Prenatal diagnosis of Niemann-Pick disease type C

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Summary

Niemann-Pick disease type C (NPC) was demonstrated in two successive pregnancies by strongly reduced activity of sphingomyelinase in amniotic fluid cells. By contrast, chorionic villi from the first pregnancy had shown normal sphingomyelinase activity. The prenatal diagnosis of NPC in the two fetuses was confirmed, after termination of the pregnancies, by (phospho)lipid analyses of the fetal livers, by the assay of sphingomyelinase in the fetal fibroblasts and by the demonstration of a defective esterification of exogenous cholesterol and of cholesterol accumulation by filipin staining. Retrospective analysis of cultured amniocytes for cholesterol esterification and filipin staining confirmed the feasibility of these methods for prenatal diagnosis. In a recent pregnancy in the same mother the three available methods were applied to amniotic fluid cells and an unaffected child was correctly predicted. Lipid analysis of liver tissue from the patient with NPC and the two fetuses showed a 3–5 times increased level of cholesterol, a 2–3 times increased level of sphingomyelin and a remarkable increase of bis (monoacylglyceryl) phosphate.

Introduction

Niemann-Pick disease type C (NPC; MIM257220) is an autosomal recessive lysosomal disorder of which the primary defect is not entirely clear. Like Niemann-Pick disease types A and B there is a lysosomal accumulation of sphingomyelin, cholesterol and bis (monoacylglyceryl) phosphate in liver and spleen of patients with...
NPC [1]. By contrast with types A and B, in which a complete deficiency of
sphingomyelinase (EC 3.1.4.12) is the basic defect, this enzyme is only partially
deficient in cultured fibroblasts of many patients with NPC but not in other types
of cells or tissues [1,2]. These findings and the demonstration of genetic complemen-
tation with respect to sphingomyelinase activity in cell fusions of NPA (or B) and
NPC have suggested that different genes are involved and that the sphingomyelinase
deficiency in NPC fibroblasts is probably secondary to the basic defect [3].

Studies in the past 6 years have demonstrated a defective intracellular transport
and metabolism of LDL-derived cholesterol, which results in an accumulation of
cholesterol in lysosomes and an inability to mobilize and esterify lysosomal
cholesterol [4–7]. Based on these findings, tests have been developed for the
diagnosis of NPC using cultured cells [8] or lymphocytes [9]. These methods have
also been applied to prenatal diagnosis of NPC [1,10], but few data have been
published so far. In this paper we describe both prospective and retrospective in-
vestigations in three successive pregnancies at risk for NPC in one family.

Case Report

The male proband was the first child of healthy parents who were consanguineous.
Pregnancy and delivery were uneventful. From birth there were severe feeding dif-
ficulties. Because of hyperbilirubinemia, necessitating phototherapy, the neonate
was admitted at the fourth day of life. After discharge however continuing feeding
difficulties necessitated a second admission at the age of four months. Growth
parameters were all below the third centiles. He had dysmorphic facial features.
Hepatosplenomegaly was present as well as a generalised hypotonia. A severe
respiratory insufficiency developed: radiologically atelectasis and overexpansion was
seen in both lungs. This pattern persisted, even after treatment with antibiotics.

These clinical manifestations and the presence of foam cells in bone marrow
suggested a lipid storage disease, possibly Niemann-Pick disease. Subsequent bio-
chemical investigations, described in this report, indicated NPC. The child died at
9 months due to heart failure secondary to the lung pathology. Histopathologic
studies showed foam cells in the alveolar walls as well as in spleen, bone marrow,
liver, lymph glands and spine marrow.

Prenatal analyses were performed in the three subsequent pregnancies of the pa-
tient's mother. In the first pregnancy chorionic villi were investigated in the 10th
week and, to corroborate the results, amniocentesis was done in the 16th week. In
the second and third pregnancy only cultured amniocytes were analyzed. Affected
fetuses were diagnosed by sphingomyelinase assay in the first two pregnancies, which
were then terminated. These pregnancies occurred in 1985 and 1986 and cholesterol
accumulation and esterification could only be studied in retrospect in the stored am-
niocytes from the second pregnancy. In the third, most recent pregnancy an unaf-
fected fetus was predicted prenatally and a healthy child was born.

