A Cell Line Derived from Sphingomyelinosis Mouse Shows Alterations in Intracellular Cholesterol Metabolism Similar to Those in Type C Niemann-Pick Disease

Kousaku Ohno, Eiji Nanba, Shigeki Miyawaki, Takeshi Sakiyama, Teruo Kitagawa, and Kenzo Takeshita

Division of Child Neurology, Institute of Neurological Sciences, Tottori University School of Medicine, Yonago 683, Research Laboratories, Nippon Shinyaku Co. Ltd., Kyoto 607, Department of Pediatrics, Nihon University School of Medicine, Tokyo 101, Japan

Key words: sphingomyelinosis/Niemann-Pick disease/cholesterol/cholesterol transport

ABSTRACT. Cell lines derived from the sphingomyelinosis (gene symbol, spm) mouse were established from homozygous (spm/spm) and heterozygous (spm/+ ) embryos according to a rigid 3T3 transfer schedule. The SPM-3T3 cells derived from a homozygous embryo showed extensive accumulation of intracellular cholesterol, attenuated esterification of exogenously added cholesterol and increased de novo cholesterol synthesis, when compared to SPMH-3T3 cells derived from a heterozygous embryo. The phenotypic abnormalities were very similar to those observed in fibroblasts from patients with Niemann-Pick disease type C (NP-C), in which a defect in the intracellular transport of unesterified cholesterol is suggested. The genetic defect in SPM-3T3 cells should be closely related to that in NP-C. The SPM-3T3 cell line is useful for biochemical and genetic studies on the regulation of intracellular cholesterol metabolism.

The sphingomyelinosis mouse (gene symbol, spm) is an animal model of Niemann-Pick disease, an autosomal recessive condition resulting in neurovisceral accumulation of unesterified cholesterol and sphingomyelin, and a partial deficiency of sphingomyelinase (13, 18). The mouse has been considered to be a model of Niemann-Pick disease types A or C from the clinical features, lipid analysis results and sphingomyelinase activity in tissues (14). Recently, it was reported that primary cultured fibroblasts from the mouse also show substantial accumulation of intracellular cholesterol and defective esterification of exogenously added cholesterol, mimicking Niemann-Pick disease type C (NP-C) (24). NP-C is an autosomal recessive disorder associated with the accumulation of sphingomyelin, cholesterol and glycolphospholipids. In contrast to Niemann-Pick disease types A and B, which are characterized by a severe deficiency of lysosomal acid sphingomyelinase activity, the activity in NP-C cells is normal or reduced to as much as 80% of the control level (for a review see ref. 22). The basic defect remains undetermined. Pentchev and colleagues (16, 17) demonstrated abnormal accumulation of intracellular cholesterol and decreased esterification of exogenously derived cholesterol in NP-C fibroblasts (but not in fibroblasts from cases of Niemann-Pick disease type A or B). A defect in the intracellular transport of unesterified cholesterol has been suggested (9, 20, 21). The sphingomyelinosis mouse, having phenotypically very similar abnormalities to those in NP-C, is especially advantageous not only for biochemical and genetic studies on cholesterol metabolism, but also for therapeutic trials for the human disease.

Cells grown in tissue culture have proven to be more useful for the study of lipid metabolism because of the availability of different nutritional conditions with media of definite compositions (2, 5). An immortalized cell line derived from the sphingomyelinosis mouse should be useful for biochemical and genetic studies on mutation producing such abnormal intracellular cholesterol metabolism. Cholesterol is an important membrane component closely related with membrane functions, and cholesterol homeostasis is changed by the cell growth state (12) and by neoplastic transformation of cells (1, 6, 25). Immortalized cells that have proliferative phenotypes similar to those of untransformed cells are especially ideal for studies on cell cholesterol metabolism. We have attempted to establish cell lines with "normal" properties as to their proliferative phenotypes by means of a 3T3 transfer schedule (23). We describe

---

1 To whom correspondence should be addressed.

Abbreviations: NP-C, Niemann-Pick disease type C; SPM, sphingomyelinosis; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum.
herein the initial characterization of the 3T3 cell lines established from sphingomyelinosis mouse embryos.

