J. Biochem. 2009;146(3)439–447
doi:10.1093/jb/mvp091

Storage of Gangliosides GM2 and Fucosyl GM1 in the Kidney of MCC Strain of Mastomys (*Praomys coucha*)

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Received March 26, 2009; accepted June 11, 2009; published online June 24, 2009

Previously, we histochemically examined the kidney of the MCC strain of mastomys (*Praomys coucha*) and found the storage of gangliosides. In the present studies, the lipid-bound sialic acid content of gangliosides in the MCC kidney was about 9- to 14-fold higher than that of the control (MWC strain). In the MCC kidney, sialic acids of male gangliosides were composed of *N*-acetylneuraminic acid at 91.5%; sialic acids of female gangliosides, however, were composed almost entirely of *N*-glycolylneuraminic acid. TLC of gangliosides showed that the MCC kidney contained four abundant gangliosides (two gangliosides each in males and females). These gangliosides isolated by HPLC were identified to be GM2(NeuAc) and fucosyl GM1(NeuAc) in the male MCC kidney and GM2(NeuGc) and fucosyl GM1(NeuGc) in the female MCC kidney by secondary ion mass spectrometry, TLC/immunostaining and TLC after enzyme treatments. Although the MCC kidney contained control levels of the activities of β-*N*-acetylhexosaminidase, α-L-fucosidase, *N*-acetylgalactosaminyltransferase and fucosyltransferase, the activity of β-galactosidase in the MCC kidney was increased to 400–500% of that in the MWC kidney. Therefore, we discussed the possibility that in the MCC kidney, GM2 was abundantly produced by the effect of increased β-galactosidase activity.

Key words: fucosyl GM1, ganglioside, GM2, kidney, mastomys.

Abbreviations: Cer, ceramide; CDH, lactosylceramide; fucosyl GM1(NeuAc), IV^{2}Fucα,II^{3}NeuAcα-Gg$_4$Cer; fucosyl GM1(NeuGc), IV^{2}Fucα,II^{3}NeuGcα-Gg$_4$Cer; Hex, hexose; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; GD1a, IV^{3}NeuAcα,II^{3}NeuAcα-Gg$_4$Cer; GD1b, II^{3}NeuAcα$_2$-Gg$_4$Cer; GD2, II^{3}NeuAcα$_2$-Gg$_3$Cer; GD3, II^{3}NeuAcα$_2$-LacCer; GM1(NeuAc), II^{3}NeuAcα-Gg$_4$Cer; GM1(NeuGc), II^{3}NeuGcα-Gg$_4$Cer; GM2(NeuAc), II^{3}NeuAcα-Gg$_3$Cer; GM2(NeuGc), II^{3}NeuGcα-Gg$_3$Cer; GM3, II^{3}NeuAcα-LacCer; GM3(NeuGc), II^{3}NeuGcα-LacCer; 4-MU, 4-methylumbelliferyl; PBS, phosphate-buffered saline; SI-MS, secondary ion mass spectrometry.

Mastomys (*Praomys coucha*) is an African rodent, with a size intermediate between those of a mouse and a rat. Since the first introduction of this species into laboratories in the 1940s, it has been used in many biomedical fields, including oncology, parasitology and epidemiology (*1*). One of the advantages of using mastomys as a laboratory rodent is that it exhibits great phenotypic variations, as do laboratory mice and rats. An inbred strain of mastomys, MCC, with pink eyes and diluted hair colour, was established from an MEC-chamois mastomys colony (Y strain) at the Institute for Medical Science, Tokyo University, and reported to have green–brown kidneys (*2*). Recently, we histochemically examined the kidney of MCC mastomys and found that the renal proximal tubular epithelial cells contain strongly PAS-positive inclusion bodies in the lysosomes. The inclusion bodies noted by electron microscopy exhibited multilamella myelin-like figures and an electron-dense amorphous matrix (*3*). These findings indicated that there is lysosomal storage of glycosphingolipids in the renal proximal tubular epithelial cells of MCC mastomys. In addition, immunohistochemical analysis showed that GM2 ganglioside was one of the glycosphingolipids accumulating in the lysosomes. In this study, we biochemically examined the MCC kidney gangliosides, and we elucidated that GM2 and fucosyl GM1 gangliosides were abundant in the kidney of MCC mastomys.

MATERIALS AND METHODS

Animals—MCC and MWC mastomyses were free of specific pathogens except pinworm. They were maintained in open-top polyolefin cages on shredded paper beddings under barrier conditions (temperature, 23 ± 2°C; humidity, 55 ± 5%; light, 5:00 a.m. to 7:00 p.m.) and provided with water and commercial laboratory mouse chow *ad libitum*. MCC mastomys, aged 20–26 weeks, were used in this study. MWC mastomys with normal kidneys were used as the control at ages 19–34 weeks. Animal experiments were conducted in accordance with Guides for animal experiments performed at NIID.

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Materials—DEAE-Sephadex A-25 was obtained from Amersham Biosciences (Uppsala, Sweden); Sep-Pak C18 reverse-phase cartridge was from Waters Associations (Milford, MA, USA); Silica gel 60 thin-layer chromatography plates (precoated 0.2-mm-thick plates) were from E. Merck (Darmstadt, Germany) and Polygram Sil G thin-layer chromatography plates (precoated 0.25-mm-thick plates) were from Marchery-Nagel (Darmstadt, Germany). Chicken antiserum against *N*-glycolylneuraminic acid-containing GM2 (GM2(NeuGc)) was provided by Dr N. Kasai, Tohoku University, Japan. Horseradish peroxidase-conjugated rabbit anti-chicken Ig G was purchased from Cappel Laboratories (Cochranville, PA, USA). UDP-*N*-acetyl-D-[6-^{3}H]galactosamine was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). GDP-[^{14}C]fucose was purchased from NEN Life Science Products, Inc. (Boston, MA, USA).

