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MOLECULAR BASIS OF ZELLWEGER SYNDROME, β-KETOTHIOLASE DEFICIENCY AND MUCOPOLYSACCHARIDOSES

Tadao ORII

Chubu Women's College, 4909-3 Mukaiyama, Kurachi, Seki 501-32, Japan

Summary 1. A human peroxisome assembly factor-1 (PAF-1) complementary DNA has been cloned that restores the morphological and biochemical abnormalities (including defective peroxisome assembly) in fibroblasts from a patient with group F Zellweger syndrome. The cause of the syndrome in this patient was a point mutation that resulted in the premature termination of PAF-1. The homozygous patient apparently inherited the mutation from her parents, each of whom was heterozygous for that mutation. Furthermore, we cloned and characterized the rat and human cDNAs for peroxisome-assembly factor-2 (PAF-2), which restores peroxisomes of the complementary group C Zellweger cells, by functional complementation, and identified two pathogenic mutations in the PAF-2 gene in two patients. 2. Seventeen mutations have been identified in 13 mitochondrial acetoacetyl-CoA thiolase-deficient patients. 3. We purified *N*-acetylgalactosamine-6-sulfate (GalNAc6S) sulfatase and cloned the full-length cDNA of human *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS). The gene encoding GalNAc6S sulfatase has been localized by fluorescence *in situ* hybridization to chromosome 16q24, and the entire genomic gene structure has been characterized. About 40 different GALNS gene mutations have been identified in the patients with mucopolysaccharidosis IVA.

1. Zellweger syndrome

Clinical findings. Newly delivered babies with Zellweger syndrome are severely hypotonic. Spontaneous movements are lacking and feeding is difficult. Some patients need assisted ventilation and gavage feeding. Typical craniofacial dysmorphism including large anterior fontanel, high forehead, hypertelorism, epicanthus, broad nasal ridge, low set ears, high arched palate and micrognathia is present. Psychomotor development is poor and patients seldom acquire a social smile or head control. Convulsions resistant to anticonvulsants usually occur. Hepatomegaly and hepatic dysfunction gradually become obvious. Renal function

is not disturbed, though renal cortical microcysts are characteristically identified at autopsy. Calcific stippling, mainly in the patella, is a typical roentogenographic finding.

Pathological findings. A typical neuropathological finding is a defect in neuronal migration. Macroscopically, polymicrogyria, pachygyria, agenesis of corpus callosum and enlargement of posterior horns of lateral ventricles are often noted.

Biochemical abnormalities. In 1982, Brown et al. reported an accumulation of VLCFA such as tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0) in patients with Zellweger syndrome and neonatal adrenoleukodystrophy (Brown *et al.*, 1982). They pointed out the relationship between the defect of peroxisomes and biochemical abnormalities. Three peroxisomal β-oxidation enzymes, acyl-CoA oxidase, bifunctional enzyme and 3-ketoacyl-CoA thiolase, are deficient (Tager *et al.*, 1985). These enzymes are synthesized normally, however, degradation occurs rapidly due to abnormal processing and localization (Suzuki *et al.*, 1988). The accumulation of VLCFA closely relates to neural dysfunction and migration disorders, since deficiencies of peroxisomal β-oxidation shared similar findings.

Catalase, a marker enzyme of peroxisomal matrix, is present in the cytosol. Other metabolic defects, including phytanic acid oxidation, pipecolic acid oxidation and polyunsaturated fatty acid synthesis, plasmalogen synthesis are present in patients with Zellweger syndrome.

Complementation group. Genetic heterogeneity among peroxisome-deficient disorders was identified by Brul *et al.* in 1988 (Brul *et al.*, 1988). Four complementation groups were noted in their cell fusion study. If peroxisomes are formed and defective enzyme activities are restored after cell fusion, defective genes in the cell lines used will differ. If restoration does not occur, the responsible gene is assumed to be identical. To date, we identified 10 complementation groups among Japanese, North American and Australian patients with peroxisome-deficient disorders (Shimozawa *et al.*, 1993; Poulos *et al.*, 1995). At least 10 genes are involved in the formation of peroxisomes and are responsible for peroxisome-deficient disorders. Ten complementation groups exist among Zellweger patients. Moreover, clinical subtypes such as Zellweger syndrome, neonatal adrenoleukodystrophy and infantile Refsum disease do not correlate with complementation grouping.

