Restoration of phytanic acid oxidation in Refsum disease fibroblasts from patients with mutations in the phytanoyl-CoA hydroxylase gene

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Abstract Refsum disease (RD) is biochemically characterized by the excessive accumulation of phytanic acid in tissues and body fluids due to deficiency of phytanoyl-CoA hydroxylase (PAHX). In this study, we screened three RD patients and identified a novel deletion (88 amino acids), and a missense mutation (Arg275Trp) in the previously reported PAHX cDNA (Jansen et al., 1997; Mihalik et al., 1997). Moreover, transfection of skin fibroblasts from two RD patients with wild-type PAHX gene restored the activity for α-oxidation of phytanic acid. Southern analysis on a somatic cell hybrid panel detected the PAHX gene on chromosome 10, corroborating radiation hybrid and homozygosity mapping data (Mihalik et al., 1997; Nadal et al., 1995).

Key words: Refsum disease; α-oxidation; Phytanoyl-CoA hydroxylase; Phytanic acid

1. Introduction

Refsum disease (RD, Heredopathia atactica polyneuritiformis) was first reported by Refsum in 1946 as a familial neurological syndrome which is characterized by retinitis pigmentosa, peripheral neuropathy, cerebral ataxia, nerve deafness and excessive accumulation of phytanic acid in tissues and body fluids [4,5]. Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a 20-carbon saturated branched-chain fatty acid, is formed from phytol which is a derivative of the chlorophyll molecule. Humans do not synthesize phytanic acid but consume 50–100 mg/day in diet containing dairy products and fat. Normal human plasma contains only trace amounts of phytanic acid (<0.3 ng/dl), whereas phytanic acid in plasma of RD patients may account for as much as 5–30% of the total fatty acids [5]. In addition to RD, an abnormality in the catabolism of phytanic acid in peroxisomes also causes its accumulation in other peroxisomal inherited metabolic disorders including Rhizomelic Chondrodysplasia Punctata (RCDP), Zellweger Syndrome (ZS), Infantile Refsum disease (IRD) and Neonatal Adrenoleukodystrophy (NALD) [6–11].

Due to the presence of a methyl group at the β-carbon position, the phytanic acid is first α-oxidized to pristanic acid, which is then catabolized by β-oxidation in peroxisomes. The oxidation of phytanic acid to pristanic acid involves activation of phytanic acid to phytanoyl-CoA, and α-hydroxylation of phytanoyl-CoA to α-hydroxyphytanoyl-CoA. This α-hydroxyphytanoyl-CoA further undergoes dehydrogenation to α-ketophytanoyl-CoA and decarboxylation to pristanic acid [2,5,12–15]. The deficient oxidation of phytanic acid as compared to normal oxidation of α-hydroxyphytanic acid in cultured skin fibroblasts of RD patients suggested a defect in the conversion of phytanic acid to α-hydroxyphytanic acid and this defect was attributed to the deficient activity of phytanoyl-CoA hydroxylase (PAHX), an enzyme which catalyzes the first reaction in the α-oxidation of phytanic acid [7,8,11,16,17].

Recently, two research groups have simultaneously reported the sequence of human PAHX cDNA by using partial amino acid sequences of purified rat PAHX protein followed by the search of EST (expressed sequence tags) database [1] or directly searching the EST database for a PTS2 (a peroxisomal matrix protein targeting sequence) signal [2]. PAHX open reading frame (1014 nucleotides) encodes a protein of 41.2 kDa (338 amino acids) and contains a PTS2 signal. PAHX encoded protein is targeted to peroxisomes, interacted with the PTS2 receptor in the yeast two-hybrid assay and had phytanoyl-CoA hydroxylase activity [2]. Radiation hybrid and homozygosity mapping have localized the PAHX gene to chromosome 10 [2,3].

Skin fibroblasts from seven RD patients were screened for mutations in the RT-PCR derived PAHX cDNA [1,2]. This mutational analysis has revealed point mutations or deletions in PAHX cDNA from all examined RD patients, including a deletion of single nucleotide (T164) causing a frame shift, a large deletion of 37 amino acids, and missense mutations (Asn269His or Arg275Trp). Demonstration of mutations in the PAHX cDNA of RD patients indicated that the metabolic abnormality may be due to defects in the PAHX gene, but whether or not such mutations directly cause the dysfunction of phytanic acid α-oxidation has yet to be determined. To answer this question, we have identified the mutations in the PAHX cDNA from three RD patients and transfected normal PAHX cDNA into diseased fibroblasts. The results demonstrated the restoration of phytanic acid α-oxidation activity in RD fibroblasts. Also, the Southern analysis on a somatic cell hybrid panel containing human monochromosomal DNA revealed the presence of PAHX gene on chromosome 10.