MATERIALS AND METHODS

Mice. A colony of CS57BL/KsJ mice carrying the mutant allele, spm, was maintained at the Research Laboratories of Nippon Shinyaku Co., Ltd., Yamashina, Kyoto. For experimental use, affected and heterozygous normal mouse fetuses were produced according to the following procedures. Ovaries of affected females derived from the sphingomyelinosis mouse (spm/spm) were transplanted into (C57BL/KsJ × CBA/J)F1 females, which were mated with known heterozygous normal males (spm/+). Eighteen 14- to 16-day-old mouse embryos from two pregnant mice were used for the experiments.

3T3 transfer of cultured cells from sphingomyelinosis mouse embryos. Independent cultures of cells from each embryo were prepared by fine mincing of the embryo after removal of the brain, followed by disaggregation with 0.05% trypsin in phosphate-buffered saline (PBS) containing 0.54 mM ethylenediaminetetraacetic acid (EDTA). The cells from each embryo were cultured in five 6cm petri dishes in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1% glucose and 0.37% NaHCO3, supplemented with 500 IU/ml penicillin G, 100 µg/ml streptomycin sulfate and 10% FBS, under a humidified atmosphere of 10% CO2-90% air. On the 10th culture day, spm/spm or spm/+ cells were differentiated by filipin staining [8, 16]. Cells from four spm/spm and two spm/+ cultures were dispersed with a 0.05% trypsin solution, inoculated into forty 6 cm petri dishes at a cell density of 3 x 10⁵ per dish and then subcultured according to a 3T3 transfer schedule (23) for more than 90 days. Cells were counted with a hemocytometer. After the attainment of stationary proliferation, colonies were picked up with a stainless steel ring. Human skin fibroblasts from patients with type A (GM0370 and GM0112) and type C (GM0110A and GM3123) Niemann-Pick disease and from apparently normal individuals (GM5659 and GM5756) were obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, NJ, USA) and maintained under the same culture conditions. The cells were negative for Mycoplasma infection, as revealed by fluorescent 32528 Hoechst staining (3).

Filipin staining. To detect intracellular unesterified cholesterol, cells were stained with filipin [8, 16]. Briefly, cells cultured in plastic petri dishes were fixed in formaldehyde (10% in PBS) for 10 min at room temperature, incubated in Nonidet P-40 (0.05% in PBS) for 10 min and then stained with 200 µg/ml filipin (Sigma, St. Louis, MS) in distilled water (stock solution, 10 mg/ml dimethyl sulfoxide) for 30 min. The stained monolayer was mounted in PBS and then observed under a fluorescent microscope (Olympus, model BH-RFL-LB, Tokyo).

Lysosomal enzyme assay. Subconfluent cells in a 90 cm dish were harvested with a rubber policeman. The cell pellet, after being washed in PBS twice by centrifugation, was suspended in about 1 ml of distilled water and then sonicated for 10–15 sec on ice. The lysate was used as the enzyme solution and for protein determination. Protein was determined by Lowry’s method (10). The activities of α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, total hexosaminidase, α-fucosidase and α-mannosidase were fluorometrically assayed, and that of acid sphingomyelinase was assayed using 14C-sphingomyelin as a substrate (19).

Chromosomal analysis. Exponentially proliferating cells were cultured in medium containing 0.1 µg/ml colcemid for 2–3 h, then harvested by trypsinization and resuspended in 75 mM KCl for 15 min at 37°C. The cells were then fixed twice with methanol-acetic acid (3 : 1, v/v), and a flame-dried preparation was prepared on a glass slide. The cells fixed on slides were stained with Giemsa, and more than 100 metaphases were examined microscopically as to the chromosome number.

