Metabolism of Sphingomyelin in Cultured Skin Fibroblasts from Patients with Different Types of Niemann-Pick Disease

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NAKASHIMA, M., KUDOH, T., SUKEGAWA, K., MARUYAMA, K. and ORII, T. Metabolism of [choline-methyl-14C] sphingomyelin in cultured skin fibroblasts from patients with different types of Niemann-Pick disease was measured 1 and 3 days after uptake from the media. The cell lines obtained from type A disease had more than 95% unhydrolyzed sphingomyelin in situ on day 3 while two cell lines obtained from type B had 36.3% and 43.3% unhydrolyzed sphingomyelin on day 3. The cell line derived from one patient with the transitory type disease had 48.1% unhydrolyzed sphingomyelin on day 3, and there was no significant difference in the sphingomyelinase activity measured in vitro or in degradation of sphingomyelin in situ between the type B and transitory type disease. In three cell lines from patients with type C disease, there was 18.5%, 29.6% and 31.1% unhydrolyzed sphingomyelin on day 3, which indicates that this type has a decreased ability to metabolize sphingomyelin. Cell from type E disease metabolized sphingomyelin normally.

Niemann-Pick disease refers to a group of genetic diseases in which sphingomyelin is stored in certain tissues. The disease is classified into several clinical types (Brady 1983; Elleder and Jirasek 1983). In types A and B of this disease, the specific activity of sphingomyelinase is markedly reduced in cultured skin fibroblasts, whereas in types C, D and E it is characteristically near normal.

The in vitro measurement of lysosomal enzyme activities is not a good predictor of the clinical course (Porter et al. 1971; Kudoh and Wenger 1982). Thus, the study of the uptake and metabolism of radiolabeled substrates by cultured skin fibroblasts has been suggested to more closely reflect the in vivo metabolism of such substrates (Kudoh et al. 1981; Kudoh and Wenger 1982).
Recently, the uptake and metabolism of radiolabeled sphingomyelin by fibroblasts from patients with Niemann-Pick disease types A, B, C and D have been examined (Beaudet and Manschreck 1982; Maziere et al. 1982; Kudoh et al. 1983; Vanier et al. 1985). No such studies have been reported for type E or the transitory type, and in type C the results are conflicting.

This study was designed to investigate the uptake and metabolism of [choline-methyl-14C] sphingomyelin by cultured skin fibroblasts from patients with A, B, C, E and transitory type Niemann-Pick disease.

**Materials and Methods**

**Materials**

[Choline-methyl-14C] sphingomyelin (45 mCi/mmol) was purchased from New England Nuclear (Boston, MA, USA). Sphingomyelin (bovine brain) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Thin layer chromatography plates (Silica gel 60) were obtained from Merck (Darmstadt, F.R.G.). Silicic acid (Wako-Gel, 100–200 mesh) and other chemicals were purchased from Wako Pure Chemical Industries (Tokyo). Eagle’s minimum essential medium (Cat. No. 410–1500) was obtained from Gibco Laboratories (Grand Island, NY). Fetal calf serum was purchased from M.A. Products (Walkersville, MI, USA). Penicillin and streptomycin were obtained from Meiji Seika Kaisha (Tokyo). X-ray film (Kodak X-Omat AR film) was purchased from Eastmann Kodak Co. (Rochester, NY, USA).

**Preparation of substrate**

[Choline-methyl-14C] sphingomyelin was diluted with unlabeled sphingomyelin and repurified by silicic acid column chromatography with increasing concentrations of methanol in chloroform. The radiopurity was 99.0% and the specific radioactivity was adjusted to 3.0 mCi/mmol.

