Clinica Chimica Acta, 165 (1987) 303–310
Elsevier

CCA 03858

Prenatal diagnosis of Zellweger syndrome by measurement of very long chain fatty acid (C26 : 0) β-oxidation in cultured chorionic villous fibroblasts: implications for early diagnosis of other peroxisomal disorders

R.J.A. Wanders [a], M.J.A. van Wijland [a], C.W.T. van Roermund [a], R.B.H. Schutgens [a], H. van den Bosch [c], J.M. Tager [b], A. Nijenhuis [a] and A. Tromp [a]

[a] *Department of Paediatrics and Clinical Chemistry and* [b] *Laboratory of Biochemistry, University Hospital Amsterdam, Academic Medical Centre, Amsterdam and* [c] *Department of Biochemistry, State University Utrecht (The Netherlands)*

Key words: Peroxisomal disorder; Prenatal diagnosis; Zellweger syndrome; Fatty acid β-oxidation; Peroxisome

Summary

In this paper we show that cultured chorionic villous fibroblasts efficiently catalyse the peroxisomal β-oxidation of hexacosanoic acid (cerotic acid), a saturated very long chain fatty acid containing 26 carbon atoms. Hexacosanoic β-oxidation was found to be strongly impaired in cultured chorionic villous fibroblasts from a Zellweger foetus. This finding indicates that measurement of peroxisomal β-oxidation can be used (in addition to measurement of acyl-CoA : dihydroxyacetone phosphate acyltransferase, de novo plasmalogen biosynthesis, the amount of particle-bound catalase and phytanic acid oxidase) for prenatal diagnosis in the first trimester of Zellweger syndrome, infantile Refsum disease and neonatal adrenoleukodystrophy. The method should be equally applicable to the early prenatal diagnosis of disorders in which there is a deficiency of a single peroxisomal β-oxidation enzyme. Such diseases include X-linked adrenoleukodystrophy (peroxisomal very long chain fatty acyl CoA ligase deficiency), 'pseudo-Zellweger syndrome' (peroxisomal 3-oxoacyl-CoA thiolase deficiency) and 'pseudo-neonatal adrenoleukodystrophy' (acyl-CoA oxidase deficiency).

Correspondence and requests for reprints to: Dr R.J.A. Wanders, Department of Paediatrics, FO-224, Academic Medical Centre, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.

0009-8981/87/$03.50 © 1987 Elsevier Science Publishers B.V. (Biomedical Division)

Introduction

The strong deficiency of peroxisomes in patients with Zellweger syndrome [1] has generally been held responsible for the multitude of biochemical abnormalities found in these patients which include elevated levels of di- and trihydroxy-coprostanoic acid, pipecolic acid, phytanic acid and very long chain fatty acids in the patients' plasma (for reviews, see refs. [2–5]). Very long chain fatty acids were initially thought to accumulate only in patients with X-linked adrenoleukodystrophy (for reviews, see refs. [6,7]). However, in 1982 Brown et al [8] reported that very long chain fatty acid levels were also elevated in plasma from patients with Zellweger syndrome and neonatal adrenoleukodystrophy. Recent studies have shown that very long chain fatty acids are also elevated in infantile Refsum disease [9,10], hyper-pipecolic acidaemia [4], pseudo-Zellweger syndrome [11] and pseudo-neonatal adrenoleukodystrophy [12]. All these inborn errors of metabolism are severe diseases usually leading to death within the first decade of life. In principle, prenatal diagnosis could be done in the first trimester by measurement of very long chain fatty acid β-oxidation in cultured chorionic villous cells. However, no information is available thus far on whether chorionic villous fibroblasts are capable of very long chain fatty acid β-oxidation. We, therefore, decided to study hexacosanoic acid (cerotic acid; C26 : 0) β-oxidation in cultured chorionic villous fibroblasts. The results described in this paper indicate that hexacosanoic acid is oxidized efficiently in these cells. In chorionic villous fibroblasts from a Zellweger foetus hexacosanoic acid β-oxidation was found to be strongly deficient. This finding indicates that the method can be used for the prenatal detection of any peroxisomal disorder in which there is a net deficiency in peroxisomal β-oxidation of very long chain fatty acids either due to the absence of one (X-linked adrenoleukodystrophy, pseudo-Zellweger syndrome, pseudo-neonatal adrenoleukodystrophy) or all (Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy) peroxisomal β-oxidation enzyme proteins.

