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

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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 complementation 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 investigations 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 difficulties. 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 biochemical 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 patient'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 amniocytes from the second pregnancy. In the third, most recent pregnancy an unaffected fetus was predicted prenatally and a healthy child was born.

Material and Methods

Human lipoprotein deficient serum (d = 1.25) (LPDS) and low density lipoproteins (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-^{14}C]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 × 10^4 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 [^{14}C]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 Broeckhuyse [16]. Cholesterol was measured according to Heider and Boyett [17].

Results

Sphingomyelinase and β-glucosidase activities

Table I 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 I

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

	Family under study		Normal controls	
	Sphingo-myelinase[a]	β-Gluco-sidase	Sphingo-myelinase	β-Gluco-sidase
Chorionic villi				
At risk 1	6.9[a]	125	4.9–12.5[a]	55–144
			($n = 10$)	($n = 9$)
Amniotic fluid cells				
At risk 1	0.8	50	3.1–8.0	65–231
At risk 2	1.5	25	($n = 11$)	($n = 18$)
At risk 3	6.7	112		
Fibroblasts				
Fetus 1	1.5	58	4.9–20.5	120–330
Fetus 2	2.9	83	($n = 12$)	($n = 33$)
			NP type A patients	
Index patient	2.7	74		
Mother	20.5	323	<0.05	
Father	14.9	235	(n = 12)	

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

[a]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 [^{14}C]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

	Formation of cholesteryl [^{14}C]oleate (pmol/h/mg protein)		
	NPC	Normal controls	
		Intra-assay	Inter-assay
Amniocytes			
Pregnancy 2	< 10	890	280–1610
Pregnancy 3	360	620	960 ± S.D. 420
		310	(*n* = 16)
Fibroblasts			
Index patient	< 10	1250	350–1370
Mother	1370	1030	810 + 340
Father	1170	740	(*n* = 10)
Fetus 1	< 10		
Fetus 2	< 10		
GM3123	< 10		

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 (monoacylglyceryl) phosphate is increased from 0.3% (of the total phospholipid content) in the controls to 4.5–9.7% in the NPC livers.
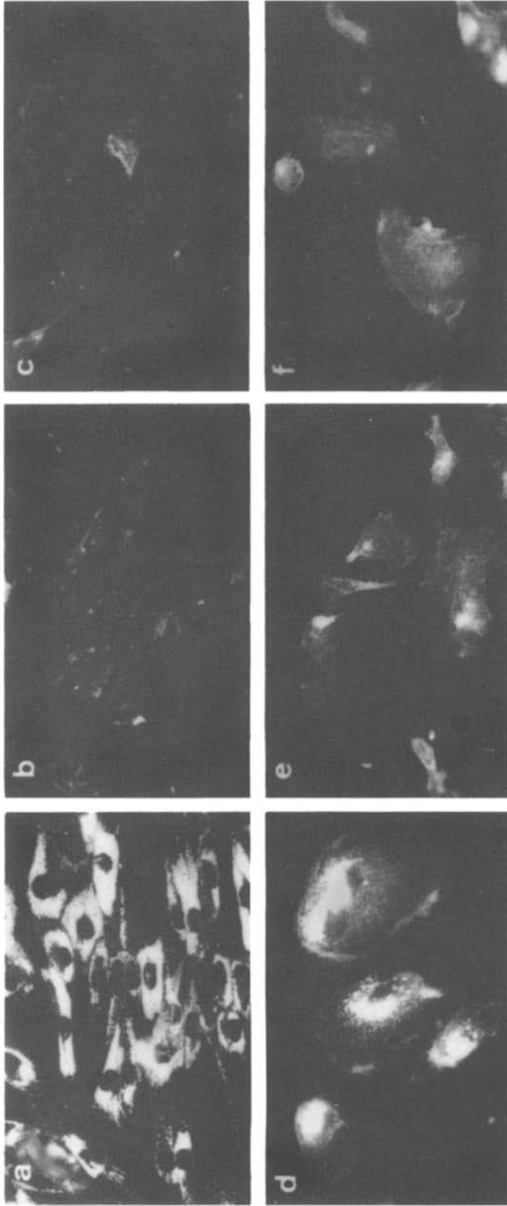
Fig. 1. Filipin staining for free cholesterol in lysosomes. (a–c) Fibroblasts of (a) index patient, (b) control, (c) mother. (d–f) Amniocytes of (d) pregnancy 2, (e) control, (f) pregnancy 3.

