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PEROXISOMAL DISORDERS: COMPLEMENTATION ANALYSIS USING BETA-OXIDATION OF VERY LONG CHAIN FATTY ACIDS

M.C. McGuinness, A.B. Moser, H.W. Moser and P.A. Watkins

Kennedy Institute and the Department of Neurology,
Johns Hopkins University School of Medicine, Baltimore, MD

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Complementation studies, using fused cell lines from patients with peroxisomal disorders, have shown correction of defective plasmalogen synthesis and phytanic acid oxidation as well as an increase in the number of peroxisomes. At least six complementation groups have been reported. We demonstrate here that complementing cell lines also acquire the ability to oxidize very long chain fatty acids (VLCFA), and that complementation groups defined with this technique are identical to those reported previously when plasmalogen synthesis was used as the criterion for complementation. This VLCFA complementation technique is of particular value in the study of patients in whom defective VLCFA is the only or major enzymatic defect, and we show complementation between cell lines from two patients each with an isolated defect in one of the peroxisomal fatty acid beta-oxidation enzymes.

Disorders of peroxisomal biogenesis, including the Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum syndrome and hyperpipecolic acidemia are characterized by an absence or diminution in the number of morphologically detectable peroxisomes in tissues from affected patients. The absence of peroxisomes in these patients is associated with a number of biochemical abnormalities (reviewed in reference 1) including impaired beta-oxidation of very long chain fatty acids (VLCFA), impaired synthesis of plasmalogens, and accumulation of bile acid intermediates, pipecolic acid and phytanic acid. The biochemical and clinical distinctions between these phenotypes, however, are unclear and it is not known whether these diverse disorders represent allelic or non-allelic mutations of peroxisomal biogenesis.

Complementation analysis has been used to examine the relationship between phenotype and genotype in the peroxisomal disorders. Fibroblasts from two different patients, both deficient in a peroxisomal metabolic process, are induced to fuse; the resulting multinuclear cells are examined for their ability to carry out this metabolic function (2). Restoration of activity can only occur if each parental cell line provides the gene product defective in the other. Other investigators have used de novo plasmalogen biosynthesis (3), dihydroxyacetone phosphate acyltransferase activity (4), particle bound catalase (4,5) or phytanic acid oxidase activity (6) as criteria for complementation.

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Singh et al. (7) showed that beta-oxidation of VLCFA, e.g. lignoceric acid ($C_{24:0}$) takes place in peroxisomes rather than mitochondria. The present study is the first to use beta-oxidation of VLCFA as the criterion for complementation when grouping cell lines from patients with peroxisomal disorders. Many of the cell lines used in this study were also used by Roscher and coworkers (3) in complementation studies where plasmalogen biosynthesis was the criterion for complementation. It was of interest, therefore, to compare complementation groups obtained using both beta-oxidation of VLCFA and plasmalogen biosynthesis as indices of complementation to see if the same genetic defect affects both metabolic pathways.

Complementation analysis, using beta-oxidation as the criterion for complementation, may be useful in the study of patients who are suspected of having a single enzyme defect in the peroxisomal beta-oxidation pathway. In many of these patients, all three beta-oxidation enzyme proteins (acyl-CoA oxidase, bifunctional enzyme and beta-ketothiolase) are detectable by immunoblot analysis (McGuinness and Watkins, unpublished observations). These patients may have an inactive form of one of the enzymes which could be detected by complementation studies using cell lines known to be deficient in each of the beta-oxidation enzyme activities.

MATERIALS AND METHODS

Materials: Cell culture reagents were obtained from GIBCO Laboratories (Grand Island, NY). [1-^{14}C]Lignoceric acid (54.0 mCi/mmol) was obtained from Research Products International Corporation and [1-^{14}C]palmitic acid (58.0 mCi/mmol) from New England Nuclear. Ficoll 400 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ) and polyethylene glycol 4000 from Merck (Rahway, NJ). All other reagents were of analytical grade and were obtained from commercial sources.

