Niemann–Pick variant disorders: Comparison of errors of cellular cholesterol homeostasis in group D and group C fibroblasts

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ABSTRACT Fluorescence microscopic examination of filipin-stained cultured skin fibroblasts derived from two brothers with group D Niemann–Pick disease revealed abnormal storage of low density lipoprotein (LDL)-derived cholesterol. LDL stimulation of intracellular cholesteryl ester synthesis was severely compromised in the Niemann–Pick D fibroblasts, as it also was in fibroblasts obtained from Niemann–Pick C patients. Cholesteryl ester synthesis was intermediate in cells derived from an obligate group-D heterozygous carrier. Activity of acyl-CoA:cholesterol acyltransferase was within the normal range in cells-free extracts of both LDL-depleted and LDL-supplemented cultures of Niemann–Pick C and D fibroblasts. Incubation of Niemann–Pick D fibroblasts with LDL did not lead to as high a level of intracellular cholesterol accumulation as the excessive storage observed with Niemann–Pick C fibroblasts. These findings suggest that the Niemann–Pick variant disorders may represent a family of specific and possibly individual mutations that disrupt cellular cholesterol homeostasis.

Between 1914 and 1927, Niemann (1) and Pick (2) correlated a series of clinical cases of progressive central nervous system deterioration and hepatosplenomegaly into a distinct disease entity. This inborn metabolic disorder came to be known as Niemann–Pick disease and was shown by Klenk (3) in 1934 to be associated with elevated tissue levels of sphingomyelin. The clinical presentation of Niemann–Pick disease is variable, and in 1961, Crocker (4) proposed the delineation of patients with varying degrees of visceral sphingomyelin accumulation into four separate and distinguishable groups, designated as groups A, B, C, and D Niemann–Pick disease. Patients with an early onset of central nervous system involvement and extensive visceral sphingomyelin accumulation were designated as group A, patients with visceral involvement but no central nervous system deficiencies as group B, patients with a moderate course of progressive neurological and visceral involvement beginning variably in late infancy as group C, and patients of French-Canadian Nova Scotian ancestry with a similar moderate neurovisceral decline developing in early or middle childhood as representing group D.

The metabolic bases of the group A and group B Niemann–Pick disorders was established in 1966 to represent gross failure of normal sphingomyelin catabolism due to a specific primary deficiency in lysosomal sphingomyelinase activity (5–7). The primary metabolic lesion(s) of the Niemann–Pick variant disorders (groups C and D; NP-C and NP-D) have yet to be defined. Moderate visceral storage of sphingomyelin and inconsistent documentation of sphingomyelinase deficiencies have led to the conclusion that sphingomyelin storage is not the primary metabolic basis of NP-C (8). A similar conclusion had previously been reached concerning NP-D disease (9).

The documentation of a primary cholesterol-storage disorder in a mutant strain of BALB/c mice (10, 11) was instrumental in the subsequent discovery that NP-C patients also manifest abnormal patterns of intracellular cholesterol processing in cultured cells developed from these individuals (8, 12–14). Potential abnormalities of cholesterol metabolism have not been reported for NP-D. The present investigations document that fibroblasts derived from two affected male siblings of a NP-D family manifested an abnormal pattern of cholesterol homeostatic responses that resembles but differs in part from the cholesterol-processing errors expressed by NP-C cell lines.

MATERIALS AND METHODS

Case Studies. The family was referred by A. Crocker (Boston, MA) with the diagnosis of NP-D in two of four children. The mother, age 42, traces her ancestry to one of the well-established NP-D families through her maternal grandmother. The father, age 44, has four great-grandparents from the Rive du Loup region of Nova Scotia, Canada. Their 14-year-old son presented with learning disability at school age, and hepatosplenomegaly was first noted at age 8. He subsequently developed dystarthis and ataxia at the age of 10, which have been progressive, and has shown progressive school difficulties, with dementia developing at age 12. Vertical gaze impairment was also noted at age 12. Their 11-year-old affected son was noted to have splenomegaly at age 6 weeks and underwent full evaluation at age 5, where lipid-laden histocytes were found in a bone marrow biopsy. He has had school performance difficulties and balance impairment since age 5 and has vertical gaze impairment.