Extraction of Lipids—The lyophilized tissue was homogenized with 100 volumes of chloroform:methanol (2:1, by volume) and the extract was obtained by centrifugation. The residue precipitated was extracted with 100 volumes of chloroform:methanol (1:2) and removed by centrifugation; these extracts were combined and evaporated to dryness (total lipids).

Preparation of Gangliosides—A portion of the total lipids was dissolved in 5 ml of chloroform:methanol:water (30:60:8). The total lipid solution was applied to a 0.5 cm i.d. × 7.5 cm column containing DEAE-Sephadex A-25 (acetate form) (*4*). The column was rinsed with 5 column volumes of the same solvent to remove neutral lipids. Acidic lipids (mainly gangliosides and acidic phospholipids) absorbed were eluted with 10 column volumes of chloroform:methanol:0.8 M sodium acetate (30:60:8). An aliquot of the eluate was taken for lipid-bound sialic acid analysis, and the rest was evaporated to dryness (acidic lipids). The amount of lipid-bound sialic acid was determined in the acidic lipid fraction by spectrophotometric measurement with the resorcinol-HCl reagent (*5*).

A portion of the kidney acidic lipids was heated with 2 ml of methanolic 0.2 N KOH at 37°C for 1 h. The reaction mixture was neutralized with 1 ml of AG50W-X8 (100–200 mesh, H^{+} form) and centrifuged. The neutralized supernatant was applied to a Sep-Pak C18 reverse phase cartridge (*6*), which was then washed with 5 ml of water. The effluent and washing contained non-lipid materials. The gangliosides absorbed were recovered, first with 5 ml of methanol and then with 10 ml of chloroform:methanol (2:1). The combined eluates were evaporated to dryness (gangliosides).

To isolate four gangliosides, A, B, C and D, as shown in Fig. 2, a portion of gangliosides (equivalent to 0.4–0.6 mg of sialic acid) prepared from the MCC kidney was dissolved in 0.8 ml of chloroform:methanol:water (60:43:9.5) and subjected to high-performance liquid chromatography (HPLC). A Japan Spectroscopic model 880 HPLC with a 2.6 mm × 100 cm stainless steel column packed with Iatrobeads 6RSP-8005 (Iatron Laboratories, Inc., Tokyo, Japan) was used. The gangliosides absorbed on the HPLC column were eluted with 30 ml of chloroform:methanol:water (60:43:9.5) at a flow rate of 0.2 ml/min; 0.5 ml effluents were collected in each tube. The aliquot from each tube was examined by thin-layer chromatography. Each ganglioside thus separated was still contaminated with other gangliosides. Therefore, the ganglioside was applied again to the Iatrobeads HPLC column of the same size as above. The column was processed as described above, except that the gangliosides absorbed on the column were eluted with 80 ml of chloroform:methanol:2.5 N NH_4OH (60:43:9.5). The pure substances thus obtained were subjected to chemical analysis.

Gas Chromatographic Analysis—For gas–liquid chromatography (GLC), a Shimadzu model GC-7A gas chromatograph (Shimadzu Co., Kyoto, Japan) with a 25 m × 0.32 mm × 0.17 μm ultra-performance capillary column packed with cross-linked methyl silicone (Hewlett Packard Co., NY, USA) was used.

The composition of sialic acids, *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc), was determined in the ganglioside fraction by GLC, as described by Yu and Ledeen (*7*). The temperature was initially maintained at 160°C for 4 min, programmed from 160°C to 240°C at 4°C/min, and maintained at 240°C for 4 min. The sialic acid composition was calculated by the use of a Chromatopac C-R1A integrator (Shimadzu Co.).

To extract the fatty acid methyl esters, the isolated gangliosides were methanolyzed with 5% methanolic HCl at 85°C overnight. Then, the fatty acid methyl esters were extracted from mathanolizates with *n*-hexane and analysed by GLC. The temperature was initially maintained at 130°C for 4 min, programmed from 130°C to 240°C at 4°C/min, and maintained at 240°C for 8 min. In determination of fatty acid methyl esters by the use of a Chromatopac C-R1A integrator, the peak areas were calculated by comparison with the areas of peaks of an authentic mixture of fatty acid methyl esters.

Mass Spectrometric Analysis—Secondary ion mass spectrometry (SI-MS) in a negative ion mode was carried out by the use of a TSQ 70 triple-stage quadrupole mass spectrometer (Finnigan MAT, Inc., San Jose, CA, USA) equipped with a cesium ion gun. Triethanolamine was used as the matrix. The primary beam was maintained at 20 kV, the ion multiplier was at 1.2 kV and the conversion dynode was at 20 kV.

Thin-Layer Chromatographic Analysis—Thin-layer chromatography (TLC) was performed with two solvent systems, chloroform:methanol:2.5 N NH_4OH (60:40:9) and chloroform:methanol:0.2% $CaCl_2$ (60:43:9.5 or 65:25:4). For the detection of glycolipids including gangliosides, the plate was sprayed with the orcinol-H_2SO_4 reagent (*8*) and heated at 100°C for 5 min.

