INTRALOBULAR AND INTRACELLULAR LOCALIZATION OF ALCOHOL DEHYDROGENASE (ADH) ISOZYMES IN MOUSE LIVER: BASIC ADH (CLASS I) AND ACIDIC ADH (CLASS III)

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ABSTRACT

Localization of alcohol dehydrogenase isozymes [basic alcohol dehydrogenase (ADH): Class I and acidic ADH: Class III] was studied at both lobular and cellular levels in mouse liver by using immunohistological methods. The immunofluorescent microscopy showed that the basic ADH is distributed more predominantly in the perivenous zone than in the periportal zone, although the enzyme was detected in every parenchymal cell within the whole hepatic lobule. By immunoelectron microscopy, the enzyme was shown to distribute within the cytoplasmic matrix of hepatocytes, except for glycogen areas. On the other hand, acidic ADH was found to localize mainly on the periphery of the parenchymal cell, showing no distinct localization within the liver lobule by the immunofluorescent microscopy. The immunoelectron microscopic study confirmed that the acidic ADH localizes in the sinusoidal endothelial cells of the liver and revealed that the enzyme is also distributed in phagocytotic vesicles within hepatocytes. Thus, the basic and acidic ADH isozymes localize differently from each other in mouse liver tissue including parenchymal and nonparenchymal cells. These findings suggest that ADH isozymes may play different roles from each other in alcohol metabolism in the liver, not only at the enzymic property level but also at the cellular level.

It is generally accepted that alcohol dehydrogenase (ADH) (EC 1.1.1.1) is a key enzyme in alcohol metabolism in mammalian liver. During metabolism of alcohol, the enzyme produces the highly toxic acetaldehyde and also large amounts of NADH, shifting the cellular redox potential to the reduced state and thus leading to heavy disturbances of the cell metabolism. The enzyme is therefore thought to play a significant role in the pathology of alcohol intoxication (27, 28, 34, 39).

Mammalian liver ADH isozymes are grouped into 3 classes (Classes I, II and III) (3, 16, 22, 38). Class I enzymes are very sensitive to pyrazole, and have a low K_m for ethanol and a basic pI [human ADH isozymes with α , β , γ subunits (38) and mouse basic ADH (16) or ADH A_2 (3)]. Class III

enzymes are insensitive to pyrazole, and have a very high K_m for ethanol and an acidic pI [human κ -ADH (33) and mouse acidic ADH (14-16) or ADH B_2 (3)]. Class II enzymes have intermediate properties between Class I and Class III and their contents in the liver are low [human π -ADH (4), mouse ADH- β (16)].

Localization of ADH within the liver lobule has been studied from a pathological point of view by various methods. The results, however, have inconsistently demonstrated its centrilobular (7, 25, 30), or periportal (11, 12, 29) localization. In these studies, the Class I ADH isozymes have been solely focused on because the isozymes have been believed to play a main role in ethanol metabolism, whereas the other two classes of ADHs have been

recently found and few studies on their roles in alcohol metabolism *in vivo* have been reported.

A recent study using ADH⁻ deer mouse, genetically deficient in ADH (Class I), has revealed the existence of a significant metabolic pathway of alcohol, which is independent on Class I ADH (8).

We have recently reported that mouse liver acidic ADH (Class III) is increased in its activity during acute alcohol intoxication (17) and that human liver α -ADH (Class III) increases its activity corresponding to the increase of total alcohol intake (24). These data suggest a relationship between Class III ADH and alcohol metabolism or alcoholic liver disease.

In this study we investigated the localization of acidic ADH (Class III) and basic ADH (Class I) in mouse liver by immunofluorescence and immunoelectron microscopy using the isozyme-specific antibodies, and discussed the relationship between isozyme localization in the liver and alcohol metabolism or alcoholic liver disease.