Cell Cultures. Superficial skin biopsy specimens were obtained by standard procedures from the mother and two affected male siblings of the NP-D family. Stable secondary cell lines established from the biopsy samples were used for the tissue culture studies. Established normal and NP-C fibroblasts were obtained from the National Institute of General Medical Sciences, Human Genetic Mutant Cell Repository (Camden, NJ) and from D. Wenger (Department of Pediatrics, University of Colorado, Health Sciences Cen-

Abbreviations: LDL, low density lipoprotein; NP-C and NP-D, Niemann–Pick group C and group D, respectively.
ter, Denver, CO). Fibroblast cultures from normal BALB/c mice and the mutant cholesterol-storage mouse (10) were established from explants of subcutis of the ventral abdominal region.

**Low Density Lipoprotein (LDL) and Lipoprotein-Deficient Serum.** Fresh human LDL in the density range of 1.019-1.063 g/ml was prepared by sequential ultracentrifugation from KBr/plasma solution (15). Lipoprotein-deficient serum (density <1.20 g/ml) was prepared from a mixture of KBr/fetal bovine serum by ultracentrifugation. Both products were obtained from Meloy Laboratories (Springfield, VA) and were used within 6 weeks of preparation.

**Radiolabeled Compounds.** [9,10-3H]Oleic acid (2-10 Ci/mmol; 1 Ci = 37 GBq) and [1,2-3H]cholesterol (60-90 Ci/mmol) were purchased from New England Nuclear. [3H]Cholesterol was repurified by reversed-phase thin-layer chromatography prior to use (12).

**Cell Culture Techniques.** Cell lines were maintained in Eagle's minimal essential medium with 10% (vol/vol) complete fetal bovine serum, 2 mM L-glutamine, 100 units of penicillin per ml, and 100 μg of streptomycin per ml, in humidified 95% air/5% CO2 at 37°C. Cells were harvested by washing monolayers three times with phosphate-buffered saline and subsequently treating the cell layers with 0.05% trypsin (Worthington) for 5 min at 37°C before final cell washing and centrifugation.

**Lipid Analysis.** Fibroblast cell pellets were suspended in 250 μl of 2-propanol and briefly sonicated with a microprobe. The 2-propanol extract was incubated at 37°C for 30 min and subsequently centrifuged at 3000 x g 10 min. The 2-propanol extract was utilized for lipid measurements, and cell pellets were dissolved in 250 μl of 0.5 M NaOH for quantitation of protein. For isotopic measurements, an aliquot of the 2-propanol extract was transferred to a small glass tube and

![Fig. 1. Fluorescent filipin detection of LDL-cholesterol loading in cultured normal and NP-D fibroblasts. Stock cell lines were maintained in McCoy's medium with 10% lipoprotein-deficient fetal bovine serum for 4 days and subsequently reseeded in single glass-slide chambers (8.6 cm²) at a density of 3.5 × 10⁴ cells. The newly plated cell cultures were maintained in 4 ml of fresh medium containing 10% lipoprotein-deficient serum for 24 hr and subsequently in fresh McCoy's medium with or without LDL (100 μg/ml) for 1 day. Washed monolayers were fixed with phosphate-buffered 10% formalin and stained with filipin as described (17). Stained slides were photographed with phase-contrast and fluorescence microscopy. Fluorescence micrographs all represent equal exposures.](image-url)
cholesterol and cholesteryl oleate (25 µg each) were added as carriers. The samples were dried under a stream of N₂ and subsequently dissolved in 50 µl of chloroform/methanol (2:1, vol/vol) and applied to silica gel 60 plates ( precoated, 0.25 mm thick, E. Merck, Darmstadt, F.R.G.). Neutral lipids were separated using a solvent mixture of hexane/ether/glacial acetic acid (90:10:1 vol/vol). The positions of free and esterified cholesterol were established with iodine vapor. These areas were scraped from the chromatograms and their radioactivity was determined by scintillation spectroscopy. Mass measurements of unesterified and esterified cholesterol were carried out in aliquots of the 2-propanol extract with an enzymatic–fluorimetric method (16).