Materials and methods

Cell culture conditions

Chorionic villous biopsies were taken transcervically at 7-9 weeks of gestation. After dissection of the biopsy under a stereo-microscope tissue was placed into plastic culture flasks and cells cultured as described for fibroblasts derived from skin [13]. At the time of confluency cells were collected by trypsinisation, as described in detail before [13]. Cells were pelleted by centrifugation ($500 \times g_{av}$, 5 min) at room temperature in a Sorvall-GLC-1 centrifuge and the pelleted cells were subsequently washed twice in phosphate-buffered saline (150 mmol/l NaCl plus 10 mmol/l potassium phosphate, pH 7.4). The final pellet was taken up in a buffer containing 250 mmol/l sucrose plus 5 mmol/l morpholinopropane sulphonic acid (MOPS)-NaOH (final pH 7.4). Skin fibroblasts were grown and processed exactly as described before [13].

Measurement of fatty acid oxidation

Incubations were carried out at 37 °C for 60 min using a medium (total volume: 200 μl) of the following composition: 300 mmol/l sucrose, 50 mmol/l morpholinopropane sulphonic acid (pH 7.4), 10 mmol/l ATP, 1 mmol/l NAD^+, 100 μmol/l FAD^+, 5 mmol/l $MgCl_2$, 200 μmol/l coenzyme A, and 10 μmol/l [1-^{14}C]hexacosanoic acid (spec. act. 51 mCi/mmol) dissolved in α-cyclodextrin (final concentration of α-cyclodextrin in the reaction mixture 1 mg/ml). Reactions were started by the addition of fibroblasts (20 μl) at a final concentration of 0.2–0.4 mg protein per ml. Reactions were terminated after 60 min and the amount of $^{14}CO_2$- and ^{14}C-radiolabelled water-soluble products generated was measured as described [14,15].

Very long chain fatty acids analysis

Very long chain fatty acids in cultured skin fibroblasts were determined as described by Moser and coworkers [16–18] with some modifications as described below. In short, about 1 mg of packed human skin fibroblasts grown as described before [13] and kept at −80 °C until analysis was suspended in 500 μl of H_2O and disrupted by sonication. Total lipid extracts were subsequently prepared as described [16,17] and taken to dryness under N_2 after prior addition of heptacosanoic (C27 : 0) methyl ester as internal standard. To each dry total lipid extract 1.5 ml of 1 mol/l hydrochloric acid in methanol was added and the capped tubes placed in an oven at 75 °C for 16 h, followed by cooling at room temperature. Samples were dried again under N_2, solubilized in chloroform/methanol in a v/v ratio of 2 : 1 and applied to prewashed 0.25 μ silica gel G-60 thin layer plates (Merck, Darmstadt, FRG). On both sides of the TLC plate, a mixture of methylesters ranging from C14 : 0 to C27 : 0 was applied and cochromatographed. Plates were developed for 1 h in toluene/ether (97 : 3 v/v). Plates were subsequently dried in air. After 10 min of air drying, methylesters were visualized by blowing over fumes of sodium. The area corresponding to the methyl ester references was scraped off and the scraped material was subsequently extracted with 1 ml of hexane. The hexane phase was collected by centrifugation (1 000 × g_{av}, 2 min) at room temp. This procedure was repeated twice. The combined hexane extracts were taken to dryness under N_2 and the final residue was dissolved in about 30 μl hexane. 1 μl portions of this extract were subsequently analysed on a Packard gas–liquid chromatograph (mode 438) equipped with a 25 m × 0.20 capillary column (100% dimethylpolysiloxane; HP-101) using a splitless capillary injection systems with a gas flow rate of 25 cm/s (helium) and flame ionization detection. Chromatography conditions were as follows. Injector temperature 275 °C; detector temperature 280 °C; initial oven temperature, 50 °C; from 50 °C to 180 °C at 20 °C/min with a final isothermal delay time of 0.3 min, then to 240 °C at 2.5 °C/min with a final isothermal delay time of 5 min, and finally to 275 °C at 2.5 °C/min with a final isothermal delay time of 10 min. Identification of individual peaks occurred by co-chromatography with authentic standards. Peaks were measured with a Shimadzu Chromatopac Data processor (Mode 604) and expressed as percentage of total fatty acids with chain length of 14 carbon atoms or more.

