GALT deficiency causes UDP-hexose deficit in human galactosemic cells

K. Lai1,2,3, S.D. Langley3, F.W. Khwaja4, E.W. Schmitt1, and L.J. Elsas3,4

1Division of Medical Genetics, Department of Pediatrics, Emory University School of Medicine, 2040 Ridgewood Drive, Atlanta, GA 30322, USA; 2Department of Genetics and Molecular Biology Program, Graduate Division of Biological Sciences, Emory University School of Medicine, 2040 Ridgewood Drive, Atlanta, GA 30322, USA

Previously we reported that stable transfection of human UDP-glucose pyrophosphorylase (hUGP2) rescued galactose-1-phosphate uridylyltransferase (GALT)-deficient yeast from “galactose toxicity.” Here we test in human cell lines the hypothesis that galactose toxicity was caused by excess accumulation of galactose-1-phosphate (Gal-1-P), inhibition of hUGP2, and UDP-hexose deficiency. We found that SV40-transformed fibroblasts derived from a galactosemic patient accumulated Gal-1-P from 1.2 ± 0.4 to 5.2 ± 0.5 mM and stopped growing when transferred from 0.1% glucose to 0.1% galactose. Control fibroblasts accumulated little Gal-1-P (0.2 ± 0.1 mM) and continued to grow. The GALT-deficient cells had 157 ± 10 μmoles UDP-glucose/100 g protein and 25 ± 5 μmoles UDP-galactose/100 g protein when grown in 0.1% glucose. The control cells had 236 ± 25 μmoles UDP-glucose/100 g protein and 82 ± 10 μmoles UDP-galactose/100 g protein when grown in identical medium. When we transfected the GALT-deficient cells with either the hUGP2 or GALT gene, their UDP-glucose content increased to 305 ± 28 μmoles/100 g protein (hUGP2-transfected) and 210 ± 13 μmoles/100 g protein (GALT-transfected), respectively. Similarly, UDP-galactose content increased to 75 ± 12 μmoles/100 g protein (hUGP2-transfected) and 55 ± 9 μmoles/100 g protein (GALT-transfected), respectively. Though the GALT-transfected cells grew in 0.1% galactose with little accumulation of Gal-1-P (0.2 ± 0.2 mM), the hUGP2-transfected cells grew but accumulated some Gal-1-P (3.1 ± 0.4 mM). We found that 2.5 mM Gal-1-P increased the apparent $K_M$ of purified hUGP2 for glucose-1-phosphate from 19.7 μM to 169 μM, without changes in apparent $V_{max}$. The $K_i$ of the reaction was 0.47 mM. Gal-1-P also inhibited UDP-N-acetylglucosamine pyrophosphorylase, which catalyzes the formation of UDP-N-acetylglucosamine. We conclude that intracellular concentrations of Gal-1-P found in classic galactosemia inhibit UDP-hexose pyrophosphorylases and reduce the intracellular concentrations of UDP-hexoses. Reduced Sambucus nigra agglutinin binding to glycoproteins isolated from cells with increased Gal-1-P is consistent with the resultant inhibition of glycoprotein glycosylation.

Key words: competitive inhibition/fibroblast/galactosemia/galactose-1-phosphate uridylyltransferase/UDP-glucose pyrophosphorylase

Introduction

In humans, deficiency of galactose-1-phosphate uridyltransferase (GALT) (E.C. 2.7.7.12) produces the disorder classic galactosemia (OMIM entry 230,400) (Isselbacher et al., 1956; Segal and Berry, 1995) (Figure 1). In the newborn period, exposure to galactose produces hepatotoxicity, Escherichia coli sepsis, and death in untreated patients. Survivors have long-term complications that include ataxia, verbal dyspraxia, and premature ovarian failure (Waggoner et al., 1990). The mechanisms producing dysfunction of these different organs are complex and remain unknown.

In addition to GALT gene mutations, epigenes and environment are important factors governing the outcome of this disorder (Robertson et al., 2000; Guerrero et al., 2000). Because intracellular accumulation of high level of galactose-1-phosphate (Gal-1-P) (up to 3.3 mM) is uniquely observed in GALT deficiency, one hypothesis is that the accumulated Gal-1-P is toxic (Gitzelmann et al., 1984). This hypothesis is based on the clinical observation that patients who suffer from galactokinase deficiency (OMIM 230,200) but have normal GALT activity have cataracts and over-produce galactitol but do not accumulate Gal-1-P. These galactokinase-deficient patients do not have the complications observed in GALT-deficiency galactosemia (Segal and Berry, 1995).