2. Materials and methods

2.1. Cell lines and cell culture

Three patients (designated as 1, 2 and 3) with clinical manifestations of RD were studied. Their cultured skin fibroblasts are defective

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Abbreviations: RD, Refsum disease; PAHX, phytanoyl-CoA hydroxylase; RPTLC, reverse phase thin layer chromatography

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in α-oxidation of phytanic acid [7,8]. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech), supplemented with 10% bovine calf serum (HyClone).

2.2. Mutation analysis

A full-length PAHX cDNA, positions −7 to +1086 bp of the published sequence [1], was amplified from total RNA (10 μg) isolated from human normal skin fibroblasts and liver tissue. Two primer sets, carrying an *Eco*RI linker, were designed based on the published sequence [1]. The sequence of forward primer was 5′-TTGAATTCG-CAGCCATGGAGCAGCTTCG-3′ and of reverse primer was 5′-GCGAATTCTTGGTTTTGGTTTTCTGTTGAAAGAGTTATAG-CAG-3′. RT-PCR amplified PAHX cDNA was digested with *Eco*RI and subcloned in the unique *Eco*RI site of pBluescript SK+ vector (designated as SK+PAHX). Complete sequence of the PAHX cDNA was obtained.

PAHX cDNA was also cloned from fibroblasts of patients 1, 2 and 3 as described for normal fibroblasts. The PAHX cDNA clones from these patients were completely sequenced.

2.3. Vector construction and transfection of fibroblasts

SK+PAHX clone was digested with *Eco*RI and the resulting full-length PAHX cDNA was cloned in the sense orientation into the unique *Eco*RI site of pcDNA3 (Invitrogen), a mammalian expression vector, and designated as pcDNA3PAHX. The resulting recombinant construct was sequenced to confirm the orientation of the PAHX gene in pcDNA3 vector.

pcDNA3 alone (negative control) and pcDNA3PAHX DNA (15 μg/75 cm^2 tissue culture flask) was transfected separately into fibroblasts from normal human and RD patients using the LipoTAXI transfection reagent, 150 μl per 75 cm^2 tissue culture flask of 50% cell confluence, according to the manufacturer's protocol (LipoTAXI mammalian transfection kit, Stratagene).

2.4. Enzyme assay for oxidation of phytanic acid

Fibroblasts were processed for PAHX activity assay 72 h after transfection. The assay for oxidation of [(2,3)-^{3}H]phytanic acid was performed essentially as described by Zenger-Hain et al. [18]. Briefly, α-cyclodextrin-solubilized [(2,3)-^{3}H]phytanic acid (3 μM) was added to fibroblasts (25–100 μg protein) suspended in 250 μl of Hank's balanced salt solution (GIBCO BRL). The reaction was stopped by adding 0.625 ml of 1 N KOH in methanol followed by incubation at 60°C in a shaking water bath for 1 h. The amount of radioactivity in the upper phase of Folch partition represented the rate of α-hydroxylation of [(2,3)-^{3}H]phytanic acid.

2.5. Determination of α-hydroxyphytanoyl-CoA formation in human skin fibroblasts

Formation of α-hydroxyphytanoyl-CoA from [1-^{14}C]phytanic acid (55 mCi/mmol) solubilized with β-cyclodextrin was studied with fibroblasts (100–150 μg protein) suspended in 50 mM Tris pH 7.4, 5 mM $MgCl_2$, 0.2 mM coenzyme A, 10 mM ATP, 0.2 mM DTT, 1 mM 2-oxoglutarate, 1 mM Fe^{2+} and 1 mM ascorbate. Reaction mixture was adjusted to pH 7.4. The total volume of the assay was 250 μl containing 20 μM [1-^{14}C]phytanic acid (5 nmol). Reactions were incubated for 60 min at 37°C. After incubation, reaction mixtures were immediately frozen at −70°C. Samples were thawed at room temperature, sonicated and applied directly on the concentrating zone of reverse phase TLC plates (octadecylsilane bonded RPTLC, 20×20 cm, Whatman Labsales Inc.). The plate was developed in MeOH:H_2O (80:20). The chromatogram was autoradiographed and spots were quantitated by densitometric scanning using GS-670 imaging densitometer (Bio-Rad). Standard samples of 1-^{14}C-labeled phytanic acid, α-hydroxyphytanic acid, phytanoyl-CoA and α-hydroxyphytanoyl-CoA were used to identify the individual products present in the reaction mixture.