Materials

Nucleotides and enzymes were from Boehringer (Mannheim, FRG) or Sigma (St. Louis, MO, USA). [1-^{14}C]Hexacosanoic acid was prepared as described by Singh et al [19] and found to be radiochemically pure as assessed by thin-layer chromatography. All other reagents were of analytical grade.

Patients

The patients studied in this paper were selected on the basis of established diagnoses based on clinical and biochemical grounds. The Zellweger patients have been described elsewhere (Heymans et al [20]; Wanders et al [21]). The neonatal adrenoleukodystrophy patients have been described in refs. [22–24]. The infantile Refsum patients studied represent the cases described by Scotto et al [25] and Poll-Thé et al [10]. Fibroblasts from a hyperpipecolic acidaemia patient (Thomas et al [26]) were obtained from the Human Genetic Mutant Cell Repository (National Institutes of Health, Camden, USA). Full details on the pseudo-neonatal adrenoleukodystrophy (acyl-CoA oxidase deficiency) patient will be described elsewhere (Poll-Thé et al, in prep.; see [12]).

Results

We studied very long chain fatty acid β-oxidation in cultured skin fibroblasts from patients with Zellweger syndrome, neonatal adrenoleukodystrophy, infantile

TABLE I

Very long chain fatty acid accumulation (C26/C22 ratio) and hexacosanoic acid (C26:0) β-oxidation in skin fibroblasts from controls and patients with different peroxisomal disorders

Fibroblast phenotype	C26/C22 ratio [a]	Hexacosanoic acid β-oxidation activity (pmol/min per mg protein)
Controls	0.067 ± 0.036 (0.01–0.15) (*n* = 39)	2.23 ± 0.41 (*n* = 10)
Zellweger syndrome	0.57 ± 0.23 (0.21–0.98) (*n* = 17)	0.18 ± 0.10 (*n* = 6)
Infantile Refsum disease	0.70 ± 0.44 (0.21–1.05) (*n* = 3)	0.22 ± 0.08 (*n* = 7)
Neonatal adrenoleukodystrophy	0.54 ± 0.27 (0.25–0.86) (*n* = 5)	0.19 ± 0.05 (*n* = 6)
Hyperpipecolic acidaemia	n.d. [b]	0.12 ± 0.05 (*n* = 3)
X-linked adrenoleukodystrophy	0.43 ± 0.15 (0.26–0.66) (*n* = 7)	0.87 ± 0.21 (*n* = 8)
Acyl-CoA oxidase deficiency	0.27; 0.30	0.22 ± 0.06 (*n* = 3)

[a] C26/C22 very long chain fatty acids and hexacosanoic acid β-oxidation were measured as described in 'Materials and Methods'. Values are mean ± SD with the range given in brackets.

[b] n.d., not determined.

TABLE II

Hexacosanoic acid β-oxidation in chorionic villous fibroblasts from controls and a Zellweger foetus [a]

Chorionic villous fibroblasts phenotype	Hexacosanoic acid β-oxidation activity (pmol/min per mg protein)
Control	2.1 ± 0.4 (5)
Zellweger syndrome	0.05; 0.10

[a] For details see 'Materials and Methods'. Results expressed as mean ± SD.

