The Urinary Bladder is Rich in Glycosphingolipids Composed of Phytoceramides

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Abstract Glycosphingolipids (GSLs) are one of the membrane lipids in mammalian cells. They are involved in a wide spectrum of cellular functions, such as growth, adhesion, migration, and death (1). Defects in GSL metabolism are associated with, for example, lysosomal sphingolipid storage diseases, neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases, and cancers (2). GSLs are composed of a polar head glycan chain and a hydrophobic tail known as ceramide. Together with variation in the glycan chain, ceramides exhibit tissue-specific structural variation in the long-chain base (LCB) and N-acyl chain moieties in terms of carbon chain length, degree of desaturation, and hydroxylation. Here, we report the structural variation in GSLs in the urinary bladders of mice and humans. Using TLC, we showed that the major GSLs are hexosylceramide, lactosylceramide, globotriaosylceramide, globotetraosylceramide, Neu5Ac-Gal-Glc-Ceramide, and Neu5Ac-Neu5Ac-Gal-Glc-Ceramide. Our LC-MS analysis indicated that phytoceramides structures with a 20-carbon LCB (4-hydroxyecosa sphinganine) and 2-hydroxy fatty acids are abundant in hexosylceramide and Neu5Ac-Gal-Glc-Ceramide in mice and humans. In addition, quantitative PCR demonstrated that DES2 and FA2H, which are responsible for the generation of 4-hydroxy sphinganine and 2-hydroxy fatty acid, respectively, and SPTLC3 and SPTSSB, which are responsible for the generation of 20-carbon LCBs, showed significant expressions in the epithelial layer than in the subepithelial layer. Immunohistochemically, dihydroceramidasesphinganine C4-hydroxylase (DES2) was expressed exclusively in urothelial cells of the urinary bladder. Our findings suggest that these ceramide structures have an impact on membrane properties of the stretching and shrinking in transitional urothelial cells.

Supplementary key words sphingolipids • ceramides • 20-carbon long-chain base • 2-hydroxy fatty acid • DES2 • FA2H • SPTLC3 • SPTSSB • epithelial cell • urothelial cell

Glycosphingolipids (GSLs) are one of the membrane lipids in mammalian cells. They are involved in a wide spectrum of cellular functions, such as growth, adhesion, migration, and death (1). Defects in GSL metabolism are associated with, for example, lysosomal sphingolipid storage diseases, and cancers (2). GSLs are composed of a polar head glycan chain and a hydrophobic tail known as ceramide. The glycan chains and ceramides show highly diverse and strictly regulated structural variations among tissues (3–5). The variations of ceramides reside in carbon chain length, degree of desaturation, and hydroxylation of the N-acyl chain and long-chain base (LCB). Six distinct ceramide synthases (CERS) encoded by CERS1 to CERS6 produce variation in N-acyl carbon chain length (6, 7). Fatty acid 2-hydroxyxlase (encoded by FA2H) catalyzes the production of 2-hydroxy fatty acids of sphingolipids in myelin (8, 9). Serine palmitoyltransferases (SPTs) are heterotrimeric proteins consisting of a large subunit dimer and one of two small subunits and responsible for the variation in carbon-chain length in LCBS (10). Dihydroceramidasesphinganine A4-desaturase (DESI) produces ceramides with sphingenine, which are the most abundant LCBs in mammalian cells (11). Dihydroceramidasesphinganine C4-hydroxylase (DES2) produces phytoceramides with an additional hydroxy group at sphinganine C-4, which are abundant LCBS in the small intestine and kidney (12). However, the biological roles of the above species are unclear.

The GSL composition in the small intestines of mice is developmentally regulated (5). The ceramide moieties of GSLs are mainly composed of phytosphingosine and 2-hydroxy fatty acid from the neonatal period to adulthood, and the polar glycan head group of GSLs is converted at around 2 weeks of age from glucosylceramide (GlcCer), Neu5Ac-Gal-Glc-Ceramide (GM3), Gal-GalNAc-(Neu5Ac-)Gal-Glc-Ceramide, and Neu5Ac-Gal-GalNAc-(Neu5Ac-)Gal-Glc-Ceramide into GlcCer and Gal-GalNAc-Gal-Glc-Ceramide (asialo GMI), which is synchronized with the expression of nutrient transporters. These results suggest the importance of GSL structures for modulating the membrane properties of intestinal epithelial cells.
Urothelial cells line the urinary tract, including the renal pelvis, ureters, and urinary bladder, and are known as a transitional epithelium because they are composed of superficial, intermediate, and basal layers (13, 14). These unique epithelial structures enable changes in urinary bladder volume depending on the accumulation and release of urine. Characteristically, terminally differentiated superficial cells called umbrella cells are large dome-shaped polyhedral cells that change their morphology according to urine volume from cuboidal to highly stretched. To enable these changes, it is speculated that the apical membrane of urothelial cells has unique protein and lipid compositions. Indeed, the apical membrane of umbrella cells is covered with highly specialized two-hexagonally packed 16 nm protein plaques composed of a transmembrane complex of uroplakins (15, 16). No study has focused on the membrane lipids of urinary bladder tissues.

In this study, we hypothesized that urothelial cells have unique GSL compositions to enable their stretching-and-shrinking membrane properties and investigated the structural diversity of GSLs in urinary bladder tissues of mice and humans.

