Molecular Basis for High Renal Cell Sensitivity to the Cytotoxic Effects of Shigatoxin-1: Upregulation of Globotriaosylceramide Expression

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Abstract. Cellular injury in post-diarrheal hemolytic-uremic syndrome (D+HUS) is related to shigatoxin (Stx) binding to globotriaosylceramide (Gb3). High renal Gb3 expression may determine renal susceptibility in D+HUS; however, the molecular mechanism(s) responsible for such relatively abundant Gb3 levels are unknown. Consequently, kidney cells expressing high Gb3 (cultured human proximal tubule cells [HPT]) were compared with non-kidney cells with low Gb3 content (cultured human brain microvascular endothelial cells [HBEC]). HPT were much more sensitive to the cytotoxic and protein synthesis inhibitory effects of Stx-1; this correlated with Gb3 content and [125I]-Stx-1 binding. HPT had greater Gb3 synthase (GalT6) and lower α-galactosidase activities than HBEC, whereas lactosylceramide synthase (GalT2) activity was higher in HBEC. Ceramide glucosyltransferase (CGT) activity was similar between the two cell types. The higher HPT GalT6 activity was associated with increased GalT6 mRNA steady-state levels, but no difference in GalT6 mRNA half-life. The lower HPT α-galactosidase activity was associated with reduced α-galactosidase mRNA steady-state levels but no difference in α-galactosidase mRNA half-life. Higher HBEC GalT2 activity was associated with increased steady-state GalT2 mRNA levels. These studies suggest that high renal Gb3 expression is due to enhanced GalT6 gene transcription and reduced α-galactosidase gene transcription and occur despite relatively low GalT2 activity.

Post-diarrheal hemolytic-uremic syndrome (D+HUS) is the leading cause of acute renal failure in children. The disorder is characterized by thrombocytopenia, microangiopathic hemolytic anemia, and acute renal insufficiency (1). Although other organ systems may become involved in D+HUS, renal injury is prominent and occurs early in the course of the disease. Renal cell damage in D+HUS has been related to enteric infection by shigatoxin (Stx)-producing organisms (typically Escherichia coli 0157:H7) (2). Stx binds to glycosphingolipids with a galactose-α-1,4, galactose moiety, the most abundant form being galactose-α-1,4, galactose-β-1,4, glucose-ceramide (Gb3). Once bound, the toxin is internalized, and ultimately is cytotoxic, at least in part, by directly inhibiting peptide elongation. The observations that intact kidney and isolated renal cells express relatively high amounts of Gb3 on the cell surface has been used to explain renal targeting in D+HUS (2–6). Although this scenario is generally accepted, it remains unknown as to why the kidney contains such large amounts of Gb3.

Several components of the Gb3 metabolic pathway could be involved in high renal Gb3 expression (Figure 1). Gb3 is synthesized from lactosylceramide (LacCer) and UDP-galactose by Gb3 synthase (GalT6) (7) and is metabolized to LacCer by α-galactosidase (8). LacCer is derived, in turn, from glucosylceramide (GlcCer) and UDP-galactose by LacCer synthase (GalT2) (9), whereas GlcCer is synthesized from ceramide and UDP-glucose by ceramide glucosyltransferase (CGT) (10). Consequently, increased renal Gb3 content could conceivably be due to increased activity of GalT6, GalT2, or CGT, and/or it could be due to decreased activity of α-galactosidase. Currently, there is no information on the activities or regulation of these enzymes in the kidney. The current study was therefore undertaken to define how these enzymes are involved in controlling the levels of renal cellular Gb3 expression.

On light microscopy, glomerular endothelial cell injury is a prominent feature of D+HUS (2). This led to the hypothesis that Stx binds directly to these cells by virtue of their high basal Gb3 expression. This hypothesis was supported by the observation that cultured human microvascular endothelial cells were much more sensitive to the cytotoxic effects of Stx compared with human umbilical vein endothelial cells (3). Subsequent studies have found, however, that cultured human glomerular endothelial cells are quite insensitive to Stx (6.11). In contrast, human proximal tubular cells (HPT) have been found to be exquisitely sensitive to Stx cytotoxicity (12).