TLC/Immunostaining Analysis—For the TLC/immunostaining experiment, a portion of the MCC kidney gangliosides was applied to two TLC plates, a Polygram Sil G plate and a Silica gel 60 plate, and the two plates were developed with chloroform:methanol:0.2% $CaCl_2$ (60:43:9.5). The Polygram Sil G plate was subjected to the immunostaining analysis, as described by Higashi *et al*. (*9*). In our experiment, the plate was incubated at 37°C for 2 h with a GM2(NeuGc)-specific chicken antiserum (*10*) diluted to 1:30 in phosphate-buffered saline (PBS) containing 1% egg albumin and 1%

polyvinylpyrrolidone, which was followed by incubation at 37°C for 2 h with a peroxidase-conjugated rabbit anti-chicken Ig G diluted to 1:250 in PBS containing 3% polyvinylpyrrolidone. After the incubation reaction, the plate was reacted with a substrate solution containing 4-chloro-1-naphthol and H_2O_2 according to the method of Hawkes *et al*. (*11*) for 15 min at room temperature. An example of the immunostained TLC is shown in Fig. 2. The Silica gel 60 plate was run simultaneously with the Polygram Sil G plate in the same chamber for comparison of R_f values.

Analytical Methods—The neuraminidase reaction was carried out at 37°C for 18 h in the assay system containing, in a final volume of 0.5 ml, 10 mM acetate buffer (pH 5.0), 50 mU of neuraminidase (EC 3,2,1,18, *Arthrobacter ureafaciens*, Sigma Chem. Co., St Louis, MO, USA) (*12*) and 0.25 mg of sodium taurodeoxycholate. The reaction was stopped by adding 2 ml of chloroform: methanol (2:1) and the reaction products were obtained in the lower phase. For the β-*N*-acetylhexosaminidase reaction, the assay system contained 50 mM sodium citrate buffer (pH 4.0), 0.1 mg of sodium taurodeoxycholate and 0.6 U of β-*N*-acetylhexosaminidase (EC 3,2,1,52, Jack bean, Seikagaku Co., Tokyo, Japan) (*13*) in a final volume of 0.5 ml. After the assay mixture was incubated at 37°C for 18 h, the reaction products were obtained in the lower phase, as described above. For the α-1,2-L-fucosidase reaction, the assay system contained 50 mM potassium phosphate buffer (pH 6.5), 0.1 mg of sodium taurodeoxycholate and 20 mU of α-1,2-L-fucosidase (EC 3,2,1,63, *Bacillus* sp. K40T, Seikagaku Co., Tokyo, Japan) (*14*) in a final volume of 0.2 ml. After incubation of the assay mixture at 37°C for 18 h, the mixture was filtered through a Sep-Pak C18 reverse-phase cartridge. The reaction products were recovered in the effluent.

Preparation of Enzyme Source—Protein was measured by the method of Bradford (*15*). The tissue was homogenized with nine volumes of 50 mM sodium citrate buffer (pH 5.0), and the supernatant obtained by centrifugation at 10,000 rpm for 20 min was used as an enzyme source for the glycosidase assay. The kidney was homogenized with 10 volumes of 50 mM HEPES buffer (pH 7.0), 0.5 M sucrose and 1 mM EDTA (homogenization buffer). The homogenate was centrifuged at 3000 rpm for 10 min and the supernatant was then centrifuged at 18,000 rpm for 30 min. The pellet obtained by centrifugation was suspended in 300 μl of the homogenization buffer and used as an enzyme source for the glycosyltransferase assay. All preparation procedures were carried out at 4°C.

Glycosidase Assay Conditions—The activities of β-*N*-acetylhexosaminidase, α-L-fucosidase and β-galactosidase in kidneys and brains were assayed with 4-methylumbelliferyl (4-MU) glycosides as substrate, in a final volume of 55 μl, in the following assay systems: for β-*N*-acetylhexosaminidase, 0.5 mM 4-MU-*N*-acetyl β-D-glucosaminide (Sigma) in a 0.1 M citrate–0.2 M phosphate buffer (pH 4.5); for α-L-fucosidase, 0.5 mM 4-MU-α-L-fucoside (Sigma) in a 0.1 M citrate–0.2 M phosphate buffer (pH 4.0) and for β-galactosidase, 0.5 mM 4-MU-β-D-galactoside (Sigma) in a 0.2 M sodium citrate buffer (pH 4.5) with 0.1 M NaCl. Five microlitres of the tissue extract, which corresponded to 15–30 μg of the kidney protein or 5–9 μg of the brain protein, was added to the assay systems. The assay mixtures were incubated at 37°C for 30 min. The reaction was stopped by adding 0.15 ml of 0.4 M glycine–NaOH (pH 10.7). The fluorescence developed was measured on a Twinkle LB970 microplate fluorometer (Bethold Technologies GmbH and Co. KG, Bad Wildbad, Germany) with the use of an excitation wavelength at 355 nm and an emission wavelength at 460 nm.

Glycosyltransferase Assay Conditions—The activities of *N*-acetylgalactosaminyltransferase and fucosyltransferase in kidneys were assayed in a final volume of 0.1 ml in the following assay systems: for *N*-acetylgalactosaminyltransferase, 0.1 M sodium cacodylate buffer (pH 7.3), 10 mM $MnCl_2$, 0.3 mg of Triton X-100, 50 μg of GM3 (NeuGc), 2 μM of UDP-*N*-acetyl-D-[6-^{3}H]galactosamine (20 Ci/mmol) and enzyme source corresponding to 0.1 mg of protein; for fucosyltransferase, 25 mM HEPES buffer (pH 7.2), 20 mM $MnCl_2$, 5 mM CDP choline, 30 μg of GM1 (NeuAc), 3.7 μM of GDP-[^{14}C]fucose (271 mCi/mmol) and enzyme source corresponding to 0.1 mg of protein. The assay mixtures were incubated at 37°C for 60 min and the reaction was terminated by adding 40 μl of 0.5 M KCl and 0.25 M EDTA. Then, 5 ml of 0.1 M KCl was added, and the solution was filtered through a Sep-Pak C18 reverse-phase cartridge described as above. The radioactivity incorporated into the lipid fraction was determined with a LSC-6100 liquid scintillation counter (ALOKA Co., Tokyo, Japan).