Esterification of exogenously added cholesterol. Esterification of [3H]cholesterol added as a quasi-soluble emulsion in

Fig. 1. Fluorescence photomicrographs (upper) after staining with filipin for intracellular unesterified cholesterol in primary cultures from spm/spm (a) and spm/+ (b) embryos, and in established SPM-3T3 (spm/spm) (c) and SPMH-3T3 (spm/+)(d) cultures. Respective phase photomicrographs (lower) of the same microscopic fields.
medium was measured according to Pentchev et al. (16), with minor modifications. Sparsely proliferating cells were dispersed in a 0.25% trypsin-EDTA solution and then inoculated at a cell density of $2 \times 10^4$ per 6 cm dish in 5 ml of DMEM containing 10% FBS. On the 3rd day the medium was replaced by 3 ml of fresh DMEM containing 10% FBS and 50 μg of $[^{3}H]$cholesterol (50 dpm/μmol., New England Nuclear, Boston, U.S.A.) dissolved in 5 μl of ethanol. Cultures were labelled for 48 h and then the cells were harvested into screw-capped conical glass tubes by trypsinization. Cellular lipids were extracted with chloroform-methanol (2:1) (4) and then separated on a precoated silica gel HPTLC plate (Merck, Darmstadt, Germany) in a solvent system of hexane-ethyl ether-acetic acid (85:15:0.8). The spots of cholesterol and cholesteryl ester, identified in comparison with authentic standards, were scraped off and the radioactivities were measured by liquid scintillation counting.

Sterol synthesis from $[^{3}H]$sodium acetate. Cells were inoculated into 12 dishes at the same density as above. On the 3rd day the medium was replaced with 3 ml of DMEM containing 10% FBS and 15 μCi of $[^{3}H]$acetate sodium salt (87 μCi/mmol., 10 μCi/ml., New England Nuclear, Boston, U.S.A.) and then the cultures were labelled for 48 h. To determine the incorporation of the precursor into free cholesterol and the cholesteryl moiety of cholesteryl ester, cells in 4 dishes were harvested into one conical tube, and then the cellular lipids were extracted and separated by HPTLC in triplicate as above. The area corresponding to the authentic standard of cholesterol was scraped and the radioactivity was counted. The area identified as corresponding to the cholesteryl oleate was scraped into a conical glass tube and then saponified in 5 ml of 2N KOH-methanol at 90°C for 1 h. Nonsaponifiable lipids were extracted into petroleum ether and then separated by HPTLC again. The area corresponding to cholesterol was scraped and the radioactivity was counted as the cholesterol moiety of cholesteryl ester.

RESULTS

Establishment of 3T3 cell lines. Because primary skin fibroblasts from homozygous (spm/spm) mice have been reported to show accumulation of intracellular cholesterol [24], we have tried to identify homozygous (spm/spm) and heterozygous (spm/+) embryo cultures through detection of the accumulation of intracellular cholesterol on the 10th day of primary culture. Eighteen primary cultures derived from each embryo could be clearly classified into the two types by filipin staining. One type showed extensive intracellular accumulation of filipin reactive materials, whereas the other accumulated almost no such materials (Fig. 1a, b). The former type of culture was considered to be homozygous (spm/spm) and the latter heterozygous (spm/+). Among the 18 embryos, there were 5 homozygous and 13 heterozygous cultures. Four of the 5 homozygous cultures and two of the 13 heterozygous cultures were then maintained with a rigid 3T3 transfer schedule according to Todaro and Green (23). As shown in Figure 2, the growth rate decreased progressively during four successive transfers. Cultures derived from homozygous embryos showed more rapid decreases than those from heterozygous embryos. During the 5th and 10th transfers the growth rates of the cultures increased progressively. Then, most cells entered a constant growth phase with some fluctuation. After being maintained on the 3T3 transfer schedule for 100 days, clones derived from a single cell colony were isolated from a homozygous culture and a heterozygous culture with higher growth rates.