MATERIALS AND METHODS

Purification of Acidic and Basic ADH from Mouse Liver

Livers (237 g) of ddY male mice (10 weeks old) were homogenized in 3 vol (w/v) of an extraction buffer [0.5 mM NAD/0.65 mM dithiothreitol (DTT)/5 mM Tris-HCl, pH 8.5] with an Ultra Turax homogenizer. The homogenate was centrifuged at 105,000 g for 1 h to obtain liver extract. The extract was dialyzed against 0.5 mM NAD/0.65 mM DTT/5 mM phosphate buffer (PB), pH 6.8, and then applied to a CM-cellulose column equilibrated with the same buffer. Acidic ADH fraction passing through the CM column was chromatographed on a DEAE-cellulose column by a linear PB gradient (5–100 mM) supplemented with 0.5 mM NAD and 0.65 mM DTT. The eluted enzyme fraction was dialyzed against 10 mM PB, pH 7.4 and applied to a Blue Sepharose column equilibrated with the same buffer. The enzyme was eluted from the column by a linear gradient of NAD (0–1.5 mM) in the PB containing 16 mM hexenol. After the affinity chromatography, the enzyme fraction was concentrated and then gel-filtered using a Toyo pearl column equilibrated with 10 mM PB, pH 7.4 (14).

Basic ADH was eluted from the CM-cellulose column by a linear PB gradient (5–100 mM) supplemented with 0.5 mM NAD and 0.65 mM DTT.

The eluted enzyme fraction was dialyzed against 10 mM PB, pH 7.4 and applied to a Blue Sepharose column. The enzyme was eluted from the column by a linear gradient of NAD (0–1.5 mM) in the buffer containing 14 mM ethanol and then gel-filtered by a Toyo pearl chromatography.

ADH activity was assayed at 37°C in 0.1 M glycine buffer (pH 10.7) containing 1.7 mM NAD and substrate (7 mM hexenol for acidic ADH, 14 mM ethanol for basic ADH) at 340 nm using a Simadzu UV-300 Autoscan spectrophotometer.

Protein concentration was determined with BCA Protein Assay Reagent (Pierce, Rockford, IL).

The purity of the final enzyme preparations was checked by electrophoresis on a gradient SDS-polyacrylamide gel (10–20%) according to the method of Laemmli (26) and molecular weight of ADH was estimated using High Molecular Weight Standard Mixture (Sigma, St. Louis, MO).

Preparation of Antibody against Acidic or Basic ADH

Antibody was raised in rabbits by subcutaneous injection of purified ADH isozyme (1 mg each protein/animal) emulsified in Freund's complete adjuvant (Difco, Detroit, MI) (1:1, v/v). After 1 week, the same dose of the emulsion was injected into the animal. Additional immunization by two intravenous injections with enzyme protein alone (0.2 mg each/animal) followed at weekly intervals. The rabbit was bled from the heart on the 5th week, and the antiserum was collected. Preimmune serum samples collected prior to the first injection served as controls.

Evaluation of Antibody Specificity

The antiserum was tested by double immunodiffusion in 1.2% agarose (See Kem LE, Marine Collids, Rockland, ME) according to Ouchterlony (32).

Western blot analysis was performed according to Towbin *et al.* (37) with nitrocellulose filter [Schleier & Schuell, cellulosenitrat (E), BA 85, 0.45 μ m, Ref. No. 401196] after electrophoresis on a gradient SDS-polyacrylamide gel. Immuno-reaction on the filter was detected by the biotin-avidin technique (20).

Immunocross-reactivities between the mouse ADH isozymes (acidic, basic and ADH- β) were studied by alkaline disc electrophoresis of antibody-treated liver extract (14, 16).

Fluorescence Microscopy

According to Ishizaki *et al.* (21), blocks of fresh mouse liver (ddY, male, 8 weeks old) were placed on a filter paper and immediately frozen in liquid *n*-hexane which was chilled with dry-ice-acetone. Tissue sections (2–3 μ m in thickness) were prepared in a cryostat at -20°C , fixed in acetone (-20°C) for 10 min and washed with phosphate-buffered saline (PBS) for 10 min at 25°C .

For indirect immunofluorescence staining, the sections were incubated with properly diluted antiserum against mouse ADH isozyme at 25°C for 30 min. After washes in PBS for 5 min (3 times), the sections were stained with FITC-labeled anti-rabbit IgG, goat antibody (Miles-Yeda, Elkhart, IN) at 25°C for 30 min, and then washed again with PBS for 5 min (3 times). All reagents were obtained from commercial sources and checked by immunoelectrophoresis.