RESULTS

In Fig. 1, the lipid-bound sialic acid content in the kidneys varied between 2.33 and 4.31 mg per g dry weight in the mastomys MCC strain and between 0.25 and 0.32 mg in the mastomys MWC strain (control). Furthermore, Fig. 1 shows the lipid-bound sialic acid content in the brain, lung and liver tissues. In these tissues, MCC showed essentially the same lipid-bound sialic acid levels as MWC. These results indicated that the kidneys

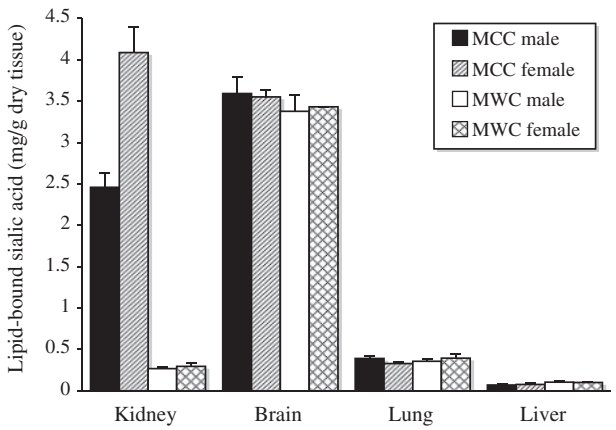

Fig. 1. **Lipid-bound sialic acid contents in the kidneys, brains, lungs and livers of MCC and MWC strains of mastomys.** The amount of lipid-bound sialic acid was determined in the acidic lipid fraction as described under MATERIALS AND METHODS. Each value represents the mean ± SD of two male and two female tissues.

of the MCC strain contained enhanced amounts of ganglioside.

The composition of the sialic acids, NeuAc and NeuGc, was determined by the GLC procedure as described under MATERIALS AND METHODS. For this determination, the ganglioside fraction was prepared separately from the male and female kidneys. The sialic acid compositions of the gangliosides from the MCC kidneys are summarized in Table 1. We compared the compositions of the sialic acids of the gangliosides in the MCC kidneys between the males and females. We found that (a) in male gangliosides, sialic acids were composed mainly of NeuAc as much as 90% and (b) in female gangliosides, sialic acids were composed almost entirely of NeuGc with NeuAc at only 2–3%.

We next compared the ganglioside composition in MCC and MWC kidneys and also between male and female kidneys. The compositions of gangliosides in these kidneys are shown in Fig. 2. One TLC plate (Fig. 2, plate I) was developed with chloroform:methanol:0.2% $CaCl_2$ (60:43:9.5) and the other (Fig. 2, plate II) was developed with chloroform:methanol:2.5 N NH_4OH (60:40:9). The TLC patterns revealed that the MCC kidney contained four gangliosides (represented as A and C in the male kidney and B and D in the female kidney) with traces of other gangliosides, apparently in a pattern different from that in the MWC kidney. The following experiments were designed to characterize gangliosides A, B, C and D.

Table 1. **Sialic acid composition of ganglioside from kidneys of MCC mastomys.**

MCC kidneys	Sialic acids (%)	
	N-acetylneuraminic acid (NeuAc)	*N*-glycolylneuraminic acid (NeuGc)
Male kidney	91.5	8.5
Female kidney	2.6	97.4

The results were obtained from two separate experiments.

Characterization of Gangliosides A and B—Ganglioside A (in the male MCC kidney) comigrated with authentic *N*-acetylneuraminic acid-containing GM2 (GM2(NeuAc)), as shown in Fig. 2, plates I and II, indicating that A was GM2(NeuAc). Ganglioside B (in the female MCC kidney) was different from ganglioside A in that it moved in a slow manner, as shown in Fig. 2, plate II; the ratio of B to GM2(NeuAc) with respect to the R_f value is about 1.0 in Fig. 2, plate I, whereas it is 0.7 in Fig. 2, plate II. This finding suggested that ganglioside B was GM2(NeuGc), so that the R_f value of B was reduced in Fig. 2, plate II by a known effect of NH_4OH. To test this possibility, ganglioside B was examined by TLC/immunostaining with an anti-GM2(NeuGc) antibody. For comparison, ganglioside A was also examined. As the results show in Fig. 2, plate III (note that TLC plates I and III were run in the same chamber), ganglioside B was indeed immunostained and ganglioside A was not, thus supporting our assumption.

Gangliosides A and B were obtained by HPLC and analysed by SI-MS, as described under MATERIALS AND METHODS. Typical SI-MS tracings (from m/z 500 to 1500) are shown in Fig. 3A and B. The spectra exhibited fragment ions, such as m/z 648 due to ceramide (Cer), m/z 810 due to Cer-hexose (Cer-Hex), m/z 972 due to Cer-Hex-Hex and m/z 1175 due to Cer-Hex-Hex-*N*-acetylhexosamine. With these fragment ions, the prominent deprotonated molecule at m/z 1466 in Fig. 3A and at m/z 1482 in Fig. 3B should be identical to GM2(NeuAc) and GM2(NeuGc), respectively.

