediate precursor of GlcNAcβ1→2Manα1→3Man|GlcNAcβ1→2 Manα1→6β1→4GlcNAcβ1→4GlcNAc. The absence of the latter, suggests that the activities of N-acetylgalcosaminyltransferase III and IV are greater than that of N-acetylgalcosaminyl transferase II. Similarly the absence of the truncated triantennary glycan suggests the activity of N-acetyl glucosaminyltransferase V is greater than that of N-acetyl glucosaminyltransferase IV. It can also be concluded that addition of the bisecting N-acetylgalcosamine prevents further branching under these conditions. These observations add to our knowledge of control points in the glycoprotein processing pathway (Schachter, 1984).

The origin of the core pentasaccharide (1) is interesting because it is not normally an intermediate in the biosynthesis or processing pathways for glycoproteins in most cell types. However, there is evidence that in nonerythroid cells there is a distinct α-mannosidase (III) that can remove two mannose residues from the ManαGlcNAc2 processing intermediate to produce compound 1 without the prior action of N-acetylgalcosaminyl transferase 1 (Bonay and Hughes, 1991; Chui et al.,

![Fig. 3. Structures and retention times of oligosaccharide standards and glycans identified by HPLC. Numbers in boldface type denote glycans released from serum transferrin from patients with galactosemia, and asterisks indicate the standards used to calibrate the HPLC column.](https://academic.oup.com/glycob/article-abstract/8/4/351/677803)
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Fig. 4. Processing of N-linked glycans in patients with galactosemia. Key: Gn, N-acetylglucosamine; M, mannose; G, galactose; and SA, sialic acid. Enzymes: 1, α3/6-mannosidase II; 2, N-acetylglucosaminyltransferase III; 3, N-acetylglucosaminyltransferase II; 4, galactosyltransferase; 5, sialyl transferase; 6, N-acetylglucosaminyltransferase IV; 7, N-acetylglucosaminyl transferase V.

1997). Alternatively compound 1 could arise by removal of N-acetylglucosamine from the most abundant glycan (2) on transferrin from serum of galactosemic patients either during processing or by the action of serum β-hexosaminidase. Theoretically, glycan (1) could be Manα1→2Manα1→3/6Manβ1→4GlcNAcβ1→4GlcNAc, but it is difficult to understand how such a structure could arise from the established specificities of human α-mannosidases (Daniel et al., 1992).

There was not a clear relationship between the length of treatment and the fall in the level of galactose-1-phosphate in red blood cells and the normalization of glycosylation of serum transferrin. There are several possible explanations for this observation. One is that there is a difference in response times for the drop in red blood cell galactose-1-phosphate level and normalizaton of serum protein glycosylation. The level of galactose-1-phosphate in red blood cells drops within a few days of commencing the galactose-free diet. Normal transferrin has a half-life of 8 days and it has been shown in alcoholics, in whom there is also an increase in undersialylated transferrin, that at least 10 days abstinence is required before transferrin patterns start to return to normal (Stibler, 1991). As all the patients had been on the diet for at least 20 days and most for much longer the glycosylation pattern of transferrin should have returned to normal. Compliance with diet may be an explanation. The faster response of red blood cell galactose-1-phosphate level may not reveal a previous lapse in diet, which is manifested by the slower normalization of serum protein glycosylation. However it has also been suggested that measurement of galactose-1-phosphate levels in red blood cells is not informative for the rest of the body organs (Segal, 1995). It has also been shown that there is no correlation between the galactose-1-phosphate levels and the IQ, health, or development of the child (Waggoner et al., 1990). There have been many studies on the intellectual impairment of galactosemic patients, but no correlation has been found between the galactose-1-phosphate levels and genotype or age of onset of treatment (Komrower, 1982; Waggoner et al., 1990; Schweitzer et al., 1993; Cleary et al., 1995). Red blood cells are not actively glycosylating proteins and measurement of the levels of galactose derivatives in cells actively synthesizing proteins would be more informative. Analysis of the glycan chains of transferrin may give
Materials and methods

Patient samples

Whole blood samples were collected from 7 patients with a proven GALT deficiency. For two patients samples were available before and after they were put on a galactose-free diet. Galactose-1-phosphate levels were measured in packed red blood cells and the serum was used for the transferrin analysis.

Purification of serum transferrin

Serum (0.5 ml) was added to 0.2 ml of water and thoroughly mixed. Rivanol (0.3 ml, 3% w/v) in water) was added drop-wise with mixing. After standing at room temperature for 10 min, the suspension was centrifuged at 1000 \times g for 10 min and the yellow supernatant collected. 0.43 ml of 25% (w/v) NaCl was added to the supernatant and after mixing thoroughly the suspension was centrifuged at 1000 \times g for 10 min. and the yellow supernatant collected. 0.43 ml of 25% (w/v) NaCl was added to the supernatant and after mixing thoroughly the suspension was centrifuged at 1000 \times g for 10 min; 0.8 ml of saturated ammonium sulfate was added per ml of the clear supernatant. The solution was vortexed and used to deduce the structures of components for which an authentic standard was not available.

Analysis of N-linked glycans of transferrin

N-Linked glycans were released from purified transferrin by sequential digestion with trypsin (Sigma) and N-peptide glycase F (Oxford Glycosciences) (Charwood et al., 1997). After removal of peptides by precipitation with acetonitrile the glycans were fluorescently labeled with 2-aminobenzamide using a kit (OGS K-404) from Oxford Glycosciences and analyzed by HPLC using a GlycoSep-N column with a gradient running from 250 mM ammonium formate, pH 4.4 (acetonitrile) to 100% 250 mM ammonium formate, pH 4.4. The effluent was monitored at an excitation wavelength of 330 nm and emission at 420 nm. The column was calibrated with fluorescently labeled dextran hydrolysate and authentic standard glycans (M3N2, NGA2, NGA3, NGA4, NGA2FB, HYBR, MAN5, and A2, Oxford Glycosciences; Figure 3). The contribution of different glycosyl residues to the retention time was calculated in terms of glucose equivalent units (Guile et al., 1996; Iourin et al., 1996) and used to deduce the structures of components for which an authentic standard was not available.

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