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# Quantitative Measurement of Low- and High-Km Aldehyde Dehydrogenase Activity in Rat Liver

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Quantitative measurement of low- and high-Km aldehyde dehydrogenase (ALDH) activity was performed in the liver acinus by combination of light microscopic enzyme histochemistry with simple video microscopy and routine spectrophotometry. After ALDH activity staining, integrated staining intensity of reference wide area and small area of interest were measured by image analysing program. The resulting relative values of the small areas were converted into absolute ALDH activity using the results of spectrophotometeric enzyme assay and staining intensity obtained from the reference wide areas. The validity of the present method was confirmed by the results showing the linear relationships between the activity and either incubation time (5~20 min) or section thickness (6~20 $\mu$m). In liver acinus, both total and high-Km ALDH activity were gradually decreased from zone 3 to 1. Total ALDH activity in zone 3, 2 and 1 was 19.6, 17.2 and 16.4 mU/mm$^2$, respectively. However, the intra-acinar distribution of low-Km ALDH activity was nearly flat; 8.6, 7.9 and 8.2 mU/mm$^2$ in zone 3, 2 and 1, respectively. Low-Km values of ALDH estimated by this histochemical method did not exhibit a significant difference between zone 1 and 3; 55 and 62 $\mu$M, respectively. In view of predominant localization of alcohol dehydrogenase activity in the zone 3, even distribution pattern and nearly equal Km values of low-Km ALDH in liver acinus may provide a metabolic background for alcohol-induced liver damage which occurs predominantly in the zone 3. (Ajou Med J 1998; 3(1): 12~19)

**Key Words:** *Aldehyde dehydrogenase, Enzyme histochemistry, Video microscopy, Liver acinus, Alcohol*

## INTRODUCTION

NAD-linked aldehyde dehydrogenase (ALDH, EC 1.2.1.3) has a broad substrate specificity and convert various aldehydes into corresponding carboxylic acids. ALDH is believed to have an important function in metabolizing aldehyde metabolites, which are either derived from physiological compounds such as corticosteroids[1] and biogenic amines[2] or produced by exogenous alcohol metabolism. Although ALDH is widely distributed in the liver, kidney, stomach, brain, and heart, hepatic ALDHs have received a special interest because of its highest activity in the liver and importance for alcohol metabolism. ALDH is divided into two broad groups according to Km value for acetaldehyde: low-Km ALDH with micromolar and high-Km ALDH with millimolar range. Up to date, several isozymes of low- and high-Km ALDH were purified from mammalian livers and their biochemical characteristics were extensively investigated.[3~5] In view of implication of ALDH in detoxification of acetaldehyde, knowledge of intralobular localization of acetaldehyde metabolism in the liver is important for understanding the hepatic damage due to alcohol. However, the distribution pattern of low- and high-Km ALDH activity has been a matter of dispute. In enzyme histochemical studies, hepatocytes in the centrilobular region exhibited significantly higher ALDH activity than in the periportal region.[6,7] In contrast, luminometric or fluorometric studies indicated no regional difference in ALDH activity.[8,9] In this respect, quantitative histochemical methods using microphotometer or image analysis system would have a greater advantage in

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determination of the distribution pattern of enzyme activity on the tissue sections *in situ*. Recently, an indirect method using immunohistochemistry in combination with a video microscopy and enzyme immunoassay has been attempted to measure the antigen content in the tissue section.[10,11] It was based on the hypothesis that staining intensity due to final reaction product in the tissue section is proportional to the amount of antigen in the section. If this simple principle is verified, this method would be readily applicable to quantitative enzyme histochemical study.

In the present study, we attempted to determine the intra-acinar distribution pattern of low- and high-Km ALDH activity in the liver by combination of light microscopic enzyme histochemistry with a simple video microscopy and spectrophotometric enzyme assay. We firstly evaluated the validity of the present method by incubating tissue sections under various experimental conditions and determined the quantitative difference in ALDH activity in the hepatic lobule.

## MATERIALS AND METHODS

### Materials

Male Sprague-Dawley rats weighing 200~250 g were used. They were allowed to access freely to foods and water, and starved for 24 hr before use.