2.6. Chromosomal localization of the PAHX gene

Human monochromosomal DNA (8 μg/somatic cell hybrid from Mapping Panel #2, version 3, Coriell Cell Repositories) was digested with *Eco*RI, electrophoresed in 0.8% agarose gel, and transferred to a Hybond N^+ membrane (Amersham). PAHX cDNA probe was labeled with ^{32}P-dCTP using Ready-To-Go DNA labeling beads (Pharmacia Biotech) and hybridized to immobilized DNA using ExpressHyb hybridization solution according to the manufacturer's protocol (Clontech).

3. Results

3.1. Mutational analysis of PAHX gene from RD fibroblasts

PAHX cDNA (1026 bp) was isolated from normal human skin fibroblasts and liver using RT-PCR. Both fibroblasts and liver cDNA clones were found 100 percent identical to the reported sequence [1,2]. This cDNA contained an open reading frame of 1014 bp encoding a 338 amino acid protein. Subsequently, PAHX cDNA was cloned from cultured skin fibroblasts of three patients (patient 1, 2 and 3) as described for normal fibroblasts. The complete sequence of PAHX cDNA clones from all three patients was obtained.

The PAHX cDNA analysis from patient 1 showed a large deletion of 88 amino acids (amino acid numbers 138 to 225). Deletion breakpoint in the cDNA sequence is indicated in Fig. 1A as Δ. PAHX cDNA from patient 2 had a single base substitution of T for C (indicated as * in Fig. 1B) at nucleotide position 823, which lead to replacement of arginine for tryptophan (Arg275Trp). The PAHX cDNA from patient 3 carried a large deletion of 88 amino acids identical to patient 1 (Δ in Fig. 1C) and a missense mutation, C823T (Arg275Trp, * in Fig. 1D) identical to patient 2. Mutational analysis on PAHX gene from all three patients suggested that the deficient activity of PAHX in RD may be due to missense mutations and/or a large deletion in this gene.

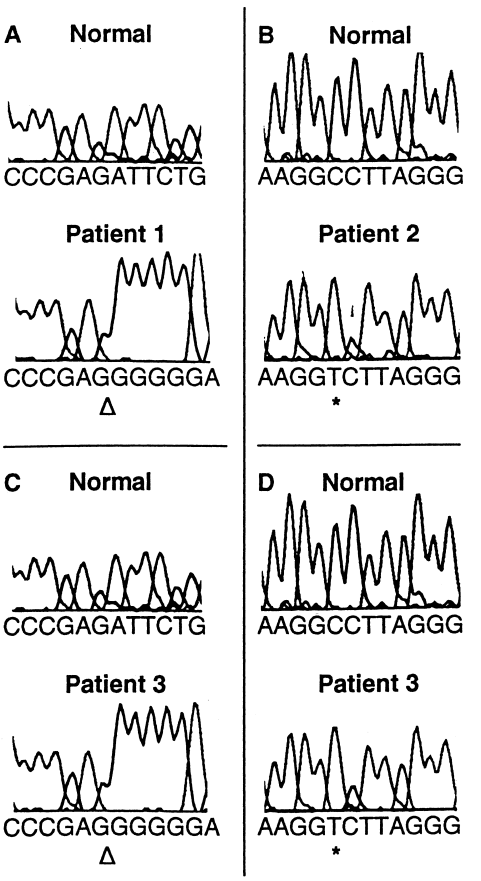

Fig. 1. Electropherograms depicting the sequence surrounding the mutations in the PAHX cDNA from three RD patients as compared with normal PAHX. A and C: Nucleotide sequence surrounding a 88 amino acid deletion (△) in PAHX cDNA from patients 1 and 3. B and D: A point mutation (*), C823T (Arg275Trp) in PAHX cDNA from patients 2 and 3.