Others have proposed that the long-term complications in GALT-deficiency galactosemia result from a deficiency of galactose-containing glycoproteins and/or glycolipids because of deficient production of UDP-galactose (Ng et al., 1989). Biochemical evidence for abnormal galactosylation is found in the altered isoform patterns of serum transferrin, β-hexosaminidase, and follicle stimulating hormone when Gal-1-P is accumulated in patients’ cells (Charlow et al., 1998; Prestoz et al., 1997; Jaeken et al., 1992). The oligosaccharide chains of the circulating transferrin and follicle-stimulating hormone were found deficient in their penultimate galactose and terminal sialic acids. Moreover, lymphocytes and brain lipids of an infant with galactosemia who died from sepsis had reduced N-acetylgalactosamine and galactosyl residues when compared to a nongalactosemic control (Petry et al., 1991). From these observations GALT-deficiency galactosemia was classified as one of the carbohydrate-deficient glycoprotein
syndromes (Jucken et al., 1992). The mechanisms for these biochemical alterations remained unclear. Because tremor, ataxia, learning disabilities, and premature ovarian failure were seen in older children with classic galactosemia, it was possible that elevated levels of Gal-1-P altered surface glycosphingolipids (the gangliosides) of the developing brain neurons, as well as the O- or N-linked glycosylation patterns of secreted glycoproteins. This pathology could occur either during embryonic or postpartum development.

Animal models of classic galactosemia failed to mimic the human pathophysiology of either the neonatal toxicity syndrome or the long-term complications associated with the disorder (Leslie et al., 1996). In one study, normal rats fed high galactose (40%) diets (Chen et al., 1981) gave birth to newborns with cataracts and female pups had fewer oocytes, but these pups remained fertile and had no long-term complications. The authors contended that the endogenous GALT genes in these rats might have protected them from galactose toxicity. Leslie and co-workers constructed a GALT double knockout transgenic mouse, but these mice were healthy and fertile despite being fed with high galactose diet (Leslie et al., 1996; Ning et al., 2000). The absence of complications in the GALT-knockout mice indicated the uniqueness of human galactose metabolism and emphasized the need to develop a human cell model system.

In an earlier study, we found that overexpression of the human UDP-glucose pyrophosphorylase (hUGP2) gene in a GALT-less yeast strain allowed it to overcome galactose toxicity and grow on galactose (Lai and Elsas, 2000). In this study, we develop a human cell model that duplicates the yeast system. We used SV40-transformed human fibroblasts that were derived from a patient with classic galactosemia. This cell line accumulated Gal-1-P and did not grow in galactose. However, when these GALT-deficient cells were transfected with hUGP2 or GALT they were rescued. We test the hypothesis that in classic galactosemia, Gal-1-P was accumulated and reduced UDP-glucose synthesis by inhibiting hUGP2. We quantified UDP-glucose and UDP-galactose and determined the kinetics of hUGP2 inhibition by Gal-1-P. We conclude that overexpression of hUGP2 in human GALT-deficient cells overcame inhibition of endogenous hUGP2 by Gal-1-P and restored normal UDP-hexose levels.

Results

Gal- phenotype in human GALT-deficient cells

The inability to grow in galactose (the Gal- phenotype) is not limited to GALT-less yeast (Douglas and Hawthorne, 1966; Lai and Elsas, 2000) but is also observed in dermal fibroblasts derived from humans with naturally impaired GALT activity (Pourci et al., 1990; this study). There are at least three possible mechanisms to explain why human GALT-deficient fibroblasts cannot grow in medium with galactose as the sole carbohydrate:

1. Lack of energy because galactose is not metabolized to form glucose-1-phosphate;
2. Toxicity resulting from accumulated toxins (e.g., Gal-1-P) in the blocked Leloir pathway;
3. Decreased production of essential uridylated hexoses, such as UDP-galactose.

In this study, we used a human cell model to differentiate 1, 2, and 3. We developed a GALT-deficient fibroblast cell line, GM00638A, that ceased to grow when 0.1% galactose was added to hexose-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Figure 2). We chose to use 0.1% galactose in this experiment because plasma galactose concentrations easily reach between 10–20 mM (1.8–3.6 mg/ml) in galactosemic infants after ingestion of a 60-ml bottle of cow milk (Greenman and Rathbun, 1948; Komrower et al., 1956; Segal and Berry, 1995; Siegel et al., 1988). The GALT-deficient fibroblasts began to perish at 24 h after the transfer to 0.1% galactose, and over 50% of cells died off by 48 h. By contrast, fibroblast cell line GM00637I with normal GALT activity was unaffected by Gal-1-P. We conclude that overexpression of hUGP2 in human GALT-deficient cells overcame inhibition of endogenous hUGP2 by Gal-1-P and restored normal UDP-hexose levels.
Expression of hUGP2 or GALT allowed GALT-deficient cells to grow in galactose

Previously, we found that overexpression of hUGP2 in a GALT-less yeast strain allowed it to overcome galactose toxicity and grow on galactose (Lai and Elsas, 2000). We transfected the GALT-deficient cell line GM00638A with hUGP2 or GALT cDNA. Stable transfectants were selected by a predetermined dose of G-418 as described in Materials and methods. The enzyme activities of hUGP2 or GALT expressed in the untransfected and stable transfectants are shown in Table I. Although there was no GALT activity detected in the galactosemic patient’s red blood cells, there was detectable but low GALT activity in the untransfected SV40-transformed fibroblasts derived from this patient. This was increased 10-fold when transfected with the GALT gene (Table I).

