Immunoelectron Microscopic Localization of Peroxisomal Enzymes in the Fibrillar Structures of Rat Liver Peroxisomes Induced by Administration of Acetylsalicylic Acid.

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Administration of acetylsalicylic acid (ASA) to rats caused an alteration in intraperoxisomal localization of enzymes in liver peroxisomes as investigated by means of immunoelectron microscopy. Rats were fed on a diet containing 0.5% ASA for 2 weeks and livers were fixed by perfusion. Vibratome sections of the liver tissue were processed to routine electron microscopy (EM), alkaline 3,3'-diaminobenzidine (DAB) reaction for catalase, and post embedding immunocytochemistry for several peroxisomal enzymes. By light microscopy (LM) of the DAB stained materials, many large spindle-form peroxisomes were noted in the cytoplasm of hepatocytes, among which normal size of peroxisomes was also observed. By EM, such large peroxisomes contained fibrillar inclusions in the matrix in addition to the crystalloid core. The fibrillar inclusions were more strongly stained than the matrix after the DAB reaction for catalase. By postembedding immunoelectron micro-

scopy, gold particles showing catalase, acyl-CoA oxidase, L- α -hydroxy-acid oxidase A and serine: pyruvate/glyoxylate aminotransferase (SPT) were closely associated with the fibrillar structures. Urate oxidase was confined exclusively to the crystalloid core, on which a few gold particles showing L- α -hydroxy-acid oxidase A was also noted. D-amino acid oxidase was associated exclusively with a clear spot located adjacent to the crystalloid core. In this clear spot, no other enzymes examined were present. The results show that the fibrillar structure induced by ASA includes at least catalase, acyl-CoA oxidase, L- α -hydroxy-acid oxidase A and SPT, but not urate oxidase and D-amino acid oxidase. D-amino acid oxidase is one of elements that constitute the clear spot. Taken together, the present results demonstrate that some subcompartments exist in the experimentally altered liver peroxisomes.

Key words: Liver peroxisomes, Fibrillar structure, Localization of enzymes, Acetylsalicylic acid.

I. Introduction

Peroxisomes are ubiquitous cell organelles that contain catalase and hydrogen peroxide-producing oxidases [2, 13]. It has been established that absence of peroxisomes or a defect in peroxisomal functions causes severe diseases such as Zellweger syndrome [25]. Morphologically, two types of peroxisomes, large type ($>0.4\ \mu\text{m}$ in diameter) and small type ($<0.4\ \mu\text{m}$), are recognized in mammalian tissues. The large type of peroxisomes exists

in the liver and kidney and the small type in many other cells and tissues including liver and kidney [20].

It is well known that liver peroxisomes are proliferated by several hypolipidemic drugs [4–6, 9, 14, 18, 21, 23, 27–29] and other chemicals [15, 16]. Some of these proliferators also cause structural alteration of liver peroxisomes, such as increase in size [10, 21, 27, 38], unusual shape [28, 34, 38], and formation of intraperoxisomal inclusions [10, 22, 27]. Hruban and coworkers have shown that administration of acetylsalicylic acid (ASA) to the rat results in formation of the fibrillar inclusion in liver peroxisomes, in addition to intermediate increase in number [5, 13, 14]. Similar structure has been observed in liver per-

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oxisomes after dimetrin treatment [5, 14]. These structures seem to be quite different from the crystalloid cores, tubular structure and marginal plates that have been observed in liver and kidney peroxisomes of rat [1, 17, 43] as well as in excretory duct of mouse salivary gland [7].

It was noted in early studies [26] that the crystalloid core exists in liver peroxisomes of animals other than primates and birds that lack the urate oxidase activity. Tsukada *et al.* have purified the crystalloid core from mitochondrial fraction of rat liver by repeated extraction with Triton X-100 [31]. The purified core contained a high enzyme activity of urate oxidase, and negative staining of them showed a structure quite similar to that seen in sections. They proposed that the crystalloid core consists of fine tubular structures. Furthermore, Yokota has shown that this tubular structure consists of small globular units that line up spirally to form a tubule [35]. Hruban *et al.* have shown that the crystalloid core is composed of urate oxidase [11]. When the urate oxidase activity of isolated core fraction was compared with that of highly purified urate oxidase, the specific activity was not identical [36]. This indicated that the crystalloid core is not composed of only urate oxidase protein. Similar results were reported by Hayashi *et al.* [8]. The fibrillar structure induced by ASA is quite different from the crystalloid core that is not induced by treatment with drugs and other chemicals so far.