Nitroblue tetrazolium, $NAD^+$, polyvinyl alcohol (MW 70,000~100,000), pyrazole, and phenazine methosulfate were purchased from Sigma (St. Louis, U.S.A.). Acetaldehyde was obtained from Fluka (Buchs, Swiss).

### Histochemical Staining of ALDH Activity

Animals were sacrificed under ether anesthesia, and livers were removed immediately and frozen in isopentane cooled at $-50^{\circ}C$. The tissue was embedded in OCT compound and 14 $\mu$m thick-sections were made with Cryocut (Reichert-Jung 1800, Cambridge, U.K.). Incubation for demonstration of ALDH activity was performed in the media containing high concentration of polyvinyl alcohol to prevent diffusion of the enzyme.[12] The incubation media contained 60 mM phosphate buffer (pH 7.4), 18% (w/v) polyvinyl alcohol, 5 mM sodium azide, 1 mM EDTA, 0.6 mM pyrazole, 1mM $NAD^+$, 0.3 mM phenazine methosulfate and 5 mM nitroblue tetrazolium. For measurment of total and low Km ALDH activity, 5 mM and 80 $\mu$m of acetaldehyde were used as substrate, respectively.[13] Incubation was carried out for 20 min at $37^{\circ}C$ in the dark. Controls were incubated in the media without substrate. After incubation, sections were immediately rinsed in hot distilled water to stop the reaction and to remove the viscous incubation media, and were mounted with glycerol jelly.

### Spectrophotometric Measurement of ALDH Activity

The total and low-Km ALDH activity in a single section was measured spectrophotometrically by monitoring formation of NADH at 340 nm[14]. Frozen sections (14 $\mu$m) for spectrophotometry were prepared without embedding by OCT compound to exclude its possible effect on enzyme activity. The sections were thawed at $4^{\circ}C$, briefly homogenized in 1 ml of 10 mM Tris and 0.5 mM EDTA (pH 7.4), followed by sonication at $0^{\circ}C$ using Vibracell (Sonics and Materials, Danbury, U.S.A.) for $3 \times 10$ sec. The enzyme assay mixture contained 50 mM sodium pyrophosphate buffer (pH 8.4), 0.5 mM pyrazole, 1 mM $NAD^+$, and 0.5% (w/v) sodium deoxycholate. For measurement of total and low-Km ALDH activity, 5 mM and 80 $\mu$m of acetaldehyde were used as substrate, respectively. The enzyme reaction was initiated by the addition of substrate, and NADH production was monitored at 340 nm with Pharmacia Ultraspec 2000 (Cambridge, U.K.). One enzyme unit was defined as the amount of enzyme which produced 1 nmol of NADH per min.

### Histochemical Measurement of ALDH Activity

For quantitative analysis of ALDH activity on the tissue section, a microscope (Zeiss Axioskop, Oberkochen, Germany) connected with a charge-coupled device (CCD) camera (AVT-Horn, Aalen, Germany) and Mackintosh Quadra 650 computer was used. The microscopic images were transferred to the image processing program, MD-Image version 1.44, and then converted into gray scale for the measurement of pixel intensity. The mean pixel intensity of image were measured as gray levels ranging from 0 (white) to 255 (black). To maintain a constant condition for image analysis, the intensity of light beam passed through a neutral density filter and the diaphragm opening of microscope were fixed throughout the study. From the staining intensity of region of interest, the enzyme activity was calculated by the following four steps.

Step 1: Calculation of enzyme activity (Er) in the reference wide area of section;

$Er = 0.126 \times Es/As$

Es: Enzyme activity (unit) measured by spectrophotometry

As: Area ($mm^2$) of section used in spectrophotometry

Reference wide area was defined as the largest rectangular area to be captured at magnification of 100. This was determined as 0.126 $mm^2$ and thus could be used as a constant value in this formula.

