Biochemical and Morphologic Aspects of Peroxisomes in the Human Rectal Mucosa: Diagnosis of Zellweger Syndrome Simplified by Rectal Biopsy

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ABSTRACT. Rectal mucosa biopsy specimens from five controls and three infants with Zellweger syndrome were investigated morphologically and biochemically to elucidate peroxisomal structure and functions in the human rectal mucosa and to develop a simple method for an early postnatal diagnosis of peroxisomal deficiency disorders. After the alkaline 3,3'-diaminobenzidine reaction, peroxisomes of the rectal mucosa from the controls could be identified, electron microscopically, but not light microscopically. However, they were strongly stained using an immunoenzyme technique applied to semi-thin Epon sections and then were clearly visible under the light microscope. However, no positive granules were observed in the specimens of infants with Zellweger syndrome, using either of the two staining techniques. On immunoblot analysis, immunoreactive proteins of peroxisomal acyl-CoA oxidase and 3-ketoacyl-CoA thiolase were present in rectal mucosae from the controls; however, these enzyme proteins were not detected in tissues from the patients. Activity of dihydroxyacetone phosphate acyltransferase was detectable in rectal mucosae from the controls, whereas in those from infants with Zellweger syndrome, the activity of this enzyme was greatly reduced. These observations indicate that the peroxisomal structure and multiple functions are present in the rectal mucosa and that rectal biopsy is of potential value for the early and less invasive detection of Zellweger syndrome and other peroxisomal disorders. (Pediatr Res 24: 723-727, 1988)

Abbreviations

ZS, Zellweger syndrome
DHAP-AT, dihydroxyacetone phosphate acyltransferase
DAB, 3,3'-diaminobenzidine
BSA, bovine serum albumin
HRP, horseradish peroxidase
LM, light microscope
SDS, sodium dodecyl sulfate
PAGE, polyacrylamide gel electrophoresis

Recent studies revealed a new group of genetic diseases that have a number of metabolic disturbances considered to be due to peroxisomal defects, including ZS (McKusick 21410) (1), neonatal adrenoleukodystrophy (McKusick 20237) (2) and infantile Refsum disease (3). The diagnosis of these peroxisomal diseases is usually made by a combination of analysis of these metabolic errors, including very long-chain fatty acids, plasmalogen, bile acids, pipecolic acid, phytanic acid, and demonstration of the absence of peroxisomes (4).

The morphologic study of peroxisomes in mammalian tissues is limited to hepatocytes and renal tubules, because this organelle, although being ubiquitous in mammalian cells (5), is particularly large and abundant in both tissues, in comparison with others (6, 7). However, liver or renal biopsies in the early postnatal period are invasive. However, biopsy specimens of the rectal mucosa can be readily obtained by a simple and noninvasive technique with the use of Watson's intestinal biopsy capsule. Using this method, we investigated the presence of immunocytochemically recognizable peroxisomes and enzyme proteins of peroxisomal β-oxidation (8): acyl-CoA oxidase (EC 1.3.99.3), a bifunctional protein that exhibits the activities of enoyl-CoA hydratase (EC 4.2.1.17), and 3-hydroxyacyl-CoA dehydrogenase (EC 1.11.1.6), and 3-ketoacyl-CoA thiolase (EC 2.3.1.16) [9] by the immunoblotting method and exhibits enzyme activity of DHAP-AT (EC 2.3.1.42), a membrane-bound peroxisomal enzyme involved in the first step in the biosynthesis of etherphospholipids (10).

We used this noninvasive biochemical and morphologic approach for the early postnatal diagnosis of patients with ZS, demonstrating the absence of peroxisomes and the defects in peroxisomal β-oxidation enzymes and DHAP-AT.

MATERIALS AND METHODS

Five control patients with conditions not associated with known peroxisomal disorders and three infants with ZS (patients 1, 2, and 3) (11) were investigated. All ZS infants were Japanese and manifested typical findings and a clinical course of this syndrome. The ratio of hexacosanoic acid (C26:0)/docosanoic acid (C22:0) in serum sphingomyelin was unusually high. Three peroxisomal β-oxidation enzyme proteins were absent in liver tissues from patients 1 and 2, and in both patients, liver peroxisomes were absent electron microscopically. Patient 3 was diagnosed by clinical and biochemical findings but a morphologic study was not performed.

