Analysis of Peroxisomes in Lymphoblasts: Zellweger Syndrome and a Patient with a Deletion in Chromosome 7

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ABSTRACT. Lymphoblasts are useful cells for the diagnosis and basic studies of several human genetic disorders. Peroxisomal disorders are usually diagnosed by using fibroblasts or blood samples. Here, we report the characterization of peroxisomes in lymphoblasts. We demonstrated that lymphoblasts from a patient with Zellweger syndrome, the prototypical disorder of peroxisome biogenesis, contained peroxisomal ghosts like those described previously in Zellweger fibroblasts. We also found that lymphoblasts that carry a deletion on chromosome 7 (q11.23q22.1), a region thought to contain one Zellweger syndrome gene, contained normal peroxisomes. (*Pediatr Res* 33: 441-444, 1993)

Abbreviations

PxIMP, peroxisomal integral membrane protein
ZS, Zellweger syndrome
VLCFA, very long chain fatty acid
NIGMS, National Institute for General Medical Sciences

A group of genetic disorders involving peroxisomal functions has been defined (1, 2). In some peroxisomal disorders, the biogenesis of the organelle is defective. ZS is the prototype of this category. This autosomal recessive disorder is characterized by metabolic disturbance, neurologic malfunction, and neonatal death (1, 2). At the cellular level, normal peroxisomes appear to be absent, as first noted by Goldfischer *et al.* (3). We found that peroxisomal membranes were present in ZS fibroblasts, but they seemed to be largely empty. This suggested to us that a defect in the import machinery for peroxisomal proteins was the basic cause of this disorder (4, 5).

Primary cultures of skin fibroblasts are the most common cell type used for the study of ZS. However, their use has significant drawbacks such as limited life-span and low efficiency of transformation and transfection with DNA. These characteristics represent important limiting factors for the identification and isolation of genes involved in the genesis of peroxisomal disorders. Therefore, immortal cell lines expressing the phenotypic defect(s) of ZS would be extremely useful. Lymphoblasts would be suitable for such studies. Lymphoblast cell lines derived from

ZS patients are currently available (NIGMS Mutant Cell Repository, Camden, NJ), but the biochemical and morphologic characterization of peroxisomal function in these cell lines is still lacking.

Recently, a putative gene for ZS has been tentatively assigned to chromosome 7, in region 7q11.23. This is based on the presence of a deletion involving 7q11.23 in one ZS patient and an inversion in the same chromosome region in another ZS patient (6, 7).

This article demonstrates the potential usefulness of lymphoblasts for the study of peroxisome deficiency disorders. We characterized peroxisomes in normal lymphoblasts and demonstrated that ZS lymphoblasts display some phenotypic changes that have been observed previously in other cell types. We also investigated a cell line obtained from NIGMS Mutant Cell Repository containing a deletion in one chromosome 7q11.23 to test whether this cell line contains abnormal peroxisomes.

MATERIALS AND METHODS

Cells. Control human lymphoblasts were obtained by *in vitro* transformation with Epstein-Barr virus of blood lymphocytes isolated from a control subject according to Nilsson *et al.* (8).

ZS lymphoblasts (GM 8764) and lymphoblasts GM10160 carrying a deletion in the long arm of chromosome 7 [46,XY,del(7) (pter>q11.23::q22.1>qter)] were obtained from the NIGMS Mutant Cell Repository. GM10160, the 7q- cell line, was established from a patient who had a seizure disorder, developmental delay, hypotonia, and congenital heart disease.

The cells were grown in suspension in RPMI medium, supplemented with glutamine, antibiotics, and 15% fetal bovine serum. Cultures were kept at 37°C in 5% CO₂.

VLCFA analysis and plasmalogen biosynthesis. Saturated VLCFA were measured according to Moser and Moser (9), and the peroxisomal contribution to plasmalogen synthesis was determined according to Roscher *et al.* (10).

