Accumulation of $G_{M2}$ Ganglioside in Niemann-Pick Disease Type C Fibroblasts

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Abstract: Niemann-Pick disease type C (NP-C) is an autosomal recessive neurovisceral lipid storage disorder biochemically characterized by a defect in intracellular transport of low-density lipoprotein (LDL)-derived cholesterol from the lysosome to other cellular sites. We have found substantial accumulation of $G_{M2}$ ganglioside in NP-C fibroblasts. The intracellular distribution of $G_{M2}$ ganglioside was similar to that of cholesterol detected by filipin staining, indicating that the accumulation of $G_{M2}$ ganglioside is mainly lysosomal. The incorporation of N-acetyl-D-[3H]mannosamine into gangliosides was also increased in NP-C fibroblasts, especially into the $G_{M2}$ and $G_{M3}$ fractions. A culture condition which eliminates cholesterol accumulation does not eliminate $G_{M2}$ accumulation. It is suggested that the accumulation of $G_{M2}$ ganglioside together with the accumulation of cholesterol is a unique abnormality in NP-C fibroblasts and that the defect in NP-C may involve intracellular transport of both cholesterol and $G_{M2}$ ganglioside.

Key words: Niemann-Pick disease type C; cholesterol; gangliosides; $G_{M2}$ ganglioside; lysosomal transport; genetic heterogeneity; sialic acid.

Introduction. Niemann-Pick disease type C (NP-C) is an autosomal recessive lipidosis characterized by a unique error in intracellular trafficking of low-density lipoprotein (LDL)-derived cholesterol. The primary molecular defect is still unknown. 1) A constant abnormality in NP-C is an impaired LDL-derived cholesterol processing in cultured cells as revealed by histochemical detection of intracellular accumulation of unesterified cholesterol and by defective LDL-induced cholesteryl ester formation.1,2) In the tissues of NP-C patients, however, pronounced glycolipid abnormalities have been reported in liver, spleen and brain from NP-C patients1–3) and from the BALB/c model mice of NP-C.4–5) Most of studies have specially emphasized the increase in neutral glycolipids, particularly of glucosylceramide and lactosylceramide. Although secondary disruption of lysosomal functions such as activities of sphingomyelinase and glucocerebrosidase has been known in NP-C6– and the model mice,4–7) deficiencies of the lysosomal hydrolases that degrade lactosylceramide or gangliosides are not known.4,7,8) There is however evidence that $G_{M3}$ ganglioside is moderately increased in liver and spleen,4,9) and above all that elevated $G_{M2}$ ganglioside, $G_{M2}$ ganglioside and asialo-$G_{M2}$ ganglioside are a common finding in brain of NP-C patients3 as well as of the mouse model.4,5) The mechanism underlying the accumulation of gangliosides in NP-C tissues has not been characterized and, as far as we know, no previous report exists on ganglioside metabolism in cultured fibroblasts from NP-C patients. In this report, we describe that NP-C fibroblasts have a defect in $G_{M2}$ ganglioside metabolism.

Materials and methods. Chemicals. [14C]Sphingo- myelin (54.5 Ci/mmol), [3H]oleic acid (9.2 Ci/mmol), N-acetyl D-[3H]mannosamine (15 Ci/mmol) were

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purchased from New England Nuclear (Boston, U.S.A.). Filipin complex, lipoprotein-deficient calf serum (LPDS), fatty acid free albumin, 2-thiobarbituric acid, and gangliosides G_{D1a}, G_{M1}, G_{M2}, G_{M3} were purchased from Sigma (St. Louis, U.S.A.). HPTLC plates were purchased from Merck (Darmstadt, Germany). For protein assay, Bio-Rad (Hercules, U.S.A.) Protein Assay Kit I was used.

