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MOLECULAR GENETICS OF PEROXISOMAL DISORDERS

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1. ABSTRACT

Twenty five human peroxisomal disorders have been defined at this time. They are subdivided into two major categories: 1) the disorders of peroxisome biogenesis, in which the organelle fails to form normally, and there are defects that involve multiple peroxisomal functions; and 2) disorders that affect single peroxisomal enzymes. During the last five years the molecular defects have been identified in nearly all. These recent advances have several important implications. They have facilitated diagnosis of affected patients. The improved capacity to provide prenatal diagnosis and heterozygote identification has been of great value for genetic counseling and disease prevention. Study of genotype-phenotype correlations has led to a new and more rational classification system. The identification of the molecular defects and the development of animal models have increased understanding of pathogenetic mechanisms, and have led to novel therapeutic approaches.

2. INTRODUCTION

The peroxisome was named in 1965 (1), and its normal function and alterations in disease states are the subject of several recent reviews, books and symposia (2-5). The peroxisome is present in all human cells other than the mature erythrocyte. It carries out a large variety of metabolic functions, including the production and degradation of hydrogen peroxide and many reactions that involve lipids (2). At least twenty-five genetically determined human peroxisomal disorders have been described and are subdivided into two major categories. The first category is Disorders of Peroxisome Biogenesis (PBD). In these disorders the organelle fails to be formed normally and this causes defects in multiple peroxisomal proteins. In the second category the defects involve single peroxisomal enzymes. The molecular defects that underlie nearly all of these disorders have been defined during the last five years. These new developments have facilitated diagnosis, increased greatly the effectiveness of genetic counseling and disease prevention, revolutionized classification, improved understanding of disease mechanisms, and have provided new therapeutic leads.

3. MATERIALS AND METHODS

Patients included in these studies were diagnosed to have one of the peroxisomal disorders listed in table 1**.** Complementation groups were established by studies of cultured skin fibroblasts with the aid of analytical, enzymatic and immunohistochemical techniques (6). The definition of molecular defects in the various human complementation groups was aided by studies of yeast mutants (7) and CHO cell lines (8) in which the molecular abnormalities leading to defective peroxisome biogenesis had been defined. Mouse models of X-linked adrenoleukodystrophy (X-ALD) were achieved by the targeted disruption of the X-ALD gene (9)(10)(11) and of Zellweger syndrome by targeted disruption of the *PEX 2* (12) or *PEX5* (13) genes.

4. MOLECULAR BIOLOGY OF THE DISORDERS OF PEROXISOME BIOGENESIS

Peroxisomal proteins are synthesized in cytoplasmic polyribosomes and imported into the organelle posttranslationally (14). The mechanisms that control

Table 1: Genetically Determined Peroxisomal Disorders

Assembly Deficiencies	Ref.	Single Peroxisomal Enzyme Deficiencies	Ref.
Zellweger syndrome	23,24	X-linked adrenoleukodystrophy	62
Neonatal adrenoleukodystrophy	25,26	Acyl-coenzyme A oxidase deficiency	40
Infantile Refsum disease	27	Bifunctional enzyme deficiency	39,43
Rhizomelic chondrodysplasia punctata	28	Peroxisomal thiolase deficiency	41,42
		DHAP alkyl transferase deficiency	47
		Alkyl DHAP synthase deficiency	46
		Glutaric aciduria type III	75
		Classical Refsum disease	49,50
		Hyperoxaluria type I	53,54
		Acatalasemia	76

import have been reviewed recently (15,16). Proteins that are "destined" for the peroxisome contain targeting sequences. Two of the sequences have been identified and are referred to as Peroxisome Targeting Sequences (PTS). PTS 1 is a carboxy-terminal tripeptide with the consensus sequence SKL or a conservative variant. This signaling sequence is used by the majority of peroxisomal matrix proteins (17). PTS 2 contains the sequence R-L/I-x5-H-L, located 2 to 12 residues from the amino-terminus of the protein (15,18). The targeting sequences of peroxisomal membrane proteins are less well understood (16). Proteins that contain one of the targeting sequences then interact with another set of proteins required for peroxisome biogenesis. These proteins are under intensive study (15,16). A new nomenclature, has been agreed upon (19). Genes and proteins required for peroxisome biosynthesis are now referred to as peroxins. They are numbered sequentially in accordance with the date of publication of their description. The latest count exceeds twenty. The gene is referred to in italicized capitals, and the gene product in lower case. In this review we will mention those that have been found to be defective in human disorders of Peroxisome Biogenesis (PBD).