We found that expression of either GALT or hUGP2 in the GALT-deficient cells allowed them to grow in hexose-free DMEM supplemented with 0.1% galactose and 10% FBS (Figure 3).

Preliminary biochemical characterization of normal, untransfected, and transfected GALT-deficient cell lines

To explore the mechanisms of rescue in the transfected cell lines, we first quantified and compared galactose metabolites of the normal, GALT-deficient, and transfected

(continued)

Table I. UGP and GALT activities measured in fibroblast cell extracts

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<tr>
<th>Sample</th>
<th>UGP activitya</th>
<th>GALT activityb</th>
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<tr>
<td>GM00637I (normal)</td>
<td>16.7 ± 2.5 (N = 4)</td>
<td>24.3 ± 1.6 (N = 4)</td>
</tr>
<tr>
<td>GM00638A (GALT-deficient)</td>
<td>14.9 ± 2.0 (N = 4)</td>
<td>1.1 ± 0.2 (N = 4)</td>
</tr>
<tr>
<td>GM00638A transfected with hUGP2</td>
<td>Not done</td>
<td>Not done</td>
</tr>
<tr>
<td>GM00638A transfected with GALT</td>
<td>Not done</td>
<td>11.0 ± 0.9 (N = 4)</td>
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</table>

UGP and GALT activities were determined in cells harvested at 80% confluency in DMEM with 0.1% glucose and 10% FBS.

a nmol UDP-glucose produced/mg cell protein/min.
b nmol UDP-galactose produced/mg cell protein/min.

Fig. 2. Galactose is toxic to human GALT-deficient fibroblasts. Human GALT-deficient cell line GM00638A grew well in DMEM with 0.1% glucose and 10% FBS (a), but culture in hexose-free DMEM supplemented with 0.1% galactose and 10% FBS resulted in cessation of growth and eventual cell death after 24 h, as illustrated by the round cells in b. Survival of the GALT-deficient cells in 0.1% galactose can be prolonged for 24 h if 0.01% glucose is added to the medium.

Fig. 3. Growth of normal, untransfected, and transfected GALT-deficient cell lines on 0.1% galactose. Wild-type (GM00637I), GALT-deficient (GM00638A), and GALT-deficient fibroblasts transfected with either GALT or hUGP2 were cultured (in replicates) in DMEM with 0.1% glucose medium and 10% fetal bovine serum prior to transfer to hexose-free DMEM supplemented with 0.1% galactose and 10% FBS at day 1. Cell growth (y-axis) was monitored directly by cell counts (expressed as number of cells per 75-cm² flask).
fibroblast cell lines before and after transfer to 0.1% galactose from 0.1% glucose (Table II).

When grown in DMEM with 0.1% glucose supplemented with 10% FBS, the untransfected GALT-deficient cell line had significantly lower UDP-hexose contents than the normal control ($p < 0.05$). Moreover, the ratio of UDP-glucose to UDP-galactose deviated significantly from the 3:1 ratio seen in normal individuals (Segal and Berry, 1995). In addition, a significant amount of Gal-1-P (1.2 ± 0.4 mM) was accumulated in the untransfected GALT-deficient cell line despite being grown in glucose. When we expressed hUGP2 or GALT cDNA in the GALT-deficient cell line, the level of UDP-hexoses increased significantly ($p < 0.05$). There was also no detectable accumulation of Gal-1-P in the transfected cells (Table II).

Although the normal control and transfected cell lines continued to grow after being transferred to hexose-free DMEM supplemented with 10% FBS and 0.1% galactose, the quantification of galactose metabolites for the GALT-deficient cells after the same transfer was technically challenging as they begin to die at 24 h after the transfer. We later realized that an addition of 0.01% glucose to the original 0.1% galactose medium could prolong their survival for another 24 h. We therefore decided to measure galactose metabolites of all cell lines at 24 h after the transfer to hexose-free medium containing 0.1% galactose, 10% FBS, and 0.01% glucose.

As shown in Table II, UDP-hexose concentration of the normal control and GALT-transfected cell lines decreased slightly at 24 h after being transferred to the 0.1% galactose medium. Although the decrease in UDP-gluucose was more striking in the hUGP2-transfected cell line, the value remained close to those found in the normal control and GALT-transfected cell lines. On the other hand, the low UDP-hexose concentrations of the GALT-deficient cells dropped further after the transfer to the 0.1% galactose medium. Additionally, Gal-1-P accumulated up to 5.2 ± 0.5 mM in the GALT-deficient cell line when it was transferred to 0.1% galactose. This high level of Gal-1-P dropped to 3.1 mM and 0.2 mM for the hUGP2-transfected and GALT-transfected cells, respectively.

To further examine the mechanisms of rescue by over-expression of hUGP2, we proposed the following two hypotheses, which are not mutually exclusive: The over-expressed hUGP2 enzyme could have metabolized toxic Gal-1-P to form UDP-galactose, and/or the overexpressed hUGP2 enzyme activity facilitated cellular functions, such as decreased UDP-hexose biosynthesis, that were inhibited by other toxic by-products of the blocked Leloir pathway.