Step 2: Calculation of mean integrated staining intensity (ISIr) in the reference wide area;

$ISIr = 0.126 \times (\Sigma SIr/N - \Sigma SIrc/N)$

SIr: Mean staining intensity at gray level in the reference wide area

SIrc: Mean staining intensity at gray level in the reference wide area in the control section

N: Number of observation

Step 3: Integrated staining intensity (ISIt) in the small area of interest;

$ISIt = At \times (SIt - SItc)$ ---------- Eq(1)

At: Area ($mm^2$) of the small area

SIt: Mean staining intensity in small area

SItc: Mean staining intensity of small area at gray level in the control section

Step 4: Enzyme activity (Et) in small area of interest;

$Et = Er \times ISIt/ISIr$----------Eq(2)

In this final calculation, enzyme activity in a small area can be obtained by using Er and ISIr as constant values. Since specific activity in small area can be expressed as Et/At, i.e., unit/$mm^2$, it can be obtained from Eq (1) and (2), as follows;

Specific Activity = $Et/At = Er \times (SIt - SItc)/ISIr$

The validity of the present quatitative method was investigated by incubating 14 $\mu$m sections for 2~40 min at 37°C and by incubating sections 6~30 $\mu$m thick for 20 min at 37°C. For quantitative analysis of intra-acinar distribution of ALDH activity, ten sections were prepared from different animals and five acini were randomly chosen in each section, and ten small areas per each acinar zone were used for analysis of total and low-Km ALDH activity.

## RESULTS

In histochemical stain, total ALDH activity defined as the sum of low- and high-Km activity showed a heterogenous distribution in the liver acinus, with higher activity in pericentral (zone 3) than in periportal area (zone 1) (Fig. 1a). However, low Km ALDH activity measured in the presence of 80 $\mu$M acetaldehyde was distributed almost evenly in pericentral and periportal area (Fig. 1b). In the absence of substrate, any final reaction product was not observed, indicating that the histochemical stain for ALDH activity is specific (Fig. 1c). For quantitation of intra-acinar distribution of enzyme activity, we attempted to calculate the activity per area on the basis that integrated staining intensity is proportional to the reaction product; that is, the enzyme activity in the tissue section. The calculation of the enzyme activity is acommplished by two main steps: firstly, calculation of enzyme activity of the reference wide area, and secondly, calculation of activity of small area using the results of spectrophotometric enzyme assay and staining intensity measured in the wide reference area. The measured or calculated parameters in the first process are shown in Table 1.

When the tissue sections were incubated for varing periods, a linear relationship between the specific reaction of ALDH and the period of incubation for 5 to 20 min was observed both in pericentral and periportal regions (Fig. 2). A linear relationship was also found for the specific ALDH reaction and the section thickness up to 20 $\mu$m (Fig. 3). When the cencentration of acetaldehyde in the incubation medium was varied, it was found that a fast increase in reaction velocity existed between 0.1 and 2 mM, followed by a plateau, showing a similar reaction curve obtained in biochemical assay (Fig. 4). These results demonstrated validity of the present method for histoche-