Two general strategies have been of great value for the definition of the molecular defects in human PBD. The combined use of these two strategies has led to astoundingly rapid advances. The first strategy was complementation analysis. The underlying principle of this technique is that fibroblasts from two different patients, both deficient in a peroxisomal process, are induced to fuse. The resulting multinucleated cells are examined for their ability to carry out this metabolic process. Restoration of activity can occur only if each cell line provides the gene product defective in the other, and cell lines that complement each other in this way thus must represent distinct genotypes. Brul *et al* were the first to apply this approach to the study of peroxisomal disorders (20). It has now been applied to large series of patients and twelve complementation groups have been identified (6,21). Table 2 lists the twelve complementation groups that have been established. Complementation analyses were conducted independently in several laboratories. Three different numbering systems were developed in Japan, the Netherlands, and the US. Table 2 lists the three systems as well as the correspondence between them.

The second strategy involved the study of defects in peroxisome biogenesis in yeast (7), and CHO cell lines (8). These cell systems facilitate the rapid creation and identification of defects of peroxisome biogenesis. The next step was to use the human expressed sequence tags (EST) data base to screen cell lines from PBD patients in each of the complementation groups for defects in genes known to be associated with yeast and CHO PBD defects. This approach led to the demonstration that complementation group 2 (US nomenclature) is due to a defect in *PEX*5, the receptor for PTS1. Table 2 shows that the molecular defects have now been defined in ten of the complementation groups. Complementation group 1 (US nomenclature) is by far the largest. Only two complementation groups, US group 8 and Japanese group G, remain undefined. Both of these groups include only small number of patients. Indeed, the molecular defect has been defined in 96% of the 182 PBD patients listed in table 2. A recent review lists the biochemical properties of the peroxins and the specific mutations that have been identified (22).

5. GENOTYPE-PHENOTYPE CORRELATIONS IN THE DISORDERS OF PEROXISOME BIOGENESIS

Most PBD patients have one of four phenotypes: Zellweger syndrome (ZS) (23,24), neonatal adrenoleukodystrophy (NALD) (25,26), Infantile Refsum Disease (IRD) (27), or Rhizomelic Chondrodysplasia Punctata (RCDP) (28). These disorders were named and their clinical features and histopathology were described before their relationship to the peroxisome was recognized. The clinical features of these disorders are summarized in Table 3. The results of the molecular studies listed in Table 2 have had a profound effect on the classification and the conceptual framework of these disorders. The ZS, NALD and IRD phenotypes can be associated with eleven different complementation groups and with nine different molecular defects. This finding, combined with the analysis of the clinical features of these disorders (6,29), indicates that they represent a clinical continuum, with ZS the most severe, IRD the least severe, and NALD intermediate. In contrast, among the PBD, RCDP is associated only with a defect in *PEX7*. Since ZS, NALD, and IRD can be associated with a variety of genetic defects, the classification system should be altered. In those instances where the molecular defect has been identified, the diagnosis that is assigned should refer to the molecular defect. Definition of the molecular defect also permits identification of heterozygotes.