RESULTS

Fluorescent Histochemical Staining for Cellular Storage of LDL-Derived Cholesterol. The specific interaction of the fluorescent dye filipin with unesterified cholesterol offers a histological means of examining the cellular uptake of exogenous cholesterol (17). In the absence of an exogenous lipoprotein source, control and NP-D fibroblasts developed little fluorescent staining (Fig. 1). When nonconfluent cells were incubated with LDL (100 µg/ml) for 24 hr, NP-D fibroblasts developed a strikingly more intense fluorescence than that seen in normal cultures. This suggested that, in contrast to a normal cellular response, the NP-D cells were primarily accumulating LDL-derived cholesterol as unesterified cholesterol that was localized within inclusion bodies. Similar findings have been reported for NP-C cell lines (12). The increase in plasma membrane filipin staining in normal fibroblasts incubated with LDL suggests that unesterified cholesterol increased in these plasma membranes. The absence of increased staining of NP-D fibroblast plasma membranes suggests that unesterified cholesterol was not released from lysosomal inclusions and consequently was not available for entry into the plasma membrane cholesterol pool.

Mass Measurements of Cellular Cholesterol. The accumulation of LDL–cholesterol by confluent cell cultures was significantly higher in NP-C cells when compared to NP-D and control fibroblasts (Fig. 2). In NP-C cells the net increase in cellular cholesterol rose by 92 nmol/mg of protein above a cholesterol-depleted baseline level of 44 nmol/mg of protein when mutant fibroblasts were incubated with LDL (50 µg/ml) for 48 hr. Under identical culture conditions, LDL-induced accumulation of cellular cholesterol in normal cells rose by only 45 nmol/mg of protein over a cholesterol-depleted level of 30 nmol/mg of protein. LDL incubation with NP-D cell cultures for 48 hr stimulated a somewhat lower than normal net cellular increase of 30 nmol of cholesterol per mg of protein over a baseline level of 35 nmol of cholesterol per mg of protein. LDL-cholesterol accumulated predominantly as unesterified cholesterol (≥90%) in both NP-C and NP-D cell cultures, confirming the fluores-

Table 1. LDL-cholesterol processing in mutant murine fibroblasts

<table>
<thead>
<tr>
<th>Culture</th>
<th>LDL addition</th>
<th>Cholesteryl [3H]oleate, nmol/mg of protein</th>
<th>Cholesterol mass, nmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Normal  (2)</td>
<td>–</td>
<td>0.8 ± 0.2</td>
<td>30 ± 1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>6.9 ± 1.0</td>
<td>53 ± 3</td>
</tr>
<tr>
<td>Mutant (2)</td>
<td>–</td>
<td>1.0 ± 0.0</td>
<td>43 ± 10</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.0 ± 0.0</td>
<td>70 ± 5</td>
</tr>
</tbody>
</table>

Stock cells were seeded at a density of 5 x 10⁴ cells in 35-mm wells and maintained with 2.0 ml of McCoy's medium with 10% fetal bovine serum for 5 days, with one change of fresh medium after 3 days of incubation. Cell cultures were subsequently treated with McCoy's medium plus 10% lipoprotein-deficient serum for 48 hr. Experiments were initiated with the addition of fresh McCoy's medium/10% lipoprotein-deficient serum, with or without LDL (50 µg/ml), containing 0.1 mM [3H]oleate (200 dpm/pmol). After 48 hr, mass levels of cholesterol and cholesteryl esters and the formation of cholesteryl [3H]oleate were quantified. Values in parentheses represent the number of separate cell lines used. Cell cultures were tested in triplicate.
ence observations that these mutant cells were deficient in their esterification of exogenously derived cholesterol. In normal cells, the LDL-induced increase in cellular cholesterol was approximately evenly distributed between esterified and unesterified cholesterol.

A murine cholesterol-storage disorder with phenotypic lesions resembling the human Niemann–Pick variant disorders has been described (9–11). Cultured fibroblasts derived from this mutant mouse displayed an impairment in cholesteryl ester synthesis, and LDL–cholesterol accumulated predominantly as unesterified cholesterol (Table 1). These mutant mouse fibroblast cultures did not accumulate excessive amounts of LDL-derived cholesterol, and consequently their pattern of abnormal cholesterol processing more closely resembles the deficiencies noted with human NP-D cell cultures than those presented by NP-C cells.