Subcellular fractionation. Cells grown in T25 culture flasks were collected by centrifugation at 1000 × *g* for 10 min at room temperature. The cell pellet was washed twice with PBS and finally resuspended in homogenization solution at a concentration of approximately 4 to 6 × 10⁷ cells/mL. The homogenization solution consisted of 0.25 M sucrose; 5 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4; 1 mM EDTA; and 0.1% ethanol to which a protease inhibitor cocktail [leupeptin, chymostatin, antipain, and pepstatin (Sigma Chemical Co., St. Louis, MO)] was added at a final concentration of 10 µg/mL each.

Homogenization was carried out in a 3-mL Teflon/glass Potter-Elvehjem homogenizer (type AA, Arthur H. Thomas). A

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postnuclear supernatant was prepared according to Santos *et al.* (11), except that the nitrogen pump step was omitted.

The postnuclear supernatant was centrifuged at 40 000 rpm for 30 min in the Beckman Ti-50 rotor ($105\,000 \times g$) to prepare a particulate fraction (containing mainly mitochondria, lysosomes, peroxisomes, and microsomes) and a supernatant (equivalent to the cell cytosol).

Enzyme measurements. Catalase and proteins were determined as described previously (11).

Immunofluorescence. Cells were collected by centrifugation at $1000 \times g$ for 10 min at room temperature and then resuspended in fetal bovine serum (about one fifth of the cell pellet volume). Cell smears were prepared using two coverslips. They were air dried, fixed with 4% paraformaldehyde for 30 min in 0.1 M cacodylate, and subsequently treated exactly as described by Santos *et al.* (4).

Affinity-purified antibodies against catalase and the 69-kD PxIMP were described previously (4).

Cytogenetics. Cytogenetic studies were performed on GM08764 and GM10160. Prophase chromosomes were obtained by synchronizing cultures with methotrexate (12). One h before harvest, actinomycin D (Calbiochem, final concentration 1 g/mL) and Velban (Eli Lilly, final concentration 0.01 g/mL) were added to the cultures. Air-dried slides were prepared by conventional techniques. Chromosome bands were visualized with a standard trypsin-Giemsa method (G-banding) (13).

RESULTS

Control lymphoblasts. Catalase, the prototypical peroxisomal marker enzyme, was present in control cells at a specific activity of approximately 2 mU/mg (Table 1). To determine the percentage of catalase that could be recovered in peroxisomes in control lymphoblasts, we separated a postnuclear supernatant into a high-speed pellet (containing all organelles) and a supernatant. Table 1 shows that two thirds of the catalase found in the postnuclear supernatant fraction is recovered in the high-speed pellet, similar to what is found in control fibroblasts (11).

Indirect immunofluorescence was carried out with an affinity-purified antibody that reacts with human catalase (4). As shown in Figure 1a, catalase was detected in small particles, similar to the appearance of peroxisomes in control fibroblasts (4). We also performed immunofluorescence with an antibody against one of the major peroxisomal integral membrane proteins: the 69-kD PxIMP. As expected, a similar immunofluorescence pattern was found with this antibody (Fig. 1d).

The level of VLCFA in control lymphoblasts was found to be within approximately the same range as that of control fibroblasts (Table 2). It is worth mentioning that lipid analysis of lymphoblast samples shows considerable variation from one culture to another (Moser AB, unpublished data). The amount of C26:0 accumulation in lymphoblast membranes is often higher than that of fibroblasts. The peroxisomal contribution to plasmalogen synthesis in control lymphoblasts was similar to that in control fibroblasts (Table 2).