Refsum disease, hyperpipecolic acidaemia, X-linked adrenoleukodystrophy and pseudo-neonatal adrenoleukodystrophy (acyl-CoA oxidase deficiency) (Table I). Fatty acid β-oxidation was determined by measuring the production of $^{14}CO_2$ plus ^{14}C-radiolabelled water-soluble products from [1-^{14}C]hexacosanoic acid. The results show that there is a substantial reduction in the rate of hexacosanoic acid β-oxidation in cultured skin fibroblasts from the patients thus explaining the elevated C26/C22 ratios in fibroblasts from these patients (see column 2 of Table I).

Table II shows that normal chorionic villous fibroblasts efficiently catalyze the β-oxidation of [1-^{14}C]hexacosanoic acid to $^{14}CO_2$ and ^{14}C-labelled water-soluble products. The activity measured is comparable to the rate of hexacosanoic acid β-oxidation in cultured skin fibroblasts from controls (cf. Table I). In chorionic villous fibroblasts from a foetus at risk for Zellweger syndrome a substantially reduced rate of cerotic acid β-oxidation was found. The diagnosis of Zellweger syndrome was confirmed in further studies showing a deficient acyl-CoA : dihydroxyacetone phosphate acyltransferase activity, a deficient de novo plasmalogen biosynthesis, a deficiency of particle-bound catalase and an elevated C26/C22 ratio in these chorionic villous fibroblasts.

Discussion

The results described in this paper indicate that control chorionic villous fibroblasts efficiently catalyze the β-oxidation of hexacosanoic acid, a very long chain fatty acid (C26 : 0), whereas in chorionic villous fibroblasts from a Zellweger foetus, this activity was found to be strongly deficient. Since it is generally accepted that initial β-oxidation cycles in the degradation of very long chain fatty acids proceed in peroxisomes [19], the deficient hexacosanoic acid β-oxidation activity in the fibroblasts reflects the strong deficiency of peroxisomes in these cells. At present several methods are available for prenatal diagnosis of Zellweger syndrome, including measurement of acyl-CoA : dihydroxyacetone phosphate acyltransferase in cultured amniotic fluid cells, chorionic villous fibroblasts or chorionic villi [27–29], measurement of C26/C22 fatty acids in cultured amniotic fluid cells or chorionic villi [28,30,31], measurement of the intracellular localisation of catalase [32] and measurement of phytanic acid oxidase activity [33]. An advantage of the present method is that relatively low amounts of fibroblast protein are required (about 150–200 μg protein).

Furthermore, actual measurement of very long chain fatty acid β-oxidation in chorionic villous fibroblasts does not require prolonged periods of incubation as for phytanic acid oxidase [33] but can be done within 3–4 h thus allowing a rapid decision to be made. Finally, an important consequence of our findings is that this technique in principle allows prenatal diagnosis of other peroxisomal disorders in which the underlying defect is at the level of one (or more) of the peroxisomal β-oxidation enzymes. These include infantile Refsum disease, neonatal adrenoleukodystrophy, hyperpipecolic acidaemia, X-linked adrenoleukodystrophy (peroxisomal very long chain fatty acyl-CoA synthetase [34,35]), pseudo-Zellweger syndrome (peroxisomal 3-oxo-acylcoenzyme A thiolase [36]) and pseudo-neonatal adrenoleukodystrophy (acyl-CoA oxidase deficiency [12]).

Acknowledgements

The authors gratefully acknowledge the help of Drs. A.B. Moser and H.W. Moser in setting up very long chain fatty acid measurements via gaschromatography and for supplying several of the fibroblast cell lines studied in this paper. We thank Dr. Bwee-Tien Poll-Thé and Prof. J.M. Saudubray for providing some of the cell lines, Annie Vandenput, Paul Bentlage, Ellen Meijboom and Wilma Smit for expert technical assistance and Truus Klebach for expert preparation of the manuscript. This research was supported by a grant from the Netherlands Foundation for Medical and Health Research (MEDIGON) and the Princess Beatrix Fund (The Hague, The Netherlands).