To examine the two hypotheses, we investigated the following:

a. Can hUGP2 enzyme convert Gal-1-P and UTP to UDP-galactose?

b. Will Gal-1-P inhibit UDP-glucose production from glucose-1-phosphate and UTP catalyzed by the hUGP2 reaction?

We determined the basic kinetic parameters of purified recombinant hUGP2 using an enzyme-linked assay (Duggleby et al., 1996). UDP-glucose production was saturated at increasing concentrations of glucose-1-phosphate from 0 to 100 μM, with an apparent $V_{\text{max}}$ of 0.12 ± 0.02 nmol UDP glucose/μg enzyme/min (Table III). This $V_{\text{max}}$ was in agreement with the values previously reported by two other groups (Duggleby et al., 1996; Knop and Hansen, 1970). The $K_M$ of hUGP2 for glucose-1-phosphate was 19.7 ± 0.4 μM.

**hUGP2 utilizes Gal-1-P as a substrate**

When we replaced glucose-1-phosphate by Gal-1-P in the assay described, we found that hUGP2, like its yeast counterpart (Lai and Elsas, 2000), produced UDP-galactose from Gal-1-P and UTP. However, the $K_M$ for Gal-1-P was found to be 1.90 ± 0.15 mM, which was 100 times higher than the $K_M$ for glucose-1-phosphate (Table III). Therefore, unless Gal-1-P is present at very high concentration, such as in untreated galactosse patients whose cellular Gal-1-P concentration can often exceed 3.3 mM (Segal and Berry, 1995; personal observation), this reaction may not be relevant under normal physiological condition. Surprisingly, this high $K_M$ for Gal-1-P is only three times that of GALT for Gal-1-P, which is 0.6 mM (Wells and Fridovich-Keil, 1997), despite the fact that Gal-1-P is the natural substrate for GALT (Figure 1).

### Table II. Galactose metabolites in normal, GALT-deficient, and transfected cells

<table>
<thead>
<tr>
<th>Glucose medium</th>
<th>Galactose medium$^a$</th>
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<tr>
<td></td>
<td>UDP-Glc</td>
</tr>
<tr>
<td>Control</td>
<td>236 ± 25</td>
</tr>
<tr>
<td>GALT-deficient</td>
<td>157 ± 10</td>
</tr>
<tr>
<td>GALT-def. + hUGP2</td>
<td>305 ± 28</td>
</tr>
<tr>
<td>GALT-def. + GALT</td>
<td>210 ± 13</td>
</tr>
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ND: not detected. UDP-Glc (UDP-glucose) and UDP-Gal (UDP-galactose) are expressed as μmoles/100 g cell protein ($N = 4$). Gal-1-P is expressed as mM ($N = 4$). Glucose medium = DMEM with 0.1% glucose + 10% FBS. Galactose medium = DMEM (hexose-free) + 0.1% galactose + 10% FBS + 0.01% glucose.

$^a$Metabolite analysis was carried out using cells harvested at 24 h after the transfer to galactose medium. No cell death was seen for another 24 h under such experimental condition.
The apparent $V_{\text{max}}$ of UDP-galactose production from Gal-1-P by hUGP2 was $0.097 \pm 0.005$ nmol UDP-galactose/µg enzyme/min (Table III). We compared the $V_{\text{max}}$ for UDP-galactose production via the hUGP2 reaction with the $V_{\text{max}}$ for UDP-galactose synthesis through the GALT reaction and found that GALT had a greater capacity. The $V_{\text{max}}$ for the GALT reaction was 80 nmol UDP-galactose produced/µg enzyme/min (Lai et al., 1999), which was 1000 times higher than the highest rate for UDP-galactose production by hUGP2 from Gal-1-P. Nonetheless, judging from the data shown in Table II, GALT-deficient cells transfected with hUGP2 had a lower concentration of Gal-1-P when compared to the untransfected cells. Therefore it is possible that overexpression of this gene in GALT-deficient cells may have helped by converting some toxic Gal-1-P to UDP-galactose.

Gal-1-P competitively inhibits hUGP2

Oliver reported that a high concentration of Gal-1-P inhibited bovine UDP-glucose pyrophosphorylase in UDP-glucose production (Oliver, 1961). We found that Gal-1-P at 2.5 mM increased the $K_M$ of hUGP2 for glucose-1-phosphate from $19.7 \pm 0.4$ to $169 \pm 21$ µM (~ eightfold) (Table III) (Figure 4). This was a significant increase in $K_M$ without a change in $V_{\text{max}}$, indicating that Gal-1-P was a competitive inhibitor of hUGP2. A $K_i$ of 0.47 mM was calculated. However, it should be noted that these data were obtained from in vitro studies of the purified protein. In regard to whether the accumulated Gal-1-P in GALT-deficient cells actually inhibits hUGP2 in vivo and to what extent, we must carry out studies such as metabolic flux analyses, which are out of the scope of this study. In the absence of in vivo kinetics, we can nevertheless infer such inhibition could have occurred in vivo in GALT-deficient cells based on the finding that restoration of GALT activity in GALT-deficient cells increased UDP-glucose and UDP-galactose concentrations (Table II).