Identification of a responsible gene for Zellweger syndrome. Tsukamoto *et al.* (1990) isolated Chinese hamster ovary (CHO) cell mutants without peroxisomes, using ethyl methanesulfonate, a mutagenic agent. They cloned a gene which restored peroxisomes in a mutant CHO cell line Z65, using a functional expression method (Tsukamoto *et al.*, 1991). They transfected a rat liver cDNA library, constructed in a mammalian expression vector pcD2, into Z65 cells. Revertant cells were selected by the 12-(1′-pyrene)dodecanoic acid/ultraviolet method (Zoeller *et al.*, 1988). Plasmid in the selected cells was recovered and peroxisome-restoring

activity was tested. Cloned cDNA has a 915 bp open reading frame which encodes 305 amino acids. Gene product, PAF-1, is a peroxisomal membrane protein with the molecular weight of 35 kDa.

To clarify whether PAF-1 is a pathogenic gene for Zellweger syndrome, we searched for patients affected in the same complementation group as the CHO mutant Z65. We used the fibroblasts from nine Japanese and American patients with peroxisome-biogenesis disorders from eight complementation groups. Fusion of the fibroblasts from all patients with Z65 resulted in the appearance of peroxisomes, thereby implying that all the patients' pathogenic genes differed from that of Z65. But at a later time, one newly screened female infant, MM, diagnosed with Zellweger syndrome, could not be classified into any of the previously characterized complementation groups. Unlike the other hybrids, the cell hybrids of MM's skin fibroblasts with Z65 cells lacked peroxisomes. We named this MM's cells group F Zellweger syndrome. When the rat PAF-1 gene was transfected to the MM's cells, peroxisomes were formed (Shimozawa *et al.*, 1992), and biochemical abnormalities were restored. Next, the human PAF-1 gene was cloned and sequenced. The human PAF-1 gene also has a 915 bp open reading frame with a high homology to the rat PAF-1 (Shimozawa *et al.*, 1992), and is mapped to chromosome 8q21.1 (Masuno *et al.*, 1994). Sequence analysis revealed a C to T transition at nucleotide position 355 and which resulted in early formation of termination codon at Arg^{119} (Shimozawa *et al.*, 1992). Thus the gene responsible for the group F Zellweger syndrome was proved to be PAF-1 gene.

Dodt *et al.* (1995) recently reported that the peroxisome-targeting signal 1 (PTS1) receptor gene, PXR1, is the gene defective in group 2 peroxisome-biogenesis disorder patients; it was isolated from expressed sequence-tagged databases, as a human homologue of PTS1 of the yeast PAS8 gene of Pichia pastoris (Dodt *et al.*, 1995).

We quite recently cloned and characterized the rat cDNA for PAF-2, which restores peroxisomes of ZP92, the model Chinese-hamster-ovary mutant for the group C cells, by functional complementation (Tsukamoto *et al.*, 1995). Rat PAF-2 is a peroxisome peripheral membrane protein of 978 amino acids containing ATP-binding sites and an AAA-protein-family signature. A homology search revealed PAF-2 to be a member of a putative ATPase family that includes two yeast genes essential for peroxisome assembly. Transformants of group C cells, as well as of ZP92 cells with the rat PAF-2 cDNA, restored peroxisomes morphologically, thereby suggesting that PAF-2 is the causal gene of peroxisome deficiency in both ZP92 and human group C cells (Tsukamoto *et al.*, 1995). To clarify the novel pathogenic gene of peroxisome-biogenesis disorders, we cloned the full-length human PAF-2 cDNA that morphologically and biochemically restores peroxisomes of group C Zellweger fibroblasts (the same as group 4 in the Kennedy-Krieger Institute) (Fukuda *et al.*, 1996c) and identified two pathogenic mutations in the PAF-2 gene in two patients with group C Zellweger syndrome

(Fukuda *et al.*, 1996c). The 2,940-bp open reading frame of the human PAF-2 cDNA encodes a 980-amino-acid protein that shows 87.1% identity with rat PAF-2 and also restored the peroxisome assembly after gene transfer to fibroblasts of group C patients. Direct sequencing of the PAF-2 gene revealed a homozygous 1-bp insertion at nucleotide 511 (511 insT) in one patient with group C Zellweger syndrome, which introduces a premature termination codon in the PAF-2 gene, and, in the second patient, revealed a splice-site mutation in intron 3 (IVS3+ 1G → A), which skipped exon 3, an event that leads to peroxisome deficiency. Chromosome mapping utilizing FISH indicates that PAF-2 is located on chromosome 6p21.1. These results confirm that human PAF-2 cDNA restores peroxisome of group C cells and that defects in the PAF-2 produce peroxisome deficiency of group C peroxisome-biogenesis disorders (Fukuda *et al.*, 1996c).