In the matrix of rat kidney peroxisomes, there are tubular structures that are located subjacent to the limiting membrane [1, 17]. This structure was stained for catalase more strongly than other matrix by alkaline DAB reaction [1] and by immunogold staining [32, 42], suggesting that the structure is composed of catalase in part. Morphologically, however, this tubular structure is different from the fibrillar structure in the peroxisomes of ASA-treated rat liver. Thus, macromolecular composition of the fibrillar structures induced by ASA treatment has not yet investigated.

Since post-embedding immunoelectron microscopic staining has high resolution, this method is very suitable for the study of localization of enzymes in intraperoxisomal structures. In this paper, immunoreactivity of the fibrillar structures to several peroxisomal enzymes was studied by postembedding immunoelectron microscopic technique.

II. Materials and Methods

Animals

Six male Wistar rats were used. Four animals weighing 107 g–115 g were fed on a laboratory diet containing 0.5% acetylsalicylic acid and water ad libitum for 15 days. The body weight and liver weight after the treatment were 182 g–186 g and 7.65 g–8.13 g, respectively. Four animals weighing 101 g–108 g were fed on a normal laboratory diet and water ad libitum for 15 days.

Antibodies

All peroxisomal enzymes were purified from rat liver. Antibodies against them were raised in rabbits, and immunoglobulin G (IgG) fraction was obtained by precipitation with 33% saturated ammonium sulfate. Characterization of these antibodies was described previously in references cited in parenthesis; anti-catalase [38], anti-acyl-CoA oxidase [39], anti-D-amino acid oxidase [38], anti-serine: pyruvate/glyoxylate aminotransferase [41], anti-L- α -hydroxy-acid oxidase A [40], anti-urate oxidase [38]. Monospecificity was tested by immunoblot analysis.

Perfusion fixation

After short anesthesia with ethyl ether, liver was perfused through the portal vein for 20 sec with 0.9% NaCl, followed by 10 min-perfusion with a fixative consisting of 4% paraformaldehyde, 0.1% glutaraldehyde, 0.01% CaCl_2 and 0.15 M cacodylate-HCl buffer (pH 7.4). After fixation, the liver was perfused with 0.9% NaCl for 1 min to remove excess fixative. The liver was cut into sections (200 μm thick) with an Oxford Vibratome (Foster City, California USA) and further cut into small pieces (approximately 1 mm $\times$ 1 mm). The tissue sections were divided into 3 groups.

Routine EM

The first group of tissue sections was fixed again in 2% glutaraldehyde in 0.1 M cacodylate-HCl buffer (pH 7.4) for 30 min at room temperature. After the fixation, sections were washed in PBS and postfixed in 1% aqueous solution of osmium tetroxide at room temperature for 1 hr. Sections were then dehydrated in graded ethanol and embedded in Epon. Thin sections were contrasted with 2% uranyl acetate for 10 min and with Reynolds' lead citrate for 3 min.

Alkaline DAB reaction for catalase

The second group of liver sections were fixed again in 2% glutaraldehyde as described above. After washing in PBS, sections were incubated at room temperature for 1 hr in alkaline DAB medium consisting of 3,3'-diaminobenzidine (DAB) tetrahydrochloride (1 mg/ml, Nacalai, Kyoto, Japan), 0.1 M Teorel-Stenhagen buffer (pH 10) and 0.01% H_2O_2 [19], and then postfixed for 1 hr in 1% osmium tetroxide that had been reduced by 1.5% potassium ferrocyanide. After dehydration in graded ethanol, sections were embedded in Epon. Semithin sections (1 μm thick) were examined with a Nikon light microscope equipped with a Nomarski lens system. Thin sections were contrasted with Reynolds' lead citrate for 3 min.

Postembedding immunoelectron microscopy

The third group of liver sections was dehydrated in graded dimethylformamide and embedded in LRWhite [38] at -20°C . Polymerization of the resin was performed

ed under UV light at -20°C . Thin sections were cut with a diamond knife using an LKB Ultratome and mounted on uncoated nickel grids. Immunostaining was carried out as described by Roth [24]. Briefly, grids with thin sections were incubated with 0.1% bovine serum albumin for 5 min, followed by overnight incubation with respective antibodies at 4°C . After washing with PBS, grids were incubated for 30 min with protein A-gold (15 nm in diameter) prepared as described by de Roe [3]. After washing with distilled water, grids were dried in air and contrasted with 2% uranyl acetate for 5 min and with Reynolds' lead citrate for 20 sec. For immunocytochemical control, IgG fraction from non-immunized rabbits was used instead of specific antibodies, followed by incubation with protein A-gold probe and other procedures described above.