More detailed analyses of the nature and the severity of the gene defects provide explanations for the variability of phenotype that may be associated with the same gene defect. The severity of phenotypic expression in *PEX1* deficiency varies with the nature of the mutation. The G843D missense mutation, which does not abolish import of peroxisomal proteins completely, was found to be associated with the NALD or IRD or still milder

Table 2. Genotypes, Phenotypes and complementation groups associated with disorders of peroxisome biogenesis

Pex Defect	Complementation Group			Number of Patients	Phenotypes
	US	Japan	Netherlands	KKI	
1	1	E	2	99	ZS, NALD, IRD
2	10	F	5	2	ZS
5	2		4	2	ZS, NALD
6	4	C	3	16	ZS, NALD
7	11		1	43	RCDP
10	7	B		5	ZS, NALD
12	3			5	ZS, NALD
13		H		2	ZS, NALD
16	9	D		1	ZS
19		J			ZS
Unidentified	8	A		7	ZS, NALD, IRD
Unidentified		G			ZS

Abbreviations: ZS : Zellweger syndrome, NALD: Neonatal adrenoleukodystrophy, IRD: Infantile Refsum Disease, KKI: Kennedy Krieger Institute.

Table 3: Major clinical features of disorders of peroxisome assembly and their occurrence in various peroxisomal disorders

Feature	ZS	NALD	IRD	Oxidase deficiency	Bifunctional enzyme deficiency	Thiolase deficiency	RCDP	DHAP synthase deficiency	DHAP alkyl transferase deficiency
Average age at death or last follow-up (year)	0.76	2.2	6.4	4.0	0.75	0.9	1.0	0.5	?
Facial dysmorphism	++	+	+	0	73%	+	++	++	++
Cataract	80%	45%	7%	0	0	0	72%	+	+
Retinopathy	71%	82%	100%	2+	+	0	0	0	0
Impaired hearing	100%	100%	93%	2+	?	?	71%	33%	100%
Psychomotor delay	4+	3 - 4+	3+	2+	4+	4+	4+	4+	?
Hypotonia	99%	82%	52%	+	4+	+	±	±	?
Neonatal seizures	80%	82%	20%	50%	93%	+	±	?	?
Large liver	100%	79%	83%	0	+	+	0	?	
Renal cysts	93%	0	0	0	0	+	0	0	0
Rhizomelia	3%	0	0	0	0	0	93%	+	+
Chondrodysplasia punctata	69%	0	0	0	0	0	100%	+	+
Neuronal migration defect	67%	20%	±	?	88%	+	±	?	?
Coronal vertebral cleft	0	0	0	0	0	0	+	+	+
Demyelination	22%	50%	0	60%	75%	±	0	0	0

Percentages indicate the percentage of patients in whom the abnormality is present.0, abnormality is absent; ± to 4+, degree to which an abnormality is present From (77).

phenotypes (30,31). In contrast, the c.2097-2098insT mutation, which abolishes peroxisome import completely, is associated with the most severe phenotype (ZS). Analogous correlations have been noted for other peroxin defects (22), including *PEX 7* deficiency. Here the L292X mutation, which abolishes completely the import of proteins that contain the PTS2 signal, is associated with the

classical and severe RCDP phenotype, whereas the A218V mutation appears to be associated with milder phenotypes (32). Mosaicism is another possible explanation for variations in phenotypic expression. Five PBD patients with relatively mild phenotypes have been reported in which the import defect showed variable expression in different tissues, or within the same tissue (33-36). At the cellular level these patients show variability of expression between the liver and cultured skin fibroblasts. In one patient, who at 23 years had a phenotype that resembles Charcot-Marie Tooth Disease, liver biopsy showed a severe defect of catalase import in all hepatic cells, while this import was normal in 90% of cultured skin fibroblasts (37). It is plausible that the admixture of a substantial number of cells with normal import mechanisms would result in a milder phenotype.

The recent creation of two mouse "knockout" models (12,13) will increase our understanding of the pathogenesis of ZS, including the characteristic defect in neuronal migration (38).