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Stx-1 potently inhibited protein synthesis in HPT, but had minimal effect on glomerular endothelial cells (11). In addition, in Fabry disease, a condition due to α-galactosidase deficiency, proximal tubule Gb3 accumulation is a major feature of the renal damage (13). Consequently, HPT were chosen for this study as the model of renal cells expressing high basal Gb3 levels. Human brain endothelial cells (HBEC), which contain very low Gb3 and are resistant to the cytotoxic effects of Stx-1, were chosen for the purpose of comparison (14,15). Several other cell types with low Gb3 expression could have been used, but HBEC have been well documented to contain little Gb3 under basal conditions (14,15).

Materials and Methods

Cell Culture

HBEC were obtained at primary culture from Cell Systems (Kirkland, WA) and studied at passages 7 to 9. Cells were grown to confluence in EGM2-MV media (Clonetics, San Diego, CA) and switched to serum-free Maintenance Formula media (Cell Systems) 24 h before all studies were initiated. In addition to characterization by Cell Systems, we determined that these cells had uniformly positive immunofluorescence for von Willebrand factor and platelet endothelial cell adhesion molecule but were negative for cytokeratin. HPT were obtained from Clonetics and studied at the third passage. HPT were maintained in 1:1 Dulbecco’s F12 media:Ham’s F12 containing 25 mM HEPES, 2 mM l-glutamine, 100 U/ml penicillin/ streptomycin, 250 µg/L amphotericin B, 1 µg/ml hydrocortisone, 10 µg/ml insulin, 5.5 mg/ml transferrin, 6.7 µg/ml selenium, 0.2 g/L ethanolamine, 6.5 mg/ml l-thyroxine, 10 ng/ml epidermal growth factor, and 10% fetal bovine serum (FBS). Cells were switched to serum-free Maintenance Formula media 24 h before all studies were initiated. The identity of these cultures was established as described previously (12).

Cytotoxicity

Cells grown in 96-well plates were analyzed for neutral red uptake as described previously (12) after 24-h exposure to varying concentrations of Stx-1. Cells were incubated in 50 µg/ml neutral red in M199 + 5% FBS for 3 h at 37°C, rinsed in 1% formaldehyde and 1% CaCl2, followed by addition of 50% ethanol and 1% acetic acid. Absorbance was determined at OD450.

Leucine Incorporation

3H-incorporation was determined as described previously (12) after 4-hr exposure to varying concentrations of Stx-1. Cells grown in 24-well plates were incubated with 1 µCi/ml 3H-leucine for 20 min at room temperature, rinsed, and solubilized in 0.1% sodium dodecyl sulfate. Protein was precipitated with 10% tricarboxylic acid, collected on GF/C filters (Whatman, Kent, England), and cpm was determined.

Gb3 Content

Gb3 content was determined as described previously (12). Cells were extracted in chloroform:methanol:water and separated on high-performance thin-layer chromatography-silica plates (Mallinkrodt Baker Inc., Paris, KY). The plates were dried, immersed in 0.5% polyisobutylmethacrylate in acetone, and sequentially incubated with Stx-1, anti-Stx-1 monoclonal antibody (purified from a hybridoma cell line, 13C4 [ATCC, Rockville, MD]), and 125I-goat anti-mouse IgG (DuPont NEN, Boston, MA). Gb3 concentrations were calculated by densitometry and standardized to total protein. Before centrifugation, a cell aliquot was solubilized in 0.1 N NaOH and mixed with Bradford reagent (Bio-Rad, Richmond, CA), and protein concentration was determined by measuring absorbance at 590 nm.

Stx-1 Binding

Cells grown in 96-well plates were used for 125I-Stx-1 binding assays as described previously (12). 125I-Stx-1 (17,000 cpm, Stx-1 iodinated according to the Iodobead manufacturer’s protocol [Pierce, Rockford, IL]) in 100 µl of M199 containing 5% FBS and 25 mM HEPES plus varying concentrations of unlabeled Stx-1 was added for 24 h at 4°C. Cells were rinsed with ice-cold HBSS and solubilized in 0.1 N NaOH, and cpm was determined.