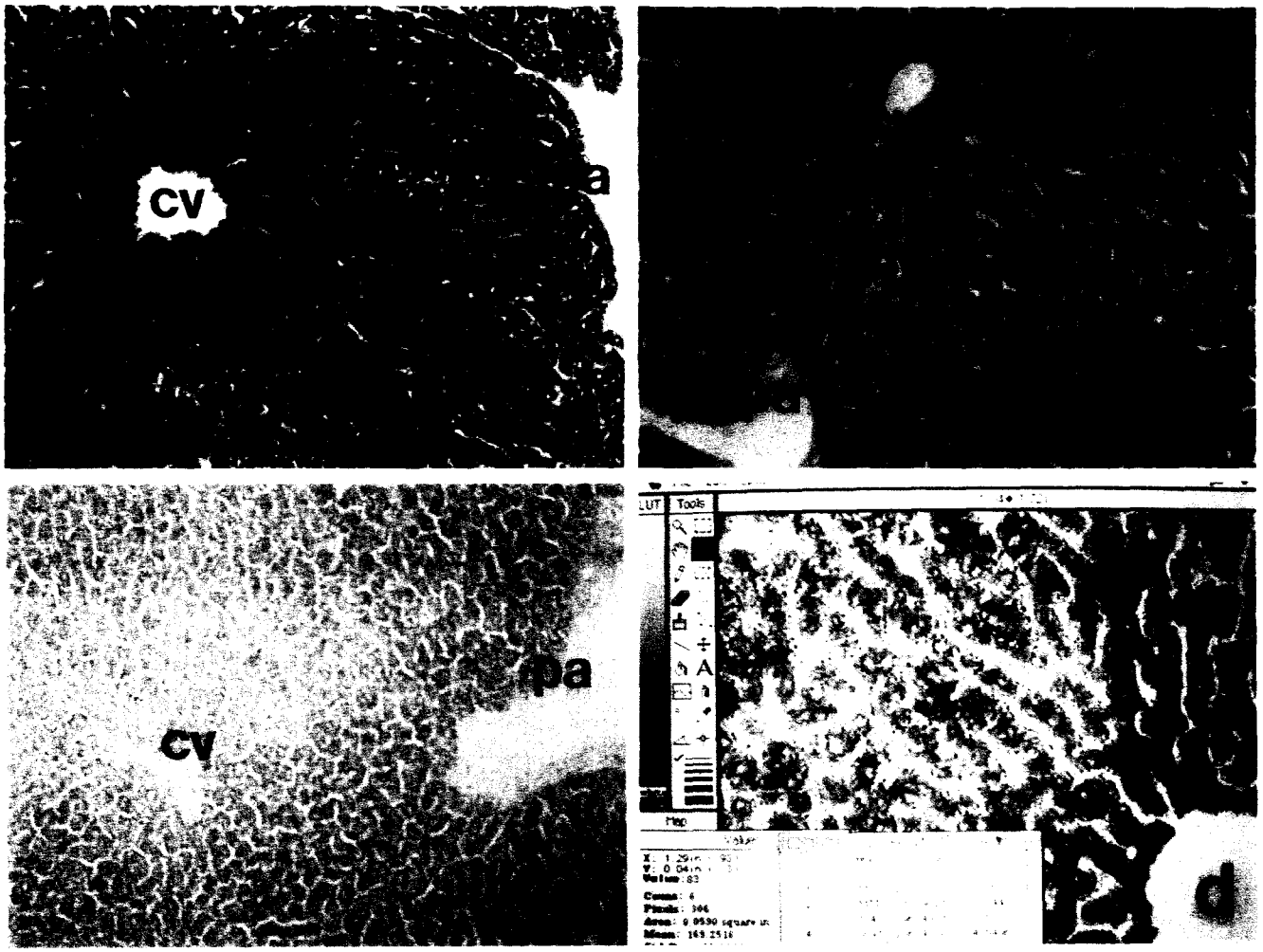


**Fig. 1.** Localization of ALDH activity in rat liver. (a) In the presence of 5 mM acetaldehyde; Total ALDH acivity is higher in the pericentral than in the periportal region. (b) In the presence of 80 $\mu$M acetaldehyde; low-Km ALDH activity is evenly distributed throughout the lobule. (c) In the absence of acetaldehyde; hardly any ALDH activity is found. (d) Microscopic image converted into gray scale (white = 0, black = 255) by image analysis program. cv: central vein, pa: portal area. Original magnification: a-c: x 125.

**Table 1.** Parameters used for calculation of total and low-Km ALDH activity

| | Definition | In measurement of | |
|---|---|---|---|
| | | Total activity[a] | Low-Km activity[b] |
| Ar | Reference wide area ($mm^2$) of rectangular image captured at 100× | 0.126 | |
| Er[c] | ALDH activity of reference wide area (Ar) (mU), | 1.16±0.21 | 0.42±0.08 |
| $\Sigma$Slr / N[c] | Mean staining intensity of reference wide area | 138.6±6.3 | 100.2±9.6 |
| $\Sigma$Slrc / N[c] | Mean staining intensity of reference wide area in control section | 28.2±1.8 | 15.0±2.4 |
| ISlr | Integrating staining intensity of reference wide area | 13.91 | 10.74 |

[a] incubation of sections were performed in the presence of 5 mM acetaldehyde as the substrate
[b] incubation of sections were performed in the presence of 80 $\mu$M acetaldehyde as the substrate
[c] mean value of 10 tissue sections prepared in different regions of liver. Means±SE