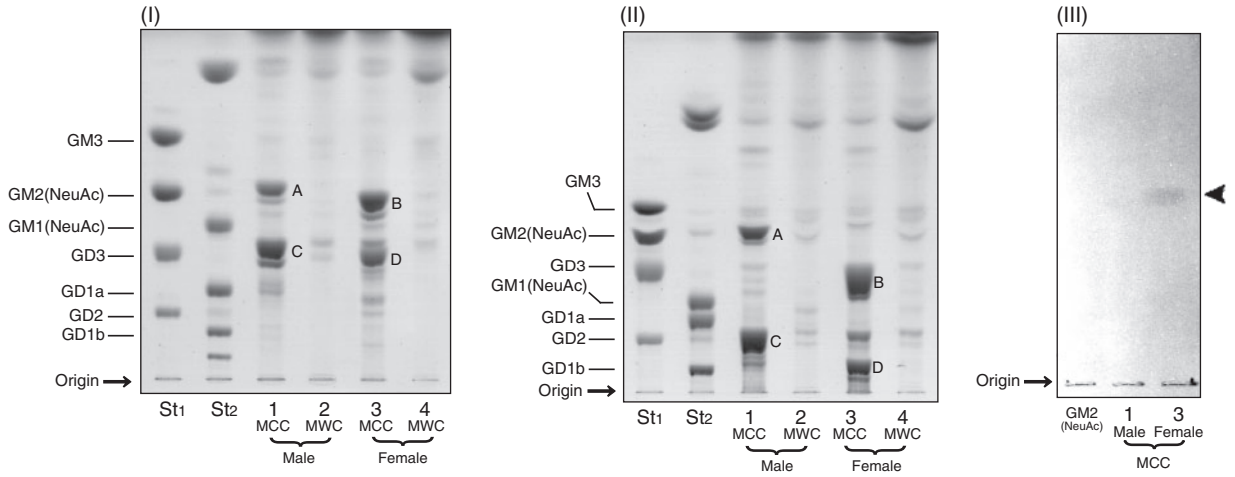

Fig. 2. **TLC of the gangliosides from mastomys kidneys.** Plate I (Silica gel 60 plate) and plate III (Polygram Sil G plate) were developed with chloroform:methanol:0.2% $CaCl_2$ (60:43:9.5) and plate II (Silica gel 60 plate) with chloroform:methanol:2.5 N NH_4OH (60:40:9). Plates I and II were subjected to the orcinol-H_2SO_4 reaction. Plate III was subjected to the TLC/immunostaining reaction with chicken anti-GM2(NeuGc)-specific antiserum, as described under MATERIALS AND METHODS. Lanes St_1 and St_2 represent standard gangliosides. St_1 includes, from top to bottom, GM3, GM2(NeuAc), GD3 and GD2. St_2 is the ganglioside fraction prepared from monkey brain, containing, from top to bottom, GM1(NeuAc), GD1a and GD1b. Lane numbers 1–4 represent the mastomys kidney gangliosides: lane 1 was obtained from male MCC, lane 2 from male MWC, lane 3 from female MCC and lane 4 from female MWC. The ganglioside fraction from 1.5 mg of dry kidney was applied on lanes 1 and 3. The ganglioside fraction from 5 mg of dry kidney was applied on lanes 2 and 4. In plates I and II, MCC samples gave four dense spots of gangliosides (represented as A, B, C and D). In plate III, ganglioside B (indicated by arrow) was immunostained.