Biosynthetic Enzyme Activity

GalT6 activity was determined as described previously (12). Briefly, cells were homogenized in 500 µl of 50 mM MES pH 6.5. Dried LacCer (25 nmol; Matreya, State College, PA) was added to sodium cholate in water (250 µg) and dried under vacuum, and the dried mixture incubated for 60 min at 4°C. A total volume of 100 µl of 50 mM MES pH 6.5 containing 10 mM MnCl2, 100 µM 5′-adenylimidodiphosphate (p(NH)ppA), 250 µM cold UDP-galactose, 44 µM UDP-14C galactose (150,000 to 400,000 cpm; Amersham Biosciences, Piscataway, NJ), and 125µg of total cellular protein was added to the dried LacCer/sodium cholate, the samples vortexed, incubated at 37°C for 1 h, and the reaction stopped by adding 1 ml of 2:1 chloroform:methanol. A Folch partition was established by adding 200 µl of 0.1 M KCl, and the upper phase re-extracted by adding 500 µl of 2:1 chloroform:methanol. The lower phase was re-extracted by adding 500 µl of 1:1 methanol:0.1 M KCl. The lower phases (containing the neutral lipids) were combined, dried under vacuum, and chromatographed as described for Gb3 above. For GalT2 and GlcCer synthase activity, cells were treated as for GalT6. Either dried GlcCer (GalT2) or ceramide (GlcCer synthase) (both reagents from Matreya) were vortexed with 2.5 µl of 10% TritonX-100 and incubated at room temperature for 30 min. A total volume of 100 µl of 50 mM HEPES (pH 6.8) containing 5 mM MgCl2, 5 mM MnCl2, 55.7 µM UDP-galactose, 44 µM UDP-14C galactose (150,000 to 400,000 cpm), and 150 µg of cell sonicate were added to the GlcCer or ceramide (100 nM final concentration/detergent mixture, vortexed, and incubated at...
37°C for 1.5 hr. A Folch partition was established, and the neutral lipids chromatographed as described for GalT6.

**α-Galactosidase Activity**

α-Galactosidase activity was determined as described previously (16). Cells were suspended in 3 mg/ml sodium taurocholate and 28 mM citric acid/44 mM Na₂HPO₄ (pH 4.4), sonicated, and centrifuged, and the supernatant was analyzed. For the reaction, supernatant was incubated with 5 mM p-nitrophenyl-α-galactopyranoside for 10 to 90 min. followed by addition of 0.2 M Na₂CO₃ and absorbance measured at 400 nm.

**Northern Analyses**

Total RNA was isolated from confluent cells, electrophoresed on a 0.9% formaldehyde gel, transferred to a nylon membrane, and prehybridized for ≥3 h at 60°C in 50% formamide, 5×SSC, 5×Denhardt's solution, 1% sodium dodecyl sulfate, and 100 μg/ml salmon sperm DNA. Fresh solution was added for hybridization along with radioactively labeled probe. For probes, cDNA was made from human proximal tubule cell total RNA using oligo dT mRNA primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA was then used as a template for PCR amplification of the coding region of the gene using specific primers: (1) GalT6 (GeneBank Accession No. AB037883): Forward, 5'-GAT CGT GGG ATA CCA TGT CCA AG –3' and Reverse, 5'-CAG TAG CGG GCA TGG AGC TGG –3', which yields a product size of 1040 bp; (2) α-galactosidase (GeneBank Accession No. XM037096): Forward, 5'-GGC TAG AGC ACT GGA CAA TGG A –3' and Reverse, 5'-CTG CGA TGG TAT AAG AGC GAG G –3', which yields a product size of 1021 bp; (3) GalT2 (GeneBank Accession No. AF097159): Forward, 5'-AAC GGT ACA GAT TAT CCC GAA G –3' and Reverse, 5'-TGG AGC TAA CTC TGG CAT GAG G –3', which yields a product size of 912 bp; and (4) Ceramide glucosyltransferase (GeneBank Accession No. AB037883): Forward, 5'-GAT CTG GGG ATA CCA TGT CCA AG –3' and Reverse, 5'-CAG TAG CGG GCA TGG AGC TGG –3', which yields a product size of 1040 bp. PCR conditions were the same for each reaction: one eighth of the RT reaction was amplified per reaction using *Taq* polymerase in a reaction mixture containing 800 uM dNTP, 1 pmol of each primer, and 50 mM MgCl₂ (all reagents and enzymes from Invitrogen). PCR conditions were as follows: 94°C for 1.5 min; 94°C for 20 s; 65°C for 25 s; 72°C for 1 min (30 cycles); 72°C for 5 min, final extension. All reactions were tested for size before combining and purifying using Wizard PCR preps (Promega, Madison, WI) as per manufacturer’s protocol. All products were purified, sequenced, and cloned into pGEM-T cloning vector (Promega). The inserts were again sequenced to ensure cloning fidelity and confirm orientation. Probes were digested with appropriate restriction enzyme to give the antisense strand, and riboprobes were made using ±³²P-UTP incorporation with either T7 or SP6 RNA polymerase (Invitrogen). The radioactive probes were purified over a 50-gram column, and specific activity was calculated. The probe was added to hybridization solution at 10 ng/ml, with a specific activity ≥10⁷ dpm/μg and incubated overnight at 60°C. Blots were washed in decreasing SSC concentrations and increasing temperature until background removed. Labeled blots were subjected to autoradiography and densitometry.