2. β-Ketothiolase deficiency

Clinical finding. Mitochondrial acetoacetyl-CoA thiolase (T2) deficiency is commonly known as β-ketothiolase deficiency (McKusick catalogue number 203750). Since the first patient was identified in 1971 (Daum *et al.*, 1971), data on more than 30 patients have been reported (unpublished included). This disorder is characterized by intermittent ketoacidotic attacks and increased urinary excretion of 2-methyl-3-hydroxybutyrate, 2-methylacetoacetate, and tiglylglycine, derived from intermediate metabolites in isoleucine catabolism, together with ketone bodies, 3-hydroxybutyrate, and acetoacetate. Severity of such symptoms varies with the patient. About one-half the number of patients develop severe attacks associated with unconsciousness, some die during an attack or are mentally retarded. Some patients who are putative candidates for this disorder have normal T2 activity (Iden *et al.*, 1990).

Thiolases and thiolase deficiencies. Mammalian tissues carry at least 5 thiolases (Middleton, 1973; Miyazawa *et al.*, 1980; Uchida *et al.*, 1992): T2, mitochondrial 3-ketoacyl-CoA thiolase (T1), mitochondrial enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (TFP), cytosolic acetoacetyl-CoA thiolase (CT), and peroxisomal 3-ketoacyl-CoA thiolase (PT). They differ in substrate specificity and/or intracellular localization. Human cDNAs for these thiolases have been cloned (T2: Fukao *et al.*, 1990; T1: Abe *et al.*, 1993; PT: Fairbairn and Tanner, 1989; CT: Song *et al.*, 1994; TFP: Kamijo *et al.*, 1994). There is 35–46% homology among the deduced amino acid sequences of these thiolases.

β-Ketothiolase deficiency was confirmed to be due to T2 deficiency, based on the following observations: (1) only T2 is responsible for cleavage of 2-methylacetoacetyl-CoA in isoleucine catabolism (Middleton and Bartlett, 1983); (2) potassium ion-activated acetoacetyl-CoA thiolase activity, a specific property of T2 (Middleton, 1973) is deficient in patients with β-ketothiolase deficiency (Robinson *et al.*, 1979); and (3) defects in T2 biosynthesis are present in fibroblasts

from patients with this disorder, as noted in pulse-chase experiments (Yamaguchi *et al.*, 1988; Nagasawa *et al.*, 1989). Deficiencies of other thiolases have also been reported and symptoms differ from those related to T2 deficiency (CT: De Groot *et al.*, 1977; Bennett *et al.*, 1984; PT: Schram *et al.*, 1987; TFP: Wanders *et al.*, 1992), although gene mutations in these deficiencies have not been detected. Therefore, the term "β-ketothiolase deficiency" seems imprecise when referring to T2 deficiency alone.

T2 protein and gene. Human T2 is a homotetramer of the 41-kDa subunit (Middleton, 1973; Fukao *et al.*, 1990). An anti-[rat T2] antibody can immunoprecipitate human T2 without cross-reactivity with other thiolases (Yamaguchi *et al.*, 1988). Rat and human T2 cDNAs were cloned and sequenced (Fukao *et al.*, 1989, 1990). Human T2 cDNA is about 1.5 kb long and is encoded for a precursor of 427 amino acids, including a 33-amino acid leader polypeptide. The T2 gene spans approximately 27 kb, contains 12 exons (Kano *et al.*, 1991), and is located at 11q22.3-23.1 (Masuno *et al.*, 1992).

Mutations and polymorphisms. No large deletion or insertion has been observed in Southern blot analysis. Seyenteen mutations were identified in 13 T2-deficient patients: nine missense (Song *et al.*, 1994), one nonsense (Fukao *et al.*, 1995), and five splice-site mutations (Fukao *et al.*, 1995), and two small deletions (Fukao *et al.*, 1990). Two polymorphic base substitutions were also detected (Kuwahara *et al.*, 1992). A common mutation in T2 deficiency has not been detected but 4 mutations (N158D, Q272X, 828+1, 1163+2) were identified in two independent families (Fukao *et al.*, 1991). Eleven of 25 mutant alleles identified caused aberrant splicing (Fukao *et al.*, 1995). *In vivo* expression analysis of 13 mutant cDNAs using a Lipofectin reagent suggested that T297M, A203P, and A380T mutant alleles retain 5–10% normal T2 activity (Wakazono *et al.*, 1995). A correlation between clinical phenotype and genotype in T2 deficiency seems unlikely.

3. *Mucopolysaccharidoses*

We investigated 3 β-glucuronidase deficient patients at the gene levels, and reported the first two mutations, both at CpG sites $Ala^{619}\rightarrow$ Val and $Arg^{382}\rightarrow$ Cys (Tomatsu *et al.*, 1990, 1991a). Furthermore, we identified one point mutation in a patient with intermediate MPS II (Sukegawa *et al.*, 1992). In this paper, however, we describe molecular aspect of mucopolysaccharidosis IVA.

