Ultrastructural and Cytochemical Demonstration of Peroxisomes in Cultured Fibroblasts from Patients with Peroxisomal Deficiency Disorders

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ABSTRACT The oxidation of very long chain fatty acids and synthesis of ether glycerolipids (plasmalogens) occurs mainly in peroxisomes. Zellweger's cerebrohepatorenal syndrome (CHRS) is a rare, inherited metabolic disease characterized by an apparent absence of peroxisomes, an accumulation of very long chain fatty acids, and a decrease of plasmalogens in tissues and cultured fibroblasts from these patients. As peroxisomes are ubiquitous in mammalian cells, we examined normal and CHRS-cultured fibroblasts for their presence, using an electron microscopic histochemical procedure for the subcellular localization of catalase, a peroxisomal marker enzyme. Small (0.08-0.20 μm) round or slightly oval peroxisomes were seen in both normal and CHRS fibroblasts. The number of peroxisomes was analyzed morphometrically and found to be significantly reduced in all CHRS cell lines. These results are discussed in relation to the underlying defect in peroxisomal function and biogenesis in this disease.

Peroxisomes are respiratory organelles that contain \( \text{H}_2\text{O}_2 \)-generating oxidases and catalase (12). They are found in plants, fungi, protozoa, and metazoan organisms, and are ubiquitous in mammalian cells (11). Their unique role in ether glycerolipid (plasmalogen) biosynthesis (7, 8) and very long chain fatty acid oxidation (23) suggests that they are essential to cellular metabolism. Further support for this concept is derived from studies of inborn errors of metabolism where expression of a hereditary abnormality results in defective peroxisomal function and severe multisystem disease (5, 6).

Zellweger's cerebrohepatorenal syndrome (CHRS) is an inherited metabolic disorder characterized by severe nervous system dysfunction, including demyelination and defective neuronal migration, hepatic fibrosis, skeletal abnormalities, and renal cortical cysts (1, 24). Affected infants rarely survive beyond one year. At the subcellular level, the most striking abnormality is an apparent lack of peroxisomes in hepatocytes (6, 14, 18) and renal proximal tubular epithelia (6). As a disease, CHRS appears to be unique in that the biogenesis of a constitutive organelle is defective. Other syndromes such as neonatal adrenoleukodystrophy share many of the clinical, biochemical, and morphological features of CHRS (1, 4). In contrast to CHRS, reduced numbers of small hepatocellular peroxisomes have been detected in these patients, who are less impaired and survive for as long as six years (4).

Biochemical evidence in support of the concept that CHRS represents a profound deficiency in peroxisomes comes from two sets of observations. In the liver, \( \beta \)-oxidation of very long chain fatty acids (C:22-26) and the initial step in ether glycerolipid (plasmalogen) synthesis occurs mainly, if not exclusively, in peroxisomes (7, 8, 23). Very long chain fatty acids accumulate in tissues and fibroblasts from CHRS patients (1), whereas plasmalogen concentrations and the activity of dihydroxyacetone phosphate acyltransferase, a key enzyme in plasmalogen synthesis, are <10% of normal (3, 9). These data have been interpreted as evidence for the absence of peroxisomes in cultured fibroblasts and tissues from patients with CHRS.

The reported absence of peroxisomes in CHRS fibroblasts, which nevertheless grow normally in vitro, and in other tissues is contrary to evidence for an essential role of this organelle in mammalian cell function. Therefore, we conducted an ultrastructural and morphometric study to determine whether peroxisomes are present in normal and CHRS fibroblasts, using a histochemical procedure for the subcellular localization of peroxisomal catalase (15). The results show that small catalase-positive peroxisomes were present in the cytoplasm of normal and CHRS fibroblasts. Morphometric analysis of...
After fixation, cells were collected by centrifugation and resuspended in 0.1 M and then fixed on ice for 2-4 h (glutaraldehyde) or 12 h (formol-calcium). The cells were then dehydrated with a graded series of ethanol and infiltrated with Epon-Araldite/ethanol (1:2, tetroxide in 0.1 M cacodylate buffer, pH 7.2. The cells were then dehydrated and embedded in Epon-Araldite/ethanol at room temperature. After polymerization, thin sections were cut with a diamond knife and ultramicrotome. Semithick sections stained with blue O and sodium borate (borax) or collected unstained and examined with bright field optics. Ultrathin sections, either unstained or stained with lead citrate (1-3 min; reference 20) and uranyl acetate (20 min) were examined in a Siemens Elmiskop 100 electron microscope (Siemens Corp., Medical/Industrial Groups, Iselin, NJ) operating at 80 kV. Whole cell profiles were photographed and the minimum sample size was calculated from the distribution of the mean for the number of peroxisomes in each sample population. Random samples from three petri dishes per culture were then morphometrically analyzed for the number of peroxisomes per 1,000 μm² cytoplasmic area (excluding the nuclear area) based upon linear intersection (13). Peroxisomes within autophagic vacuoles were excluded from such quantitation. Final image magnifications of 8,000–10,000 were calibrated with a 54,480 line diffraction grating replica (Poly sciences Inc.).