MATERIALS AND METHODS

Urinary bladder tissues of mice and humans

Whole urinary bladder tissues were collected from male and female wild-type mice of the C57BL/6j background at the age of 120 days. The tissues were immediately frozen in liquid nitrogen and stored at −80°C until use for glycolipid or RNA extraction. For histopathological analysis, the tissues were immersed in fixative (4% paraformaldehyde in 0.1 M sodium phosphate buffer [pH 7.4]) overnight at 4°C. Mice were housed under standard conditions with ad libitum access to food and water. All animal procedures were approved by the Animal Care and Use Committee of Kawasaki Medical School (approval number: 20-013) and were conducted in accordance with institutional guidelines.

Human urinary bladder tissues were collected from 10 patients (seven males and three females) diagnosed with urinary bladder cancer who underwent radical cystectomy (Table 1). The urinary bladder tissues (1 cm³) with all layers were dissected from macroscopically benign lesions. Half of the tissue was further dissected to the urinary epithelial layer (Epi) and subepithelial layer (SubEpi). The tissues were immediately frozen in liquid nitrogen and stored at −80°C until use for glycolipid or RNA extraction. For histopathological analysis, a small portion of tissue with all layers was immersed in fixative (10% formaldehyde in 0.1 M sodium phosphate buffer [pH 7.4]) overnight at 4°C. Table 1 shows the clinicopathological features of the patients. Several patients underwent chemotherapy before radical cystectomy. Tumor staging was based on the Union for International Cancer Control TNM classification. The T category describes the primary tumor site and size, the N category describes regional lymph node involvement, and the M category describes the presence or otherwise of distant metastatic spread. T1 and T2 tumors are categorized as superficial, and T3, T3, and T4 tumors are invasive. The human study was conducted in compliance with the principles of the Declaration of Helsinki and approved by the Ethics Committee of Kawasaki Medical School (approval number: 3884), and written informed consent was obtained from all patients.

GSL analysis by TLC

Sphingolipids were extracted from urinary bladder tissues of mice and humans as described previously (5). Briefly, 100 mg wet weight of mouse whole urinary bladder tissues pooled from four mice of each sex, or 100 mg wet weight of human urinary bladder tissues (total layer, Epi, and SubEpi) of case 4 (male), case 8 (female), and case 2 (male, treated by intravesical Bacille Calmette-Guerin [BCG] infusion, which depleted Epi before total cystectomy) were homogenized in four volumes of ice-cold distilled water in a Potter-Elvehjem glass homogenizer using a glass pestle. The 500 μl of each homogenate was combined with 4.5 ml chloroform/methanol (1:2, by volume) in a glass centrifuge tube and mixed thoroughly by vortexing. The sample tubes were left for 30 min at room temperature with occasional shaking and centrifuged at 1500 g for 10 min. The supernatants were collected in fresh glass tubes and evaporated under nitrogen flow. The dried residues were suspended in 0.9 ml chloroform/methanol (1:2, by volume) and 0.1 ml 1 N NaOH and incubated at 37°C for 2 h. The homogenates were neutralized with 6 μl acetic acid and evaporated under nitrogen flow. The samples were suspended in 6 ml chloroform/methanol (1:2, by volume) and purified using a reverse-phase column (Bond Elut C-18; 3 ml/500 mg; Agilent Technologies, Inc., CA). Lipid fractions equivalent to 30 mg wet weight tissues were spotted onto silica

| Case no. | Age (year) | Sex | Histology | Grade | T | N | M | Stage | Treatment history before total cystectomy |
|----------|------------|-----|-----------|-------|---|---|---|-------|-------------------------------------------|
| 1        | 73         | Male| iUC with trophoblastic differentiation | High  3b | 0 | 0 | 0 | III   | TUR-Bt                                    |
| 2        | 72         | Male| iUC       | High  3a | 1 | 0 | 0 | IV    | TUR-Bt, GEM + CBDCA, BCG for 7 months    |
| 3        | 49         | Male| iUC       | High  2  | 0 | 0 | 0 | II    | TUR-Bt                                    |
| 4        | 69         | Male| iUC       | High  2  | 0 | 0 | 0 | II    | TUR-Bt, GEM + CDDP                       |
| 5        | 79         | Male| iUC with squamous differentiation    | High  2  | 0 | 0 | 0 | II    | TUR-Bt, BCG for 15 months                |
| 6        | 71         | Male| iUC       | High  2  | 0 | 0 | 0 | II    | TUR-Bt                                    |
| 7        | 75         | Male| iUC       | High  2b | 0 | 0 | 0 | II    | TUR-Bt                                    |
| 8        | 62         | Female| iUC      | High  4a | 1 | 0 | 0 | III   | TUR-Bt, BCG for 76 months                |
| 9        | 75         | Female| iUC with glandular differentiation  | High  2  | 0 | 0 | 0 | II    | TUR-Bt                                    |
| 10       | 77         | Female| iUC      | High  4a | 1 | 0 | 0 | III   | TUR-Bt                                    |

CBDCA, carboplatin; CDDP, cisplatin; GEM, gemcitabine; iUC, invasive urothelial carcinoma; M, distant metastasis; N, lymph node involvement; T, tumor site and size; TUR-Bt, transurethral resection of the bladder tumor.
Glycosphingolipids in the urinary bladder