The stained sections were mounted with 50% glycerine in 0.5 M carbonate buffer, pH 9.5, and examined on an Olympus BHF fluorescent microscope.

As controls, tissue sections were incubated with normal rabbit serum.

Immunoelectron Microscopy

Immunoelectron microscopy using peroxidase-labeled antibody was performed as described by Yamada and Nakane (41). Fresh mouse liver was immediately fixed in periodate-lysine-paraformaldehyde fixative (PLP) (0.01 M NaIO_4 , 0.075 M lysine, 0.0375 M sodium phosphate buffer, 2% paraformaldehyde) for 6 h. The tissue was then washed overnight in 0.05 M PBS (pH 7.4) with 10% sucrose, PBS with 15% sucrose for 12 h, and PBS with 15% sucrose and 10% glycerol for 2 h. The resulting tissues were embedded in OCT compounds (Lab-Tek Products, Naperville, IL) with 10% glycerol, quick-frozen in liquid nitrogen and stored at -70°C . The 4 μ m frozen sections cut in a cryostat were mounted on glass slides coated with egg albumin and washed 3 times in PBS with 10% sucrose. For indirect immunohistochemical staining, the tissue sections were incubated at 4°C overnight with antiserum against mouse ADH isozyme.

The sections were washed with PBS containing 10% sucrose (8 times) at 4°C for 12 h, then incubated at 4°C overnight with horseradish peroxidase

(HRPO)-labeled anti-rabbit IgG, goat antibody (Miles, Elkhart, IN) diluted 1:16 with the buffer. The tissue sections were washed 8 times at 4°C for 12 h with the buffer. After postfixation in 2% glutaraldehyde for 20 min, the sections were reacted with 0.02% diaminobenzidine (DAB)/0.05% hydrogen peroxide for 10 min, fixed in 2% osmium tetroxide for 30 min, dehydrated in a graded series of ethanol to 100%. The sections were embedded by inverting a capsule filled with SPUR (Polysciences, Warrington, PA) onto the sections and polymerized in an oven. The blocks were examined by light microscopy to select positively stained areas. Ultrathin sections of these areas were cut on an LKB ultramicrotome and examined without additional staining or with uranyl acetate staining under a Hitachi 12A electron microscope. As controls, tissue sections were incubated with normal rabbit serum diluted 1:16 with PBS containing 10% sucrose.

RESULTS

Purification of Acidic and Basic ADH Isozymes

Results of purification of both ADH isozymes were summarized in Table 1, a and b. Each purified preparation of ADH isozyme formed a single protein band around 38 K (acidic ADH) or 37 K (basic ADH) by electrophoresis on a gradient SDS-polyacrylamide gel (Fig. 1). The molecular weight value of acidic ADH was almost the same as that reported previously (14) and the value of basic ADH was close to that of Class I ADHs (5).

Evaluation of Antibody Specificity against Mouse ADH Isozyme

Double immunodiffusion test of each antiserum against acidic or basic ADH showed a single precipitation line with mouse liver extract (data not shown). Western blot analysis using each antiserum also formed a single band around 38 K (acidic ADH) or 37 K (basic ADH) on nitrocellulose filter where liver extract proteins and purified acidic ADH or purified basic ADH were transferred after SDS-gradient gel electrophoresis (Fig. 2, A and B).

To check immunocross-reactivity between ADH isozymes of mouse liver, the antibody-treated liver extract was applied to alkaline electrophoresis. As shown in Fig. 3B, the antiserum against acidic ADH reacted only with that enzyme, not with the other isozymes (basic ADH and ADH- β). The anti-

Table 1a *Purification of Acidic ADH from Mouse Liver*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Liver extract	13,827.5	1,054.3	0.076	1	100
CM-chromatography	11,300.3	839.5	0.074	0.97	79.6
DEAE-chromatography	432.2	349.3	0.81	10.7	33.1
Blue Sepharose-chromatography	26.4	285.4	10.81	142.2	27.1
Toyo pearl gel filtration	20.0	222.5	11.13	146.4	21.1

Table 1b *Purification of Basic ADH from Mouse Liver*

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Liver extract	13,827.5	871.2	0.063	1	100
CM-chromatography	793.5	335.4	0.42	6.7	38.5
Blue Sepharose-chromatography	23.6	132.2	5.60	88.9	15.2
Toyo pearl gel filtration	18.0	101.9	5.66	89.8	11.7

Activities of acidic ADH and basic ADH were measured for hexenol (7 mM) and ethanol (14 mM) as substrates, respectively. For details, see Materials and Methods.

serum against basic ADH also reacted only with that enzyme, not with the others (Fig. 3C).