General characteristics of the established cell lines. Clones derived from a homozygous embryo were designated as SPM-3T3 and those from a heterozygous embryo as SPMH-3T3. The isolated clones showed patterns of intracellular accumulation of cholesterol similar to those seen in the primary cultures (Fig. 1c, d). The morphology of sparsely proliferating cells was fibroblastic. Both SPM-3T3 and SPMH-3T3 cells exhibited serum-dependent growth (Fig. 3), with population doubling times of 17 to 20 hours, in medium containing 10% FBS. In confluent cultures both SPM-3T3 and SPMH-3T3 cells yielded a typical cobblestone-like monolayer. SPM-3T3 clone 1 and SPMH-3T3 clone 3 were chosen as standard clones. Hereafter, SPM-3T3 clone 1 and SPMH-3T3 clone 3 are referred to as SPM-3T3 and SPMH-3T3, respectively. The modal chromosome numbers in SPM-3T3 and SPMH-3T3 cells were near tetraploidy (Fig. 4).

Lysosomal enzyme activities. The sphingomyelinase activity in sparsely proliferating SPM-3T3 cells in DMEM containing 10% FBS was 4.2% that of SPMH-3T3 cells (Table I). When sphingomyelinase activity was determined in various tissues from 8-week-old animals,
Fig. 3. Growth curves for each cell line in DMEM containing 10% (○, ●) 5% (□, ■) and 2% (△, ▲) fetal bovine serum. (a) and (b) are SPM-3T3 clones 1 and 4, respectively, and (c) and (d) SPMH-3T3 clones 3 and 5, respectively. Each cell line was inoculated at a density of 2 × 10^4/cm dish in DMEM containing 2%, 5% or 10% FBS, and the medium was replaced by DMEM containing 2%, 5% or 10% FBS at 24 h after cell seeding. The cell number per dish was determined with a hemocytometer at the times indicated.

the activities in the liver, spleen and brain of spm/spm mice were found to be 30%, 50% and 70-80% of normal (+/+ mouse), respectively, and those of heterozygous (spm/+ mouse) were almost the same as those of normal (+/+ mouse). The reduction of sphingomyelinase activity in SPM-3T3 cells seems to be severe when compared to the activity in various tissues. Similarly,

Fig. 4. Numbers of chromosomes in SPM-3T3 clone 1 and SPMH-3T3 clone 3.

the reduced activity of sphingomyelinase in Niemann-Pick disease type C cells, at 38% of the normal level, was more marked than previously reported (normal or reduced by as much as 80%) (22). The marked reduction of sphingomyelinase activities in both SPM-3T3 cells and Niemann-Pick type C cells may be caused by our assay conditions with sparsely proliferating cells as an enzyme source. The activities of other lysosomal hydrolases in SPM-3T3 cells were almost the same as those in SPMH-3T3 cells (Table I).

Defective cholesterol metabolism in a SPM cell line.

To determine whether esterification of exogenously added cholesterol in SPM-3T3 cells is defective, as ob-

### Table 1. Lysosomal enzyme activities in SPM-3T3 cells and fibroblasts from patients with types A and C Niemann-Pick disease.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>SPM-3T3</th>
<th>SPMH-3T3</th>
<th>NP-A (n=2)</th>
<th>NP-C (n=2)</th>
<th>Normal* (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sphingomyelinase</td>
<td>1.32</td>
<td>13.1</td>
<td>0.71</td>
<td>9.62</td>
<td>25.5</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>95.1</td>
<td>125.8</td>
<td>321.4</td>
<td>384.9</td>
<td>366.9</td>
</tr>
<tr>
<td>α-galactosidase</td>
<td>119.4</td>
<td>56.1</td>
<td>37.1</td>
<td>56.1</td>
<td>45.5</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>10.5</td>
<td>28.2</td>
<td>44.5</td>
<td>18.2</td>
<td>40.8</td>
</tr>
<tr>
<td>β-hexosaminidase</td>
<td>2313</td>
<td>1471</td>
<td>2984</td>
<td>3380</td>
<td>3005</td>
</tr>
</tbody>
</table>

1) The activities are expressed as nmole/mg protein/h.
2) Cell strains used, GM0370 and GM0112.
3) Cell strains used, GM0110A and GM3123.
4) Cell strains used, GM5659 and GM5756.
Abnormal Cholesterol Metabolism in SPM-3T3 Cell Line

Table II. Incorporation of [3H]cholesterol into cholesterol and cholesteryl ester in SPM-3T3 cells and fibroblasts from a patient with type C Niemann-Pick disease.