**Cell lines**

Cell lines of Niemann-Pick disease type A (GM 112), type B (GM 3252) and type C (GM 3123 and GM 110) were obtained from Human Genetic Mutant Repository (Camden, NJ). The cell line of type C (594N) was obtained from Laboratoire de Culture Cellulaire, Hospital Debrousse (France). The cell line from a patient with the transitory type disease was provided by Dr. Takada (Akita University School of Medicine, Department of Pediatrics). When this patient was 1 year old, hepatosplenomegaly was noticed. She was admitted for investigation at 1 year and 3 months of age. She had hepatosplenomegaly and cherry-red spots at the optic fundi. An ultrastructural examination of the rectal mucosa revealed small membranous cytoplasmic bodies in Schwann cells. Neurological findings were normal at age 2 years and 5 months. Control fibroblasts and cell lines from patients with types A, B and E and with I cell disease were established in our laboratory. Cell lines of types B and E were described as Cases 2 and 3, respectively, by Minami et al. (1979), but skin biopsy was repeated for this study. All cultures were used before their tenth passage except transitory type (14 passage) and I cell disease (12 passage). They were grown in 25 cm² flasks containing 4 ml Eagle’s minimum essential medium supplemented with 10% fetal calf serum, penicillin (10 units/ml), and streptomycin (1.0 mg/ml).

**In vitro enzyme assay**

Confluent cells were harvested with a rubber policeman after washing three times with 0.9% NaCl. The cell pellet was homogenized in distilled water, and the total homogenate was assayed for enzymatic activity. As a control, lysosomal β-hexosaminidase activity was...
measured using 4-methyl-umbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Koch-Light Laboratories, England) as described previously (Orii et al. 1972). Sphingomyelinase activity was determined using [choline-methyl-14C] sphingomyelin (Wenger et al. 1975). The activities were expressed as nmol substrate hydrolyzed/mg protein per hour. Protein was determined according to the method of Lowry et al. (1951).

Uptake and metabolism studies in cultured cells

[choline-methyl-14C] sphingomyelin was suspended in the cultured medium at a final concentration of 10 nmol/ml culture medium (Kudoh et al. 1983). After the addition of 4 ml of radio labeled media to a confluent flask, the cells were incubated at the 37°C for 1 to 3 days.

The amount of sphingomyelin derived from fetal calf serum used in this experiment was 20 nmol in 4 ml of media. The specific radioactivity was calculated using the dilution method. Cell harvest and subsequent processing are described elsewhere (Kudoh et al. 1983). After the extraction of lipids, thin layer chromatography and autoradiography were carried out. The radioactive regions, sphingomyelin and phosphatidylcholine, were scraped from the plate and counted by a liquid scintillation counter. The metabolism was expressed as a percentage of unhydrolyzed sphingomyelin.

**Results**

In vitro enzyme activities

As shown in Table 1, patients with type A, type B and transitory type Niemann-Pick disease had markedly reduced sphingomyelinase activity. Residual sphingomyelinase activity in cells from type B was definitely higher than that in type A. Sphingomyelinase activity in the transitory type was in the range of type B. The cell lines obtained from patients with type C had partially reduced

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Sphingomyelinase (nmol/mg protein/hr)</th>
<th>Hexosaminidase (nmol/mg protein/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=8)</td>
<td>85.9±18.0</td>
<td>1505±673</td>
</tr>
<tr>
<td>Niemann-Pick disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.67</td>
<td>1254</td>
</tr>
<tr>
<td>A (GM 112)</td>
<td>0.90</td>
<td>1247</td>
</tr>
<tr>
<td>B</td>
<td>4.95</td>
<td>1524</td>
</tr>
<tr>
<td>B (GM 3252)</td>
<td>3.80</td>
<td>1135</td>
</tr>
<tr>
<td>Transitory</td>
<td>4.07</td>
<td>955</td>
</tr>
<tr>
<td>C (GM 3123)</td>
<td>56.3</td>
<td>1359</td>
</tr>
<tr>
<td>C (GM 110)</td>
<td>23.6</td>
<td>1996</td>
</tr>
<tr>
<td>C (594N)</td>
<td>37.6</td>
<td>1303</td>
</tr>
<tr>
<td>E</td>
<td>90.4</td>
<td>1448</td>
</tr>
<tr>
<td>I cell disease</td>
<td>7.30</td>
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</tr>
</tbody>
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sphingomyelinase activity, ranging from 27% to 65% of control activity. The cell line obtained from a patient with type E had normal sphingomyelinase activity.

Uptake into and metabolism of [choline-methyl-\(^{14}\text{C}\)] sphingomyelin by cultured cells

The mean uptake on day 3 in controls was calculated to be 13.4 mmol/mg protein, not significantly different from the levels in the patients.