Inhibition of AGX1 by Gal-1-P

We assessed the effect of Gal-1-P on another closely related human UTP-dependent hexose pyrophosphorylase, UDP-N-acetylglucosamine pyrophosphorylase (AGX1) (E.C. 2.7.7.9). AGX1 and hUGP2 share over 21% amino acid sequence and structural similarities, supporting the notion that they belong to the same family of UDP-sugar pyrophosphorylases (Mio et al., 1998). Because the AGX1 cDNA was not available to us, we measured AGX1 activity in protein extracts obtained from human NIH:OVCAR-3 cell lines in the presence and absence of pathological concentration of Gal-1-P. In the absence of exogenous Gal-1-P, AGX1 enzyme activity was $1.12 \pm 0.03$ nmol UDP-N-acetylglucosamine/mg cell protein/min ($N = 4$). In the presence of 2.5 mM Gal-1-P, AGX1 activity dropped 18% to $0.92 \pm 0.04$ nmol UDP-N-acetylglucosamine/mg cell protein/min ($N = 4$) ($p < 0.05$). Thus Gal-1-P was an inhibitor of AGX1.

Effect of galactose on protein glycosylation in the GALT-deficient cell line

As mentioned in the Introduction, biochemical evidence for abnormal galactosylation is found in the altered isoform patterns of serum transferrin, β-hexosaminidase, and follicle-stimulating hormone when Gal-1-P is accumulated in patients’ cells (Charlwood et al., 1998; Prestoz et al., 1997; Jaeken et al., 1992). We wanted to use our newly developed cell model to examine the effect of galactose on protein glycosylation in GALT-deficient cells.

In Figure 5, by using digoxigenin-labeled Sambucus nigra agglutinin (SNA) that specifically recognizes sialic acid linked α(2-6) to the penultimate galactose molecule of the oligosaccharide chains found in complex glycoproteins (Shibuya et al., 1987), we revealed that the abundance of SNA-positive glycoproteins present in cell extracts prepared from the GALT-deficient cell line and its GALT-transfected counterpart were identical when the cell lines were cultured in 0.1% glucose. However, when the GALT-deficient cells were transferred to 0.1% galactose for 24 h, we saw a reduced abundance of SNA-positive glycoproteins of higher molecular weight (> 66 kDa) (lane 3, Figure 5).
We isolated yeast revertants that arose spontaneously from the GALT-knockout yeast that could grow on galactose-containing media. Gene expression microarray analysis showed that these revertants down-regulated the expression of genes that encoded the enzymes of the Leloir pathway and the galactose transporter (Lai and Elsas, 2000). The results of the down-regulation were reduced accumulation of galactose and Gal-1-P seen in these revertants (Lai and Elsas, 2000), suggesting that Gal-1-P was detrimental to the growth of the GALT-less yeast cells. These findings were later confirmed by Ideker et al. (2001). Earlier genetic studies found that S. cerevisiae deleted for both galactokinase and GALT genes could grow on galactose medium, but S. cerevisiae with a single deletion in either gene could not (Douglas and Hawthorne, 1966). These data, together with the lack of hepatotoxicity and long-term complications seen in patients with galactokinase deficiency (Segal and Berry, 1995), supported our hypothesis that Gal-1-P was toxic in GALT-deficient yeast and in humans with GALT-deficiency galactosemia. The present study extends these findings to human cells and addresses the mechanisms for Gal-1-P toxicity.

We utilized SV40-transformed human fibroblasts derived from a galactosemic patient that accumulated Gal-1-P concentrations above 3 mM when transferred to 0.1% galactose. These cells would not grow under these conditions. When the GALT-deficient cell line was cultured in glucose, they had decreased levels of UDP-glucose and UDP-galactose when compared with a control cell line (Table II). We then test the hypothesis that GALT deficiency caused decreased UDP-glucose and UDP-galactose. Earlier reports found UDP-galactose deficiency in the red cells, liver, and fibroblasts of galactosemic subjects and demonstrated by high-performance liquid chromatography (HPLC) that the concentration of UDP-glucose in two galactosemic fibroblast cell lines was only 25% of normal (Ornstein et al., 1992; Ng et al., 1989). A reduction of 38% in the mean UDP-galactose level in red cells was confirmed in 19 galactosemic children compared with normal children (Berry et al., 1992). Nevertheless, the authors of the latter studies argued that there was considerable overlap between the values of the galactosemics and controls; therefore, the results were not conclusive. To control for interindividual epigenetic and environmental factors, we overexpressed the GAL gene in the GALT-deficient cell line and repeated the experiments under standardized conditions. We found that restoration of GALT activity resulted in increased UDP-glucose and UDP-galactose concentrations and decreased Gal-1-P accumulation (Table II). We conclude that GALT deficiency caused UDP-hexose deficits.