References

1 Goldfischer S, Moore CL, Johnson AB, et al. Peroxisomal and mitochondrial defects in the cerebro–hepato–renal syndrome. Science 1973;182:62–64.
2 Kelley RI. The cerebro–hepato–renal syndrome of Zellweger, morphologic and metabolic aspects. Am J Med Gen 1983;16:503–517.
3 Goldfischer S, Reddy JK. Peroxisomes (microbodies) in cell pathology. Int Rev Exp Pathol 1984;26:45–84.
4 Schutgens RBH, Heymans HSA, Wanders RJA, van den Bosch H, Tager JM. Peroxisomal disorders: a newly recognized group of genetic diseases. Eur J Pediatr 1986;144:430–440.
5 Wanders RJA, Schutgens RBH, Heymans HSA, et al. Biochemical analysis of peroxisomal disorders. In: Fahimi HD, Sies H, eds. Peroxisomes in biology and medicine. Berlin: Springer Verlag, 1987;341–352.
6 Moser HW, Moser AB, Singh I, O'Neill BP. Adrenoleukodystrophy: survey of 303 cases: biochemistry, diagnosis and therapy. Ann Neurol 1984;16:628–641.
7 Powers JM. Adreno-leukodystrophy (adreno–testiculo–leuko–meyelo–neuropathic complex). Clin Neuropathol 1985;4:181–199.
8 Brown FR III, McAdams AJ, Cummins, JW, et al. Cerebro-hepatorenal (Zellweger) syndrome and neonatal adrenoleukodystrophy: similarities in phenotype and accumulation of very long chain fatty acids. Johns Hopkins Med J 1982;151:344–351.
9 Poulos A, Sharp P. Plasma and skin fibroblasts C26 fatty acids in infantile Refsum's disease. Neurology 1984;34:1606–1609.
10 Poll-Thé BT, Saudubray JM, Ogier H, et al. Infantile Refsum's disease: biochemical findings suggesting a generalized dysfunction of peroxisomes. J Inher Metab Dis 1986;9:169–174.