**Esterification of Exogenously Derived Cholesterol.** Cholesteryl [3H]oleate formation was seen to be severely restricted in both NP-C and NP-D cell cultures when fibroblasts were incubated with LDL and [3H]oleate (Fig. 3). This observation is consistent with the cholesterol mass measurements that indicated a deficiency of cholesteryl ester accumulation in both mutant cells. The level of cholesteryl [3H]oleate formation in cells derived from the obligate NP-D carrier was intermediate between the levels found in normal and homozygous affected cells when fibroblasts were cultured with LDL and [3H]oleate for 6 hr (Table 2). A lesion in cellular cholesterol esterification was also observed in NP-D and NP-C fibroblasts when these mutant cells were incubated with nonlipoprotein [3H]cholesterol (Table 3). Internalization of nonlipoprotein cholesterol does not require interaction with the LDL receptor (18).

Levels of cholesterol acyltransferase (acyl-CoA:cholesterol O-acyltransferase, EC 2.3.1.26) activity in cell-free extracts of fibroblasts incubated with LDL (50 μg/ml) for 24 hr were in the normal range (1000 ± 30 pmol of cholesteryl [3H]oleate per mg of protein per hr) in NP-C (840 ± 100 pmol per mg of protein per hr) and NP-D (700 ± 200 pmol per mg of protein per hr) cultures.

![Fig. 3. Stimulation of cholesteryl [3H]oleate synthesis in cultured fibroblasts incubated with LDL. Stock cells were seeded and incubated as described for Fig. 2. In addition to LDL (50 μg/ml), the medium included 0.1 mM [3H]oleate (200 dpm/pmol) bound to fatty acid-free bovine serum albumin (0.11%). Cells were incubated for the indicated times and subsequently analyzed for cholesteryl [3H]oleate formation. , Normal cells; ▲, NP-C cells; ■ and ○, cells from the two NP-D-affected brothers. Values represent the average of three separate cell cultures for each genotype.](image)

### Table 2. LDL stimulation of cholesteryl ester synthesis in normal and NP-D fibroblasts

<table>
<thead>
<tr>
<th>Culture</th>
<th>LDL addition</th>
<th>Cholesteryl [3H]oleate, pmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (2)</td>
<td>−</td>
<td>40 ± 0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1050 ± 90</td>
</tr>
<tr>
<td>NP-D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous (2)</td>
<td>−</td>
<td>145 ± 10</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>190 ± 10</td>
</tr>
<tr>
<td>Heterozygous (1)</td>
<td>−</td>
<td>20 ± 0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>400 ± 30</td>
</tr>
</tbody>
</table>

Stock cells maintained in McCoy’s medium with 10% fetal bovine serum were seeded in 35-mm wells at a density of 5 × 10⁴ cells and incubated with 2.0 ml of McCoy’s medium with 10% fetal bovine serum for 3 days and then for 2 days with fresh medium of identical composition. Cultures were depleted of cholesterol by incubation for 48 hr with 2.0 ml of McCoy’s medium with 10% lipoprotein-deficient serum. Experiments were initiated by the addition of 2.0 ml of fresh McCoy’s medium/10% lipoprotein-deficient serum containing LDL (50 μg/ml) and 0.1 mM [3H]oleate (200 dpm/pmol) bound to bovine serum albumin (0.11%). Cells were incubated for 6 hr and then were analyzed for protein and cholesteryl [3H]oleate formation. Cultures were tested in triplicate.