6. MOLECULAR BIOLOGY OF DEFECTS THAT AFFECT SINGLE PEROXISOMAL ENZYMES

6.1. Defects that affect single enzymes in peroxisomal fatty acid oxidation

The clinical manifestations associated with defects of the peroxisomal bifunctional enzyme (39), peroxisomal acyl-CoA oxidase (40), and peroxisomal thiolase (41,42) mimic those of the ZS-NALD-IRD continuum associated with PBD. Biochemical and histocytochemical studies permit distinction, since peroxisome structure is preserved in the single enzyme defects, catalase import is preserved, and biochemical studies show that all of biochemical disturbances are attributable to the single enzyme defect (6,29). The defect in peroxisomal bifunctional enzyme is the most frequent and in our series accounted for 18% of patients with the ZS-NALD-IRD phenotype. It has been shown recently that the defect in most, and probably all, patients with this disorder involves the D-bifunctional and not the L-bifunctional protein. Mutations in the D-bifunctional protein were found in all of the nine patients who were tested (43). Grunsven *et al* (44) reported a patient homozygous for a missense mutation (N457Y) which led to inactivation of the enoyl-CoA hydratase domain of the enzyme, but left intact the capacity to metabolize bile acids.

Defects of single enzymes involved in plasmalogen synthesis mimic the clinical manifestations of *PEX7* deficiency (Rhizomelic Chondrodysplasia Punctata).

The first two steps in the synthesis of plasmalogens take place in the peroxisome (45). Defects that involve either of these enzymes, dihydroxyacetonephosphate synthase (46) or acyl-CoA dihydroxyacetonephosphate acyltransferase (47) are associated with the clinical manifestations of RCDP. They cannot be distinguished clinically from *PEX7* deficiency. They can be distinguished biochemically, since in patients with *PEX 7* deficiency the capacity to metabolize phytanic acid and pristanic acid is impaired but is intact in the patients with defects confined to plasmalogen synthesis. The gene that codes for acyl-CoA dihydroxyacetone acyltransferase has been cloned recently (48).

7. THE MOLECULAR BIOLOGY OF REFSUM DISEASE

The gene that is deficient in Refsum disease was identified in 1997 simultaneously in two laboratories (49,50) more than fifty years after the disease was defined as a clinical entity (51). The deficient enzyme, phytanoyl-CoA hydroxylase is localized to the peroxisome (52). It is a 41.2 kd protein that contains a cleavable PTS2 signal. Several types of inactivating mutations have been identified in patients with the disease (49,50).

8. THE MOLECULAR BIOLOGY OF HYPEROXALURIA TYPE 1

Primary hyperoxaluria type 1 (PH1) is a rare autosomal recessive disorder of glyoxylate metabolism caused by deficiency of the liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase 1(AGT). In normal individuals, AGT catalyzes the transamination of glyoxylate to glycine. In individuals with PH1, the deficiency of AGT results in the increased oxidation of glyoxylate to oxalate. The increased excretion of oxalate, and the low solubility of calcium oxalate lead to progressive kidney damage (53). Approximately 60% of PH1 patients have undetectable levels of AGT. However, the remainder have activities that vary from 2 to 48% of the mean normal activity. In these patients a portion of the enzyme is mistargeted to the mitochondrion (54). ALT is found exclusively in the peroxisomal matrix in the livers of most normal individuals. In the subset of PH1 patients in whom some ALT activity is retained, the majority of AGT (90 to 95%) is found in the mitochondrial matrix, with only a minority (5 to 10%) in the peroxisome. Danpure *et al* have investigated the mechanism of this mistargeting (54). While AGT contains the PTS1 signal, and thus would be expected to be targeted to the peroxisome, they demonstrated that the mistargeting of AGT was associated with a set of three amino acid substitutions P11L, G170R, and I340M. They have examined the molecular mechanism of this phenomenon in detail and found that targeting is also related to protein folding and the capacity to form dimers. The mechanisms that control AGT targeting are of general biological interest. In humans, baboons, macaques, rabbits, and guinea pigs it is normally targeted to the peroxisome. In marmosets, rats, mice, hamsters, and opossums it is evenly divided between the peroxisome and mitochondrion, whereas in cats and dogs >90% is in the mitochondrion. Targeting characteristics appear to be the result of dietary selection pressure. In herbivorous animals on high carbohydrate, high oxalate diet, the glyoxylate detoxifying mechanisms in the peroxisome would be favored, whereas in carnivorous animals who