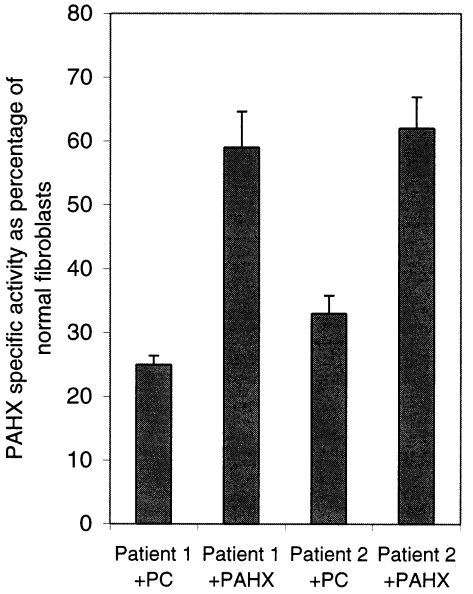

Fig. 2. Restoration of peroxisomal α-oxidation of phytanic acid in RD fibroblasts transfected with normal PAHX gene as determined by the release of tritium following oxidation of [(2,3)-^{3}H]phytanic acid. PC: RD fibroblasts transfected with plasmid only (negative control). PAHX: RD fibroblasts transfected with normal PAHX gene. Data is presented as an average of measured specific activity of PAHX as compared to control from two independent transient transfection assays.

3.2. Correction of phytanic acid metabolism in RD fibroblasts

In an attempt to restore the PAHX activity in RD fibroblasts carrying mutations in the PAHX gene, RD fibroblasts from patients 1 and 2 were transiently transfected with the wild-type PAHX cDNA. The results are the average of two experiments in triplicate (Fig. 2). The α-oxidation of phytanic acid in cultured skin fibroblasts is reduced to 25% in patient 1 and 33% in patient 2 as compared with normal fibroblasts (Fig. 2). An increase of 136% and 88% PAHX activity was observed in patients 1 and 2 (Fig. 2, patient 1+PAHX, patient 2+PAHX), respectively, as compared with RD fibroblasts transfected with plasmid only in transient transfection assays (Fig. 2, patient 1+PC, patient 2+PC). This increase accounts for 59% (patient 1) and 62% (patient 2) of normal PAHX enzyme activity.

α-Hydroxyphytanoyl-CoA is synthesized from phytanoyl-CoA, the first reaction in the catabolism of phytanic acid to pristanic acid. To further confirm that the transgene PAHX is functional in RD fibroblasts, α-hydroxyphytanoyl-CoA was determined in human cultured skin fibroblasts by reverse phase TLC. The results demonstrated an increase of 95% in the level of α-hydroxyphytanoyl-CoA in transfected RD fibroblasts (Fig. 3, patient 2+PAHX) as compared to RD fibroblasts transfected with only plasmid (Fig. 3, patient 2+PC). This increase in the metabolite is comparable to 88% increase in PAHX enzyme activity observed in transfected RD fibroblasts (Fig. 2, patient 2+PAHX) over negative control (Fig. 2, patient 2+PC).

These transfection studies demonstrate that transfection of RD fibroblasts with the PAHX cDNA restores the α-oxidation of phytanic acid in RD patients and that it is responsible for the deficient oxidation of phytanic acid in RD patients.

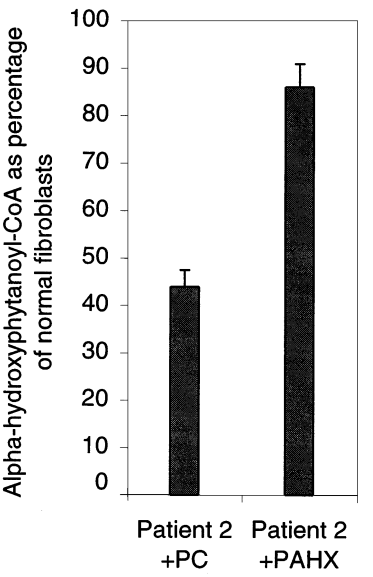

Fig. 3. Levels of α-hydroxyphytanoyl-COA in RD and PAHX-transfected RD cultured human skin fibroblasts relative to normal fibroblasts as determined by reverse phase TLC. PC: RD fibroblasts transfected with plasmid only (negative control). PAHX: RD fibroblasts transfected with normal PAHX gene.