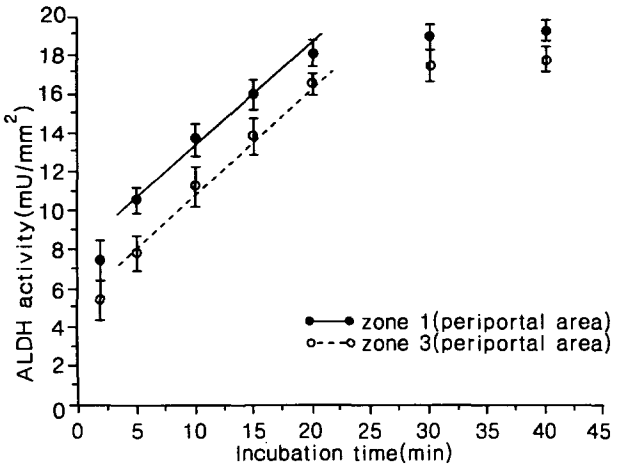


**Fig. 2.** Relationship between total ALDH activity calculated from integrated staining intensity measured in liver sections 14 $\mu$M-thick and time of incubation. Ten measurements in each zone of ten acini were analyzed. A linear relationship was found between the activity and incubation time from 5 min to 20 min; r=0.992 in zone 1and r=0.988 in zone 3. Values are means±SE.

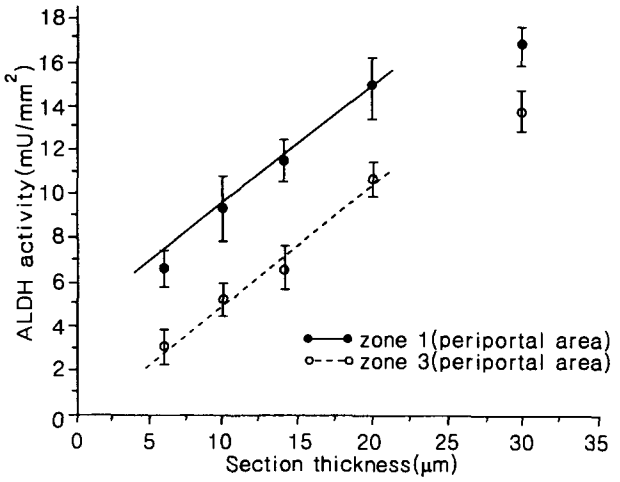


**Fig. 3.** Relationship between total ALDH activity calculated from integrated staining intensity measured in liver sections and section thickness after incubation for 20 min. Ten measurements in each zone of ten acini were analyzed. A linear relationship was found between the activity and section thickness up to 20 min; r = 0.985 in zone 1and r = 0.983 in zone 3. Values are means±SE.

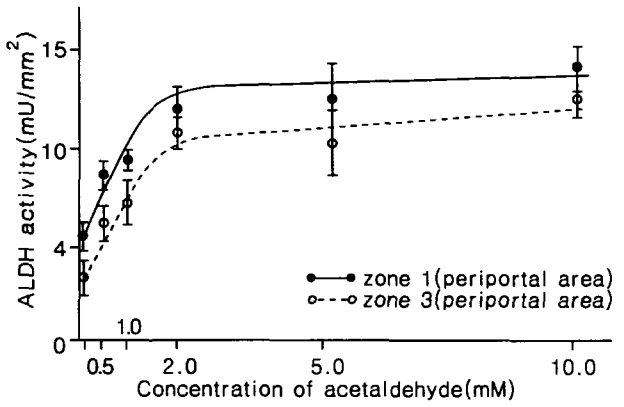


**Fig. 4.** Relationship between total ALDH activity calculated from integrated staining intensity measured in liver sections and concentration of acetaldehyde as substrate. Sections 14 mm thick were incubated for 20 min. Ten measurements in each zone of ten acini were analyzed. A fast increase in the activity up to 2 mM of substrate concentration is shown. Values are means±SE.