<table>
<thead>
<tr>
<th>Cells (Status)</th>
<th>Free cholesterol</th>
<th>Cholesteryl ester</th>
<th>Ester/Free x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPM-3T3 (spm/spm)</td>
<td>42.7</td>
<td>0.44</td>
<td>1.0</td>
</tr>
<tr>
<td>SPMH-3T3 (spm/+ )</td>
<td>26.2</td>
<td>1.73</td>
<td>6.6</td>
</tr>
<tr>
<td>GM0123 (NP-C)</td>
<td>103.8</td>
<td>0.63</td>
<td>0.6</td>
</tr>
<tr>
<td>GM5756 (Normal)</td>
<td>156.9</td>
<td>14.55</td>
<td>9.2</td>
</tr>
</tbody>
</table>

1) nmole/mg protein/48 h; values are means for triplicate samples.

Table III. Incorporation of [3H]acetate into cholesterol and the cholesterol moiety of cholesteryl ester in SPM-3T3 cells and fibroblasts from patients with type C Niemann-Pick disease.

<table>
<thead>
<tr>
<th>Cells (Status)</th>
<th>Free cholesterol</th>
<th>Cholesteryl ester</th>
<th>Ester/Free x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPM-3T3 (spm/spm)</td>
<td>472.4</td>
<td>0.66</td>
<td>0.14</td>
</tr>
<tr>
<td>SPMH-3T3 (spm/+ )</td>
<td>65.7</td>
<td>0.64</td>
<td>0.97</td>
</tr>
<tr>
<td>GM0123 (NP-C)</td>
<td>217.0</td>
<td>2.79</td>
<td>1.28</td>
</tr>
<tr>
<td>GM5756 (Normal)</td>
<td>61.5</td>
<td>1.03</td>
<td>1.67</td>
</tr>
</tbody>
</table>

3) dpm x 10^{-2}/mg protein/48 h, values are means for triplicate samples.

 observed in NP-C fibroblasts (16), sparsely proliferating cells were labelled with [3H]-cholesterol for 48 hr in DMEM containing 10% FBS. Cholesterol and cholesteryl ester, identified using authentic standards, were scrapped off and their radioactivities were counted. The amount of esterified cholesterol derived from exogenously added cholesterol and the ratio of esterified to free cholesterol in SPM-3T3 cells were about 1/4 and 1/6 of those in SPMH-3T3 cells, respectively (Table II). The decrease in the cholesteryl ester formation in SPM-3T3 cells compared with that in SPMH-3T3 cells seems less than the decrease of cholesteryl ester formation in NP-C cells compared with that in normal cells. The difference may be due to the fact that SPMH-3T3 cells are derived from a heterozygous (spm/) embryo. The de novo cholesterol synthesis from a dicarbon precursor is also of interest. The incorporation of [3H]acetate into cholesterol and the cholesterol moiety of cholesteryl ester was measured in sparsely proliferating cultures (Table III). Incorporation of [3H]acetate into free cholesterol in SPM-3T3 cells was sixfold higher than that in SPMH-3T3 cells. Increased incorporation of [3H]acetate into free cholesterol was also observed in NP-C cells. The poor esterification of exogenously added cholesterol and the increased de novo cholesterol in SPM-3T3 cells, bear a striking resemblance to those in NP-C fibroblasts.

**DISCUSSION**

The SPM-3T3 cell line, derived from a homozygous mouse embryo (spm/spm), and the SPMH-3T3 line, derived from a heterozygous mouse embryo (spm/+) were established. Both lines show near tetraploid chromosome numbers, but it is not clear whether a normal allele (+) in the SPMH-3T3 cells (spm/) becomes physically and functionally homozygous (spm/spm/+/+, or spm/+/+), making explanation of the data quite complex. However, even in comparison with SPMH-3T3 cells, the SPM-3T3 cells showed abnormalities observed in tissues of the sphingomyelinosis mouse (13, 14, 18) and primary cultured fibroblasts (24); a reduction in acid sphingomyelinase activity, accumulation of intracellular unesterified cholesterol and a decrease in the esterification of exogenously added cholesterol. Since cloning the SPM-3T3 cell line, we have continued to culture the cells for more than 1 year without a change in the phenotypes. Thus it is beyond doubt that the SPM-3T3 cells comprise an immortalized cell line expressing the cellular markers of the sphingomyelinosis mouse.