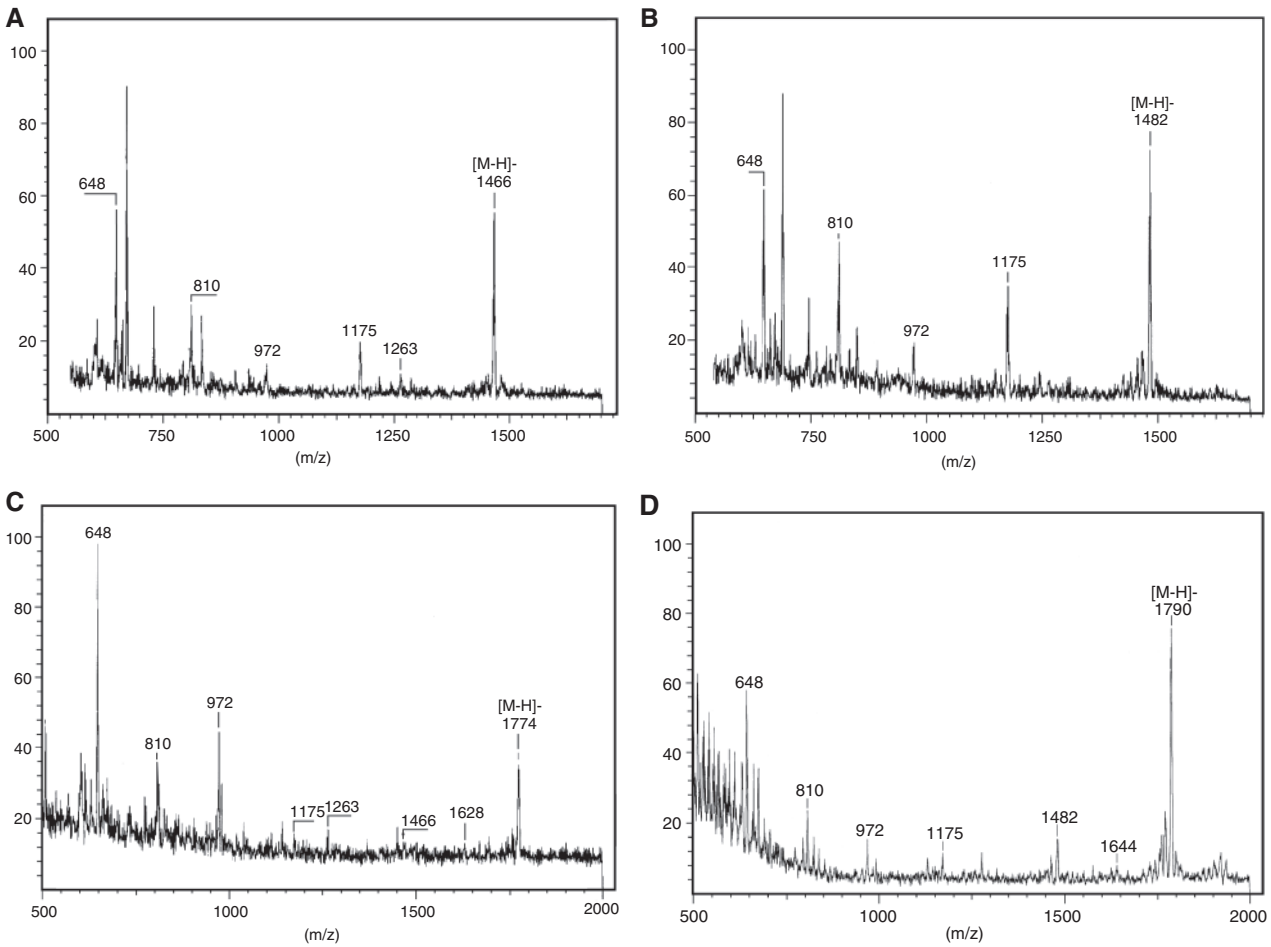

Fig. 3. **A portion of the SI-MS tracing of the four gangliosides (represented as A, B, C and D in Fig. 2) isolated from the MCC kidney.** (A) Ganglioside A, (B) ganglioside B, (C) ganglioside C and (D) ganglioside D. Preparation of these gangliosides and SI-MS conditions were as described under MATERIALS AND METHODS.

Ganglioside A (or B) was further analysed by determining the products after incubation, first with neuraminidase and second with β-*N*-acetylhexosaminidase. As shown in Fig. 4 (lanes 2 or 5), asialo GM2 (Cer-Hex-Hex-*N*-acetylhexosamine) was the product in the incubation with neuraminidase. Although the product asialo GM2 moved faster than the authentic asialo GM2 in the TLC plate, this difference was due to the difference in the fatty acid composition as shown in Table 2: thus, ganglioside A or B contained about 95% of longer-chain fatty acids (C22–C24). The product asialo GM2 was then incubated with β-*N*-acetylhexosaminidase. CDH (Cer-Hex-Hex) was produced in this incubation (Fig. 4, lanes 3 or 6). From these experimental findings, we identified ganglioside A as GM2(NeuAc) and ganglioside B as GM2(NeuGc).

Characterization of Gangliosides C and D— Ganglioside C (in the male MCC kidney) and ganglioside D (in the female MCC kidney) did not comigrate with any standard gangliosides on TLC (Fig. 2, plates I and II). In the SI-MS examination, ganglioside C (Fig. 3C) showed a pattern similar to that of ganglioside A (see Fig. 3A), except that C exhibited a fragment ion at m/z 1628, probably due to Cer-Hex-Hex(-NeuAc)-*N*-acetylhexosamine-Hex (GM1(NeuAc)). We estimated that this fragment ion was derived by eliminating 146 mass units from the prominent deprotonated molecule at m/z 1774. In Fig. 3D, ganglioside D showed an SI-MS pattern similar to that of ganglioside B (see Fig. 3B). However, ganglioside D exhibited a fragment ion at m/z 1644, probably due to Cer-Hex-Hex(-NeuGc)-*N*-acetylhexosamine-Hex (GM1(NeuGc)). Again, we estimated that this fragment ion was derived by eliminating 146 mass units from the prominent deprotonated molecule at m/z 1790. We therefore considered the possibility that GM1(NeuAc) was derived by eliminating fucose from ganglioside C and GM1(NeuGc) from ganglioside D. For this reason, we determined the products after incubation with fucosidase. After ganglioside C (or D) was incubated with α-1,2-L-fucosidase, GM1 was obtained (Fig. 5, lanes 2 or 5); our assumption was therefore supported. The product GM1 moved faster than authentic GM1 in the TLC plate, in a manner similar to the product asialo GM2 in Fig. 4 (see text). When the product GM1 was successively incubated with neuraminidase, asialo GM1 was produced (Fig. 5, lanes 3 or 6). From the characteristics both in

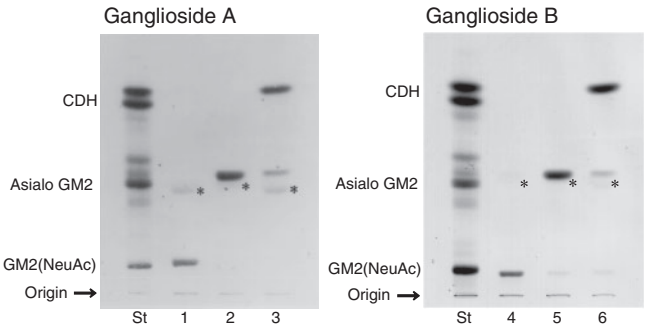

Fig. 4. **TLC of the products from gangliosides A and B after incubation with neuraminidase or β-*N*-acetylhexosaminidase.** The assay conditions were as described under MATERIALS AND METHODS. The TLC plates were developed with chloroform:methanol:0.2% $CaCl_2$ (65:25:4). The spots were detected by spraying with the orcinol-H_2SO_4 reagent followed by heating at 100°C for 5 min. The spots marked with the asterisk indicate sodium taurodeoxycholate used as the detergent. Lane St represents standard glycosphingolipids, from top to bottom, two spots of lactosylceramide (CDH), asialo GM2 and GM2(NeuAc). Lanes 1–6 represent the incubation products. Ganglioside A remained intact when the incubation was carried out in the absence of enzyme (lane 1). Asialo GM2 was produced in the incubation of the assay mixture with ganglioside A and neuraminidase (lane 2). CDH was produced in the incubation of the assay mixture with the product asialo GM2 and β-*N*-acetylhexosaminidase (lane 3). Ganglioside B remained intact when incubation was carried out in the absence of enzyme (lane 4). Asialo GM2 was produced from ganglioside B with neuraminidase (lane 5), and CDH from the product asialo GM2 with β-*N*-acetylhexosaminidase (lane 6).