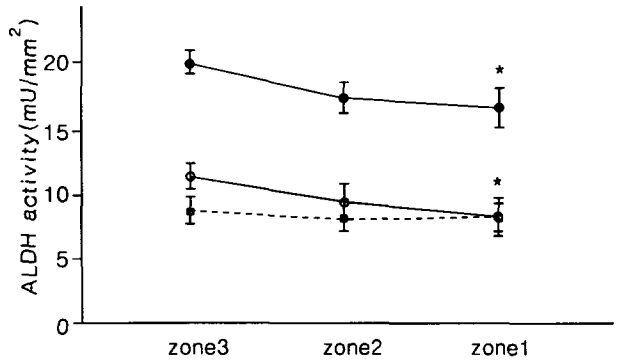


**Fig. 5.** Intra-acinar distribution of ALDH activity in rat liver. Sections 14 $\mu$m thick were incubated for 20 min. Ten measurements in each zone of 50 acini randomly chosen in different sections were analysed. Total (●—●) and high-Km ALDH activity (○—○) were decreased from zone 3 to zone 1 whereas low-Km ALDH activity (□···□) was evenly distributed. Asterisks indicate that the value is significantly different ($p<0.05$) from the values in zone 3. Values are means±SE.

mical quantitation of an enzyme activity in tissue section *in situ*.

Total ALDH activity in the liver acinus decreased gradually from zone 3 to 1. The mean values of total ALDH activity in zone 3, 2, and 1 were 19.6, 17.2, and 16.4 mU/mm$^2$, respectively (Fig. 1a, Fig. 5). In contrast to the total ALDH activity, the intra-acinar distribution of low-Km ALDH activity was nearly even; 8.6, 7.9, and 8.2 mU/mm$^2$ in zone 3 to 1, respectively (Fig. 1b, Fig. 5). These mean values corresponded to 44%, 46%, and 50% of total activity in zone 3 to 1, respectively. The mean value of high-Km ALDH activity, the value obtained by subtracting of low-Km activity from the total activity, showed a distribution pattern decreasing from zone 3 to 1. Since low-Km ALDH was reported to play an important role in acetaldehyde oxidation in ethanol metabolism in liver, the Km values of low-Km ALDH in zone 1 and

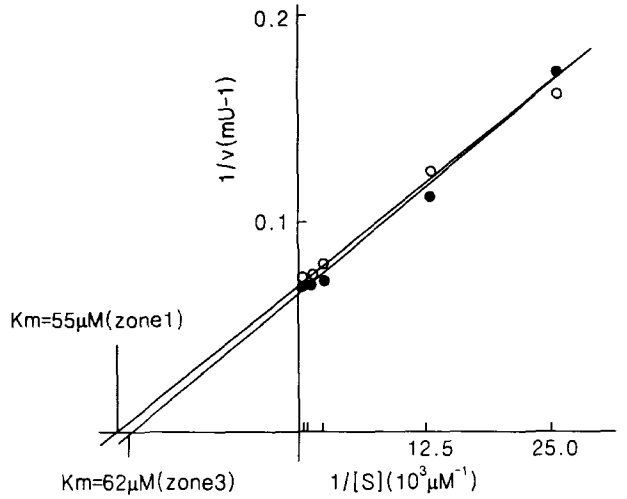


**Fig. 6.** Double reciprocal plot of low-Km ALDH activity with respect to acetaldehyde concentration(40 $\mu$M, 80 $\mu$M, 320 $\mu$M, 1 mM and 2 mM). Km values was 55 $\mu$M and 62 $\mu$M in zone 1 and zone 3, respectively. ○—○; zone 1, ●—●; zone 3.

3 were calculated using Lineweaver-Burk plotting in varing substrate concentrations (40 $\mu$M to 2 mM). The low-Km values of zone 1 and 3 were estimated to be 55 and 62 $\mu$M, respectively (Fig. 6).

## DISCUSSION

Hepatic NAD-linked ALDH has received an attention because acetaldehyde produced during alcohol degradation is very toxic to cells. It is well known that a small amount of ethanol taken by hepatic cells is oxidized to acetaldehyde by alcohol dehydrogenase in cytoplasm, which is further oxidized by mitochondrial ALDH. However, when a large amount of ethanol is taken, microsomal ethanol oxidizing system (MEOS) is induced which leads to ethanol oxidation to acetaldehyde.[15] Biochemically, ALDH is divided into two classes, high- and low-Km ALDH according to their Km values for acetaldehyde and has been reported that ALDH is present as various isoforms in different subcellular locations. High Km ALDH was reported to be present in cytoplasm and microsomes. Recently, two isoforms of the high Km ALDH were identified as glutamic-g-semialdehyde dehydrogenase[16] and succinic semialdehyde dehydrogenase.[17] Low-Km ALDH is present mainly in mitochondia and responsible for oxidation of acetaldehyde. Therefore, knowledge of intralobular distribution of Low-Km ALDH in the liver can provide a useful information on liver damage due to alcohol uptake.