The accumulation of intracellular cholesterol and defective esterification of exogenously added cholesterol are the specific cellular abnormalities observed in NP-C cells. The same abnormalities may be observed in cultured fibroblasts from patients with I-cell disease (7). I-cell disease, which is characterized by a deficiency of N-acetylglucosaminyl-1-phosphotransferase activity, shows low levels of multiple lysosomal enzymes due to the defect in targeting of newly synthesized lysosomal hydrolases to lysosomes (for a review see ref. 15). The SPM-3T3 cells exhibit normal lysosomal enzyme activities except for that of acid sphingomyelinase. With
respect to the intracellular accumulation of unesterified cholesterol, defective esterification of exogenously added cholesterol and no multiple lysosomal enzyme defects, SPM-3T3 cells are very similar to NP-C cells.

Sphingomyelinase activity in the liver, spleen or brain of the sphingomyelinosis mouse is reduced to about 30%-80% of normal (+/-+), while the activity in heterozygotes (spm/+) is normal (14). The absence of a partial deficiency of sphingomyelinase in heterozygous mice is not in accordance with the biochemical lesions observed in Niemann-Pick disease types A and B (22). The activity in tissues of sphingomyelinosis mouse is also similar to that in NP-C. However, the deficiency of the activity in the SPM-3T3 cells (4.2% activity of SPMH-3T3 cells) was severe. One of the intriguing observations in NP-C cells is the fluctuating partial sphingomyelinase deficiency (22). Vanier et al. (26) observed near normalization of the sphingomyelinase activity in NP-C cells switched to medium containing lipoprotein-depleted serum for 4 days. When LDL was added to these cells maintained in lipoprotein-depleted serum, a considerable decrease was observed 24 h after the LDL addition. NP-C cells proliferating in LDL-containing medium may show reduced sphingomyelinase activity, but quiescent cells deprived of LDL may show normal or mildly reduced sphingomyelinase activity. Our assay conditions, for which sparsely proliferating cells were used as the enzyme source, may be the reason why the sphingomyelinase activity in NP-C cells is more severely reduced than previously reported (22), and the activity in SPM-3T3 cells is more deficient than that in the tissues of the sphingomyelinosis mouse.

In NP-C cells, a defect in the transport of cholesterol from lysosomes to other cell membrane sites, which is specific to exogenously derived cholesterol, has been suggested (9, 17, 20, 21). If this defect does exist, NP-C cells must depend on the de novo cholesterol synthetic pathway for their structural cholesterol requirements, as do fibroblasts from patients with familial hypercholesterolemia (5). We have observed a marked increase in cholesterol synthesis from acetate, which is in good accordance with the findings of Maziere et al. (11). The increased cellular cholesterol synthesis in both SPM-3T3 and NP-C cells must compensate for the defect in the transport of exogenously derived cholesterol.

Acknowledgements. We wish to thank Taeko Yorita and Atsue Matsuda for their assistance in the tissue culture and lysosomal enzyme assay. This investigation was supported in part by a Grant-in-Aid for Scientific Research, No. 04670597, from the Ministry of Education, Science and Culture (Japan), and by grants from the National Center of Neurology and Psychiatry (NCNP) of the Ministry of Health and Welfare (Japan), and the Mita Research Foundation, Tottori University.

REFERENCES

Abnormal Cholesterol Metabolism in SPM-3T3 Cell Line

low-density lipoprotein uptake and cholesterol storage in cultured fibroblasts. FASEB J., 1: 40-45.


(Received for publication, January 14, 1992 and in revised form, June 12, 1992)