Multiple reaction monitoring analysis by LC-MS

The amounts of GSLs in whole urinary bladder tissues of four mice (two males and two females) and in Epi and SubEpi of human cases 2, 4, and 8 were determined independently via multiple reaction monitoring (MRM) using triple quadrupole LC-MS (Shimadzu LCMS-8060NX) and LabSolutions, version 5.113. Total lipids were extracted from tissue homogenate with 1 ml chloroform/methanol (1:2, by volume) and 20 μl 1 N NaOH and incubated at 37°C for 2 h and then dried up under nitrogen flow. Total lipids were resuspended with 180 μl of chloroform/methanol (12, by volume) and 20 μl 1 N NaOH and incubated at 37°C for 2 h and then dried up under nitrogen flow. GSLs were purified using a reverse-phase column (MonoSpin C18 FF; GL Sciences). LC conditions were described in "Structural analysis of GSLs by LC-MS" section. The MS conditions were interface nebulizer gas flow rate 2.0 l/min, heating gas flow rate 10 l/min, interface temperature 200°C, dissolved temperature 555°C, DL temperature 250°C, heat block temperature 300°C, drying gas flow rate 10 l/min, probe voltage 1.0 kV, and focus voltage 2.0 kV. MRM transitions and collision energy are shown in Supplemental Table S2 (20). Peak area of each GSLs was integrated using LabSolutions Insight, version 3.8 SP1, and the amount of GSLs is expressed as relative to internal standards indicated in Supplemental Table S2.

Quantitative RT-PCR

Total RNA was extracted from mouse whole urinary bladder tissues (n = 4, two males and two females) and human urinary bladder tissues (total layer of case 1, and total, Epi, and SubEpi of cases 2, 4, 8) using the RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer’s protocol. Total RNA was quantified by NanoDrop One spectrophotometer (Thermo Fisher Scientific, MA). Human total RNA Master Panel II (catalog no: 656643; Clontech, WI), and total RNA extracted from total layer of case 1 was used to examine differential expression levels among human tissues. Complementary DNA (cDNA) was synthesized from 1 μg total RNA using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa Bio, Kusatsu, Japan).
SPT small subunit B (SPTSSB), six different CER isoforms (CERS1–6), and GAPDH was performed using FastStart Universal Probe Master (Rox) and Universal ProbeLibrary (Roche, Basel, Switzerland) in a StepOnePlus™ Real-Time PCR System (Applied Biosystems/Thermo Fisher Scientific, MA). The primer sequences and probe numbers are listed in Supplemental Table S1. TaqMan Rodent GAPDH Control Reagents (Applied Biosystems/Thermo Fisher Scientific) were used to quantify Gapdh expression in mice. PCR conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 30 s. mRNA levels were calculated using the comparative threshold cycle method and normalized to that of Gapdh. Data in human cases were shown with technical triplicate.

Histopathological analysis

Formaldehyde-fixed urinary bladder tissues of mice and humans were processed into paraffin-embedded blocks, sectioned, and stained with hematoxylin and eosin. For immunohistochemical studies, the slides were dewaxed and subjected to antigen retrieval by boiling for 10 min in 10 mM citric acid (pH 6.0). The slides were incubated with 1% bovine serum albumin/phosphate-buffered saline with 0.5% Triton X-100 for 1 h to block nonspecific binding and increase the penetration of antibodies. The slides were incubated with the primary antibodies overnight at 20°C followed by the secondary antibodies for 2 h at room temperature. The primary antibodies were rabbit polyclonal anti-human DES2 (dilution rate 1:50, catalog no.: PA5-24082; Thermo Fisher Scientific) and mouse monoclonal anti-mouse Uroplakin III, which is the marker of apical membrane of umbrella cell (dilution rate 1:50, catalog no.: ab78197; Abcam, Cambridge, UK) antibodies. The primary antibody was omitted as a negative control. The species-specific secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 546 (Thermo Fisher Scientific) were used at a dilution of 1:200. After nuclear staining with Hoechst 33342 (Dojindo, Tokyo, Japan), slides were mounted in Vectashield (Vector Laboratories, CA) and examined with a confocal laser scanning microscope (LSM700; Carl Zeiss, Oberkochen, Germany). Quantification of mean fluorescent intensity was done using ImageJ (National Institutes of Health) (21).

Statistical analysis

Statistical analysis was performed using Student’s t-test in Prism, version 7.00 for Windows (GraphPad Software, CA). Statistical significance was defined as \( P < 0.05 \). Data are means ± SDs.

RESULTS

GSLs in urinary bladder tissues of mice and humans

TLC indicated that in mouse urinary bladders the major GSLs were hexosylceramide (HexCer), Gb3Cer, GB4Cer, and GM3. In human samples, the major GSLs were nFA-GalCer, hFA-GalCer, and nFA-GlcCer. GSLs in mouse and human urinary bladder tissues were analyzed using HPTLC with different solvent systems to separate GlcCer and GalCer. The GSLs in mouse urinary bladder tissues in male and female (A). The GSLs in human urinary bladder tissues in case 1 (B). The GSLs in human urinary bladder tissues in case 2 (C). Lipid fractions equivalent to 60 mg wet weight urinary bladder tissues of mice and human (Epi of case 4) were spotted onto the borate-impregnated silica gel-coated HPTLC plate and developed with a solvent system of chloroform/methanol/concentrated NH4OH (280:70:6, by volume) to separate GlcCer and GalCer. (*) and (**) denote HexCer and GM3, respectively. The GSLs in human urinary bladder tissues were visualized using orcinol sulfate reagent. Fig. 1. GSL analysis of urinary bladder tissues in mice and humans using TLC. Lipid fractions equivalent to 30 mg wet weight urinary bladder tissues of mice and human were spotted and developed with a solvent system of chloroform/methanol/0.2% CaCl2 (60:35:8, by volume). GSLs were visualized using orcinol sulfate reagent. ▲ and □ denote HexCer and GM3, respectively. The GSLs in mouse urinary bladder tissues in male and female (A). The GSLs in human urinary bladder tissues in male (case 4) and female (case 8) (B). The GSLs in human urinary bladder tissues in case 2 (C). Lipid fractions equivalent to 60 mg wet weight urinary bladder tissues of mice and human (Epi of case 4) were spotted onto the borate-impregnated silica gel-coated HPTLC plate and developed with a solvent system of chloroform/methanol/concentrated NH4OH (280:70:6d, by volume) to separate GlcCer and GalCer. (*), and (**) denote HexCer (D). STD, standard.
Glycosphingolipids in the urinary bladder