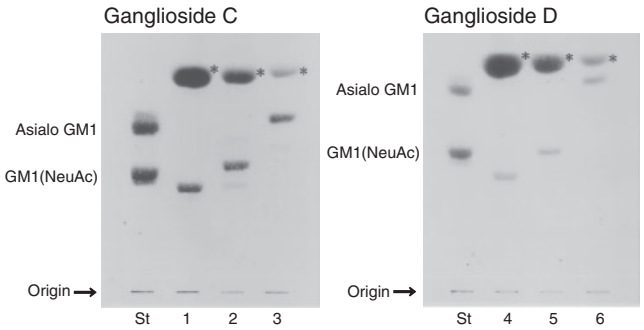

Fig. 5. **TLC of the products from gangliosides C and D after incubation with α-1,2-L-fucosidase or neuraminidase.** The assay conditions were as described under MATERIALS AND METHODS. The TLC plates were developed with chloroform: methanol:0.2% $CaCl_2$ (60:43:9.5). The spots were detected by spraying with the orcinol-H_2SO_4 reagent followed by heating at 100°C for 5 min. The spots marked with the asterisk indicate sodium taurodeoxycholate used as the detergent. Lane St represents standard glycosphingolipids, asialo GM1 and GM1(NeuAc). Lanes 1–6 represent the incubation products. Ganglioside C remained intact when the incubation was carried out in the absence of the enzyme (lane 1). GM1 was produced in the incubation of the assay mixture with ganglioside C and α-1,2-L-fucosidase (lane 2). Asialo GM1 was produced in the incubation of the assay mixture with the product GM1 and neuraminidase (lane 3). Ganglioside D remained intact when the incubation was carried out in the absence of the enzyme (lane 4). GM1 was produced from ganglioside D with α-1,2-L-fucosidase (lane 5), and asialo GM1 from the product GM1 with neuraminidase (lane 6).

Table 2. **Fatty acid composition of gangliosides from kidneys of MCC mastomys.**

	Fatty acids (%)					
Gangliosides	16:0	18:0	22:0	23:0	24:0	24:1
Ganglioside A	3.5	1.8	5.8	2.5	70.1	16.3
Ganglioside B	2.4	1.5	7.8	2.1	60.3	26.0
Ganglioside C	1.7	0.7	6.8	2.6	76.1	12.1
Ganglioside D	3.1	1.1	11.1	2.3	66.2	16.2

The results were obtained from two separate experiments.

the SI-MS tracings and in the TLC patterns of incubation products, we concluded that ganglioside C was fucosyl GM1(NeuAc) and ganglioside D was fucosyl GM1(NeuGc).

Determination of the Activities of Glycosidases and Glycosyltransferases in the MCC Tissues—In Table 3, we determined the activities of β-*N*-acetylhexosaminidase, β-galactosidase and α-L-fucosidase in the kidneys and brains of the MCC strain to investigate the cause of the abundance of GM2 and fucosyl GM1 in the MCC kidney. The kidneys of MCC males and females contained normal levels of both β-*N*-acetylhexosaminidase and α-L-fucosidase activities, similar to the results with the MWC kidneys. MCC kidneys, however, contained β-galactosidase activity (about 2000 nmol/mg protein/h) at 400–500% of that in the MWC kidneys, although β-galactosidase activity was not increased in the MCC brains. It appears, therefore, that β-galactosidase activity was enhanced specifically in the MCC kidneys. To confirm whether excess GM2 and fucosyl GM1 are synthesized in the MCC kidney, we determined the activities of *N*-acetylgalactosaminyltransferase (to transfer *N*-acetylgalactosamine to GM3) and fucosyltransferase (to transfer L-fucose to GM1) in the MCC kidneys (Table 4). In the determination, there was no difference in the activities of both enzymes between MCC and MWC.

DISCUSSION

We previously reported that gangliosides accumulate in the lysosomes of the renal proximal tubular epithelium of MCC mastomys (*3*). In the present study, we found that the lipid-bound sialic acid content in the MCC kidney was 9- to 14-fold higher than that in the MWC kidney (control), thus indicating that the MCC kidney contained enhanced amounts of gangliosides. In general, ganglioside storage disease (gangliosidosis) occurs by the inherited (autosomal recessive) defect or absence of the lysosomal enzyme and causes progressive loss of the nerve system function. It is likely that ganglioside storage disease causes death. However, MCC mastomys shows no clinical sign and may live with a normal life span (*3*).

It is generally accepted that the ganglioside accumulating in gangliosidosis possesses a particular sugar chain which should be cleaved at the carbohydrate terminal by the defective enzyme. The MCC kidney contained two abundant gangliosides, GM2 (GM2(NeuAc) and GM2(NeuGc)) and fucosyl GM1 (fucosyl GM1(NeuAc) and fucosyl GM1(NeuGc)). Since GM2 and fucosyl

Table 3. **The activities of glycosidases in the kidney and brain of MCC and MWC mastomys.**

	β-*N*-acetylhexosaminidase (nmol/mg protein/h)		β-galactosidase (nmol/mg protein/h)		α-L-fucosidase (nmol/mg protein/h)
	Kidney	Brain	Kidney	Brain	Kidney
MCC male	721.4 ± 33.9	2832.9 ± 662.0	1931.8 ± 80.4	88.7 ± 18.1	190.2 ± 33.2
MCC female	854.2 ± 121.6	2298.7 ± 219.1	2267.8 ± 15.6	68.3 ± 2.2	201.9 ± 12.9
MWC male	582.9 ± 67.3	2710.0 ± 488.5	383.2 ± 56.6	89.4 ± 17.9	126.4 ± 18.4
MWC female	883.9 ± 45.8	2656.1 ± 407.1	557.3 ± 49.0	66.1 ± 18.8	134.6 ± 13.7

Each value represents the mean ± SD of 3 tissues.