**Cells and cell culture.** Fibroblast strains used in this study were derived from four normal 2- to 9-year-old Japanese children, one normal Caucasian infant (GM05659C, obtained from NIGMS Human Genetic Mutant Cell Repository, Camden, U.S.A.), two Japanese NP-C children \(^{10}\) (YON; 4-year-old; UCH, 2-year-old), one Japanese NP-C adult \(^{10}\) (KAI, 38-year-old) and four Caucasian NP-C patients (82017, 88057, 93059, and GMO11OA). The 93059 strain (case 13 in ref. 11) is representative of the major complementation group of NP-C (complementation group 1) while the strains 82017 and 88057 (case 27 and case 19, respectively, in ref. 11) represent the second minor complementation group. \(^{11}\) In some experiments fibroblast strains derived from patients with infantile sialic acid storage disease (ISSD), Tay-Sachs disease, Niemann-Pick disease types A and B, Gaucher disease type II, I-cell disease, and Krabbe disease were also used. Unless otherwise specified, the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum.

**Cholesteryl ester formation after stimulation with fresh medium containing 20% fetal bovine serum.** Cells were cultured in DMEM containing 10 % LPDS. On the third day, the medium was replaced with fresh DMEM containing 20 % FBS and \[^{3}H\]oleic acid (250 \(\mu\)Ci/dish) and cultured for 24 hours. Cholesteryl ester was separated on HPTLC according to Pentchev et al. \(^{12}\) and the radioactivity was measured by a radio-scanner (Aloca, Radiochromanalyzer).

**Sialic acid assay.** For the quantitation of sialic acid, cell lysates were subjected to the thiobarbituric acid assay \(^{13}\) both before (=free sialic acid) and after acid hydrolysis of bound sialic acid (=total sialic acid).

**Complementation analysis.** Two slightly different procedures, both based on cell hybridization followed by filipin staining, were used in parallel as indicated. Method A closely followed the procedure described by Steinberg et al. \(^{14}\) Method B was similar to that described in Vanier et al. \(^{11}\) except that coculture was performed in 30-mm Petri dishes and that the Ficoll gradient step was omitted.

**Immunofluorescence staining of intracellular G_{M2} ganglioside and filipin-staining of intracellular cholesterol.** Intracellular cholesterol was stained with filipin (300 \(\mu\)g/ml) as described before. \(^{15}\) Intracellular G_{M2} was stained using monoclonal antibody to G_{M2} gangliosides. \(^{15}\) To examine the effects of LDL on G_{M2} ganglioside accumulation, cells were incubated in 10 % LPDS/DMEM or 10% FBS/DMEM. On the third day, intracellular accumulation of cholesterol and G_{M2} ganglioside was examined by staining with filipin and anti-G_{M2} antibody. Fluorescence of filipin-labeled cholesterol was observed under a fluorescent microscope (Olympus IX70 FLA) using U excitation (330–385 nm) and that of FITC-labeled G_{M2} gangliosides was observed using B excitation (450–480 nm).

**Lysosomal enzyme activities.** Acid sphingomyelinase activity in fibroblasts was measured using \[^{14}C\]sphingomyelin as a substrate. \(\beta\)-Galactosidase, total hexosaminidase, hexosaminidase A and neuraminidase activities were measured using 4-methylumbelliferyl (4MU)-\(\beta\)-D-galactopyranoside, 4-MU-2-acetamido-2-deoxy-\(\beta\)-D-glucopyranoside, 4MU-\(\beta\)-N-acetylglucosaminide-6-sulfate, and 4-MU-\(\alpha\)-D-N-acetyl neuraminic acid, respectively, as substrates. \(^{16}\)

**Incorporation of N-acetyl D-[\(^{3}H\)]mannosamine into gangliosides.** Cells were cultured for 72 hours in medium containing N-acetyl D-[\(^{3}H\)]mannosamine (100 \(\mu\)Ci/dish). Gangliosides were isolated as reported previously \(^{17}\) developed on HPTLC plates in a mixture of chloroform/methanol/0.02% \(\text{CaCl}_2\cdot2\text{H}_2\text{O}\) (55:45:10 v/v) and the radioactivities of each ganglioside identified by authentic standards were measured by a radio-scanner.