In human urinary bladders, the major GSLs were HexCer, LacCer, Gb3Cer, Gb4Cer, GM3, and GD3 both in males and females (Fig. 1A). In human urinary bladder tissues, the major GSLs were HexCer and GM3 were enriched in HexCer and GM3 but were not clearly separated from GalCer (Fig. 1D). Considering these results together with the results of normal-phase TLC, the major band of HexCer (***) is likely GlcCer with phytoceramide.

**TABLE 3.** Assigned GSL molecules of urinary bladder in mouse and human

| A: Mouse urinary bladder | d18:0 | d18:1 | t18:0 | t20:0 | t24:0 |
|--------------------------|-------|-------|-------|-------|-------|
| HexCer                   | d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| LacCer                   | d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| Gb3Cer                   | d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| Gb4Cer                   | d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| GM3                      | d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| GD3                      | d18/1| t18/0 | d20/1 | t20/0 | t24/0 |

B: Human urinary bladder

| d18:0 | d18:1 | t18:0 | t20:0 | t24:0 |
|-------|-------|-------|-------|-------|
| HexCer| d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| LacCer| d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| Gb3Cer| d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| Gb4Cer| d18/1| t18/0 | d20/1 | t20/0 | t24/0 |
| GM3   | d18/1| t18/0 | d20/1 | t20/0 | t24/0 |

Gb4Cer, and GM3 both in males and females (Fig. 1A). In human urinary bladders, the major GSLs were HexCer, LacCer, Gb3Cer, Gb4Cer, GM3, and GD3 both in male (case 4) and female (case 8) (Fig. 1B). In humans, bands in the HexCer and GM3 migrated slower in Epi than in SubEpi. In case 2, of which Epis were depleted by the intravesical BGC infusion before total cystectomy, the bands in the HexCer and GM3 migrated similarly in Epi and SubEpi (Fig. 1C). These findings suggest structural differences in GSL between Epi and SubEpi. It is possible that epithelial cells have a more hydrophilic structure, such as phytoceramide or 2-hydroxy fatty acid. By the TLC with the borate-impregnated silica gel-coated HPTLC plates, the minor upper bands of HexCer (**) in the urinary bladder of both mice and human (Epi of case 4), migrated faster than the standard of GalCer, suggesting that these bands are GlcCer. On the other hand, the major band of HexCer (**) in both mice and human migrated slightly faster but was not clearly separated from GalCer (Fig. 1D).

Structural characterization and quantification of GSLs in mouse urinary bladder tissue

Thirty-five GSL species were characterized by LC-MS in lipid extracts from whole urinary bladder tissues pooled from four male mice. Phytoceramide structures with 18- and 20-carbon LCBs and 2-hydroxy fatty acid were enriched in HexCer and GM3 but were not detected in LacCer, Gb3Cer, and Gb4Cer (Table 3).

Five GM3 species were detected (Table 3). Figure 2A shows the mass chromatograms of five peaks for GM3, which were confirmed by MS² analysis to be GM3 (t200-h26:0) for m/z 1353.90 peak 1, GM3 (d18:1-16:0) for m/z 1151.71 peak 2, GM3 (d18:1-22:0) for m/z 1235.80 peak 3, GM3 (d18:1-24:1) for m/z 1201.82 peak 4, and GM3 (d18:1-24:0) for m/z 1263.85 peak 5. The MS² spectrum of peak 1 identified GM3 with a 20-carbon phyto-type LCB (4-hydroxyeicosaphasphinganine, t20:0) and 2-hydroxyhexacosanoic acid (h26:0), which are characterized by reporter fragment ions of m/z 283.26 P and m/z 365.38 H (Fig. 2B). H and P ions were reporter fragments for 2-hydroxy fatty acid and LCB, respectively (Table 2) (19).

Sixteen HexCer species were detected (Table 3). Figure 3A shows the mass chromatograms of eight peaks for HexCer, which were confirmed by MS² analysis to be HexCer (t18:0-h24:0) for m/z 844.69 peak 1, HexCer (t20:0-h24:0) for m/z 872.72 peak 2, HexCer (t20:0-h26:0) for m/z 900.75 peak 3, HexCer (d18:1-16:0) for m/z 698.56 peak 4, HexCer (d18:1-24:1) for m/z 808.67 peak 5, HexCer (d18:1-24:0) for m/z 824.66 peak 6, HexCer (d18:1-24:0) for m/z 810.68 peak 7, and HexCer (d18:1-24:0) for m/z 826.68 peak 8. The MS² spectrum of peak 1 for m/z 844.69 indicated HexCer with an 18-carbon phyto-type LCB and 2-hydroxy tetracosanoic acid (t18:0-h24:0), which are characterized by reporter fragment ions of m/z 255.23 P and m/z 337.35 H for 4-hydroxyeicosaphasphinganine (t18:0) carrying h24:0 fatty acid (Fig. 3B). The MS² spectrum of peak 2 for m/z 872.72 indicated HexCer with a 20-carbon phyto-type LCB and 2-hydroxytetracosanoic acid (t20:0/h24:0), which are characterized by reporter fragment ions of m/z 283.26 P and m/z 337.35 H (Fig. 3C). The MS² spectrum of peak 3 for m/z 900.75 indicated HexCer with a 20-carbon phyto-type LCB and 2-hydroxyhexacosanoic acid (t20:0/h26:0). The MS² spectrum of peak 4 for m/z 698.56 indicated HexCer with a 20-carbon phyto-type LCB and 2-hydroxyhexacosanoic acid (h26:0), which are characterized by reporter fragment ions of m/z 283.26 P and m/z 365.38 H (Fig. 2B). H and P ions were reporter fragments for 2-hydroxy fatty acid and LCB, respectively (Table 2) (19).
acid (t20:0-h26:0), which are characterized by reporter fragment ions of m/z 283.26 P and m/z 365.38 H (Fig. 3D).