Mucopolysaccharidosis IVA. Mucopolysaccharidosis IVA (MPS IVA) is an autosomal recessive lysosomal storage disorder caused by a deficiency of *N*-acetylgalactosamine-6-sulfate (GalNAc6S) sulfatase (EC 3.1.6.4), also known as galactose-6-sulfate sulfatase. GalNAc6S sulfatase is a lysosomal enzyme which hydrolyzes the sulfate ester group of GalNAc6S at the nonreducing end of chondroitin-6-sulfate, and that of galactose-6-sulfate at the nonreducing end of keratan sulfate. A defect in this enzyme leads to accumulation of undegraded glycosamino-

glycans in lysosomes. Clinically, MPS IVA can be divided into several subtypes: besides the severe (classical) form (so-called Morquio disease) (Morquio, 1929), there also exists an intermediate (Glössl *et al.*, 1981) and a mild form (Orii *et al.*, 1981; Fujimoto and Horwitz, 1983; Hechit *et al.*, 1984; Beck *et al.*, 1986). Classical Morquio disease is a prototype of chondroosteodystrophy (spondyloepiphyseal dysplasia), characterized by specific spondyloepiphyseal dysplasia, short trunk dwarfism, coxa valga, odontoid hypoplasia, corneal opacities, preservation of intelligence, and excessive urinary excretion of keratan sulfate and chondroitin-6-sulfate.

Biochemical studies of the genetic heterogeneity in MPS IVA have been hampered because GalNAc6S sulfatase is difficult to purify and specific antibodies have been difficult to produce. Available enzymatic assays do not differentiate the mild from the severe form. Although the enzymatic diagnosis of affected patients with MPS IVA can be made, enzymatic carrier detection is less reliable because of a marked overlap of GalNAc6S sulfatase activity in fibroblasts or leukocytes from obligate heterozygotes and normal controls. We purified GalNAc6S sulfatase from human placenta and obtained a polyclonal antibody against the enzyme (Masue *et al.*, 1991). Some patients with classical MPS IVA have no detectable cross-reacting material (CRM) (Masue *et al.*, 1991), while others have CRM with no detectable enzyme activity (unpublished data). These findings and the heterogeneity of phenotypes suggest different mutations, each of which may have different effects on the active enzyme. Investigations into the molecular basis of the genetic heterogeneity in MPS IVA have been facilitated by the isolation and characterization of the full-length cDNA encoding human GalNAc6S sulfatase (Tomatsu *et al.*, 1991b). The 2,339-bp cDNA contains an open reading frame of 1,566 bp, which encodes 522 amino acids, including a signal peptide of 26 residues. The gene encoding GalNAc6S sulfatase has been localized by fluorescence *in situ* hybridization to chromosome 16q24 (Masuno *et al.*, 1993; Baker *et al.*, 1993), and the entire genomic gene structure has been characterized (Nakashima *et al.*, 1994).

Mutations and polymorphisms of mucopolysaccharidosis IVA. We have identified two different exonic mutations in two unrelated Japanese families, in one patient with classical Morquio disease, and in two brothers with a mild form of MPS IVA (Fukuda *et al.*, 1992). The nucleotide sequence of the full-length cDNA derived from a patient with classical Morquio disease revealed a two-base deletion at nucleotide position 1343–1344 (1344–1345 or 1345–1346) that altered the reading frame (designated 1342delCA). This mutation, inherited from the proband's consanguineous parents, was revealed by *Taq*I restriction analysis of a cDNA fragment amplified by the polymerase chain reaction. In the proband with the mild form of the disease, a C to G transversion at nucleotide 667 predicted the substitution of Lys for Asn (N204K). Since a new *Alu*I site was created by the N204K mutation, restriction analysis indicated that the affected brothers were homozygous for this mutation, as confirmed by the finding that both their parents

had this lesion. Transient expression in GalNAc6S sulfatase deficient fibroblasts of these two mutant alleles showed completely deficient or markedly decreased enzyme activities, thereby indicating that these two mutations were responsible for the enzyme deficiency (Fukuda *et al.*, 1992).

To date, about 40 different *N*-acetylgalactosamine-6-sulfate sulfatase (GALNS; EC 3.1.6.4) gene mutations have been identified, including missense, frame-shift, and splice-site mutations in patients with various MPSIVA phenotypes (Tomatsu *et al.*, 1994a, b, 1995a, b, 1996; Ogawa *et al.*, 1995; Fukuda *et al.*, 1996a, b). Large structural rearrangements, which cause a double gene deletion (Hori *et al.*, 1995) and total gene deletion involving adjacent genes have also been identified (Fukuda *et al.*, 1996a). Two different common mutations, I113F and double gene deletion, were identified in specific populations, respectively (Tomatsu *et al.*, 1995b, 1996), suggesting that each of these two mutations probably arose from a single founder.

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