**mRNA Half-Life Determination**

Confluent cells in 6-well plates were treated with 10 μg/ml actinomycin D for 0 to 24 h followed by determination of GalT6 and α-galactosidase mRNA levels as described under Northern analysis above.

**Statistical Analyses**

Data were analyzed by one-way ANOVA using the Bonferroni correction. Where only two data points were compared, the unpaired t test was used. *P < 0.05* was taken as significant. Data are expressed as mean ± SEM.

**Results**

The work to be described was conducted using HPT and HBEC. At the outset, it is important to note why human glomerular endothelial cells (HGEC), which have been implicated as a primary target in HUS, were not studied. First, in our hands, pure cultures of HGEC are difficult to obtain—the yield is quite small, and the cells rapidly lose endothelial cell markers with passageing. Additionally, the one group (6) that has published studies using HGEC reports that these cells are quite insensitive to Stx and contain relatively little Gb3. We have also obtained several different batches of commercially available HGEC (Cell Systems, Kirkland, WA) and have found these cells, regardless of donor age, to express little Gb3 and to be resistant to Stx. Thus, in our opinion, it remains unknown whether HGEC really are sensitive to Stx in *vivo*; this issue is problematic until a better experimental system can be devised to assess HGEC function *in vitro*.

To establish that HPT and HBEC serve as models of high and low Gb3-expressing cells, respectively, Stx-1 cytotoxicity, Stx-1 protein synthesis inhibition, Stx-1 binding, and Gb3 content were determined. Stx-1 potently killed HPT cells (LD₅₀, approximately 100 pg/ml), whereas HBEC were highly resistant (Figure 2). Similar results were obtained with Stx-1 inhibition of protein synthesis (Figure 2). This high HPT sensitivity was associated with over 100-fold greater Gb3 content and 125I-Stx-1 binding (Figure 3) as compared with HBEC. Hence, these data confirm that HPT and HBEC should serve as valid models.

CGT, GalT2, GalT6, and α-galactosidase activities were next determined in HPT and HBEC to compare Gb3 biosynthetic pathways between the two cell types. No differences were detected in CGT activity (Figures 4 and 5). GalT2 activity was lower in HPT than in HBEC (Figures 4 and 5), an unexpected finding that indicated that GalT2 activity was not rate-limiting for Gb3 formation. GalT6 activity was approximately twofold greater in HPT (Figures 4 and 5). In addition, α-galactosidase was about fivefold less in HPT than in HBEC (Figure 5). Thus, when the relative degrees of GalT6 and α-galactosidase activities are considered together, it is evident that there is about a tenfold greater drive for Gb3 accumulation in HPT than in HBEC.