MRM analyses revealed that the amounts of phytoceramide structures with a 20-carbon LCB and with 2-hydroxy fatty acid (t20:0-h24:0 and t20:0-h26:0) were abundant in HexCer and GM3 but not in LacCer, Gb3Cer, and Gb4Cer (Fig. 4). Considering these results together with the results of TLC using borate-impregnated HPTLC plates (Fig. 1D), it can be concluded that the major HexCer molecular species in the mouse urinary bladder is GlcCer with phytoceramide structure not GalCer with ceramide structure.

### Structural characterization and quantification of GSL in human urinary bladder Epi

Forty GSL species were detected by LC-MS in Epi of case 4. Phytoceramide structures with 18- and 20-carbon LCBS and 2-hydroxy fatty acid were enriched in HexCer, GM3, and GD3 but were not detected in LacCer, Gb3Cer, and Gb4Cer (Table 3).

Ten GM3 species were detected (Table 3). Figure 5A shows the mass chromatograms of six peaks for GM3 containing a phyto-type LCB, which were confirmed by MS² analysis to be GM3 (t18:0-h22:0) for m/z 1269.80 peak 1, GM3 (t18:0-h23:0) for m/z 1283.872 peak 2, GM3 (t18:0-h24:1) for m/z 1295.82 peak 3, GM3 (t18:0-h24:0) for m/z 1297.83 peak 4, GM3 (t20:0-h24:1) for m/z 1323.85 peak 5, and GM3 (t20:0-h24:0) for m/z 1325.86 peak 6. The MS² spectrum of peak 4 for m/z 1297.83 indicated GM3 with an 18-carbon phyto-type LCB and 2-hydroxytetraicosanoic acid (t18:0-h24:0), which are characterized by reporter fragment ions of m/z 255.23 P and m/z 337.35 H (Fig. 5B). The MS² spectrum of peak 6 for m/z 1325.87 indicated GM3 with a 20-carbon phyto-type LCB and 2-hydroxytetraicosanoic acid (t20:0-h24:0), which are characterized by reporter fragment ions of m/z 283.26 P and m/z 337.35 H (Fig. 5C).

Four GD3 species were detected (Table 3). Supplemental Fig. S1A shows the mass chromatograms of two peaks for GD3, which were confirmed by MS² spectra to be GD3 (d18:1-24:1) for m/z 775.95 peak 1 and GD3 (t18:0-24:1) for m/z 784.95 peak 2. The molecular ions of GD3 were double charged [M-2H]²⁻. The MS² spectrum of peak 1 for m/z 775.95 indicated GD3 (d18:1-24:1), which are characterized by fragment ions from ceramide of m/z 237.22 P, m/z 347.33 V, m/z 365.45 U, m/z 390.37 T, and m/z 406.36 S (Supplemental Fig. S1B). The MS² spectrum of peak 2 for m/z 784.95 indicated GD3 (t18:0-24:1), which were characterized by fragment ions from ceramide of m/z 347.33 V, m/z 364.34 U, m/z 390.37 T, and m/z 420.38 X (Supplemental Fig. S1C).
Nineteen HexCer species were detected (Table 3). Figure 6A shows the mass chromatograms of six peaks for HexCer containing a phyto-type LCB, which were confirmed by MS² analysis to be HexCer (t18:0-h24:0) for m/z 844.69 peak 1, HexCer (t18:0-h22:0) for m/z 872.72 peak 2, HexCer (t18:0-h23:0) for m/z 837.35 peak 3, HexCer (t18:0-h24:0) for m/z 844.68 peak 4, HexCer (t20:0-h24:1) for m/z 900.75 peak 5, and HexCer (t20:0-h24:0) for m/z 872.71 peak 6. The MS² spectrum of peak 4 for m/z 844.68 indicated HexCer with a 18-carbon phyto-type LCB and 2-hydroxytetracosanoic acid (t18:0-h24:0), which were characterized by reporter fragment ions of m/z 255.23 P and m/z 337.35 H. Peaks 4 to 8 in (A) represent HexCer (d18:1-16:0) for m/z 698.56, HexCer (d18:1-24:1) for m/z 808.67, HexCer (d18:1-24:1) for m/z 824.66, HexCer (d18:1-24:0) for m/z 810.68, and HexCer (d18:1-h24:0) for m/z 826.68, respectively, by MS² analysis (data not shown).