RESULTS AND DISCUSSION

Small (0.08–0.20 μm) round or irregularly oval bodies delimited by a single membrane and containing the coarsely fibrillar matrix typical of anucleoid animal and plant peroxisomes were identified in both normal and CHRS fibroblasts (Fig. 1, a–d). These particles were histochemically reactive for catalase activity, a marker enzyme for peroxisomes (12, 15). We confirmed that this reaction was due to catalase by incubating fibroblasts in 0.01 M 3-amino-1,2,4-triazole, which inhibits this enzymes activity (10). After treatment with aminotriazole, reaction product was absent from these organelles. The peroxisomes in cultured fibroblasts were similar in morphology and catalase activity to those found in a human hepatocellular carcinoma cell line incubated under identical conditions (Fig. 1 e) and in numerous other mammalian cell types previously described (2, 11). In a few CHRS, but not normal, cells the cisternae of rough endoplasmic reticulum were dilated and moderately electron dense (Fig. 1 b). Otherwise, CHRS and normal fibroblast cells were morphologically comparable.

The detection of peroxisomes in CHRS fibroblasts, albeit in reduced numbers, was surprising as this organelle has not been reported in either liver or kidney from patients with this disease (6, 14, 18). However, the original description of the peroxisomal deficiency in CHRS cautioned that it is impossible to demonstrate unequivocally the absence of an organelle by morphological criteria (6). Some published micrographs suggest that a few small (0.2 μm) structures resembling peroxisomes, normally 0.5 μm, are present in hepatocytes of CHRS patients (18). Despite the fact that biochemical and fractionation studies failed to demonstrate particulate (peroxisomal) enzymatic activity in CHRS (22, 25), the resolution and sensitivity of ultrastructural cytochemistry for catalase activity allowed us to identify this organelle in CHRS fibroblasts. The number of peroxisomes was analyzed morphometrically and found to be substantially diminished in all CHRS lines as compared with the control (Table I). The reduced quantities of plasmalogens (9) and diminished activity of catalase and sensitivity of ultrastructural cytochemistry for catalase activity by the deposition of electron-dense reaction product (a) A normal human cell (WI-38) containing several peroxisomes with variable staining for catalase. (Inset) Heterogeneous distribution of reaction product within a peroxisome. (b) and (c) CHRS (GM4340) cells. (d) Peroxisomes adjacent to vesicular-tubular portion of smooth endoplasmic reticulum. (b) A CHRS (GM4340) cell with peroxisomes adjacent to or within autophagic vacuoles (arrows). (c) A peroxisome of a human hepatocellular carcinoma cell (P3Tg-2). Cells in a and inset fixed with formol–calcium; all other material fixed in glutaraldehyde. Bars: (a, b, and d) 0.5 μm; (inset, c, and e) 0.2 μm. (a) × 18,900; (inset) × 60,000; (b) × 12,500; (c) × 20,000; (d) × 32,500; (e) × 90,000.

**TABLE I**

| No. of peroxisomes | % control | Student's t test |
|---------------------|---------|-----------------|
| Sample | P | Age | n | area | mean ± SE |
| WI-38 | 8 | Fetal | 12 | 10.1 ± 1.2 | 100.0a |
| GM4340* | 7 | 6 mo | 14 | 0.7 ± 0.4 | 6.9b |
| GM0228* | 6 | 6 mo | 10 | 0.9 ± 0.5 | 8.9b |
| LF† | 9 | 5 yr | 10 | 2.5 ± 0.2 | 24.8c |
| SC‡ | 9 | 3 yr | 14 | 5.4 ± 0.7 | 53.5d |

Wi-38 fibroblast culture derived from normal fetal lung was kindly supplied by Dr. Ora Rosen, Sloan Kettering Institute, NY. Statistically significant (P < 0.01) variation between the samples is indicated by differences between the letters; use of the same letter represents the absence of statistically significant variation. P, passage number at time of sampling for ultrastructural morphometry; n, number of whole cell profiles analyzed.

* CHRS fibroblast cell cultures from the Mutant Cell Repository, Camden, NJ, derived from primary cultures of skin biopsy samples.

† CHRS fibroblast cultures derived from primary cultures of skin biopsy samples provided by Dr. Ann Moser, Johns Hopkins University School of Medicine, Baltimore, MD.

‡ Patient still living.

these cells revealed that in all CHRS fibroblasts the number of peroxisomes was markedly diminished.