Rectal biopsy. Specimens from the rectal mucosa were taken using Watson's intestinal biopsy capsule (Ferraris Development

and Engineering Co. Ltd., London, U.K.). The capsule was placed about 5 cm inside the anus. The size of these specimens was about 2 to 3 mm in diameter and 2 mm thick. One of a few specimens thus obtained from the patients and controls was immediately fixed in 0.1% glutaraldehyde, 4% paraformaldehyde, 0.2 M cacodylate buffer, pH 7.4, at 4° C for use in electron microscopy, and the remaining tissues frozen and stored at −80° C until the use for biochemical analysis.

Alkaline DAB reaction and immunocytochemistry of catalase. The fixed specimens at rectal biopsy were refixed in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 1 h at 4° C and divided into two groups; the specimens of group 1 were incubated in alkaline DAB medium consisting of 2 mg/ml diaminobenzidine, 0.02% H_2O_2, and 0.1 M Teorell-Steuhagen buffer, pH 9.3, for 2 h at room temperature, postosmicated, and embedded in Epon (12). The specimens of group 2 were dehydrated and embedded in Epon without postosmication.

Semi-thin sections of group 2 were stained using an immunoenzyme technique as previously described (13). Briefly, the sections were treated with 10% NaOH in absolute ethanol for 1 h at 30° C to remove the epoxy resin. After a 5 min-incubation with BSA (5 mg/ml), the sections were incubated with anti-catalase IgG (20 μg/ml) for 1 h, followed by a 30-min incubation with HRP-conjugated Fab' of goat anti-rabbit IgG. HRP activity was visualized by the DAB reaction (14). The sections were air-dried and mounted with Entellan (Merck, Darmstadt, Germany) and examined using a Nikon LM. For the immunocytochemical control, the sections were incubated with an IgG fraction from a nonimmunized rabbit, followed by the HRP-conjugated Fab'. Some sections were incubated directly with the HRP-Fab' without the preceding step of the specific antibody.

Immunoblotting. The specimens of rectal mucosa were homogenized with 5 vol of 50 mM potassium phosphate buffer (pH 8) and 0.5% Triton X-100. The supernatant obtained by centrifugation (12,000 rpm, 5 min) at 4° C was taken up in the same volume of sample buffer (125 mM Tris-HCl, pH 7.4, 5% SDS, 0.32 M 2-mercaptoethanol, 20% glycerol, and 0.0025% bromophenol blue) and boiled for 3 min. These extracts, containing 30 μg of proteins, were applied to SDS/PAGE, then Western blotting was performed as described (15). Acyl-CoA oxidase, peroxisomal 3-ketoacyl-CoA thiolase, and catalase were located on the membrane as follows. The membrane was first blocked with 3% BSA followed by incubation with antibody against each of these enzymes, then incubated with goat anti-rabbit IgG alkaline phosphatase conjugate (Promega Biotec, Madison, WI). Color development was performed with the use of 5-bromo-4-chloro-3-indolyl phosphate. Purified rat enzymes and antibodies were prepared as described (16, 17). Catalase was purified from human erythrocytes.

Measurement of DHAP-AT activity. DHAP-AT activity was measured by the method of Schutgens *et al.* (18) with slight modifications. The specimens of rectal mucosa were homogenized in 5 mM Tris-HCl, pH 7.5, 50 mM NaCl to give a protein concentration of approximately 2 mg/ml. [U-^{14}C]DHAP was generated from [U-^{14}C]glycerol 3-phosphate (Amersham, Buckinghamshire, U.K.). The activity of DHAP-AT in the rectal mucosa was assayed by incubation of 20–40 μg homogenate at 37° C for 2 h in 75 mM sodium acetate buffer, pH 5.4, 8 mM NaF, 8 mM $MgCl_2$, 0.8 mg BSA, 0.3 mM palmytoyl-CoA and 0.1 mM DHAP in a total volume of 0.12 ml. Radioactivity incorporated into the chloroform-soluble lipid was determined.

RESULTS

Staining of peroxisomes by alkaline DAB and immunoenzyme techniques. In the specimens from controls, peroxisomes of rectal mucosa were stained positively by the alkaline DAB reaction (Fig. 1A), whereas in those from ZS infants, no positive granules were observed (Fig. 1B). With LM, it was difficult to identify these small peroxisomes in the materials stained by the alkaline

DAB technique (data not shown). When the semi-thin Epon sections were stained using the immunoenzyme technique after the removal of epoxy resin, discrete granular reaction deposits were noted in epithelial cells of the controls (Fig. 2A), but not in those from the ZS infants (Fig. 2B). In the immunocytochemical control sections, no granular staining was observed.