### DISCUSSION

The eponym Niemann–Pick disease has served to classify a series of divergent genetic disorders that display varying degrees of abnormal sphingomyelin storage. Although the enzymatic basis for this sphingomyelin accumulation is well established in the classic Niemann–Pick diseases (groups A and B), a similarly direct identification of the primary metabolic mutation of the Niemann–Pick variant disorders (groups C and D) is lacking at present. Relevant biochemical lesions of NP-C have recently begun to be characterized. It has been shown that fibroblasts derived from NP-C patients show unique lesions in the processing of exogenously derived cholesterol. The metabolic errors associated with this human disorder in tissue culture include (i) blocked esterification of exogenously derived cholesterol in mutant cells (8, 12, 13); (ii) partial blockage of LDL–cholesterol esterification in heterozygous mutant fibroblasts (14); (iii) excessive accumulation of LDL-derived cholesterol in mutant cells (14); and (iv) a delay in the initiation of LDL-receptor down-regulation and cholesterol acyltransferase activation in mutant cells (19). These metabolic errors in NP-C mutant fibroblasts appear to be closely linked to abnormal lysosomal trapping of exogenously derived cholesterol.

### Table 3. Esterification of nonlipoprotein [3H]cholesterol in cultured normal, NP-C, and NP-D fibroblasts

<table>
<thead>
<tr>
<th>Culture</th>
<th>Unesterified</th>
<th>Esterified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (2)</td>
<td>266 ± 6</td>
<td>15.5 ± 1.0</td>
</tr>
<tr>
<td>NP-C (2)</td>
<td>212 ± 15</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>NP-D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homozygous (2)</td>
<td>282 ± 46</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Heterozygous (1)</td>
<td>174 ± 10</td>
<td>9.3 ± 1.1</td>
</tr>
</tbody>
</table>

Stock cell cultures were seeded in 35-mm wells at a density of 10⁵ cells and incubated in 3 ml of McCoy’s medium with 10% lipoprotein-deficient fetal bovine serum for 48 hr. Subsequently, cells were incubated with 3.0 ml of fresh medium containing [3H]cholesterol (50 μg/ml, 31 dpm/pmol) in 12 μl of ethanol. Cells were incubated at 37°C for 24 hr, after which the monolayers were washed three times with phosphate-buffered saline. The harvested cell pellet was analyzed for protein, [3H]cholesterol, and [3H]cholesteryl esters. The number of cell lines employed is indicated in parentheses, and all cultures were tested in triplicate.
The widely divergent geographical origins and diverse clinical presentations of the Niemann–Pick variant disorders suggest the possibility that these diseases may represent a series of specific genotypic subtypes. The availability of fibroblasts from a confirmed NP-D family presented an opportunity to begin to examine this particular mutant for possible errors in cholesterol processing. Fibroblasts derived from clinically affected male children were found to be grossly deficient in their ability to esterify cholesterol derived from LDL and nonlipoprotein sources. The heterozygous mutant cell culture obtained from an obligate carrier was partially deficient in esterification of LDL–cholesterol. The homozygous mutant NP-D cell cultures accumulated LDL-derived cholesterol primarily as unesterified cholesterol. No deficiency of cholesterol acyltransferase activity was seen in cell-free extracts of NP-D fibroblasts. The deficiency of the NP-D cells in initiating normal cholesterol ester synthesis parallels a similar pattern of blocked cholesterol esterification observed in NP-C fibroblasts.

One notable feature of abnormal cholesterol processing common to NP-C cells was, however, not noted with the NP-D cells under comparable culture conditions. Incubation with LDL caused confluent NP-C fibroblast cultures to accumulate excessive intracellular cholesterol that was 2- to 3-fold greater than normal. Similarly treated confluent cultures of NP-D cells did not manifest the excessive uptake of the lipoprotein-derived cholesterol observed with NP-C cells. A lesion in the esterification of internalized cholesterol, but not in the regulation of LDL-cholesterol uptake, was also seen in fibroblasts derived from the mutant cholesterol-storage mouse. Although the molecular basis of this murine disorder remains to be identified, the primary mutation has been shown to be closely linked to a lesion in esterification of exogenously derived cholesterol (13). The similar profiles of abnormal cholesterol processing in the mutant mouse and human NP-D cells encourage further comparative studies.

The appearance of apparent varying expressions of abnormal cellular cholesterol processing in the NP-C and NP-D cells suggests that genotypically distinct lesions may exist within the family of Niemann–Pick variant disorders that disrupt specific segments of the cholesterol homeostatic pathway. Further elucidation of this possibility will have to await a detailed examination of the reactions involved in intracellular cholesterol processing.

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