MATERIALS AND METHODS

**Culture and Fixation Conditions:** Four CHRS cell lines were examined by electron microscopy and cytochemistry, one from each of two classical CHRS patients who had died at 6 mo and one from each of two clinically atypical patients who had features of both CHRS and neonatal adrenoleukodystrophy (Table I). One of the latter group died at 5 yr; the other is 3 yr old. Fibroblast cell cultures were grown in tissue culture flasks containing Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal calf serum at 37°C in a 5% CO₂ environment. For ultrastructural and cytochemical studies cells were harvested at near confluence with 0.2% (wt/vol) trypsin-ethylenediaminetetraacetate, or in a reaction medium without 3,5-diaminobenzidine and hydrogen peroxide. Incubation in the reaction medium was followed by from three to five washes in 0.1 M cacodylate buffer, pH 7.2. After fixation, cells were centrifuged and resuspended in 0.1 M sodium cacodylate buffer containing 0.5% sucrose. This step was repeated three or four times to remove excess fixative.

**Ultrastructural Cytochemistry and Morphometry:** Fixed cells were incubated for 90–120 min at 37°C in a histochemical medium (15) adjusted to pH 9.8–10.2 for the in situ demonstration of peroxisomal catalase activity. Control cells were first treated with 0.01 M 3-amino-1,2,4-triazole, which inhibits this enzymes activity (10). After treatment with aminotriazole, reaction product was absent from these organelles. The peroxisomes in cultured fibroblasts were similar in morphology and catalase activity to those found in a human hepatocellular carcinoma cell line incubated under identical conditions (Fig. 1 e) and in numerous other mammalian cell types previously described (2, 11). In a few CHRS, but not normal, cells the cisternae of rough endoplasmic reticulum were dilated and moderately electron dense (Fig. 1 b). Otherwise, CHRS and normal fibroblast cells were morphologically comparable.

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ities of dihydroxyacetone phosphate acyltransferase (3) and 
$\beta$-oxidation of very long chain fatty acids in cultured fibro-
blasts and tissues from CHRS patients (1) are consistent with 
the numerical decrease of peroxisomes reported here. Analysis 
of variance with the Student's t test revealed significant dif-
ferences ($P < 0.01$) between the number of peroxisomes in 
normal and CHRS cell lines (Table I). In patients with the 
classical form of CHRS the number of peroxisomes was 
reduced by 91–93%, whereas in longer-lived and clinically 
atypical CHRS patients this reduction was only 46–75% of 
the control value. Variations in the number of peroxisomes 
in different CHRS lines may be related to the clinical severity 
of the disease (Table I). However, more extensive studies are 
required to determine whether this is a consistent and pre-
dictable feature. The quantity of peroxisomes in cultured 
CHRS fibroblasts may differ from in vivo fibroblast popu-
lations; identification and quantitation of peroxisomes in fibro-
blasts of skin biopsy samples from CHRS patients should 
clarify this point and extend our in vitro observations.

In liver and kidney, peroxisomes are usually degraded by 
autophagocytosis (17, 19). In all cell lines that we studied 
peroxisomes were found in vacuoles, some of which contained 
cytoplasmic debris (Fig. 1, b and d). Autophagy of peroxi-
somes may reflect a cellular response to altered peroxisomal 
membrane constituents during organelle maturation and sen-
escence. A membrane defect has been hypothesized in 
related syndromes but evidence for such an anomaly has 
not been obtained. The only peroxisomal enzyme whose 
activity is known to be deficient in CHRS is dihydroxyacetone 
phosphate acyltransferase, which catalyzes the initial step 
during the synthesis of plasmalogens (3, 7, 8). This group of 
ether glycerolipids comprises >20% of the lipid in the human 
brain (16) and is widely distributed in cellular membranes.
Except for platelet activating factor, their function in animal 
cells is poorly understood.

Little is known of the factors that regulate the formation of 
peroxisomes. Peroxisomal enzymes are synthesized on free 
polysomes and transported through the cytosol into the target 
organelle (12). In CHRS, the total activity of matrix enzymes, 
e.g., catalase, $\epsilon$-amino acid oxidase, and $\alpha$-hydroxy acid 
oxidase is not reduced in liver or cultured fibroblasts (22, 23).
In contrast, the activity of a peroxisomal membrane-associated 
enzyme (dihydroxyacetone phosphate acyltransferase) is 
declined to <10% of the control value (3). Whether this 
enzymatic deficiency is of pathogenetic significance in CHRS 
or is simply a reflection of the quantitative decrease in 
peroxisomes reported here remains to be determined. How-
ever, the fact that this activity is not reduced in parents of 
CHRS patients (3) indicates that the basic defect lies else-
where. Further investigations of fibroblasts from this disorder 
of peroxisomal biogenesis should contribute to our under-
standing of peroxisomal formation and their role in cellular 
metabolism.

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