Material and Methods

Human lipoprotein deficient serum (d = 1.25) (LPDS) and low density lipopro-
teins (d = 1.019–1.063) (LDL) were a gift of Drs. R. de Crom and R. van Haperen,
Rotterdam and were prepared according to Havel et al. [11]. [1-14C]Oleic acid (Amersham, 1.88 GBq/mmol; radiochemical purity 97.6%) was bound to bovine serum albumin (fatty acid poor, Fluka) according to Goldstein et al. [12].

Chorionic villi were obtained by transcervical aspiration, selected under a microscope, washed in 0.15 M NaCl, homogenized in water in a Potter tube and by sonication on ice (10s amplitude 9 μm). Mutant NPC fibroblasts (GM 3123) were purchased from the Human Mutant Cell Repository (Camden, NJ, USA). Fibroblasts and amniotic fluid cells were cultured in Ham's F10 with 10% and 20% fetal calf serum (FCS; Gibco), respectively; for enzyme assay they were harvested with trypsin 7 days after the last subculture and homogenized by sonication in water.

For the cholesterol esterification assay cells were seeded at a density of 4 × 10⁴ cells in 2 ml Ham's F10 + 10% FCS with penicillin and streptomycin in culture dishes (3.5 mm diameter). Just before reaching confluency, the monolayer was washed with phosphate buffered saline (0.01 M phosphate/0.15 M NaCl, pH 7.4) and then given 1 ml F10 + 10% LPDS for 4 days. Esterification was initiated by adding fresh medium containing 0.1 mM [14C]oleic acid (30,000 dpm/nmol) in albumin with or without LDL. Incubation, harvesting and lipid analysis of the cells was performed as described [8].

Fluorescent staining of cholesterol in intact cells was performed using filipin (filipin complex, Sigma) as described [13] except that the cells were seeded on cover slips in standard medium (Ham's F10/10% FCS) at one-third of confluency. After 3 days cultivation, the cells were fixed and stained. Sphingomyelinase, β-glucosidase and β-galactosidase activities were measured as previously described [14]. Lipid analyses in liver were performed as described by Wenger et al. [15] except that the phospholipids were separated on thin-layer chromatography as used by Broeckhuysen [16]. Cholesterol was measured according to Heider and Boyett [17].

Results

Sphingomyelinase and β-glucosidase activities

Table 1 summarizes the activities of sphingomyelinase and β-glucosidase in chorionic villi and cultured amniocytes from the three successive pregnancies at risk and in cultured skin fibroblasts from the two affected fetuses, the previous affected child (the proband) and the parents. Fibroblasts of the proband exhibited a substantially reduced activity of sphingomyelinase and to some extent of β-glucosidase as well; the parents' cells showed normal activities for both enzymes. Sphingomyelinase and β-glucosidase activities were normal in the chorionic villi from pregnancy 1, when compared to the reference range. By contrast sphingomyelinase activity in the cultured amniocytes from the same pregnancy was substantially reduced; β-glucosidase was slightly less than the lower limit of the reference range. Similar results were obtained in the amniocytes of pregnancy 2; both pregnancies were terminated and the diagnosis of an affected fetus was confirmed by the demonstration of reduced sphingomyelinase and β-glucosidase activities in the fetal fibroblasts and later by the analysis of lipid accumulation in the fetal livers and of abnormal cholesterol metabolism in the fibroblasts (see below). Sphingomyelinase activity in amniocytes of the third pregnancy was normal.
TABLE 1

Sphingomyelinase and β-glucosidase activities in fetal cells from three successive pregnancies at risk for Niemann-Pick disease type C.

<table>
<thead>
<tr>
<th>Family under study</th>
<th>Normal controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sphingomyelinase</td>
</tr>
<tr>
<td>Chorionic villi</td>
<td></td>
</tr>
<tr>
<td>At risk 1</td>
<td>6.9*</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>Amniotic fluid cells</td>
<td></td>
</tr>
<tr>
<td>At risk 1</td>
<td>0.8</td>
</tr>
<tr>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>At risk 2</td>
<td>1.5</td>
</tr>
<tr>
<td>At risk 3</td>
<td>6.7</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td></td>
</tr>
<tr>
<td>Fetus 1</td>
<td>1.5</td>
</tr>
<tr>
<td>(n = 12)</td>
<td></td>
</tr>
<tr>
<td>Fetus 2</td>
<td>2.9</td>
</tr>
<tr>
<td>Index patient</td>
<td>2.7</td>
</tr>
<tr>
<td>Mother</td>
<td>20.5</td>
</tr>
<tr>
<td>Father</td>
<td>14.9</td>
</tr>
</tbody>
</table>

The presented sphingomyelinase activities were obtained with [14C]sphingomyelin as substrate; a complete set of parallel results with the artificial substrate 2-hexadecanoylamino-4-nitrophosphorylcholine showed a similar pattern.