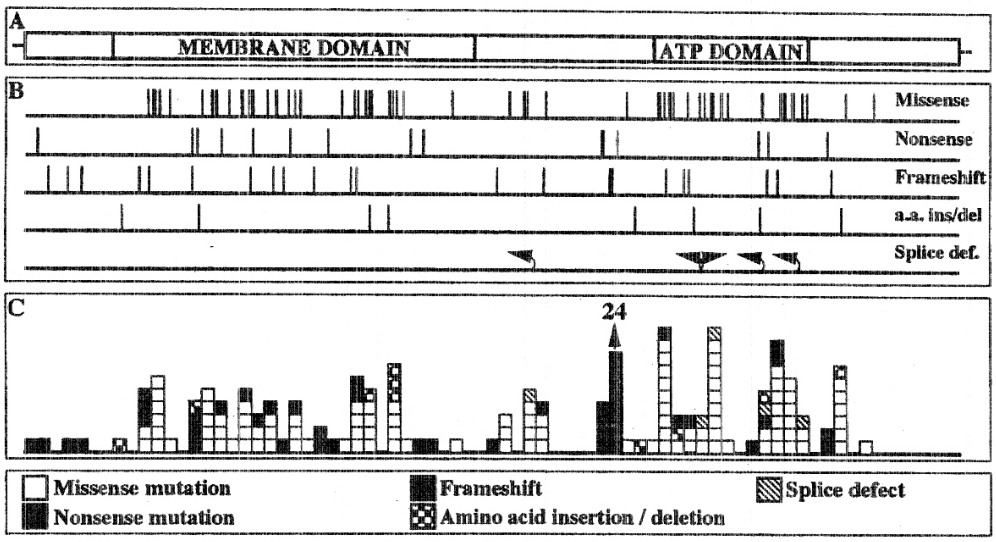

Figure 1. Graphic presentation of mutations in the X-linked adrenoleukodystrophy (ALD) gene identified worldwide, including those reported by Takano *et al* (78). A. Schematic presentation of the ALD open-reading frame. The putative transmembrane and adenosine triphosphate (ATP)-binding domains are shown. B. Distribution of nonrecurrent mutations in the ALD gene. Each vertical bar represents the location of a "private" mutation. Mutations are grouped by type. The density of missense mutations is greatest in the transmembrane and ATP-binding domains. The arrows in the splice defect column indicate whether the defect affect the splice donor or splice acceptor site. C. Presentation of all mutations identified in the ALD gene other than the 15 large chromosomal deletions that have been identified. The common dinucleotide deletion that was observed in 24 families is not to scale. From (79).

consume high-protein, low-carbohydrate, low-oxalate diets the gluconeogetic role of AGT in the mitochondrion is desirable.