Immunoblot analysis. Acyl-CoA oxidase consists of three components of different sizes: component A (Mr = 72000), B (Mr = 52000), and C (Mr = 21000). All three components were detected in both liver and rectal mucosae from the controls, yet only the component A was detected in rectal mucosa from patients with ZS, as in the case of liver tissue. This can be explained by the finding that component A was synthesized by pulse labeling of the fibroblasts from the patients, but components B and C were not formed by processing of component A (19) (Fig. 3A).

As shown in Figure 3B, the human peroxisomal 3-ketoacyl-CoA thiolase seen in controls is slightly larger than the rat enzyme. In tissues from the patients, we observed a faint band of protein that was slightly higher in mol wt than the mature enzymes of human peroxisomal 3-ketoacyl-CoA thiolase. These bands are considered to be a precursor form of the thiolase, inasmuch as the pulse-chase experiments of fibroblasts revealed that the precursor form of peroxisomal 3-ketoacyl-CoA thiolase was not processed to its mature form in such patients (19). However, catalase (Fig. 3C) was present in tissues from the patients and from the controls. Bifunctional protein, another peroxisomal β-oxidation enzyme, was present only in a minute amount in rectal mucosae even from the controls (data not shown).

DHAP-AT activity. The DHAP-AT activity in the rectal mucosa from the controls was about half of that in the liver whereas in both tissues from patients, the activity of this enzyme was reduced, as compared to those of the controls. Only patient 3 seemed to have a partial activity of this enzyme, but the activity in the liver from the patient was strongly reduced as in the other two cases (Table 1).

DISCUSSION

Recent immunocytochemical studies clarified localization of the individual enzymes of peroxisomal β-oxidation in the human liver and kidney (20, 21). In other tissues, except for cultured skin fibroblasts, biochemical or cytochemical studies of this organelle were limited because of the small number and size of the peroxisomes.

We have now obtained evidence for not only the presence of peroxisomal functions, including proteins of β-oxidation enzymes and activities of DHAP-AT, but also immunocytochemical demonstration of this organelle in the human rectal mucosa. In the present study, we have stained the peroxisomes of rectal mucosa using two different methods: the alkaline DAB technique and the immunoenzyme technique applied to semi-thin Epon sections. With the former, peroxisomes could be observed electron microscopically, but not light microscopically. However, with the latter technique peroxisomes were stained strongly and light microscopical identification presented no difficulty. These results indicate that the immunoenzyme technique applied to the semi-thin Epon sections is most useful for detection of peroxisomes in specimens of the rectal biopsy.