To determine the mechanism of enhanced Gb3 biosynthetic enzyme activity, CGT, GalT2, GalT6, and α-galactosidase mRNA levels were assessed in the two cell types. In general, steady-state mRNA levels directly correlated with enzyme activity (Figures 6 and 7). CGT mRNA tended to be higher in HPT, but this did not achieve statistical significance. GalT2 message was reduced in HPT as compared with HBEC. GalT6...
mRNA content was approximately twofold greater in HPT, whereas α-galactosidase mRNA was about threefold less in PT. Again, as for the enzyme activities, the combination of relative GalT6 and α-galactosidase mRNA levels indicate that HPT have a substantially greater Gb3 biosynthetic drive than do HBEC.

The mRNA half-lives for GalT6 and α-galactosidase were evaluated to determine the mechanism of increased mRNA levels of these two enzymes. The half-lives for both GalT6 and α-galactosidase (Figure 8) were similar between HPT and HBEC. Consequently, these data suggest that the differences in GalT6 and α-galactosidase activities between HPT and HBEC are controlled, at least in part, at the transcriptional level.

Discussion

High renal Gb3 expression is likely to play an important role in kidney damage in at least two diseases, namely D+HUS and Fabry’s disease. In D+HUS, Gb3 serves as the major receptor for the putative pathogenic toxin, shigatoxin (2). In Fabry disease (α-galactosidase deficiency), Gb3 accumulates in lysosomes and eventually causes severe tubulointerstitial damage and renal failure (13). Despite the central importance of Gb3, to date there has been no information on the mechanisms responsible for predominant renal expression of this glycosphingolipid. Such information would potentially be of benefit in designing therapeutic strategies for these disorders. This is
particularly important in that current treatments have not proven highly effective in ameliorating D+HUS (enteral toxin-absorbing resins) (2) or Fabry disease (intravenous recombinant \( \alpha \)-galactosidase) (13). Indeed, efforts are now underway toward developing and/or using inhibitors of glycosphingolipid biosynthesis in these diseases (17). Clearly, an understanding of which enzymes are responsible for high renal Gb3 levels, as well as an understanding of how these enzyme levels are regulated, would be crucial in the development of rational therapies.

The present study constitutes the first description of factors involved in high renal Gb3 expression. First, it was not surprising that CGT activity and mRNA were not upregulated in HPT in that this constitutes the earliest step in synthesis of all glycosphingolipids based on glucosylceramide. However, the findings that GalT2 activity and mRNA content were reduced in HPT were unexpected. This clearly suggests that GalT2 activity and resultant LacCer levels are not rate-limiting for Gb3 accumulation in HPT. The reasons why GalT2 activity is lower in HPT are unknown because the factors responsible for directly or indirectly modifying GalT2 activity and/or mRNA levels have not been determined. One might speculate that downstream glycosphingolipid products derived from LacCer could modulate GalT2 activity, but the biologic significance of renal glycosphingolipids is also poorly understood. This issue will therefore need to await additional studies.

In contrast to GalT2, GalT6 activity was increased in HPT. This observation is as predicted and serves to explain, at least in part, elevated HPT Gb3 levels. Furthermore, the combina-

Figure 4. High-performance thin-layer chromatography of GalT6, GalT2, and CGT activities in human PT and HBEC. Gb3 bands are GalT6 activity, lactosylceramide (LacCer) bands are GalT2 activity, and glucosylceramide (GlcCer) bands are CGT activity. Standards confirming glycosphingolipid location were run on the same gels but are not shown. All data are normalized to total cell protein.

Figure 5. Densitometry of GalT6, GalT2, and CGT activities in human PT and HBEC. Ordinate values are expressed as percent of HBEC enzyme activity. \( n = 6 \) each data point; * \( P < 0.005 \) versus HBEC; ** \( P < 0.001 \) versus HBEC.

Figure 6. Northern blot of GalT6, GalT2, CGT, and \( \alpha \)-galactosidase mRNA steady-state levels in human PT and HBEC. All products are the predicted size.