All thin sections from all groups were examined in a Hitachi H600 electron microscope at an accelerating voltage of 80 kv.

III. Results

Effect of ASA on body and liver weights

As shown in Table 1, the body weight gain of rats fed on ASA-containing diet was similar to that of rats fed on normal diet. The body weights of rats fed for 15 days increased 1.66 times in ASA feeding group and 1.67 times in the normal control. After the 15 days feeding, liver weight evaluated as percentage of body weight was higher in ASA-fed rats ($4.3 \pm 12\%$) than clearly increased in control rats ($3.1 \pm 0.10\%$), indicating that the ASA-feeding caused an increase in liver weights.

Light microscopy of alkaline DAB staining

DAB reaction for catalase was observed with Nomarski lens system. As shown in Fig. 1a and 1b, two types of peroxisomes were noted in hepatocytes of ASA-fed rats; one is round peroxisomes with normal size, and the other is spindle-form. The long diameter of the latter type reached 5 to $18\text{ }\mu\text{m}$. The number of peroxisomes also increased

Table 1. Body and liver weights of rats fed on ASA for 15 days

	Original body wt.	Final body wt.	Liver wt.	Liver wt. in % of final body wt.
0.5% ASA	111 ± 2.9	183.8 ± 1.5	7.9 ± 0.17	4.3 ± 0.12
Chow	105 ± 2.9	175.8 ± 1.5	5.6 ± 0.19	3.1 ± 0.10

Fig. 1. Light microscopic profile of liver peroxisomes after ASA treatment. Light micrographs of sections from alkaline DAB-stained material were taken by a Nikon light microscope with Nomarski lens system. **a:** low power view of rat liver. Proliferated peroxisomes form clusters (arrows). $\times 630$ **b:** high power view of a rat hepatocyte. Large spindle-form peroxisomes are observed (arrowheads). N: nucleus $\times 2,400$

in ASA-fed rats, compared with those of untreated animals and made clusters in the cytoplasm.

Routine EM

Most of spindle-form peroxisomes in hepatocytes of ASA-fed rats contained electron dense fibrillar inclusions that ran in parallel with the long axis. In the longitudinal

Fig. 2. Routine EM of peroxisomes in ASA-treated rat liver. In addition to crystalloid cores (*), many fibrillar structures are present in the peroxisomal matrix. In the cross section of them, 3 to 4 arched plates are closely associated with each other and each plate is separated with an electron lucent line (arrowheads). In the longitudinal section, fibrillar structures extend along the longitudinal axis. Electron lucent spot is noted (arrows) in some peroxisomes. **a.** $\times 27,000$ **b.** $\times 43,000$

Fig. 3. Peroxisomes stained by alkaline DAB reaction. Reaction deposits are present exclusively in peroxisomes. In a low power view (**a**) peroxisomes are stained homogeneously, whereas in a high power view (**b**) fibrillar structures (arrowheads) are stained more densely than other part of matrix. A core (*) and electron lucent clear spot (arrows) are negative for DAB reaction. **a.** $\times 16,000$ **b.** $\times 37,000$

Fig. 4. Protein A-gold staining for catalase. Gold particles showing catalase antigen are present in peroxisomes and most of particles are closely associated with the fibrillar inclusions produced by ASA treatment. Crystalloid core (long arrow) and clear spot (short arrows) are mostly negative for labeling. $\times 29,000$

Fig. 5. Protein A-gold staining for acyl-CoA oxidase. Most gold particles are present on fibrillar structures. Some particles are observed on crystalloid cores (long arrow). Clear spots (short arrows) are largely negative for labeling. $\times 35,000$

Fig. 6. Immunogold labeling for SPT. Gold particles are present on fibrillar structures. Crystalloid cores (long arrows) and clear spots are mostly negative for labeling (short arrows). A few gold particles are seen on mitochondria (M). $\times 35,000$