These data demonstrate that each antiserum is monospecific for each ADH isozyme.

Immunofluorescent Microscopic Study on Localization of ADH Isozyme

Basic ADH Strong and almost even staining of the parenchymal cells around the pericentral area was found when sections of normal mouse liver were incubated with the antiserum against basic ADH (Fig. 4A). The fluorescence around the periportal area tended to be darker, indicating a lower amount of basic ADH in this area than in the pericentral area. No fluorescence was seen in nuclei of the parenchymal cells.

Acidic ADH With the antiserum against acidic ADH, strong stainings were seen on the periphery of the parenchymal cells, and cells themselves were also faintly stained, showing no distinct localization within the liver lobule (Fig. 4B).

Control Almost even darkness was seen in all tissue area, when the section was incubated with normal rabbit serum prepared from preimmune rabbit (data not shown).

Immunoelectron Microscopic Study on Localization of ADH Isozyme

Basic ADH By the treatment of the sections with the antiserum against basic ADH, granular depositions of the peroxidase reaction product were almost evenly distributed in the cytoplasm of the hepatocyte, except for the glycogen areas (Fig. 5).

Acidic ADH With the antiserum against acidic ADH, strong reaction products were seen on sinusoidal walls, which were constituted mainly of sinusoidal endothelial cells (Fig. 6).

Fig. 7 shows that the peroxidase reaction products derived from the antiserum were mainly found in sinusoidal endothelial cells. The products were additionally seen in spaces between endothelial cells and hepatocytes (Disse space), and also in phagocytotic vesicles of the hepatocyte.

DISCUSSION

Localization of Basic ADH

The previous studies of the intralobular localization of ADH in the liver have yielded conflicting results when the enzyme activity was used as an indicator, partly because the substrate conditions

Fig. 1 SDS electrophoresis of purified acidic ADH and basic ADH on 10–20% gradient polyacrylamide gel. Protein samples were treated with 1% 2-mercaptoethanol/1% SDS at 100°C for 2 min and subjected to electrophoresis with a slab gel (8.4×9.0×0.1 cm) and 0.1% SDS/25 mM Tris-glycine buffer, pH 8.4. Protein bands were stained with 0.25% Coomassie brilliant blue R in 10% acetic acid/50% methanol. Lanes 1, 2, 10, 11, liver extract proteins (86, 43, 43, 86 μ g); lanes 3, 4, 5, acidic ADH (1.9, 4.8, 9.6 μ g); lanes 7, 8, 9, basic ADH (2.6, 6.5, 12.9 μ g); lane 12, molecular size markers

were different (11, 12, 25, 29, 30). High concentrations of ethanol (0.5–1.2 M) (25, 40) or 0.4 M propanol (11) used as substrate to detect ADH in the several papers, are not specific for detection of each ADH isozyme. In general the techniques using enzyme activity as an indicator [histochemistry (11, 25, 40), microdissection (30), separation of isolated hepatocytes (12, 29)] have the common difficulty in discriminating various isozymes (23). If ADH isozymes localize differently in liver tissue, the results of the localization studies could be changed depending on substrate conditions, because the ADH isozymes have the different substrate specificities from each other (16). Therefore, it is necessary to apply immunohistochemical methods using isozyme-specific antibody to identify each isozyme in the localization study. Immunohistochemical techniques have demonstrated the perivenous localization of ADH both in human