Could the decrease in UDP-hexose concentrations be a result of Gal-1-P toxicity? Our findings support this notion. Gal-1-P did not accumulate to as high a concentration in the hUGP2-transfected cells as in the GALT-deficient parent cell line (Table II). Gal-1-P competitively inhibited the conversion of glucose-1-phosphate to UDP-glucose by purified hUGP2 (Table III, Figure 4). Thus Gal-1-P at pathological concentrations seen in galactosemia directly reduced UDP-glucose synthesis and could account for decreased concentrations of both UDP-glucose and UDP-galactose in GALT deficiency.

The abundance of these high-molecular-weight SNA-positive glycans returned to normal in the GALT-transfected cells (lane 4, Figure 5). Interestingly, the abundance of the lower-molecular-weight SNA-positive glycoproteins (below 66 kDa) in the GALT-deficient was not affected by the presence of galactose.

It should be noted that this is a preliminary study of cellular glycosylation in the GALT-deficient cells because we did not examine glycolipids, nor did we use agglutinins that recognize other specific linkages in the oligosaccharide chains of the glycoproteins. Thus, even though the data suggested that decreased UDP-hexoses in the glucose-grown GALT-deficient fibroblasts was not sufficient to cause changes in the SNA-positive glycoproteins, it did not preclude any qualitative and quantitative changes in other types of glycans. Nevertheless, we showed that when these GALT-deficient cells were further challenged with galactose, it caused a further decrease in UDP-hexose, a sharp increase in Gal-1-P, and reduced abundance of the high molecular weight SNA-positive glycoproteins.

**Discussion**

We previously studied the mechanisms of galactose toxicity using the model system *Saccharomyces cerevisiae* deleted for the endogenous GALT gene (Lai and Elsas, 2000). These GALT-knockout yeast cells could not grow when galactose was the sole carbon source in growth medium.

**Fig. 5.** Effect of carbohydrate source on glycosylation profiles of GALT-deficient fibroblasts. Proteins extracts were prepared from GALT-deficient fibroblasts grown in media that contained either 0.1% glucose or 0.01% galactose. Equal amount of proteins (100 µg) were harvested from each cell culture and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being immobilized onto nitrocellulose membrane. The membrane was incubated with buffer containing digoxigenin-labeled SNA that recognized sialic acid linked (2→6) to galactose of glycans (Shibuya et al., 1987). The digoxigenin-labeled lectin bound was identified by incubation with alkaline phosphatase–labeled anti-digoxigenin antibody, followed by a chromogenic reaction resulted from the reaction of alkaline phosphatase with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate. M: molecular weight marker; 1: GALT-deficient cells in 0.1% glucose; 2: GALT-def cells transfected with GALT gene in 0.1% glucose; 3: GALT-deficient cells in 0.01% glucose + 0.1% galactose; 4: GALT-deficient cells transfected with GALT gene in 0.01% glucose + 0.1% galactose.
Could a reduction in cellular UDP-glucose and UDP-galactose affect membrane-bound glycoproteins/glycolipids? Flores-Diaz and colleagues showed that UDP-glucose deficiency caused by impaired UGP activity in a Chinese hamster cell line led to hypersensitivity to the cytotoxic effect of Clostridium perfringens phospholipase C (1997, 1998). The authors attributed this effect to alterations in the composition of plasma membrane glycolipids or glycoproteins. Daran et al. (1997) showed that reduced UDP-glucose formation in S. cerevisiae led to reduction in the β-glucan and mannann component of the cell wall. But how could decreased UDP-glucose synthesis also decrease the UDP-galactose concentration? In living cells, there are two known pathways for the synthesis of UDP-galactose: (1) uridyltransfer of an UMP moiety to Gal-1-P by GALT (LeLor, 1951) (Figure 1), and (2) epimerization of UDP-glucose by UDP-galactose-4-epimerase (GALE) (Saló et al., 1968) (Figure 1). The first reaction is blocked in GALT deficiency galactosemia (Isselbacher et al., 1968) (Figure 1). The WV1 reaction is only 0.097 ± 0.005 nmol UDP-galactose formed per μg enzyme per minute (Table III). This rate is 1000 times slower than that of the GALT reaction, and the production of fentomolar amounts of UDP-galactose via this route would not be sufficient to sustain normal cellular growth and posttranslational processing. Thus overexpression of the hUGP2 gene is necessary to rescue the GALT-less yeast and reduce Gal-1-P accumulation (Lai and Elsas, 2000). We can thus predict that in human cells cultured from patient with galactosemia with Gal-1-P concentrations above 2.5 mM endogenous hUGP2 cannot produce sufficient UDP-glucose to overcome the reduced rates of UDP-galactose production caused by deleterious mutations in the GALT gene (Figure 1).

One could argue that UDP-galactose could be formed from high concentrations of Gal-1-P in galactosemics via the hUGP2 reaction (Figures 1 and 4). Although theoretically feasible, our kinetic analysis of the human UGP2 enzyme indicated that its apparent Vmax for UDP-galactose formation is only 0.97 ± 0.005 nmol UDP-galactose formed per μg enzyme per minute (Table III). This rate is 1000 times smaller than that of the GALT reaction, and the production of fentomolar amounts of UDP-galactose via this route would not be sufficient to sustain normal cellular growth and posttranslational processing. Thus overexpression of the hUGP2 gene is necessary to rescue the GALT-less yeast and reduce Gal-1-P accumulation (Lai and Elsas, 2000). We can thus predict that in human cells cultured from patient with galactosemia with Gal-1-P concentrations above 2.5 mM endogenous hUGP2 cannot produce sufficient UDP-glucose to overcome the reduced rates of UDP-galactose production caused by deleterious mutations in the GALT gene (Figure 1).