Cell lines: Cultured skin fibroblasts from twelve patients with disorders of peroxisomal biogenesis were originally obtained for diagnostic purposes. They were maintained in Eagle's Minimum Essential Medium with 10% fetal bovine serum, 1% glutamine and penicillin/streptomycin. Clinical findings for patients SED1 and SED2, with single enzyme defects, have been reported previously by Watkins et al. (8) and Poll-The et al. (9) respectively. The major clinical findings of patients Z2, Z4, Z5, Z6, Z7, N7 and N8 included in this study, where Z and N represent Zellweger and neonatal adrenoleukodystrophy patients, respectively, were reported by Roscher et al. (3). Skin fibroblasts from patients Z9, Z10 and Z11 were also studied.

Cell fusion: Two parental cell lines (1.5 X 10^6 cells each) were seeded in 10cm dishes in a 1:1 ratio and fused with polyethylene glycol 4000 using the method of Roscher et al. (3). Multi- and mono-nucleated cells were separated on a discontinuous Ficoll gradient. After separation of the cells, "fused" and "unfused" cell fractions were examined microscopically. In the present study at least 80% of the cells in the fused cell fraction were multinucleate. The unfused cell fraction contained greater than 95% mononuclear cells. This fraction was equivalent to cocultured cells. Fused cells and those that failed to fuse were recultivated and beta-oxidation measured after a 48-h recovery period.

Fatty acid oxidation: Oxidation of [1-^{14}C]palmitic acid and [1-^{14}C] lignoceric acid to water soluble products was measured as previously described (10).

RESULTS AND DISCUSSION

Fatty acid beta-oxidation was measured in fibroblasts from controls and from two patients known to be deficient in one of the peroxisomal beta-oxidation enzymes (Table 1). The ratio of oxidation of lignoceric acid ($C_{24:0}$) to palmitic acid ($C_{16:0}$) was taken as an indication of the ability of the cells to oxidize VLCFA (8,10,11). VLCFA oxidation in SED1 (immunologically deficient in peroxisomal bifunctional enzyme) and SED2 (immunologically deficient in peroxisomal acyl-CoA oxidase) was 11 and 16% of control, respectively (Table 1), in agreement with previously published results (8,9).

To determine whether complementation analysis could be used in the study of patients with peroxisomal beta-oxidation disorders, we investigated the ability of cell lines SED1 and SED2 to complement each other. Fibroblasts were briefly

TABLE 1

Beta-oxidation of VLCFA in fibroblasts before and after fusion

	Beta-Oxidation of Fatty Acids		C_{24}/C_{16}	% of Control
	C_{16}	C_{24}		
	(pmol/mg/min)			
C1	62.9	14.3	0.228	(100)
C2	61.9	15.2	0.245	93
C1XC2 cc	49.8	11.1	0.222	91
C1XC2 fused	57.1	14.5	0.253	103
SED1	55.7	1.4	0.026	11
SED2	82.9	3.1	0.038	16
SED1XSED2 cc	72.6	4.1	0.056	23
SED1XSED2 fused	64.3	14.3	0.223	91

Oxidation of palmitic acid (C_{16}) and lignoceric acid (C_{24}) was measured in parental fibroblast cell lines, fused and cocultured (cc) cells as described in Methods. In the last column, the C_{24}/ C_{16} ratio is expressed as percentage of control C1. C1 and C2, Controls; SED1, bifunctional protein single enzyme defect; SED2, acyl-CoA oxidase single enzyme defect.

TABLE 2

Complementation analysis of fibroblasts from patients with peroxisomal disorders using beta-oxidation of VLCFA as the criterion for complementation

		1		2		3	4			6
	Z4	Z9	Z2	Z5	N7	Z6	Z7	Z10	Z11	N8
Z4		24/24	21/28						33/104	
Z9		22/16	18/18	42/101					26/69	
Z2			15/15	15/76			12/63	27/77	29/77	
Z5						34/87				
N7							25/129		30/96	
Z6										
Z7								11/19		
Z10								8/10		
Z11									10/13	18/160
N8										

The ratio of oxidation of lignoceric acid (C_{24}) to palmitic acid (C_{16}) is expressed as percentage of a control measured in the same experiment. Numbers above the diagonal represent cocultured cells and those below this line represent fused cells. Z, Zellweger syndrome; N, neonatal ALD. The numbers above the brackets refer to the original group numbers of Roscher et al. (3).

incubated with polyethylene glycol to induce cell fusion. Beta-oxidation of VLCFA in the cocultured, mononucleate cell fraction and the fused, multinucleate cell fraction was measured. Cocultured SED1 and SED2 fibroblasts had a low oxidation ratio but the fused cells oxidized VLCFA at 91% of the control ratio (Table 1), indicating that complementation had occurred.