*Sphingomyelinase activity in chorionic villi is expressed as nmol/17 h/mg protein; all other activities are in nmol/h/mg protein. The activity of a reference enzyme, β-galactosidase, was normal in all samples.

Cholesterol esterification and accumulation

Cholesterol uptake and esterification was studied retrospectively in amniotic fluid cells from pregnancy 2 after storage for several years in liquid nitrogen and prospectively in amniocytes from pregnancy 3. After growth in medium with 10% LPDS the cells were incubated for 6 h in the presence of [14C]oleate with or without LDL as described in Methods. The results were compared with esterification levels in control amniocytes and in fibroblasts of controls, a known patient with NPC (GM3123), the index patient, the parents and the two fetuses from the terminated pregnancies 1 and 2.

Table II shows the esterification levels at an LDL concentration of 30 μg protein/ml. The esterification levels in fibroblasts of the index patient and the two affected fetuses of the family under study and the GM3123 cell line were negligible. The absence of cholesterol esterification in the amniocytes of pregnancy 2 clearly confirmed the earlier prenatal diagnosis of NPC whereas the amniocytes of pregnancy 3 showed a low-normal level of esterification which indicated that the fetus was not affected but could be a heterozygote.

Lysosomal accumulation of cholesterol was demonstrated in amniotic fluid cells
### TABLE II

Cholesterol uptake and esterification in amniotic fluid cells and fibroblasts

<table>
<thead>
<tr>
<th></th>
<th>NPC</th>
<th>Normal controls</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-assay</td>
<td>Inter-assay</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Amniocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy 2</td>
<td>&lt;10</td>
<td>890</td>
<td>280–1610</td>
<td></td>
</tr>
<tr>
<td>Pregnancy 3</td>
<td>360</td>
<td>620</td>
<td>960 ± S.D. 420</td>
<td>(n = 16)</td>
</tr>
<tr>
<td><strong>Fibroblasts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index patient</td>
<td>&lt;10</td>
<td>1250</td>
<td>350–1370</td>
<td></td>
</tr>
<tr>
<td>Mother</td>
<td>1370</td>
<td>1030</td>
<td>810+340</td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>1170</td>
<td>740</td>
<td>(n = 10)</td>
<td></td>
</tr>
<tr>
<td>Fetus 1</td>
<td>&lt;10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetus 2</td>
<td>&lt;10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GM3123</td>
<td>&lt;10</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

and in fibroblasts of NPC patients by histochemical staining with filipin. Figure 1 shows the intensive perinuclear fluorescence in fibroblasts of the index patient (Fig. 1a) and in amniocytes from the affected pregnancy 2 (Fig. 1d). The same pattern of fluorescence was present in fibroblasts of the fetuses 1 and 2 (not shown), which confirmed the earlier prenatal diagnosis of NPC in both cases. The mother’s cell culture was negative with the exception of very few cells with some fluorescence (Fig. 1c). In cultures of normal controls some aspecific peripheral fluorescence was occasionally observed (Figs. 1b and 1e). In the amniocytes from pregnancy 3 only aspecific (non-lysosomal) staining was present (Fig. 1f as in the control Fig. 1e) which indicated, together with the normal cholesterol esterification and sphingomyelinase activity, that the fetus was not affected.