Fig. 2 Western blot analyses of acidic ADH (A) and basic ADH (B). Enzyme protein was electrophoresed on a gradient SDS-gel as in Fig. 1, and transferred to a nitrocellulose filter by electroblotting at 0.8 mA/cm² for 1 h in 25 mM Tris-glycine buffer (pH 8.4) containing 20% methanol. The filter blot was blocked with 3% bovine serum albumin (BSA)/10% horse serum (HS)/TBS (0.2 M NaCl/0.5 M Tris-HCl buffer, pH 7.4) for 1 h at 25°C, washed with 0.1% HS/TBS for 10 min, 3 times and then incubated with isozyme-specific antiserum in 3% BSA/10% HS/TBS at 4°C overnight. After rewashing and reblocking, the blot was incubated with biotin-conjugated anti-rabbit IgG in 3% BSA/5% HS/TBS for 1.5 h at 25°C, washed with TBS (10 min, 3 times) and then incubated with avidin-labeled horseradish peroxidase in 3% BSA/5% HS/TBS for 45 min at 25°C. Colouring reaction was done with 0.1% 3,3'-diaminobenzidine tetrahydrochloride/15 mM imidazole/0.01% H₂O₂/TBS after washing with 0.05% Tween 20/TBS for 6 min, 2 times and with TBS for 6 min, 3 times. A: antiserum against acidic ADH, lanes 1, 2, 3, liver extract proteins (4.4, 0.8, 0.4 μ g); lane 4, purified acidic ADH (0.2 μ g); B: antiserum against basic ADH, lane 1, purified basic ADH (0.2 μ g); lanes 2, 3, 4, liver extract proteins (0.4, 0.8, 4.4 μ g)

(7) and rat (25) livers.

Our immunohistological study using the isozyme-specific antibody also confirmed the perivenous distribution of basic ADH (Class I) in mouse liver lobule, although the perivenous localization was not so predominant (Fig. 4A), when compared with the results in rat (25) and human (7) livers.

From these immunohistological results in mammalian livers, it may be possible to conclude that Class I ADH proteins localize predominantly around the perivenous area (Zone III) in the liver lobule.

However, it is necessary to take into consideration that the localization of Class I ADH may be changed within the liver lobule, depending on the condition of liver. Several reports have demonstrated the periportal localization of ADH activity in isolated hepatocytes (12, 29), even under the exclusive substrate conditions for Class I ADH. Several enzymes involved in glycolysis or glycogenesis are known to shift their localizations within the liver lobule as a result of starvation (35) or hepatectomy (6).

The intracellular localization of ADH in hepatocyte has been investigated by Watabiki (40) using ultracytochemical staining. ADH within mouse hepatocyte localizes unevenly in the cytoplasmic matrix, especially in close proximity to the rough endoplasmic reticula or mitochondria, and within the mitochondria (40).

In Watabiki's study, however, ADH was detected by enzyme activity with 1.2 M ethanol as substrate, with which acidic ADH shows predominant activity rather than basic ADH. In addition, the ultracytochemical technique may be seriously affected by endogenous NADH or diffusion of the enzyme-reaction products.

Our data by immunoelectron microscopy showed that basic ADH localizes evenly within the cytoplasmic matrix of the hepatocyte, except for glycogen areas, and not in nuclei and mitochondria (Fig. 5). This result is supported by the biochemical data that ADH activity was negligible in organelle fractions when ethanol was used as substrate (9, 31).

Localization of Acidic ADH

Our study using the immunofluorescent microscopy showed that acidic ADH localized mainly on the periphery of the parenchymal cells and slightly within the cells themselves, although the intra-

Fig. 3 Disc electrophoresis of alcohol dehydrogenase isozymes in the mouse liver extract after treatment with isozyme-specific antibody. The liver extract was treated with antiserum or control serum at 4°C for 2 h and then centrifuged at 8,000 g for 10 min. The supernatant was subjected to electrophoresis on 7.5% acrylamide gel (0.1 M L-arginine buffer, pH 9.7) using 0.5 mM NAD/40 mM β -alanine/3.5 mM L-arginine buffer, pH 9.0. The enzyme was stained with 2.2 mM NAD/7 mM hexenol/0.8 mM neotetrazolium/1.4 mM PMS/0.1 M glycine buffer, pH 10.7. A, control serum; B, anti-acidic ADH antibody; C, anti-basic ADH antibody

lobular localization was almost even both in the peripheries and in the cells (Fig. 4B). The immunoelectron microscopy revealed precisely that the enzyme was localized in sinusoidal endothelial cells (Figs. 6 and 7). In addition, the enzyme was distributed in Disse spaces and in hepatocyte, where the enzyme was in phagocytotic vesicles (Fig. 7). These findings suggest that acidic ADH is synthesized in the sinusoidal endothelial cell and then transported to the hepatocyte.