**Results.** Impairment of LDL-derived cholesterol metabolism. All NP-C cells in this study showed typical abnormalities in NP-C fibroblasts; an accumulation of intracellular cholesterol and a decreased cholesteryl \[^{3}H\]oleate formation (Fig. 1 and Table I). In one adult NP-C strain (KAI), accumulation of intracellular cholesterol was confirmed but was found to be very mild (Fig. 1), while cholesteryl \[^{3}H\]oleate formation was also intermediate between other NP-C cells and normal cells.

**Cellular sialic acid levels.** Total sialic acid levels were significantly increased in NP-C cells (Table II). In the childhood NP-C cells, the total sialic acid was increased 2 folds over the control level. This elevation was caused by an increase of the bound form. In the adult patient, however, both the free and bound sialic...
acids were only mildly increased. We have questioned whether the mild difference might be associated with the recently recognized genetic heterogeneity in NP-C. 11,14 Complementation analysis. As shown in Table III, UCH and YON as well as the GMO110A fibroblasts were clearly assigned to complementation group 1 (major group). Because cholesterol accumulation in the KAI strain was very mild, it was particularly difficult to differentiate filipin-positive and filipin-negative hybrids. By method A, the results did not exclude that the KAI strain might belong to a third complementation group. Repeated crosses using the method B, however, gave results in the non-complementing range in hybrids between KAI and 93059, suggesting that the KAI strain is actually belonging to complementation group 1 (Table III).

Table III. Complementation Analysis

<table>
<thead>
<tr>
<th>Cells</th>
<th>CG1 93059</th>
<th>CG2 82017</th>
<th>CG2 88057</th>
<th>GMO110A</th>
<th>UCH</th>
<th>YON</th>
<th>KAI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM0110A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCH</td>
<td>5^a</td>
<td></td>
<td>1^a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YON</td>
<td>70^b</td>
<td>25^b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>93059</td>
<td></td>
<td></td>
<td></td>
<td>22^a</td>
<td>11^a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>82017 juvenile</td>
<td>2^a</td>
<td>22^a</td>
<td>&lt;1^a</td>
<td>1^a</td>
<td>3^a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KAI adult</td>
<td>40^a</td>
<td>29^a</td>
<td>30^a</td>
<td>1^a</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Immunofluorescence staining of intracellular GM2 ganglioside. Then, we have investigated ganglioside metabolism to characterize the increased levels of bound sialic acid. First, we have studied histochemo-}

cial detection of intracellular GM2 using anti-GM2 ganglioside antibody. Intracellular GM2 staining revealed perinuclear granular fluorescent in Tay-Sachs cells as reported previously15 (Fig. 2A). Surprisingly, all childhood NP-C fibroblasts studied were stained with anti-GM2 antibody at the same levels of intensity and distribution as seen in Tay-Sachs fibroblasts (Fig. 2D). In the juvenile NP-C cells belonging to the second

![Fig. 1. Intracellular cholesterol revealed by filipin staining. Note that the accumulation in adult NP-C (KAI) cells (B) is milder than that in the childhood NP-C (YON) cells (A). Note also that normal cells (C) are not stained.](image)

Table I. Cholesteryl[3H]oleate formation in NP-C fibroblasts used in this study

<table>
<thead>
<tr>
<th>Case</th>
<th>Clinical type</th>
<th>dpm/mg protein/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM0110A</td>
<td>childhood</td>
<td>4140 ± 679^a</td>
</tr>
<tr>
<td>UCH</td>
<td>childhood</td>
<td>661 ± 182^a</td>
</tr>
<tr>
<td>YON</td>
<td>childhood</td>
<td>2080 ± 72^a</td>
</tr>
<tr>
<td>93059</td>
<td>childhood</td>
<td>323 ± 2^a</td>
</tr>
<tr>
<td>82017</td>
<td>juvenile</td>
<td>1160 ± 296^a</td>
</tr>
<tr>
<td>KAI</td>
<td>adult</td>
<td>21200 ± 2670^a</td>
</tr>
<tr>
<td>Controls</td>
<td>children</td>
<td>42200 ± 6200</td>
</tr>
</tbody>
</table>

Values are mean ± SD obtained from two independent dishes. ^a P<0.001 from the level of controls (Student’s t-test).