Figure 7. Densitometry of GalT6, GalT2, CGT, and \( \alpha \)-galactosidase mRNA steady-state levels in human PT and HBEC. Ordinate values are expressed as percent of HBEC mRNA levels. \( n = 3 \) each data point; * \( P < 0.025 \) versus HBEC; ** \( P < 0.01 \) versus HBEC.
tion of increased GalT6 mRNA steady-state levels, together with no difference in GalT6 mRNA half-life, suggests that GalT6 gene transcription is upregulated in HPT. The GalT6 gene has only recently been cloned (7), and relatively little is known about its regulation. Tumor necrosis factor/TNF-α and protein kinase C induce GalT6 enzyme activity in endothelial cells (5). Interestingly, the GalT6 promoter contains an AP-1 domain 200 bp 5’ to the transcription start site; whether this is relevant to heightened renal Gb3 expression remains to be determined. Studies are currently underway involving transfection of HPT with GalT6 promoter-reporter constructs to begin to identify promoter domains critical for gene expression.

α-Galactosidase activity was markedly reduced in HPT compared with HBEC. α-Galactosidase steady-state mRNA levels were also greatly reduced in HPT and, similar to GalT6, α-galactosidase mRNA half-life was not different between the two cell types. These data suggest that decreased α-galactosidase activity in HPT is a result of decreased gene transcription. As for GalT6, relatively little is known about α-galactosidase gene regulation. There are CRE-BP1/c-Jun and Pax-2 (the latter may be involved in renal development and Wilms tumor formation [18]) binding domains in the first 200 bp of the α-galactosidase promoter immediately 5’ to the transcription start site. However, whether these are involved in renal-specific downregulation of Gb3 accumulation remains to be determined.

The combination of reduced α-galactosidase and enhanced GalT6 activities clearly provides a strong net driving force toward Gb3 accumulation in HPT. Such a scenario is not without precedent. For example, the increase in rabbit intestinal microvillus Gb3 expression that occurs with aging is associated with coordinate increases in GalT6 and decreases in α-galactosidase activities (19). These investigators did not, however, determine mRNA levels of these enzymes; it is therefore unclear if alterations in gene transcription similar to those seen in the present study are involved. It is nonetheless tempting to speculate that coordinated and inverse regulation of GalT6 and α-galactosidase gene expression may involve common regulatory factors.

In summary, using HPT as a model of high Gb3-containing renal cells, these data indicate that the combination of increased GalT6 and decreased α-galactosidase enzyme activities and gene expression is responsible for enhanced renal Gb3 expression. Further characterization of the molecular mechanisms responsible for this pattern of enzyme activity may ultimately lead to development of therapies that reduce renal Gb3 levels and prevent and/or ameliorate renal injury in D+HUS and Fabry’s disease.

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Mauro Abbate and Giuseppe Remuzzi
See related article by Boffa et al. (pp. 1132–1144).

Cardiovascular Risk Factors and Urinary Albumin: Vive la Petite Difference
Eberhard Ritz
See related article by Verhave et al. (pp. 1330–1335).

ANNOUNCEMENTS
Cover picture: Photograph of sacred Corn Mountain, located in Zuni Pueblo, NM. Zuni Pueblo is the home to the Zuni Indians, who are experiencing both diabetic and nondiabetic renal disease. The work described in the article by Shah et al. (page 1320–1329) is the population-based cross-sectional survey of renal disease among Zuni Indians living in Pueblo. This photograph is published with the written permission from the Zuni tribal government.

Errata

A. An author name, Z. Ergonul, was incorrectly reported in the article by Hughes et al. (pp 2239–2245) in the September 2002 issue of JASN. The correct citation is as follows:


B. The title of the article by Vallon et al. in the February 2003 issue of JASN contained a typographical error. The online version has been corrected in departure from print. The correct citation is as follows:


C. In the March issue of JASN, several articles were incorrectly categorized in the Table of Contents. The online version has been corrected in departure from print. Please consult the online version for proper categorization.

D. In the April issue, the unit of measure for SPT Activity was incorrectly reported in Tables 2 and 3 of the article by Takayama et al. (pp 939–946). The correct unit of measure is ug/liver.

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