We found that these peroxisomal structures and functions were absent in rectal mucosa from infants with ZS. Ultrastructural and cytochemical demonstration of peroxisomal defects in ZS has been noted in hepatocytes, renal proximal tubules (22), and cultured skin fibroblasts (23). In the fibroblasts, small round or slightly oval peroxisomes were seen in both control and ZS infants, although the number of peroxisomes were significantly reduced in all ZS cell lines. Deficient proteins of peroxisomal β-oxidation enzymes and activities of DHAP-AT in ZS infants have been reported, the former in the liver (24, 25) and in fibroblasts (26), the latter in the liver, fibroblasts (18), and
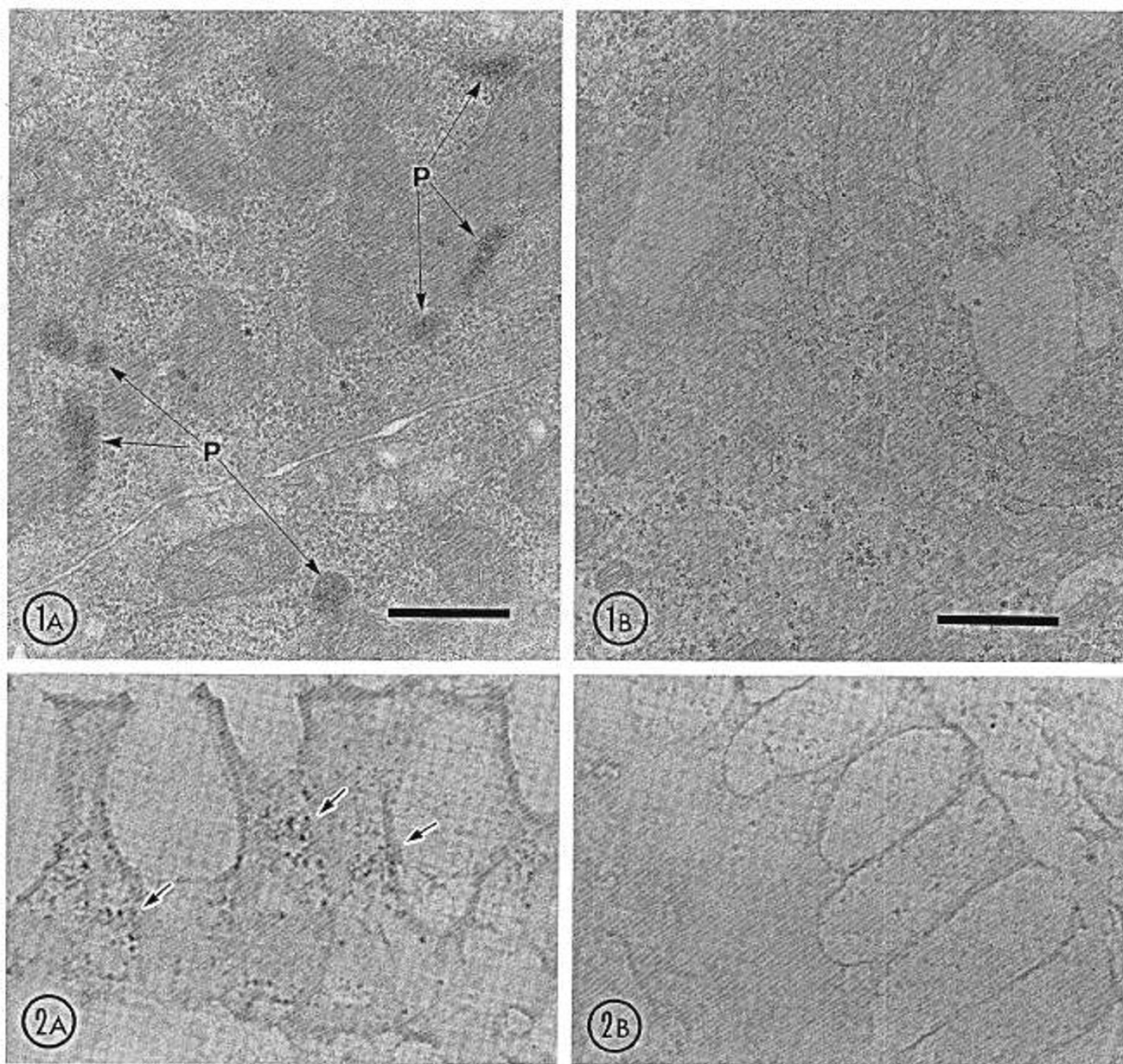
Fig. 1. Electron micrographs of mucous epithelial cells from human rectal biopsy. The cells were stained by alkaline DAB method. *A*, specimen from the control. Small peroxisomes (*P*) were stained. Other cell organelles are negative for the reaction. *Bar* = 0.5 μm. ×36,000. *B*, specimen from ZS infant. Note that there are no DAB reaction granules. Bar = 1 μm. ×18,000.

Fig. 2. Light micrographs of mucous epithelial cells from human rectal biopsy. Semi-thin Epon sections of the materials were stained for catalase by immunoenzyme technique after removal of epoxy resin. *A*, specimen from the control. Small cytoplasmic granules are stained and they are crowded in some part of the cytoplasm (*arrows*). ×1400. *B*, specimen from ZS infant. Note that there are no positively stained cytoplasmic granules. ×1400.

thrombocytes (27). These structural and functional defects in ZS infants have apparently never been identified in the rectal mucosa.

Rectal biopsy using Watson's intestinal biopsy capsule has been done in case of ultrastructural studies of neuronal storage disease (28, 29), and with this method, there are no complications such as infection, bleeding, and perforation. This method is potentially superior to the previously used methods including liver and renal biopsy with the demerits of involved techniques and invasiveness, and skin biopsy with those of time-consuming culturing of fibroblasts, as a diagnostic test for neonates with ZS. It is simple, safe, rapid, and reliable.