Table 4. **The activities of glycosyltransferases in the kidney of MCC and MWC mastomys.**

	N-acetylgalactosaminyl-transferase (pmol/mg protein/h)	Fucosyltransferase (pmol/mg protein/h)
MCC male	1.50	1.30
MCC female	1.34	0.68
MWC male	1.35	0.93
MWC female	1.23	0.73

Each value represents the mean of two tissues.

GM1 possess β-*N*-acetylgalactosamine and fucose, respectively, at the carbohydrate terminal, we first considered the possibility that in the MCC kidney, both β-*N*-acetylhexosaminidase (to cleave β-*N*-acetylgalactosamine) and α-L-fucosidase (to cleave fucose) are abnormal. The experimental findings that neither β-*N*-acetylhexosaminidase nor α-L-fucosidase was deficient in the MCC kidney excluded this possibility. In determining the activity of β-galactosidase, on the other hand, the MCC kidney showed about 4- to 5-fold higher activity than the MWC kidney. Because β-galactosidase degrades GM1 to GM2, it is reasonable to consider that high rates of the β-galactosidase reaction produced abundant GM2 in the MCC kidney. Nevertheless, an additional finding that the β-galactosidase activity did not increase in the MCC brain (in Table 3) suggested us that the accumulation of GM2 is kidney specific. These characteristics in MCC mastomys are, therefore, entirely different from those of GM2 gangliosidosis (*16–19*).

The accumulation of fucosyl GM1 has been reported in rat hepatoma H35 cells (*20*). With rat hepatoma H35 cells, Holmes and Hakomori (*21*) found that not only α-L-fucosidase activity was deficient but also fucosyltransferase activity was enhanced. Therefore, we determined the fucosyltransferase activity to confirm the possibility that enhanced activities of fucosyltransferase cause abundant fucosyl GM1 in the MCC kidney. In addition, the activities of *N*-acetylgalactosaminyltransferase were determined in the MCC kidney because this enzyme is involved in the syntheses of both GM2 and fucosyl GM1. Unexpectedly, the activities of both enzymes were not enhanced in the MCC kidney as shown in Table 4. This result suggested that neither *N*-acetylgalactosaminyltransferase nor fucosyltransferase was involved in the accumulation of gangliosides in the MCC kidney. The cause of the accumulation of fucosyl GM1 in the MCC kidney was not revealed in this study. Therefore, further study of other factors such as an intracellular transport of ganglioside may be necessary to clarify the cause of the accumulation of fucosyl GM1 in the MCC kidney in the future. Also, whether the increase of β-galactosidase activity affects fucosyl GM1 accumulation remains to be examined.

In MCC mastomys, the gangliosides accumulating in the kidney contained mostly NeuGc in females but those in males contained mostly NeuAc. Although the expression of NeuGc is age specific (*22*), strain specific (*23*) or tissue specific (*24*), there has never been any report of an animal species like MCC mastomys that has gangliosides with different sialic acids between males and females. It was reported that the major pathway of NeuGc biosynthesis is the conversion of CMP-NeuAc to CMP-NeuGc by hydroxylation (*25–27*). Moreover, it has been suggested that CMP-NeuAc is converted to CMP-NeuGc by an electron transport system consisting of NADH-dependent cytochrome *b*5 reductase, cytochrome *b*5 and CMP-NeuAc hydroxylase (*28–30*). Based on the results of others (*31*, *32*), it is accepted that high amounts of NeuGc exist together with high rates of CMP-NeuAc hydroxylase reaction. We consider that since the sialic acids of the gangliosides in the female kidney of the MCC strain were composed of NeuGc at 97.4% (Table 1), the female MCC kidney should possess high rates of the CMP-NeuAc hydroxylase reaction. The differences between males and females were reported in the glycosphingolipid composition in the kidneys of mice (*33*) and rats (*34*) or in the ganglioside composition in rat kidneys (*34*). We also noticed that the ganglioside composition in the MCC kidney was considerably different between males and females: fucosyl GM1 was predominant in males and GM2 in females (in Fig. 2).

The present studies were initiated to investigate the gangliosides accumulating in the MCC kidney. We described that the male kidney contained two abundant gangliosides, GM2(NeuAc) and fucosyl GM1(NeuAc); the female kidney contained two abundant gangliosides, GM2(NeuGc) and fucosyl GM1(NeuGc) and we then found that the activity of β-galactosidase was enhanced in the MCC kidney. If high levels of β-galactosidase activity were involved in the abundance of GM2 and fucosyl GM1 in MCC kidneys, there must be a regulatory mechanism by which GM2 and fucosyl GM1 are kept abundant in a normal life span: such a mechanism remains to be demonstrated. An additional finding that the expression of NeuGc in the MCC kidney was female

specific stimulates us to investigate (a) the regulation of NeuGc formation by female hormones and (b) the physiological significance of NeuGc at high levels in the female kidney.

ACKNOWLEDGEMENT

We would like to thank Dr Masaharu Naiki, deceased in 1996, for his advice and technical support while he was alive.

CONFLICT OF INTEREST

None declared.

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