**Lipid analyses**

Stored livers of the index patient and of the two affected fetuses were subjected to extraction, fractionation and quantitative analysis of lipids and the results were compared with the lipid contents of three control fetal livers (Table III). The cholesterol content in the three NPC livers is significantly increased compared to the level in controls: the ratio cholesterol/total phospholipids is 4–5 times increased in the NPC livers compared to controls. The total phospholipid content in the NPC livers was not different from the controls but there are at least two remarkable differences in the composition of the phospholipid fraction: (a) sphingomyelin content in NPC is 2–3 times higher than in controls and (b) the relatively small fraction of bis (monoacylglycerol) phosphate is increased from 0.3% (of the total phospholipid content) in the controls to 4.5–9.7% in the NPC livers.
Fig. 1. Filipin staining for free cholesterol in lysosomes. (a–c) Fibroblasts of (a) index patient, (b) control, (c) mother. (d–f) Amniocytes of (d) pregnancy 2, (e) control, (f) pregnancy 3.
### TABLE III

Lipid contents* of liver tissue of the patient and two fetuses with Niemann-Pick disease type C and controls

<table>
<thead>
<tr>
<th></th>
<th>Index patient</th>
<th>Fetus 1</th>
<th>Fetus 2</th>
<th>Controls (means: n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol</strong></td>
<td>79</td>
<td>116</td>
<td>168</td>
<td>32</td>
</tr>
<tr>
<td><strong>Phospholipids — Total</strong></td>
<td>119</td>
<td>187</td>
<td>190</td>
<td>192</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>29</td>
<td>22</td>
<td>33</td>
<td>11</td>
</tr>
<tr>
<td>Phosphatidylserine + Inositol</td>
<td>3</td>
<td>13</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>19</td>
<td>44</td>
<td>42</td>
<td>64</td>
</tr>
<tr>
<td>Bis(monoacylglyceryl)phosphate</td>
<td>12</td>
<td>11</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

*Values in nmol/mg protein.

### Discussion

Prenatal diagnosis of Niemann-Pick disease type A and B is reliably possible by the demonstration of the primary defect, the deficiency of sphingomyelinase, in amniotic fluid cells [18,19] or chorionic villi [20]. For NPC this approach may not be generally applicable because the often observed deficiency of sphingomyelinase in cultured cells of NPC patients is only partial, variable and is not the primary defect; in fact prenatal diagnosis for NPC has, to our knowledge, not been reported until the defect in cholesterol transport was discovered. Nevertheless sphingomyelinase assay may occasionally be useful as we have demonstrated in the present two affected pregnancies in a family where the proband had quite low sphingomyelinase activity in fibroblasts. Very low sphingomyelinase activity in amniotic fluid cells and reduced β-glucosidase activity as well, indicated that the fetuses were affected and since the involvement of cholesterol metabolism in NPC was not yet known at that time, the diagnosis and the decision to terminate the pregnancies was based on the sphingomyelinase results only. By contrast with the cultured amnionctyes the un-cultured chorionic villi did not express the sphingomyelinase deficiency, which is in accordance with normal sphingomyelinase activity in other types of tissues and cells of NPC patients [1].

Vanier et al. [10] described the successful use of methods to measure cholesterol esterification and accumulation for the prenatal diagnosis of NPC using cultured chorionic villus cells and the experience has expanded since then [2]. We have applied these methods to amniotic fluid cells from an affected pregnancy and demonstrated that these cells are suitable for prenatal diagnosis as well: the NPC amniocytes were completely deficient in cholesterol esterification and showed an intense fluorescent staining by filipin. Thus, in principle three methods are available for use for prenatal diagnosis: measurement of cholesterol esterification and/or accumulation may be preferred because of the probable nearness of the primary defect but sphingomyelinase may be a useful second or third parameter. Unfortunately
none of the three methods can be applied directly to chorionic villi for technical reasons or because the defect (sphingomyelinase deficiency) is not expressed in the villi. The use of cultured chorionic villus cells still allows an early diagnosis but has some disadvantages. These cultures may be contaminated with maternal cells and this risk of probably about 1% [21; also our unpublished observations] can be excluded only in case of a male fetus. Growth of chorionic villus cells is rather variable which increases the variation of results of the esterification test. These may be reasons to recommend occasionally amniocentesis to confirm the initial results on the villus cells or even to choose for amniocentesis only.

Lipid analyses of liver tissue of the two affected fetuses and of the proband showed that already during the fetal development the lipid profile is typical for NPC with moderately increased levels of cholesterol and sphingomyelin but a strong elevation of bis(monoacylglycerol)phosphate.

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