TABLE III

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

	Index patient	Fetus 1	Fetus 2	Controls (means; $n = 3$)
Cholesterol	79	116	168	32
Phospholipids — Total	119	187	190	192
Sphingomyelin	29	22	33	11
Phosphatidylcholine	54	95	94	87
Phosphatidylserine + Inositol	3	13	8	19
Phosphatidylethanolamine	19	44	42	64
Bis(monoacylglyceryl)phosphate	12	11	9	1
Cardiolipin	2	2	4	10

[a]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 amniocytes 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(monoacylglyceryl)phosphate.

Acknowledgements

References

1 Spence MW, Callahan JW. Sphingomyelin-cholesterol lipidoses: The Niemann-Pick group of diseases. In: Scriver CR, Beaudet AL, Sly WS, Valle D. The Metabolic Basis of Inherited Disease. McGraw-Hill, New York, 6th ed. 1989;1655.

2 Vanier MT, Pentchev PG, Rodriguez-Lafrasse C, Rousson R. Niemann-Pick disease type C: An update. J Inher Metab Dis 1991;14:580–595.

3 Besley GTN, Hoogeboom AJM, Hoogeveen A, Kleijer WJ, Galjaard H. Somatic cell hybridisation studies showing different gene mutations in Niemann-Pick variants. Hum Genet 1980;54:409–412.

4 Pentchev PG, Comly ME, Kruth HS et al. A defect in cholesterol esterification in Niemann-Pick disease (type C) patients. Proc Natl Acad Sci USA 1985;82:8247–8251.

5 Blanchette-Mackie EJ, Dwyer NK, Amende LM et al. Type-C Niemann-Pick disease: low density lipoprotein uptake is associated with premature cholesterol accumulation in the Golgi complex and excessive cholesterol storage in lysosomes. Proc Natl Acad Sci USA 1988;85:8022–8026.

6 Sokol J, Blanchette-Mackie EJ, Kruth HS, Dwyer NK et al. Type C Niemann-Pick disease: lysosomal accumulation and defective intracellular mobilization of low density lipoprotein cholesterol. J Biol Chem 1988;263:3411–3417.

7 Liscum L, Ruggiero RM, Faust JR. The intracellular transport of low density lipoprotein-derived cholesterol is defective in Niernann-Pick type C fibroblasts. J. Cell Biol 1989;108:1625–1636.

8 Vanier MT, Wenger DA, Comly ME, Rousson R, Brady RO, Pentchev PG. Niemann-Pick disease group C: clinical variability and diagnosis based on defective cholesterol esterification. Clin Genet 1988;33:331–348.

9 Argoff CE, Kaneski CR, Blanchette-Mackie EJ et al. Type C Niemann-Pick disease: documentation of abnormal LDL processing in lymphocytes. Biochim Biophys Res Commun 1990;171:38–45.

10 Vanier MT, Rousson RM, Mandon G, Choiset A, Lake BD, Pentchev PG. Diagnosis of Niemann-Pick disease type C on chorionic villus cells. The Lancet 1989;i:1014–1015.

11 Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 1955;34:1345–1353.

12   Goldstein JL, Basu SK, Brown MS. Receptor-mediated endocytosis of low density lipoprotein in cultured cells. Methods Enzymol 1983;98:241–260.

13   Kruth HS, Vaughan M. Quantification of low density lipoprotein binding and cholesterol accumulation by single human fibroblasts using fluorescence microscopy. J Lipid Res 1980;21:123–130.

14   Galjaard H. Genetic metabolic diseases, early diagnosis and prenatal analysis. Amsterdam: Elsevier/North Holland, 1980:819.

15   Wenger DA, Kudoh T, Sattler M, Palmieri M, Yudkoff M. Nieman-Pick disease type B: prenatal diagnosis and enzymatic and chemical studies on fetal brain and liver. Am J Hum Genet 1981;33:337–344.

16   Broeckhuyse RM. Quantitative two-dimensional thin-layer chromatography of blood phospholipids. Clin Chim Acta 1969;23:457–461.

17   Heider JG, Boyett RL. The picomole determination of free and total cholesterol in cells in culture. J Lipid Res 1978;19:514–518.

18   Epstein CJ, Brady RO, Schneider EL, Bradley RM, Shapiro D. In utero diagnosis of Niemann-Pick disease. Am J Hum Genet 1971;23:533.

19   Patrick AD, Young E, Kleijer WJ, Niermeijer MF. Prenatal diagnosis of Niemann-Pick disease type A using chromogenic substrate. The Lancet 1977;ii:144.

20   Vanier MT, Boué J, Dumez Y. Niemann-Pick disease type B: first-trimester prenatal diagnosis on chorionic villi and biochemical study of a foetus at 12 weeks of development. Clin Genet 1985;28:348–354.

21   Roberts E, Duckett DP, Lang GD. Maternal cell contamination in chorionic villus samples assessed by direct preparations and three different culture methods. Prenat Diagn 1988;8:635–640.