Fig. 7. Immunogold staining for L- α -hydroxy-acid oxidase A. Gold particles showing this enzyme are closely associated with fibrillar structures and crystalloid cores (long arrow) but not with clear spots (short arrows). $\times 35,000$

section, the fibrillar inclusion was composed of 3 to 4 dense lines that were separated by 2 to 3 clear lines (Fig. 2a, b). The length of the structure varied with cutting plane. The longest one reached 2–3 μm . The width of the structure was approximately 40–50 nm. In cross section this structure seemed to be composed of 3 to 4 rings that have a common center (Fig. 2a). The crystalloid core was observed in the matrix of these experimentally altered peroxisomes. They were located in a middle bulge of each spindle-form peroxisome. One peroxisome included only one crystalloid core. The electron density of the core was similar to that of the fibrillar structures but their profile was quite different from the fibrillar structure. The core was a lump with a size of approximately $0.2 \times 0.2 \mu\text{m}$ on average. In addition to these structures, electron lucent clear spot was discernible in the matrix of peroxisomes. The electron density of this spot was clearly lower than that of surrounding other peroxisomal matrixes. In many cases, the clear spot was located adjacent to the crystalloid core, but occasionally existed between the fibrillar structures (Fig. 2a, b). The spot was never surrounded by membranous structure. Therefore, the border between the clear spot and other matrix was not distinct.

Alkaline DAB staining under EM

DAB reaction deposits showing peroxidatic activity of catalase were exclusively located in peroxisomal matrix. At a low magnification the matrix of peroxisomes was

stained homogeneously, so that the fibrillar inclusion and crystalloid core were not discernible (Fig. 3a). At higher magnification, however, the fibrillar inclusions were distinguished and were stained stronger than the matrix and crystalloid core (Fig. 3b). Furthermore, the clear spot was also discernible and was not stained by the DAB reaction (Fig. 3b).

Immunoelectron microscopy of peroxisomal enzymes

Catalase

Gold particles showing catalase antigenic sites were confined exclusively to peroxisomes. In peroxisomes, most gold particles were present on the fibrillar structures or closely associated with the edge of these structures (Fig. 4). Consequently, gold particles lined up along the long axis of the spindle-form peroxisomes. A small number of gold particles were present free in other part of the matrix. The crystalloid core and the clear spot were devoid of gold particles (Fig. 4).

Acyl-CoA oxidase

This enzyme was also detected on the fibrillar structure extending along the long axis of the spindle-form peroxisomes (Fig. 5). Gold particles were not present on the entire length of the long fibrillar inclusion and some parts of them lacked gold labeling. The gold labeling in the matrix was very weak. The crystalloid cores were

Fig. 8. Protein A-gold labeling for urate oxidase. Gold particles are confined exclusively to crystalloid cores (long arrow). No gold labeling is present on fibrillar structures and clear spots (short arrows). $\times 35,000$

Fig. 9. Protein A-gold labeling for D-amino acid oxidase. Gold particles showing the antigenic sites for this enzyme are closely associated with clear spots (short arrows), but not with cores (long arrow) and fibrillar structures induced by ASA treatment. $\times 35,000$

weakly labeled (Fig. 5). The clear spot was largely devoid of gold particles (Fig. 5).

SPT

Gold particles showing this enzyme were present in peroxisomes and mitochondria, but the labeling intensity was very weak, compared with other enzymes tested (Fig. 6). In peroxisomes, gold labeling was confined to the fibrillar inclusions. The crystalloid core and the clear spot were devoid of gold particles (Fig. 6).

L- α -hydroxy-acid oxidase A

Gold particles showing this enzyme were confined exclusively to peroxisomes, in which most of the gold particles were associated with the fibrillar structures and in part with the crystalloid cores (Fig. 7). The clear spot was devoid of gold particles.

Urate oxidase

This enzyme was present exclusively in peroxisomes. Within the peroxisomes the gold particles showing the urate oxidase antigenic sites were confined exclusively to the crystalloid core (Fig. 8). The fibrillar structures induced by ASA treatment as well as the clear spot were consistently negative for this enzyme.

D-amino acid oxidase

The enzyme was localized in peroxisomes. Within the peroxisomes experimentally altered by ASA treatment, gold particles showing this enzyme were detected exclusively in the clear spot. The other matrix as well as the fibrillar structures and the crystalloid core were completely devoid of gold particles (Fig. 9).

Immunocytochemical control

When thin sections were incubated with the IgG fraction from non-immunized rabbits instead of specific antibodies, no gold particles were noted in peroxisomes (data not shown), indicating that gold labeling for each peroxisomal enzyme was specific.

Results of the immunoelectron microscopy of peroxisomal enzymes are summarized in Table 2.