Glycosphingolipids in the urinary bladder

Figure 6A shows the mass chromatograms of six peaks for HexCer containing a phyto-type LCB, which were confirmed by MS² analysis to be HexCer (t18:0-h16:0) for m/z 732.56 peak 1, HexCer (t18:0-h22:0) for m/z 816.65 peak 2, HexCer (t18:0-h23:0) for m/z 830.67 peak 3, HexCer (t18:0-h24:0) for m/z 844.68 peak 4, HexCer (t20:0-h24:1) for m/z 870.70 peak 5, and HexCer (t20:0-h24:0) for m/z 872.71 peak 6. The MS² spectrum of peak 4 for m/z 844.68 indicated HexCer with an 18-carbon phyto-type LCB and 2-hydroxytetracosanoic acid (t18:0-h24:0), which are characterized by reporter fragment ions of m/z 255.23 P and m/z 337.35 H. Peaks 4 to 8 in (A) represent HexCer (d18:1-16:0) for m/z 698.56, HexCer (d18:1-24:1) for m/z 808.67, HexCer (d18:1-24:1) for m/z 824.66, HexCer (d18:1-24:0) for m/z 810.68, and HexCer (d18:1-h24:0) for m/z 826.68, respectively, by MS² analysis (data not shown).

MRM analyses revealed that the amounts of phytoceramide structures with a 20-carbon LCB and 2-hydroxy fatty acid (t20:0-h24:0) were abundant in HexCer and GM3 in case 4 (Fig. 7) and case 8 (Supplemental Fig. S2), but not in case 2, of which Epis were depleted by intravesical BCG infusion before total cystectomy (Supplemental Fig. S3). These findings indicate that phytoceramide structures with a 20-carbon LCB and 2-hydroxy fatty acids are abundant in particular GSL species, HexCer and GM3 in urinary epithelial cells in human. Considering these results together with the results of TLC using borate-
impregnated HPTLC plates (Fig. 1D), it can be concluded that the major HexCer molecular species in the human urinary bladder is also GlcCer with phytoceramide structure and not GalCer with ceramide structure.

Des1, Des2, Fa2h, Sptlc1-3, SptssA, SptssB, and Cers1-6 expressions in mouse urinary bladder tissues

The expressions of Des1, Des2, Fa2h, Sptlc1-3, SptssA, SptssB, and Cers1-6 were analyzed by qPCR in mouse whole urinary bladder tissues (n = 4). The expressions of Des2 and Fa2h were lower than Des1 but detected in significant amount (Fig. 8A). The expressions of Sptlc1, Sptlc2, and Sptlc3 were detected in this order, and the expression of SptssB, which are responsible for the generation of 20-carbon LCBs, was significantly higher than SptssA and highest among tissues analyzed (Fig. 8B). Among the expressions of Cers1-6, the expressions of Cers2, Cers3, Cers5, and Cers6 were dominant (Fig. 8C). Immunohistochemical analysis of mouse urinary bladder tissues showed that the expression of DES2 was found exclusively in urinary epithelial cells (Fig. 8D, E). These findings support that GSLs containing phytoceramide structures with 20-carbon LCBs and 2-hydroxy fatty acid are abundant in mouse urinary epithelial cells.

DES1, DES2, FA2H, SPTLC1-3, SPTSSA, SPTSSB, and CERS1-6 expression in human urinary bladder tissues

The expressions of DES1, DES2, FA2H, SPTLC1-3, SPTSSA, SPTSSB, and CERS1-6 were analyzed by qPCR in human urinary bladder tissues (total layer, Epi, and SubEpi) from case 4 (male), case 8 (female), and case 2 (female, treated by intravesical BCG infusion, which...
depleted Epi before total cystectomy). In case 4 and 8, the expressions of DES2 and FA2H in Epi were significantly higher than SubEpi (Fig. 9A). The expressions of SPTLC1-3 were detected and higher in Epi than SubEpi (Fig. 9B). The expression of SPTSSB was significantly higher in Epi than SubEpi. Among the expressions of CERS1-6, the expression of CERS2 was dominant (Fig. 9C). In case 2, most of these expressions were decreased compared with cases 4 and 8, and the abundance of DES2, FA2H, SPTLC3, and SPTSSB expressions in Epi was not observed (Fig. 9A–C). Immunohistochemical analysis of human urinary bladder tissues showed that the expression of DES2 was found in urinary epithelial cells in both cases 4 and 8, but not in case 2, where negative staining for uroplakin III demonstrated epithelial cell depletion (Fig. 9D, E). These findings confirm that GSLs containing phytoceramides with 20-carbon LCBs and 2-hydroxy fatty acids are abundant in human urinary epithelial cells.

**DES1, DES2, FA2H, SPTLC1-3, SPTSSA, and SPTSSB expression in human tissues**

The expressions of DES1, DES2, FA2H, SPTLC1-3, SPTSSA, and SPTSSB in the human tissues were analyzed by qPCR using the human cDNA library and cDNA synthesized from RNA extracted from total layer of urinary bladder of case 1 (Fig. 10). The expression of DES2 in urinary bladder was higher than that in brain and similar to gastrointestinal tract. The expression of FA2H in urinary bladder was similar to brain. The expressions of SPTLC3 and SPTSSB in urinary bladder were higher than brain. The significant expression of
SPTSSB was also detected in colon. These findings support that GSLs containing phytoceramides with a 20-carbon LCB are produced predominantly in human urinary bladder.