We consider this of potential value for diagnosis of other peroxisomal deficiency disorders with multiple dysfunction such as infantile Refsum disease (30, 31) and neonatal adrenoleukodystrophy (32, 33). Biochemical studies of peroxisomal β-oxidation and ether phospholipid in biopsied rectal mucosa will lead to an accurate diagnosis of other peroxisomal disease such as rhizomelic chondrodysplasia punctata (McKusick 21510) with a deficiency of DHAP-AT (34), pseudo-ZS with a single deficiency of peroxisomal 3-ketoacyl-CoA thiolase (35), pseudoneonatal adrenoleukodystrophy with acyl-CoA oxidase deficiency (36), and Zellweger-like syndrome with deficient proteins of peroxisomal β-oxidation enzymes and detectable peroxisomes (37).
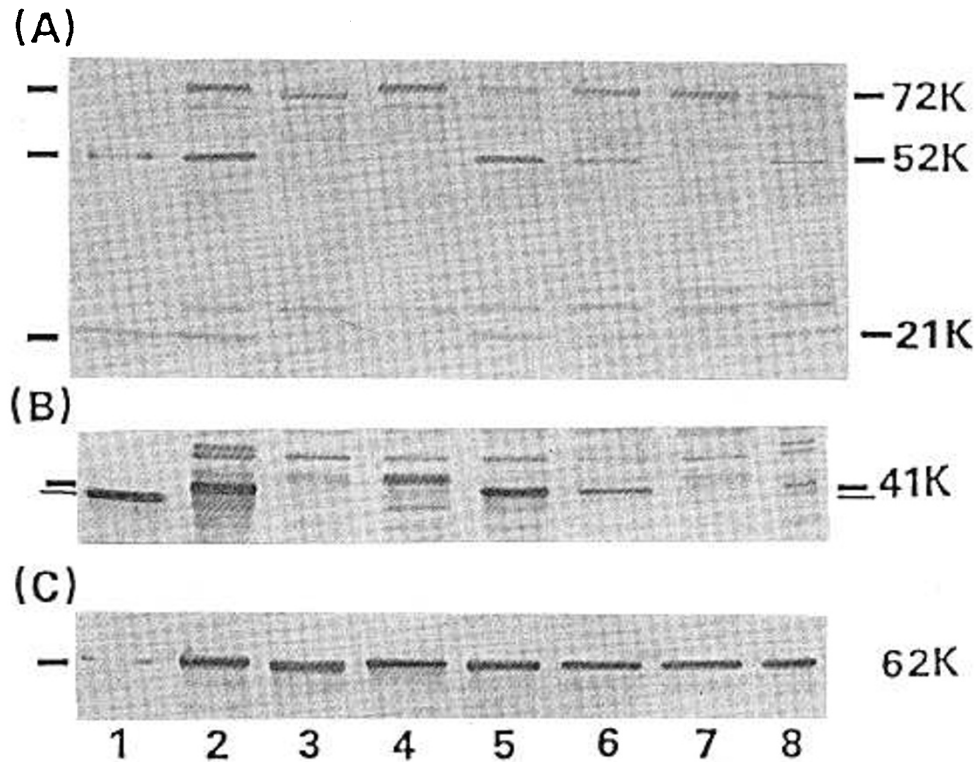
(A)

(B)

(C)

Fig. 3. Immunoblot analysis. *A*, peroxisomal acyl-CoA oxidase (72K, component A; 52K, component B; 21K, component C); *B*, peroxisomal 3-ketoacyl-CoA thiolase; *C*, catalase; *lane 1:* purified rat enzyme; *lanes 2* and *5:* liver homogenates from the controls; *lanes 3* and *4:* liver homogenates from ZS infants; *lanes 6* and *8:* rectal mucosal homogenates from the controls; *lane 7:* rectal mucosal homogenate from ZS infant.

Table 1. *DHAP-AT activity in tissues*

Tissue	Rectal Mucosa	Liver
	(nmol/2 h mg protein)	
Controls ($n = 5$)	1.35 ± 0.34	2.90 ± 0.76
	(0.94–1.79)	(2.15–4.64)
ZS		
Patient 1	0.06	0.05
Patient 2	0.07	0.02
Patient 3	0.29	0.05

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