3.3. Chromosomal localization of the PAHX gene

Full-length PAHX cDNA probe (^{32}P-labeled) was hybridized to *Eco*RI-digested DNA from a human/rodent somatic cell hybrid panel. Four signals (12 kb, 7 kb, 3 kb and 2.5 kb) were detected in chromosome 10 (Fig. 4, lane 10) and human DNA (Fig. 4, lane 25). Two signals (12 kb and 10 kb) detected in mouse DNA (Fig. 4, lane 26) were also visible in DNA samples representing human chromosome numbers 1, 16, 17, 20 and 21 (Fig. 4, lanes 1, 16, 17, 20 and 21) where DNA was from human/mouse somatic cell hybrids. These results clearly indicate the presence of PAHX gene on chromosome 10.

4. Discussion

The identification of a number of inherited metabolic disorders associated with peroxisomal dysfunction has provided

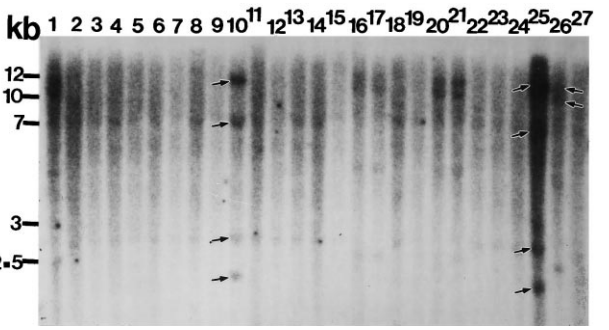

Fig. 4. Chromosomal localization of the PAHX gene. *Eco*RI-digested human monochromosomal DNA was hybridized with ^{32}P-dCTP-labeled PAHX cDNA. Lanes 1 to 27 represent DNA (8 μg/lane) for human chromosomes number 1 to 22, X, Y, human, mouse and Chinese hamster, respectively. Molecular sizes (kb) for hybridization signals discussed in the text are indicated on the left and with arrows on lanes.

a stimulus to studies for elucidation of molecular events associated with the biogenesis, disease process and biochemistry of peroxisomes [9]. Peroxisomal α-oxidation of phytanic acid attracted attention of several research groups because the deficient activity of PAHX enzyme causes an accumulation of phytanic acid, presumably in cytotoxic amounts, in RD and other peroxisomal disorders including RCDP, ZS, IRD and NALD [1,2,6,8,10,11]. In an effort to understand the metabolic role of already sequenced PAHX gene [1,2], mutational analysis was performed on three RD patients followed by transient transfection assays in RD fibroblasts.

Sequence analysis of PAHX cDNA from all three investigated patients revealed mutations involving a novel deletion of 88 amino acids and a missense mutation (Arg275Trp). Deletion identified in PAHX gene of patients 1 and 3 is distinct in sequence from an already reported deletion of 37 amino acids in four RD patients [1,2]. However, a missense mutation (Arg275Trp) identified in patients 2 and 3 is identical to one reported by Mihalik et al. [2] in a RD patient. A search for exon-exon junctions using a computer software, BCM Gene Finder (Baylor College of Medicine, Houston, Texas) indicated the presence of exon-exon junctions surrounding the 88 amino acid deleted region. Thus, this deletion might have been caused by an exon-skipping. When an altered cDNA, carrying a point mutation (Arg275Trp), was expressed in *E. coli*, the purified protein was enzymatically inactive suggesting that Arg275Trp is responsible for the lack of normal activity of PAHX enzyme [2]. We observed the same mutation (Arg275Trp) in one patient.

In transient transfection assays, an increase of PAHX activity by 136% (patient 1) and 88% (patient 2) in PAHX-transfected fibroblasts as compared to control RD fibroblasts (measured as release of ^{3}H from [(2,3)-^{3}H]phytanic acid [18]) suggests that the PAHX restored the PAHX activity in RD peroxisomes. This finding was further confirmed by the observation of a comparable increase (95%) in formation of α-hydroxyphytanoyl-CoA in PAHX-transfected RD fibroblasts (patient 2) relative to RD fibroblasts transfected with plasmid only. This observation clearly demonstrates that abnormality in phytanic acid metabolism in RD is due to defect in the α-hydroxylation of phytanic acid to α-hydroxyphytanoyl-CoA.

PAHX gene has been placed on human chromosome 10 based on radiation hybrid data and homozygosity mapping [2,3]. This finding was corroborated by using a human/rodent somatic cell hybrid panel representing DNA for individual human chromosomes. In addition, present results suggested genomic heterogeneity between human and mouse PAHX gene.

In conclusion, the studies reported here have identified a novel deletion in PAHX gene, not reported previously, in RD. Moreover, these studies have provided an evidence of involvement of PAHX in RD.

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