DISCUSSION

GSLs are composed of a polar head glycan chain and a hydrophobic tail ceramide (1–3). The structure of ceramide varies among tissues in terms of the carbon chain length, degree of desaturation, and hydroxylation in the N-acyl chain and LCB. However, the biological roles of these variabilities are unclear. Six distinct mammalian CERS (CERS1-6) that have preferences for fatty acyl-CoAs of different carbon chain lengths as substrates produce variation in the N-acyl carbon chain length (6, 7). CERS1–6 produce 2-hydroxy fatty acids containing ceramide as same as nonhydroxy fatty acids containing ceramide (22). Mutations in CERS1 cause autosomal recessive progressive myoclonic epilepsy (23). Mutations in CERS3 cause autosomal recessive congenital ichthyosis (24, 25). Fatty acid 2-hydroxylation, encoded by FA2H, produces 2-hydroxy fatty acids containing sphingolipids in myelin (8, 9). Indeed, mutations in FA2H cause autosomal recessive leukodystrophy with spastic paraplegia in humans and mice (26, 27). Structural variation in LCBs is produced by mammalian SPTs, which are heterotrimeric proteins consisting of a large subunit dimer (SPTLC1 and SPTLC2 or SPTLC3) and one of two small subunits (SPTSSA or SPTSSB). The SPTLC1, SPTLC2, and SPTSSA complex condenses a palmitoyl-CoA and serine to generate an 18-carbon LCB, the most abundant LCB in mammalian cells. By contrast, the SPTLC1, SPTLC2, and SPTSSB complex favors 225.0

Fig. 6. Structural analysis of HexCers containing 4-hydroxysphinganine in the human urinary bladder using LC-MS. A: Mass chromatograms of six HexCer molecules. B: MS² spectrum of peak 4 for m/z 844.68 represents HexCer (t18:0-h24:0), which is characterized by reporter fragment ions of m/z 255.23 P and m/z 337.34 H. C: MS² spectrum of peak 5 for m/z 870.70 represents HexCer (t20:0-h24:1), which is characterized by reporter fragment ions of m/z 283.26 P and m/z 335.33 H. D: MS² spectrum of peak 6 for m/z 872.71 represents HexCer (t20:0-h24:0), which is characterized by reporter fragment ions of m/z 283.26 P and m/z 337.35 H. Peak marked with * provides a fragment ion at m/z 168.04, demonstrating that the molecule is not a GSL but sphingomyelin.
stearoyl-CoA as a substrate, leading to a 20-carbon LCB, and that of SPTLC1, SPTLC3, and SPTSSB favors myristoyl-CoA or stearoyl-CoA, leading to a 16- or 20-carbon LCB, respectively (10, 28). Mutations in SPTLC1 and SPTLC2 cause autosomal dominant hereditary sensory neuropathy (29, 30). Twenty-carbon LCBs are present in gangliosides in human and mouse brains (31). In mice, a gain-of-function mutation in Sptssb enhances production of 20-carbon LCBs and results in profound neuropathological changes in the brain and retina, suggesting the importance of 20-carbon LCBs in vivo (32). DES1 produces ceramide with sphinganine, which is the most abundant LCB in mammalian cells. Mutations in DES1 cause autosomal recessive hypomyelinating leukodystrophy in humans (33). DES2 produces phytoceramide with an additional hydroxy group at sphinganine C-4 (11). Phytoceramide structures are known to be abundant in mammalian small intestinal and renal epithelial cells (12). Although, their distinct functions are still unknown, because of the absence of human diseases or animal models for DES2 deficiency, their important role in the various epithelial cells is presumed.

In this study, we hypothesized that urothelial cells have unique GSL compositions to enable their stretching-and-shrinking membrane properties and examined the structural variation in the GSLs in the urinary bladders of mice and humans. Few studies have focused on membrane lipid GSLs in urinary bladder tumor or cell lines. Superficial urinary bladder tumors in humans show massive accumulation of GM3 (34). Human urothelial cell lines have HexCer, LacCer, Gb3Cer, Gb4Cer, GM3, and GD3. Data are shown by mean ± SD with technical triplicate.

Fig. 7. Amounts of assigned molecular species in GSLs in the Epi of human urinary bladder (case 4). The amounts of assigned molecular species in HexCer, LacCer, Gb3Cer, Gb4Cer, GM3, and GD3 in the Epi of human urinary bladder tissue (case 4) were determined via MRM analysis as shown in Supplemental Table S2. Phytoceramide structures with 20-carbon LCBs and 2-hydroxy fatty acids denoted by * are present in significant amount of HexCer and GM3. Glycosphingolipids in the urinary bladder
some of the HexCer and gangliosides contain more complex unsaturated and 2-hydroxy fatty acids of h16:0 and h24:0 (35). We found that phytoceramide structures with a 20-carbon LCB and 2-hydroxy fatty acid are enriched in HexCer and GM3 in the urinary bladder tissues of mouse and urinary Epis of human by TLC and LC-MS analysis (Figs. 4 and 7). These findings are supported by the gene expression of key enzymes involved in the synthesis of phytoceramides with a 20-carbon LCB and 2-hydroxy fatty acids (DES2, FA2H, SPTLC3, and SPTSSB) in the Epis (Figs. 8 and 9) (5, 26, 27).

In addition, the immunohistochemical studies showed that DES2 was expressed exclusively in urothelial cells in both mouse and human.

The inner surface of the urinary bladder is lined by urothelial cells (16). The apical surface of urothelial cells is composed of umbrella cells, which dynamically change their morphology according to urine volume from cuboidal to highly stretched. The 70–90% of apical membrane of umbrella cells is covered by transmembrane protein called uroplakin (16, 36). Uroplakin forms highly specialized hexagonally packed protein plaques of 16 nm. Uroplakin plaque is thought to contribute to the barrier function and the stretching and shrinking of the apical membrane through the endocytosis and exocytosis of uroplakin-containing membrane.