The metabolism of [choline-methyl-\(^{14}\text{C}\)] sphingomyelin on day 3 was analyzed by thin layer chromatography (Fig. 1). The radioactive metabolite was phosphatidylcholine. In cells from controls, the rate of unhydrolyzed sphingomyelin was only 13.3% (Fig. 2a). In contrast, cells from a patient with \(\text{I}\) cell disease, as a negative control, showed significantly decreased ability to metabolize
sphingomyelin (Fig. 2b), the percent unhydrolyzed sphingomyelin being 42.8% on day 3. Cells from patients with Niemann-Pick disease type A showed an even greater reduction of \([\text{choline-methyl-14C}]\text{sphingomyelin}\) hydrolysis (95%). Two cell lines from patients with type B showed a greater rate of \([\text{choline-methyl-14C}]\text{sphingomyelin}\) hydrolysis compared to type A; the rates of sphingomyelin unhydrolyzed were 36.3% and 43.3% on day 3.

There was no difference in the ability to metabolize \([\text{choline-methyl-14C}]\text{sphingomyelin}\) between cells obtained from patients with type B and those from a patient with the transitory type (Fig. 2a and b). This indicates that this case is classified into type B.

Fig. 2b shows a small decrease in the ability to degrade \([\text{choline-methyl-14C}]\text{sphingomyelin}\) in three type C cell lines. In cell lines GM 3123, GM 110 and 594N the rates of sphingomyelin unhydrolyzed were 18.5%, 31.1% and 29.6% by day 3. The cell line obtained from a patient with type E disease metabolized sphingomyelin normally (Fig. 2b).

**DISCUSSION**

Both patients with type A and type B disease exhibited a marked reduction of in vitro sphingomyelinase activity; the cell lines obtained from patients with type A disease had more than 95% unhydrolyzed sphingomyelin on day 3, but those obtained from patients with type B disease had about 40% unhydrolyzed sphingomyelin on day 3 in situ. These results are in agreement with those of
others (Beaudet and Manschreck 1982; Kudoh et al. 1983; Vanier et al. 1985). The in vitro sphingomyelinase activity and in situ capacity of sphingomyelin degradation in the cells obtained from the patient with the transitory type disease were similar to those of type B.

The results of studies on the metabolism of [choline-methyl-\(^{14}\text{C}\)]sphingomyelin in cultured skin fibroblasts obtained from patients with the type C Niemann-Pick disease are conflicting. Beaudet and Manschreck (1982) reported normal hydrolysis of sphingomyelin in a preliminary study. Maziere et al. (1982) noted a slight but significant decrease of sphingomyelin hydrolysis. Two of the cell lines with type C used in our study are the same as those of Maziere et al. (1982). We found a significant decrease of sphingomyelin hydrolysis in situ in those two patients.

Kudoh et al. (1983) reported a clear decrease in sphingomyelin hydrolysis in two type C patients, and normal hydrolysis in a patient with juvenile Niemann-Pick disease by using [stearoyl-1-\(^{14}\text{C}\)]sphingomyelin as a substrate. Vanier et al. (1985) reported an abnormally low rate of sphingomyelin degradation in 16 patients and normal hydrolysis in 4 patients with type C disease. Thus, this type appears to be a heterogeneous group and can be divided into at least two subgroups.

Christomanou (1980) proposed the possible defect in an activator protein required for in vivo sphingomyelin degradation in the type C disorder. In our study, a cell line with type C (GM 3123) was confirmed to have subnormal activity of sphingomyelinase in vitro and inability to hydrolyze labeled sphingomyelin in situ. Under the in vitro condition Triton x-100 was considered to be an activator. We added a heat-treated extracts from Gaucher spleen in the culture media of type C (GM 3123) with labeled sphingomyelin. No correction was found in sphingomyelin metabolism (unpublished data). To elucidate this problem, the studies on biosynthesis, intracellular transport, and processing of the enzyme should be done.

Cells obtained from the patient with type E disease had normal sphingomyelin metabolism, as well as normal sphingomyelinase activity in vitro. Further study is necessary to classify this disorder.

Differential diagnosis of different types of Niemann-Pick disease may be made biochemically at the earlier stage of the disease and more precisely by measuring sphingomyelinase activity in vitro and metabolism of [choline-methyl-\(^{14}\text{C}\)] sphingomyelin in cultured skin fibroblasts at the same time.

Acknowledgments

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A Study on Niemann-Pick Disease

References