IV. Discussion

Administration of ASA causes a slight hepatomegaly. The effect is less than that caused by clofibrate and di-(2-ethylhexyl)phthalate (DEHP) [14, 37]. A numerical increase of peroxisomes is associated with formation of fibrillar structures within peroxisomes. These results were quite consistent with those reported so far [5, 12, 14]. The effect of ASA administration on the numerical increase of peroxisomes is also less than that of other peroxisome proliferators [9, 18, 21–23, 29, 30 37].

Light microscopy of semithin sections after alkaline DAB reaction revealed the presence of many spindle-form peroxisomes. Similar peroxisomes were observed in duct epithelial cells of mouse parotid gland [7], other than the drug-treated animals. The long diameter of the spindle-form peroxisomes occasionally exceeded the diameter of hepatocyte nucleus. By routine EM, numerous fibrillar structures were confirmed in the matrix of such experimentally altered peroxisomes. They ran in parallel to the long axis of peroxisomes. A crystalloid core was also noted in the matrix. In addition to these structures, electron less dense clear spot was distinguished near the crystalloid core that was located in a bulge at the middle of the spindle-form peroxisomes.

In the present study, localization of catalase, acyl-CoA oxidase, L- α -hydroxy-acid oxidase A, serine: pyruvate/glyoxylate aminotransferase (SPT), urate oxidase and D-amino acid oxidase was studied to characterize macromolecular composition of the fibrillar structures induced by ASA treatment. Four enzymes such as catalase, acyl-CoA oxidase, L- α -hydroxy-acid oxidase A and SPT were found to be associated with this structure. Urate oxidase was confined exclusively to the crystalloid core. D-amino acid oxidase was not associated with any of these structures but closely with the clear spot. These results indicate that the fibrillar inclusion induced by ASA treatment contains four of the enzymes examined. The result that the crystalloid core contains urate oxidase completely agrees with that of early studies [11, 26].

It is still unclear why the fibrillar structure is formed after ASA administration. As shown in the present study

Table 2. Localization of enzymes in intraperoxisomal structures of rat liver peroxisomes after ASA treatment

	Matrix	Clear spot	Core	Fibrillar structure
Catalase	+	—	—	++
Acyl-CoA oxidase	+	—	—	++
L- α -hydroxy-acid oxidase	+	—	++	++
Serine; pyruvate/glyoxylate aminotransferase	+	—	—	++
Urate oxidase	—	—	++	—
D-amino acid oxidase	—	++	—	—

—: no labeling, +: weak, ++: intermediate, +++: strong

as well as previous studies [5, 12, 14], the fibrillar structures might be regularly polymerized enzyme molecules. Hruban *et al.* supposed that catalase is a possible candidate for the polymerized molecules [14]. Catalase is the most abundant enzyme in rat liver peroxisomes [2], therefore, catalase may crystallize. However, they excluded it from the candidates, since the DAB reaction deposits were present between the fibrillar structures but not on them. These authors proposed that isocitrate dehydrogenase, which is one of the major components in peroxisomes, polymerize to form the fibrillar structure [14]. This has not yet been demonstrated. In the present study, we have found the DAB reaction deposits are present on the fibrillar structures, unlike the results of Hruban *et al.* [11]. Our immunogold staining confirmed the association of catalase with the fibrillar structures as well as other enzymes. We propose that the fibrillar structure induced by ASA treatment may be formed by polymerization of multiple enzymes rather than a single enzyme. Therefore, other peroxisomal enzymes may also be detected in the fibrillar structure. It is unknown why ASA induces such polymerization. Nevertheless the concentrations of enzymes are not high.

Previous reports have clarified that there are three subcompartments where distinct enzymes are localized within peroxisomes of rat liver and kidney. They are the crystalloid core that contains urate oxidase, the matrix where many peroxisomal enzymes are present, and the electron lucent clear spot where D-amino acid oxidase is associated [32, 33, 42]. In the present study, this compartmentalization is also confirmed in the experimentally altered peroxisomes. Especially, the electron lucent spot was more clearly visible than normal peroxisomes that had been shown previously [33]. Hayashi *et al.* showed that solubility of D-amino acid oxidase to Triton X-100 is different from other peroxisomal enzymes and suggested that this enzyme is localized neither in the area where catalase is present nor in the crystalloid core [8]. The present study and our previous results clearly showed the precise area where D-amino acid oxidase is localized.

In conclusion, the fibrillar structures induced by administration of ASA consist of, at least, catalase, acyl-CoA oxidase, SPT, and L- α -hydroxy-acid oxidase. There is clear spot in the matrix, where D-amino acid oxidase is associated.

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