Although there is no documented information on the cellular concentrations of UDP-glucose and UDP-galactose in the developing fetus, these metabolites are important in posttranslational processing during rapid fetal growth and differentiation. The developing brain and ovary of GALT-deficient embryo may well have limited amounts of glycoproteins and glycolipids. Accumulation of Gal-1-P may effectively compete with glucose-1-phosphate or N-acetylglucosamine-1-phosphate for UTP-dependent pyrophosphorylases and reduce the synthesis of their corresponding UDP-hexoses. The resultant decrease in concentrations of UDP-glucose and UDP-galactose would affect the properties of membrane glycoproteins/glycolipids, as found in patients with uncontrolled galactosemia. Charwood et al. (1998) and Petry et al. (1991) showed that galactose and N-acetylgalactosamine molecules were missing from the oligosaccharide chains of glycoproteins and gangliosides isolated from galactosemic patients with elevated Gal-1-P. We add to this notion by observing the reduced abundance of SNA-positive glycoproteins present in the GALT-deficient fibroblasts challenged with galactose (Figure 5). Thus, the critical gene (GALT), epigenes (hUGP2), and the environment (carbohydrate source) interact in producing the phenotype (cell growth) in human cells.

Materials and methods

Cell culture and transfection of DNA plasmids

We acquired a primary fibroblast cell line derived from a GALT-deficient patient with classic galactosemia who had no detectable GALT activity in red blood cells (Reichardt and Berg, 1988). The primary cell line (GM00054) and GM00638A, the SV40 large T-antigen transformed cell line derived from GM00054, were obtained from the NIGMS Human Genetic Cell Repository/Coriell Institute for Medical Research (Camden, NJ).

As a normal control, we obtained another SV40-transformed fibroblast line, GM00637I, which had normal GALT activity. We confirmed that GM00638A (GALT-deficient) manifested the Gal⁻ phenotype and did not grow in 0.1% galactose. Fibroblasts were cultured in DMEM (Sigma, St. Louis, MO) at 37°C in 5% CO₂.

Human UGP2 and GALT cDNAs were subcloned into BamHI- and Xhol-digested pCDNA3.0 (Invitrogen, Carlsbad, CA), an episomal mammalian expression vector with a CMV promoter. The inserts were verified by sequencing. The resulting recombinant vectors expressing either the hUGP2 or GALT gene were introduced into the GALT-deficient fibroblasts using the FuGene6 transfection reagent (Invitrogen). Stable transfectants were selected through resistance to a predetermined dose of 100 μg/ml genetin (G-418) over 4–6 weeks. Clones of stable transfec-
tants were isolated and expanded subsequently.

Enzyme assays

GALT assay. GALT activity was determined using methods previously published (Lai et al., 1999).

hUGP2 assay. Human UGP enzyme activity was assayed at room temperature (25°C) in a 1-ml volume containing 2 mM glucose-1-phosphate, 1 mM MgCl₂, 1 mM dithiothreitol, 0.8 mM Nicotinamide adenosine diphosphate (NAD), 0.2 mM UTP, 2.5 μl (0.5 U/μl) UDP-glucose dehydrogenase, and 1 mM Tris·HCl (pH 8.0). After incubating the reaction mixture at 25°C for 15 min, the reaction was stopped by a 3 min incubation at 95°C. The reactions were centrifuged for 10 min to remove insoluble matter. The formation of NADH was quantified by the absorbance change at 340 nm. The quantitative relationship was quantified using the Beer-Lambert equation as follows:

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\Delta \text{absorbance} = \Delta \text{concentration of solutes} \times \text{optical path length} \times \text{molar coefficient of extinction}
\]

In this case, optical path length is 1 cm, and the molar coefficient of extinction is 6220 M⁻¹ cm⁻¹.

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Kinetic studies

All reactions were carried out in a final volume of 1 ml. Each reaction contained 1 mM Tris-HCl, 1 mM MgCl₂, 0.8 mM NAD, 1 mM dithiothreitol, 0.2 mM UTP, and 2.5 µl (0.5 U/µl) of UDP-glucose dehydrogenase. Glucose-1-phosphate, Gal-1-P, or both were added at various concentrations as indicated. The reaction was incubated at room temperature (25°C) after 15 µg of the semi-purified hUGP2 enzyme was added, and spectrophotometric readings were taken at 1-, 2-, 3-, 4-, 5-, 7-, 10-, 15-, and 20-min time intervals at 340 nm.

The change in absorbance was used to quantify the formation of NADH. The formation of UDP-galactose in the reactions containing Gal-1-P was confirmed by the addition of another enzyme, UDP-galactose-4-epimerase, in addition to UDP-glucose dehydrogenase and NAD. UDP-galactose-4-epimerase converted the UDP-galactose (produced from the Gal-1-P and UTP) to UDP-glucose, which was subsequently acted on by UDP-glucose dehydrogenase as described.