Table II. Sialic acid levels in cultured fibroblasts (nmol/mg protein)

<table>
<thead>
<tr>
<th>Case</th>
<th>Total</th>
<th>Bound</th>
<th>Free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>10.6 ± 0.6</td>
<td>8.9 ± 0.5</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>NP-C (children)</td>
<td>18.5 ± 1.7^a</td>
<td>16.8 ± 1.0^a</td>
<td>1.8 ± 0.8</td>
</tr>
<tr>
<td>YON</td>
<td>23.6 ± 3.8^a</td>
<td>21.7 ± 3.1^a</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>UCH</td>
<td>20.5 ± 1.1^a</td>
<td>19.4 ± 1.0^a</td>
<td>1.1 ± 0.1^a</td>
</tr>
<tr>
<td>GM0110A</td>
<td>13.4 ± 1.1^b</td>
<td>10.2 ± 0.2^b</td>
<td>3.2 ± 1.0^b</td>
</tr>
<tr>
<td>KAI</td>
<td>82.4 ± 6.0</td>
<td>10.8 ± 7.7</td>
<td>71.5 ± 14.0</td>
</tr>
<tr>
<td>I-cell disease</td>
<td>59.7 ± 7.3</td>
<td>56.7 ± 6.9</td>
<td>3.0 ± 0.4</td>
</tr>
</tbody>
</table>

Values are mean ± SD triplicated samples. ^a P<0.001, ^b P<0.01, ^c P<0.05 (Student’s t-test).
complementation group, an accumulation was milder than in childhood NP-C cells. (Fig. 2E). In cells from the adult NP-C patient, intracellular G\(_{M2}\) was not detected histochemically (Fig. 2F). In cells derived from patients with Niemann-Pick disease types A (Fig. 2C) and B, Gaucher disease type II, G\(_{M1}\) gangliosidosis, and Krabbe disease, we could not detect any accumulation of G\(_{M2}\) ganglioside. But in cells from I-cell disease, which have multiple lysosomal enzyme deficiencies, substantial G\(_{M2}\) ganglioside-positive granules were observed (data not shown).

Intracellular distribution of G\(_{M2}\) ganglioside follows the same pattern as that of cholesterol but the accumulation is not corrected in cells cultured in LPDS medium. In order to examine whether the G\(_{M2}\) ganglioside is accumulating in the same subcellular organelles as cholesterol and whether the G\(_{M2}\) ganglioside accumulation decreases when cells are cultured in LPDS/DMEM, double staining of both cholesterol and G\(_{M2}\) ganglioside was studied. The double staining revealed that distributions of intracellular cholesterol-positive granules and G\(_{M2}\) ganglioside-positive granules showed almost the same pattern (Figs. 3A and 3B), suggesting that the accumulation is mainly in lysosomes.\(^{18}\) In some cells with cholesterol-positive granules, the G\(_{M2}\) ganglioside-positive granules were not seen. In cells cultured in 10% LPDS/DMEM for 3 days, the cholesterol-positive granules disappeared (Fig. 3D), but the G\(_{M2}\) ganglioside-positive granules were still substantially present (Fig. 3E).

Activities of lysosomal enzymes. Mild to moderate reductions in acid sphingomyelinase activities were found in NP-C fibroblasts as described previously.\(^{2}\) The other lysosomal enzymes, known to hydrolyze G\(_{M1}\) (\(\beta\)-galactosidase) and G\(_{M2}\) (hexosaminidase A) were not deficient (Table IV). Normal hexosaminidase A activity, in combination with the sustained accumulation of G\(_{M2}\) ganglioside in cells with diminished cholesterol accumulation, suggests that the accumulation of G\(_{M2}\) ganglioside does not arise from a secondary deficiency of lysosomal hydrolases caused by the storage of excess cholesterol.