9. MOLECULAR BIOLOGY OF X-LINKED ADRENOLEUKODYSTROPHY

The principal biochemical abnormality in X-linked adrenoleukodystrophy (X-ALD) is the abnormal accumulation of saturated very long chain fatty acids (VLCFA) due to the impaired capacity to degrade these substances in the peroxisome (55). At the biochemical level the step that is deficient is the impaired capacity to form the Co-A derivative of VLCFA (56). This reaction is catalyzed by the enzyme VLCFA acyl-CoA synthetase (VLCS). One VLCS was purified by Uchida *et al* (57). The gene that is deficient in X-ALD was identified and cloned in 1993 (58). It codes for a protein referred to as ALDP. It came as a surprise that ALDP is a member of a family of proteins referred to as ATP-Binding Cassette (ABC) proteins, and has no homology to VLCS. There are more than fifty ABC proteins, most of which are involved in transport functions (59). The cystic fibrosis protein also is an ABC protein. More than 200 different mutations have been identified in X-ALD patients (60,61). Figure 1 shows the location and nature of the mutations. The phenotypic expression of X-ALD varies widely. It ranges from the childhood cerebral form, which causes severe disability and often death prior to the tenth year, to milder adult forms, referred to as adrenomyeloneuropathy (AMN), and forms that manifest with adrenal insufficiency only (62). Detailed analysis has failed to demonstrate a correlation between phenotype and the nature of the mutation. In one family five different phenotypes were associated with the same mutation (63). Large deletions, in which the gene product is absent, may be associated with mild phenotypes, whereas the severe childhood cerebral form may be associated with missense mutations in which ALDP protein (albeit enzymatically inactive) can be demonstrated immunologically. Figure 1 shows that most mutations are non-recurring, that is, unique to a particular kindred. Boehm *et al* have developed a DNA-based assay that permits identification of the mutation in X-ALD heterozygotes (64) and is available on a service basis. This technique is of great value for genetic counseling and disease prevention. It can also be applied for prenatal diagnosis (65).

ALDP by itself does not have VLCS activity. However, when it is added to cultured skin fibroblasts of X-ALD patients, it restores their capacity to metabolize VLCFA (66,67). The mechanism by which ALDP restores VLCFA degradation is still unknown. Postulated mechanisms include a role in the transport of VLCFA or of VLCS into the peroxisome. Steinberg *et al* (68) have cloned the human ortholog of the rat liver VLCS described by Uchida *et al* (57) and developed an antibody to it. They then performed immunocytochemical studied in cultured skin fibroblasts of patients with X-ALD and demonstrated that it was located in the peroxisome matrix as it is in normal cells (69). This makes it unlikely that ALDP is required for the transport of VLCS into the peroxisome. It should be noted, however, that there appear to be more than one human VLCS. Recent data by Watkins *et al* (70) suggest that there exists a family of human VLCS. Their tissue distribution and interaction has not yet been fully

defined. The intriguing recent report by Min and Benzer (71) enhances the interest of such studies. These authors showed that disruption of the *bubblegum* gene in the fruitfly leads to a disease state that resembles X-ALD and is associated with VLCFA accumulation. *Bubblegum* has homology to fatty acid synthetases, is present in normal human brain and the possibility exists that it plays a role in the pathogenesis of X-ALD.

It has been demonstrated also that mammalian peroxisome contain three other ABC transport proteins in addition to ALDP. A protein referred to as ALDR (ALD-related) is of greatest interest (72). ALDR has been mapped to 12q11, has 66% identity to ALDP and its gene structure is similar to that of ALDP (73). Of greatest significance is the fact that ALDR can substitute for the function of ALDP in restoring the capacity of X-ALD cells to metabolize VLCFA (67). Kemp *et al* have utilized this finding to develop a possible new therapy for X-ALD (74). They demonstrated that the pharmacological agent 4-phenylbutyrate (4PB) leads to a three-fold increase in the expression of ALDR and restores the capacity of X-ALD cells to metabolize VLCFA. Of greatest interest is their finding that administration of 4PB to a mouse model of X-ALD (9) normalized the levels of VLCFA in the brain and adrenal of these animals. This raises the possibility of pharmacologic gene therapy based upon the principle of increasing the expression of redundant genes. Studies to test this approach in X-ALD patients are in progress.

10. CONCLUDING REMARKS

During the last five years the molecular basis of the majority of peroxisomal disorders has been defined. The disorders of peroxisome biogenesis have been subdivided into twelve complementation groups and the molecular defects have been defined in ten. These findings permit the identification of heterozygotes, facilitate prenatal diagnosis and provide a new and rational classification system. Definition of the molecular defect in X-linked adrenoleukodystrophy permits accurate identification of female carriers, adds to the capacity for genetic counseling and disease prevention, and has provided a lead for a new therapeutic approach. Animal models of Zellweger syndrome and X-linked adrenoleukodystrophy have been developed and provide the opportunity to define pathogenetic mechanisms and evaluate new therapies.

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