Competition assay

In all competition assays, Gal-1-P was added at a final concentration of 2.5 mM.

Human AGX1 assay

Human AGX1 enzyme activity in cell extracts prepared from NIH-OVCAR3 cell lines was assayed using the color reagent containing 0.03% (w/v) malachite green and 0.2% (w/v) ammonium molybdate described by Mio et al. (1998). NIH-OVCAR3 cell lines were cultured in Corning 75 cm² flask in DMEM at 37°C in 5% CO₂.

Assay of protein concentration

Protein concentration in samples was quantified using BioRad Protein Assay Reagent (Hercules, CA). Solutions of bovine serum albumin of known concentrations were used to construct the standard curve.

Measurement of metabolites

UDP-glucose and UDP-galactose were measured using enzyme-linked assay described by Ng et al. (1989). Although this method gave variable results when it was used to measure these metabolites in patients' red blood cells (Ng et al., 1989; Kirkman, 1992; Segal, 1995; Gibson et al., 1995), it had been shown to give results identical to HPLC when it was used to measure these metabolites in cultured human fibroblasts (Xu et al., 1995).

Gal-1-P contents were determined using methods previously published (Lai and Elsas, 2000). The intracellular volume was calculated from the equilibrium distribution of 3-O-methyl-D-glucose using a protocol established in our laboratory by Longo et al. (1988). It was determined that the intracellular volume of patient fibroblasts ranged from 6.5 to 8 µl/mg cell protein.

Purification of recombinant hUGP2 protein

We purified recombinant hUGP2 enzyme from a bacterial host BL21 (DE3) (Novagen, Madison, WI). The hUGP2 open reading frame was amplified by PCR (polymerase chain reaction) and primers 5’-GGCCCTCGAG-GATCCATGTCCTGAGGGTGC-3’ and 3’-GGCCCTCGAGTTCGAATCTCAGTGTCACAGTGC-3’ using plasmid pHC377 (Duggleby et al., 1996) as template. The amplified PCR product was cut with restriction enzymes BamHI and Xhol and subcloned into the bacterial expression vector pET30a (+) (Novagen). The nucleotide sequence of the subcloned hUGP2 open reading frame in the recombinant vector was determined using an ABI 310 automated sequencer (Perkin-Elmer, Boston, MA) to confirm that no mismatch errors were introduced during PCR. The recombinant vector was transfected into the bacterial strain BL21 (DE3), which has its endogenous gal operon deleted by transposon mutagenesis. The transfected bacteria were selected on Luria Bertani medium supplemented with 30 µg/ml kanamycin (Sigma). To induce hUGP2 enzyme production in the bacterial host, bacteria harboring the recombinant plasmid were grown in Luria Bertani medium with antibiotics to an optical density A₆₀₀ of 0.6. Isopropylthiogalactoside was added at a final concentration of 1 mM, and the culture was grown for another 2 h at 37°C. Cells were harvested by centrifugation at 4000 × g for 20 min. and stored at −70°C prior to purification of the recombinant hUGP2 protein.

We followed the protocol established by the manufacturer (Qiagen, Valencia, CA) and purified the hUGP2 enzyme based on the principle of affinity chromatography. The bacterial expression vector pET30a (+) enables co-transcription of a hexamer of histidines (His6) in frame at the amino terminus of the cloned hUGP2 cDNA. A nickel-charged affinity column was used to purify recombinant hUGP2 from bacterial cell lysates using modifications of the manufacturer's method (Qiagen). Lysates of bacterial pellets were incubated with 20 µl/ml nickel-charged resin (Qiagen) at 4°C for 2 h with gentle shaking. Non-His6-tagged proteins were eluted with 20 mM imidazole buffer. The His6-tagged hUGP2 protein was then sequentially eluted with buffers containing 50, 100, 150, 200, and 250 mM imidazole. Elutes were concentrated and desalted using Centricon-300 (Millipore, Bedford, MA) and resuspended in a small volume of 1 mM Tris buffer (pH 8.0). Protein concentrations were determined by Bradford assay (Bio-Rad) with bovine albumin standards. From 227.5 mg of cell extracts, we recovered 77% of hUGP2 that gave a single band of 60 kDa on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. This represented a 111-fold purification.

Qualitative analysis of SNA-positive glycoproteins

Detection of SNA-positive glycoproteins using digoxigenin-labeled SNA was performed using protocol developed by Roche Molecular Biochemicals (Indianapolis, IN).

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Abbreviations

AGX1, UDP-N-acetyl-glucosamine pyrophosphorylase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; Gal-1-P, galactose-1-phosphate; GALE, UDP-galactose-4-epimerase; GALT, galactose-1-phosphate uridylyltransferase; HPLC, high-performance liquid chromatography; hUGP2, human UDP-glucose pyrophosphorylase; PCR, polymerase chain reaction; SNA, Sambucus nigra agglutinin

References