Incorporation of N-acetyl D-[\(^3\)H]mannosamine into ganglioside fractions. Cells were labeled with N-acetyl D-[\(^3\)H]mannosamine for 72 hours and labeled gangliosides were separated on HPTLC. The labeling patterns in normal cultured fibroblasts for 72 h were
similar to those described by Fishman et al. 17) In NP-C fibroblasts, incorporation of total ganglioside fractions was 1.8- to 2.5-fold higher than that in normal fibroblasts, except for the adult NP-C strain, KAI (Table V). The radioactivities in GM2 ganglioside fractions in three young NP-C fibroblasts were 2.9- to 7-fold higher than controls and those in GM3 ganglioside fractions 3- to 4-fold higher (Table V). When the percentages of each radiolabeled fraction were compared (normal 6.8±2.5%), three NP-C fibroblasts had high percentages of GM2 gangliosides: 93017 10.5±3.1%, YON 23.6±8.2% (P<0.05, Student t-test) and 82017 22.0±1.2% (P<0.01). The incorporation study also revealed that the metabolism of GM2 ganglioside is abnormal in NP-C fibroblasts and that GM3 ganglioside may also participate in the increase of bound sialic acid.

Discussion. In the present study we have shown that in addition to abnormalities in intracellular transport of lipoprotein-derived cholesterol metabolism, NP-C fibroblasts have an abnormal ganglioside metabolism. Abnormalities consist of an intracellular accumulation of G<sub>M2</sub> ganglioside and of an increased incorporation of a precursor into G<sub>M2</sub> and G<sub>M3</sub> gangliosides. Because the accumulation was not corrected in cells cultured in lipoprotein-deficient medium, the accumulation of G<sub>M2</sub> ganglioside did not appear to be caused by the excess lysosomal accumulation of unesterified cholesterol. It is also unlikely that the G<sub>M2</sub> ganglioside accumulation is due to a deficiency of ganglioside hydrolases. An increased uptake of the radiolabeled precursor into G<sub>M3</sub> fractions was another observation of this study. It is thus possible that both G<sub>M2</sub> and G<sub>M3</sub> gangliosides are accumulating in NP-C fibroblasts.

Genetic heterogeneity has also recently been described: although a gene localized on human chromosome 18q11-1220,21) is assigned in a majority of NP-C patients, a second minor complementation group is known.10,15) In this study we have shown that NP-C fibroblasts belonging to both complementation groups exhibit a ganglioside accumulation. This is not totally surprising, since no biochemical abnormality studied so far has permitted differentiation of the two complementation groups. On the other hand, a considerable variation in abnormal regulation of cholesterol homeostasis has been observed within both groups.11)

Interestingly, in our study, one adult NP-C cell strain showed only a mild increase of cellular sialic acid levels, with neither accumulation of lysosomal G<sub>M2</sub> nor increased incorporation of the precursor. In this particular case, the accumulation of cholesterol and defective LDL-induced cholesteryl oleate formation were very mild, corresponding to what has also be described as the “variant” phenotype. The clinical features including the age of onset and the course of the disease were milder than other patients. Among the few cell lines studied, a good correlation between the degree of ganglioside accumulation and of block in cholesterol transport was observed, and it is possible that the accumulation of G<sub>M2</sub> ganglioside is also closely related to the primary NP-C defect.

Because glycosphingolipids are important components of the plasma membrane, as well as phospholipids and cholesterol, their metabolism and intracellular transport have been believed to be maintained under a stringent control.22) Gangliosides are synthesized in the endoplasmic reticulum (ER) and transported from ER through the Golgi cisternae to the plasma membrane, presumably by a vesicle-bound exocytotic membrane flow, and the gangliosides on the
plasma membrane are transported to the lysosomes by an endocytotic membrane flow along the early and late endosomal reticulum. The nature and the regulation of vesicular systems involved in the intracellular traffic of gangliosides are not well-understood. The several inhibitors of intracellular cholesterol movement, which produce the same biochemical abnormalities as the NP-C, have been shown to inhibit the movement of LDL-derived cholesterol from lysosomes to plasma membranes and the movement of plasma membrane cholesterol to the cell interior. It is postulated that a molecule defective in the NP-C cells is participating in intracellular transport of both cholesterol and gangliosides.

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References