Lipid rafts are cholesterol- and sphingolipid-enriched microdomains in the plasma membrane (37). The sphingolipids in the lipid rafts are known to be enriched in ceramide structures containing 2-hydroxy fatty acids. FA2H-knockdown in 3T3-L1 adipocytes increased the membrane mobility of raft-associated lipids and decreased transporter 4 and lipogenesis (38). Phase transition temperature of sphingomyelin composed of a hydroxy LCB and 2-hydroxy fatty acids was higher than that composed of a nonhydroxy LCB and fatty acids (39, 40). These findings suggest that the hydroxylation of LCB or fatty acid in ceramide structure plays important role for stabilization of lipid raft. The increase of chain length in LCB from 18-carbon to 20-carbon may decrease the membrane fluidity. It is speculated that the enrichment of phytoceramide structures with a 20-carbon LCB and 2-hydroxy fatty acids reduces membrane fluidity and allows tight plaque formation of uroplakin in the apical membrane of umbrella cells.

In conclusion, GSLs in urothelial cells of mouse and human urinary bladder are enriched in phytoceramide structures with a 20-carbon LCB and 2-hydroxy fatty acids in GlcCer and GM3. Our findings implicate that these unique ceramide structures play important role in stretching-and-shrinking membrane properties of transitional urothelial cells. Further research to address the biological significance and the pathophysiological contribution of these structures is warranted.

Data Availability
The data generated or analyzed during this study are included in this published article (and its supplemental data files) or are available from the corresponding author upon reasonable request.

Supplemental Data
This article contains supplemental data.
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Fig. 9. Expressions of DES1, DES2, FA2H, SPTLC1-3, SPTSSA, SPTSSB, and CERS1-6 in human urinary bladder tissues. The expression of DES1, DES2 and FA2H (A), SPTLC1-3, SPTSSA, and SPTSSB (B), and CERS1-6 (C) in the total layer, Epi, and SubEpi in human urinary bladders in case 4, case 8, and case 2 by qPCR. The expression of DES2 in the human urinary bladder tissues in case 4, case 8, and case 2 by immunohistochemical study (D). Quantification of DES2 immunofluorescence between Epi and SubEpi in case 4, case 8, and case 2 (E). Data are shown by means ± SDs by technical triplicate. *P < 0.05. MFI, mean fluorescent intensity; UP III, uroplakin III, DNA, nuclear staining.
Author Contributions
J. M. conceptualization; T. M. methodology; T. W., A. S., S. G., Y. I., and J. M. formal analysis; S. O., Y. M., T. N., K. H., and A. N. resources; J. M. writing—original draft; T. W., A. S., S. O., S. G., Y. I., T. M., Y. M., T. N., K. H., A. N., and J. M. writing—review & editing; J. M. and S. G. funding acquisition.

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Conflict of Interest
The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations
BCG, Bacille Calmette-Guerin; cDNA, complementary DNA; CERS, ceramide synthase; DES1, dihydroceramide-sphinganine Δ4-desaturase; DES2, dihydroceramide-sphinganine C4-hydroxylase; Epi, epithelial layer; FA2H, fatty acid 2-hydroxylase; GalCer, galactosylceramide; Gb3Cer, globotriaosylceramide; Gb4Cer, globotetraosylceramide; GD3, Neu5Ac-Neu5Ac-Gal-Glc-Ceramide; GlcCer, glucosylceramide; GM1, Gal-GalNAc-(Neu5Ac-)Gal-Glc-Ceramide; GM3, Neu5Ac-Gal-Glc-Ceramide; GSL, glycosphingolipid; HexCer, hexosylceramide; HPTLC, high-performance TLC; LacCer, lactosylceramide; LCB, long-chain

Fig. 10. Expressions of DES1, DES2, FA2H, SPTLC1, SPTLC2, SPTCL3, SPTSSA, and SPTSSB in a human cDNA library. The expressions of DES1, DES2, and FA2H (A), SPTLC1~3 (B), SPTSSA and SPTSSB (C) by qPCR in a human cDNA library (Human total RNA Master Panel II, Clontech, WI) and cDNA synthesized from RNA extracted from total layer of case 1. Data are shown by means ± SDs with technical triplicate. *P < 0.05. n.d., not detected.

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Fig. 10. Expressions of DES1, DES2, FA2H, SPTLC1, SPTLC2, SPTCL3, SPTSSA, and SPTSSB in a human cDNA library. The expressions of DES1, DES2, and FA2H (A), SPTLC1~3 (B), SPTSSA and SPTSSB (C) by qPCR in a human cDNA library (Human total RNA Master Panel II, Clontech, WI) and cDNA synthesized from RNA extracted from total layer of case 1. Data are shown by means ± SDs with technical triplicate. *P < 0.05. n.d., not detected.
base; MRM, multiple reaction monitoring; qRT-PCR, quantitative RT-PCR; SPT, serine palmitoyltransferase; SPTLC1, serine palmitoyltransferase LCB subunit 1; SPTLC2, serine palmitoyltransferase LCB subunit 2; SPTLC3, serine palmitoyltransferase LCB subunit 3; SPTSSA, serine palmitoyltransferase small subunit A; SPTSSB, serine palmitoyltransferase small subunit B; SubEpi, subepithelial layer.

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