Having determined that restoration of VLCFA oxidation was a valid indicator of complementation, we investigated the ability of fibroblasts from patients with disorders of peroxisomal biogenesis to complement (Table 2). Results are expressed as a percentage of the oxidation ratio in control cells. The numbers above the diagonal represent cocultured cells and those below this line represent fused cells. Oxidation ratios in parent cell lines (data not shown), cocultured cells or non-complementing cell lines were generally less than 30% of the ratio of normal control cells. An increase of at least 2.5-fold in the oxidation ratio of fused cells over cocultured cells was considered to indicate

complementation. Fusion of control cells (Table 1) or patients' cells (Table 2) with themselves did not affect the oxidation ratio.

Cell lines representing five of the six complementation groups reported by Roscher et al. (3) were studied. As can be seen from Table 2, complementing cell lines using VLCFA oxidation as the criterion segregated into groups that were identical to the groups obtained previously (indicated by brackets at the top of Table 2) when plasmalogen synthesis was used as the criterion for complementation (3). Unfortunately, fibroblasts from patient Z8 (complementation group 5 of Roscher and coworkers) were no longer available.

Complementation analysis was also performed on cell lines from three Zellweger patients which were not included in the study of Roscher et al. (3) (designated Z9, Z10 and Z11). Both VLCFA oxidation and plasmalogen synthesis were measured in fused and cocultured cells. Patient Z9 and patients Z10 and Z11 were found to belong to complementation groups 1 and 4, respectively, of Roscher and colleagues when both beta-oxidation of VLCFA (Table 2) and restoration of plasmalogen synthesis (data not shown) were used as criteria for complementation.

To date, no correlation between genotype and phenotype has been found in studies of fibroblasts from patients with peroxisomal disorders. Roscher et al. (3) showed that all known phenotypes were represented within their complementation group 1 and the most common phenotype, classical Zellweger syndrome, was found in five of their six complementation groups. There is genetic heterogeneity, therefore, not only within the group of peroxisomal disorders but also between different Zellweger cell lines. Complementation groups obtained in the present study, when beta-oxidation of VLCFA was used as the criterion of complementation, were found to be identical to the complementation groups obtained by Roscher and coworkers, supporting the hypothesis that defects in peroxisome assembly affect both VLCFA oxidation and plasmalogen biosynthesis. Imanaka et al. (12) have shown that import of peroxisomal matrix proteins requires the presence of certain membrane proteins, possibly acting as receptors. It is probable, therefore, that a single import mutation may affect both metabolic pathways.

Complementation analysis, using beta-oxidation of VLCFA as the criterion for complementation, will prove useful in the study of patients who, based on clinical diagnosis, are suspected of having a single enzyme defect in the peroxisomal beta-oxidation pathway. The laboratory findings common to these patients include elevated plasma VLCFA, impaired VLCFA oxidation in fibroblasts and normal plasmalogen synthesis (8,9,13). Some patients, with suspected single enzyme defects, were found to be immunologically positive for all three beta-oxidation enzymes (11, and McGuinness and Watkins, unpublished observations).

These patients presumably have an inactive form of one of the oxidation enzymes.

Fusing fibroblasts from patients, with suspected single enzyme defects, with fibroblasts from patients SED1 and SED2 will allow identification of patients who are likely to be deficient in the third peroxisomal beta-oxidation enzyme, beta-ketothiolase. A beta-ketothiolase deficient cell line, along with the two lines known to be deficient in acyl-CoA oxidase and bifunctional enzyme activities, will enable us to study and classify fibroblasts from patients who are suspected of having a single enzyme defect in the peroxisomal beta-oxidation pathway.

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