ZS lymphoblasts. Catalase activity in the ZS cell homogenates

Table 1. Specific activity and subcellular distribution of catalase in control, Zellweger, and 7q- lymphoblasts

	Control	Zellweger	7q-
Sp act in homogenates (mU/mg protein)*	2.1 $\pm$ 1.0	4.9 $\pm$ 0.6	3.2 $\pm$ 0.7
Subcellular distribution			
% in pellet†	66 $\pm$ 7	7 $\pm$ 2	63 $\pm$ 9
% in supernatant	34 $\pm$ 7	93 $\pm$ 2	37 $\pm$ 9

* Approximately 1×10^6 cells represent 1 mg of protein. Experiments were done in triplicate on each patient cell line.

† $105\,000 \times g$ pellets were prepared from postnuclear supernatants as described in Materials and Methods.

was more than twice that in control cells. Its subcellular distribution is abnormal. As shown in Table 1, most of the catalase is found in the cell cytosol. This distribution in lymphoblasts is similar to what has been reported in other ZS cell types (3, 11, 14–17).

The absence of particulate catalase was also observed by immunofluorescence (Fig. 1b). The cytoplasm of ZS lymphoblasts had a more intense fluorescence than did control cells, consistent with the cytosolic localization of this enzyme in ZS.

In contrast with the catalase immunofluorescence results, treatment of the ZS cells with the antibody against the 69-kD peroxisomal integral membrane protein revealed the presence of membrane particles, similar to our finding in ZS fibroblasts (4). This finding suggests that peroxisomal membrane ghosts (lacking catalase) are also present in lymphoblasts.

The ZS lymphoblasts are markedly deficient in the peroxisomal contribution to plasmalogen synthesis (Table 2). The amount of VLCFA appeared to be somewhat increased in ZS lymphoblasts, but the extent of any increase was much less than has been observed previously in fibroblast cells. Because of the variability in VLCFA determinations in control lymphoblasts (see above), no conclusion may be drawn from these data about any possible increase of VLCFA in ZS lymphoblasts.

Cytogenetic analysis of high-resolution G-banded chromosomes of the ZS cells (GM08764) revealed a normal female karyotype. No abnormalities of chromosome 7 were observed (Fig. 2).

7q- syndrome lymphoblasts. GM10160 cells were found to contain an interstitial deletion of one chromosome 7 (q11.23q22.1). The deletion begins in 7q11.23 and ends in 7q22.1 (Fig. 2).

Catalase is present in 7q- cells at approximately normal levels. Its subcellular distribution is very similar to that found for control lymphoblasts, *i.e.* the majority of catalase of the postnuclear supernatant fraction is recovered in the high-speed pellet (Table 1). Immunofluorescence of the 7q- cells showed that the particulate appearance of catalase and the 69-kD PxIMP is indistinguishable from control cells (Fig. 1c and f). Furthermore, the level of VLCFA and the rate of plasmalogen synthesis were in the same range as in control lymphoblasts.

DISCUSSION

Lymphoblast cell lines have several advantages over other cell types commonly used for diagnostic and basic studies of inborn errors of metabolism, such as primary cultures of fibroblasts. Lymphoblasts are easily obtained by transformation of blood lymphocytes (8). They are immortal cell lines. Several refined cytogenetic techniques are effectively used on these cell lines (such as prophase chromosome banding techniques) (18). They have a high efficiency of transfection with DNA (19).

No information about the presence and function of peroxisomes in lymphoblasts was available when this work was begun. Therefore, it was necessary first to establish the presence of functional peroxisomes in control cells. This was achieved by visualizing peroxisomes by immunofluorescence with antibodies against matrix and membrane proteins of peroxisomes. These peroxisomes are functional, as shown by their activity in plasmalogen synthesis. Also, catalase activity is present and is localized predominantly in peroxisomes.

To evaluate whether lymphoblasts could be used for the diagnosis and basic studies of peroxisomal disorders, we evaluated the peroxisomal phenotype showed by lymphoblasts derived from a ZS patient, the prototype of the peroxisomal disorders. We found that ZS lymphoblasts showed the absence of morphologic peroxisomes and a striking deficiency in the peroxisomal enzyme activities involved in plasmalogen biosynthesis. Furthermore, ZS lymphoblasts contained structures like peroxisomal membrane ghosts, which were previously described in fibroblasts (4).