11 Goldfischer S, Collins J, Rapin I, et al. Pseudo-Zellweger syndrome: Deficiencies of several peroxisomal oxidative activities. J Pediatr 1986;108:25–32.
12 Pool-Thé BT, Ogier H, Saudubray JM et al. A peculiar peroxisomal disorder: two siblings previously diagnosed as having neonatal-onset adrenoleukodystrophy. Eur J Cell Biol 1986;4:56 (abst.).
13 Wanders RJA, Van Roermund CWT, Van Wijland MJA et al. Peroxisomal very long chain fatty acid β-oxidation in human skin fibroblasts: activity in Zellweger syndrome and other peroxisomal disorders. Clin Chim Acta 1987;:in press.
14 Wanders RJA, Van Roermund CWT, De Vries CI, et al. Peroxisomal β-oxidation of palmitoyl-CoA in human liver homogenates and its deficiency in the cerebro–hepato–renal (Zellweger) syndrome. Clin Chim Acta 1986;159:1–10.
15 Wanders RJA, Van Roermund CWT, Van Wijland MJA, et al. Studies on the peroxisomal oxidation of palmitate and lignocerate in rat liver. Biochim Biophys Acta 1987;in press.
16 Kawamura N, Moser AB, Moser HW, et al. High concentration of hexacosanoate in cultured skin fibroblast lipids from adrenoleukodystrophy patients. Biochem Biophys Res Commun 1978;82:114–120.
17 Moser HW, Moser AB, Kawamura N, et al. Adrenoleukodystrophy: elevated C26 fatty acid in cultured skin fibroblasts. Ann Neurol 1980;7:542–549.
18 Moser HW, Moser AB, Frayer K, et al. Adrenoleukodystrophy: increased plasma content of saturated very long chain fatty acids. Neurology 1981;31:1241–1249.
19 Singh I, Moser AE, Goldfischer S, Moser HW. Lignoceric acid is oxidized in the peroxisome: implications for the Zellweger syndrome and adrenoleukodystrophy. Proc Natl Acad Sci USA 1984;81:4203–4207.
20 Heymans HSA, Van den Bosch H, Schutgens RBH, et al. Deficiency of plasmalogens in the cerebro–hepato–renal (Zellweger) syndrome. Eur J Pediatr 1984;142:10–15.
21 Wanders RJA, Smit W, Heymans HSA, et al. Age-related accumulation of phytanic acid in plasma from patients with the cerebro–hepato–renal (Zellweger) syndrome. Clin Chim Acta, 1987; :in press.
22 Kelley RI, Datta NS, Dobyns WB, et al. Neonatal adrenoleukodystrophy: new cases, biochemical studies and differentiation from Zellweger and related polydystrophy syndromes. Am J Med Genet 1986;23:869–901.
23 Wolff J, Nyhan WL, Powell H, et al. Myopathy in an infant with a fatal peroxisomal disorder. Pediatr Neurol 1986;2:141–146.
24 Wanders RJA, Schutgens RBH, Schrakamp G, et al. Neonatal adrenoleukodystrophy: impaired plasmalogen biosynthesis and peroxisomal β-oxidation due to a deficiency of catalase-containing particles (peroxisomes) in cultured skin fibroblasts. J Neurol Sci 1987;77:331–340.
25 Scotto JM, Hadchouel M, Odièvre M, et al. Infantile phytanic acid storage disease, a possible variant of Refsum's disease: three cases, including ultrastructural studies of the liver. J Inher Metab Dis 1982;5:83–90.
26 Thomas GH, Haslam HA, Gatschan MO, et al. Hyperpipecolic acidaemia associated with hepatomegaly, mental retardation, optic nerve dysplasia and progressive neurological disease. Clin Genet 1975;8:376–382.
27 Schutgens RBH, Schrakamp G, Wanders RJA, et al. The cerebro–hepato–renal (Zellweger) syndrome: prenatal detection based on impaired biosynthesis of plasmalogens. Prenatal Diagn 1985;5:337–344.
28 Hajra AK, Datta NS, Jackson LG, et al. Prenatal diagnosis of Zellweger cerebro–hepato–renal syndrome, N Engl J Med 1985;312:445–446.
29 Carey WF, Robertson EF, Van Crugten C, et al. Prenatal diagnosis of Zellweger syndrome by chorionic villus sampling — and a caveat. Prenatal Diagn 1986;6:227–229.
30 Solish GI, Moser HW, Ringer LD, et al. The prenatal diagnosis of the cerebro–hepato–renal syndrome of Zellweger. Prenatal Diagn 1985;5:27–34.
31 Moser AE, Singh I, Brown FR III, et al. The cerebro–hepato–renal (Zellweger) syndrome. Increased levels and impaired degradation of very long chain fatty acids and their use in prenatal diagnosis. N Engl J Med 1984;310:1141–1146.

32 Wanders RJA, Schrakamp G, Van den Bosch H, et al. Pre- and postnatal diagnosis of the cerebro–hepato–renal (Zellweger) syndrome via a simple method directly demonstrating the presence or absence of peroxisomes in cultured skin fibroblasts, amniocytes and chorionic villi fibroblasts. J Inher Metab Dis 1986;9 (Suppl 2):317–320.
33 Poulos A, Van Crugten C, Sharp P, et al. Prenatal diagnosis of Zellweger syndrome and related disorders: impaired degradation of phytanic acid. Eur J Pediatr 1986;145:507–510.
34 Hashmi M, Stanley W, Singh I. Lignoceroyl-CoA ligase: enzyme defect in fatty acid β-oxidation in X-linked adrenoleukodystrophy. FEBS Lett 1986;196:247–250.
35 Wanders RJA, Van Roermund CWT, Van Wijland MJA, et al. Peroxisomal fatty acid β-oxidation in human skin fibroblasts: X-linked adrenoleukodystrophy, a peroxisomal very long chain fatty acyl-CoA synthetase deficiency. J Inher Metab Dis 1987;in press.
36 Schram AW, Goldfischer S, Van Roermund CWT, et al. Human peroxisomal 3-oxoacyl-coenzyme A thiolase deficiency. Proc Natl Acad Sci USA 1987;in press.