Different from Class I and Class II ADH iso-

Fig. 4 Indirect immunofluorescent staining of ADH isozyme in sections of mouse liver. Section was stained with FITC-conjugated goat anti-rabbit IgG after incubation with antiserum against ADH isozyme. A, anti-basic ADH antibody; B, anti-acidic ADH antibody; PV, portal vein; CV, central vein. A: $\times 300$, B: $\times 600$

Fig. 5 Electron micrograph of immunoperoxidase reaction in mouse liver section incubated with anti-basic ADH antibody. Electron-dense reaction products are present within cytoplasmic matrix of hepatocyte except for glycogen areas (GA). M, mitochondrion; N, nucleus. $\times 13,200$

Fig. 6 Electron micrograph of immunoperoxidase reaction in mouse liver section incubated with anti-acidic ADH antibody. Electron-dense reaction products are present mainly on sinusoidal wall where endothelial cells locate. S, sinusoid. $\times 11,000$

Fig. 7 Electron micrograph of immunoperoxidase reaction in mouse liver section incubated with anti-acidic ADH antibody. Electron-dense reaction products are present in endothelial cells (E), Disse space (*) and phagocytotic vesicles (arrow) in hepatocyte. S, sinusoid. $\times 34,000$

zymes, Class III ADHs including acidic ADH are known to distribute in all mammalian tissues examined by electrophoretic analysis (1, 18, 19). Therefore, our data on the localization of acidic ADH in the sinusoidal endothelial cell suggests the universal localization of Class III ADHs in the endothelial cell of the capillary.

Alcohol Metabolism and Alcoholic Liver Disease

We previously discussed the possible roles of acidic ADH and basic ADH in alcohol metabolism during acute alcohol intoxication, based on their different K_m for ethanol (14, 18), and the difference in the

change of the two enzyme activities in the liver of ethanol-intoxicated mice (17).

Basic ADH shows maximum activity around 15 mM ethanol which corresponds to the blood alcohol concentration on intaking 180 ml (Ichi-go) of sake (Japanese wine) (14). On the other hand, acidic ADH increases its activity up to 3 M of ethanol, corresponding to the concentration of ethanol in sake (14).

It is known that ethanol in concentrations considerably higher than in the peripheral blood reaches the sinusoid of liver through the portal vein after drinking (10, 13).

In this study the two ADH isozymes were found

to localize in different cell types (parenchymal and sinusoidal endothelial cell) in liver (Figs. 5, 6 and 7). By combining the enzymological and localization data, it is suggested that acidic ADH in sinusoidal endothelial cells may function as the first step in ethanol metabolism in liver to protect the parenchymal cells from the toxicity of high concentrations of ethanol. Basic ADH may mainly participate in the second step in ethanol metabolism in parenchymal cells, after the ethanol concentration was reduced by acidic ADH in sinusoidal endothelial cells.

Thus, the two ADH isozymes may have different roles in liver alcohol metabolism not only at the enzymatic property level, but also at the cellular level.

Furthermore, our co-worker demonstrated previously that corresponding to the increase in the total alcohol intake, the activity of human liver α -ADH (Class III) increases in alcoholic liver diseases, while the activity of Class I ADH decreases (24). Such reverse changes in the two ADH isozyme activities in the liver diseases may be due to the difference in localization of the isozymes in liver tissue. Alcoholic liver diseases are known to involve capillarization of the sinusoid accompanied by fibrosis and breakdown of the parenchymal cells (36). The site-specific injury in liver lobule is also known to be characteristic of the alcoholic liver diseases and has been discussed in relation to the localization of ADH in the tissue (7, 25, 30). Taken together, Class III ADH in endothelial cells may play a more important role in alcohol metabolism in alcoholic liver diseases and may relate to the site-specific injury of the liver diseases such as pericellular fibrosis, which is frequently seen in alcoholic liver diseases (2).

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