Fig. 1. Immunofluorescence localization of peroxisomal proteins in control, ZS and 7q- lymphoblasts. The anti-catalase immunofluorescence revealed the peroxisomes in control (a) and in 7q- (c) cells. No catalase-containing particles were found in ZS cells (b). The anti-69 kD PxIMP immunofluorescence revealed the presence of particles bearing this protein in control (d), ZS (e), and 7q- (f) cells. Panels a, c, and d were taken with a 40 $\times$ objective lens and panels b, e, and f, with a 100 $\times$ objective lens.

Table 2. VLCFA and peroxisomal plasmalogen synthesis in control, Zellweger, and 7q- lymphoblasts*

	Lymphoblasts			Fibroblasts	
	Control	Zellweger	7q-	Control	Zellweger
VLCFA					
C22 ($\mu\text{g}/\text{mg}$)	0.39	0.28	0.52	0.90 ± 0.40	0.39 ± 0.20
C26:0 ($\mu\text{g}/\text{mg}$)	0.12	0.18	0.05	0.07 ± 0.04	0.78 ± 0.24
C26:1 ($\mu\text{g}/\text{mg}$)	0.03	0.11	0.03	0.09 ± 0.07	0.88 ± 0.55
C26/C22	0.31	0.65	0.091	0.08 ± 0.03	2.0 ± 1.2
Peroxisomal plasmalogen synthesis					
$^{14}\text{C}/^3\text{H}$	1.45	0.01	1.27	1.65 ± 0.66	0.11 ± 0.04

* Lymphoblast values were determined in triplicate for each cell line. Fibroblast values correspond to the mean $\pm$ SD of 84 control and 55 Zellweger cell lines.

Fig. 2. Cytogenetic findings in ZS and 7q- lymphoblasts. G-banding of prophase chromosomes was carried out on ZS and 7q- lymphoblasts as described in Materials and Methods. No abnormalities were found in chromosome 7 in the ZS cell line. The 7q- cell line shows the deletion comprising the 7q11.23 through 7q22.1 region.

There are at least eight different complementation groups that show the ZS phenotype (20–22). Cytosolic catalase and abnormally large peroxisomal membrane ghosts have been observed in all ZS patient fibroblasts examined (3, 4, 11, 14–17, 23–25), including representatives of all five American complementation groups (24). In view of the phenotypic similarity among fibro-

blasts from different ZS complementation groups, it seems likely that the data reported here are representative of ZS lymphoblasts.

One putative gene for ZS has been assigned to chromosome band 7q11.23, on the basis of an association of chromosomal abnormalities involving this area and the ZS clinical phenotype (6, 7). A second Zellweger gene has been mapped to chromosome 1 (26). We decided to explore the peroxisomal phenotype of lymphoblasts carrying a deletion in chromosome 7, band q11.23, in the hopes that this might clarify and confirm the tentative assignment. These lymphoblasts showed a normal peroxisomal phenotype, indistinguishable from the one displayed by control lymphoblasts. There are several possible explanations. The start of the deletion in q11.23 may have missed the putative Zellweger gene. The homologous gene on the other copy of chromosome 7 may be normal; since ZS is an autosomal recessive disorder, both copies of the putative gene would have to be defective and/or inactive to produce symptoms. In any event, analysis of this 7q- cell line with a potentially useful deletion did not in fact shed new light on the mapping of a Zellweger gene. The ZS lymphoblasts analyzed here had a normal karyotype, as did four other ZS lymphoblast lines studied by Drwinga and Hoffman (27).

In conclusion, lymphoblasts represent a suitable cell line for diagnostic tests (by measurement of plasmalogen biosynthesis) and for basic scientific studies of the Zellweger syndrome.

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