In histochemical study to study distribution of the enzyme in tissue, one of the major difficulties is to quantify the reaction products on the tissue section *in situ*. Recently, this difficulty was overcome by development of microphotometer system which measures optical density of reaction product on the section at fixed wavelength of light.[18] This analysis system, however, requires complex and expensive equipments. For this reason, several investigators have attempted an another analytical method using video microscopy for quantitative histochemistry.[10,19] This method is based on a simple principle that staining intensity of reaction product in tissue section is proportional to the amount of reaction product in the section. In earlier quantitative immunohistochemical study, the enzyme content measured by the image processing method which depends on data from ELISA, coincided with the microphotometric results, which are independent of the data of ELISA.[11] However, there are little informations on usefulness of a combination of image analysis with conventional spectrophotometry for quantitation of the enzyme activity *in situ*. Thus, in the present study, we investigated the validity of this method before estimating the intra-acinar distribution of ALDH activity. It was based on the assumption that the integrated pixel intensity in a defined area (defined area × average staining intensity) would represent the enzyme activity in the same area. As results indicated, the production of formazan granules due to ALDH activity was proportional to the incubation time (5～20 min) and section thickness (6～20 $\mu$m). In addition, when the concentration of acetaldehyde was increased, it was found that ALDH activity followed zero-order kinetics at substrate concentrations higher than 2 mM. This kinetic pattern is in good agreement wth the findings by the biochemical assay. These results showed that the present method was valid for histochemical quantitation of enzyme activity *in situ*.

Heterogenous distribution patterns of enzymes in liver are well studied in many metabolic pathways, which formed the basis for the theory of metabolic zonation in the hepatic lobule.[20] In the present study, heterogenous distribution of ALDH activity was obseved: Total ALDH activity determined with millimolar concentration of substrate was the highest in the pericentral region, zone 3 and the lowest in the periportal region, zone 1. The

distribution pattern of the total ALDH activity presented here was consistent with earlier qualitative studies.[6~7] However, in contrast to the above, measurement of ALDH activity by luminometry in freezing-dried liver strips showed that ALDH activity decreased from periportal to pericentral zone in female rats and did not show a zonal difference in male rats, whereas low-Km ALDH activity equally distributed in all three zones of liver acinus.[9] The present finding that low-Km ALDH activity was evenly distributed throughout the acinus was confirmed by the immunohistochemical study that revealed the even distribution of mitochondrial ALDH isozyme ($ALDH^2$) in the lobule.[21] Furthermore, low-Km values determined by the present histochemical method did not show a significant difference between pericentral and periportal zones although these values were much higher than those obtained from biochemical analysis of purified mitochondrial enzyme.[5,22] Therefore, it is conceivable that the rate of acetaldehyde oxidation is similar in the pericentral and periportal regions.

Intra-acinar distribution of low-Km ALDH activity must be closely related to aldehyde hepatotoxicity following ethanol ingestion, since acetaldehyde is mainly oxidized by low-Km ALDH in mitochondria. It has been known for a long time that alcohol-induced liver damage occurs predominantly in the pericentral area.[23] In contrast to the even distribution of low-Km ALDH, it was reported that alcohol dehydrogenase (ADH) activity was higher in pericentral than in periportal region,[24] suggesting a possibility that acetaldehyde production in pericentral zone surpass the capacity for oxidation of acetaldehyde and the regional accumulation of acetaldehyde occurs in the presence of a large amount of ethanol. Acetaldehyde is highly reactive so that it is readily bound with intracelluar macromolecules as well as cell membranes and alters cellular functions.[25~26] The other conceivable cause of hepatocellular damage by ethanol is thought to be an excess production of NADH and subsequent alteration of intracellular redox state.[27] In this respect, the pericentral region may be more vulnerable to ethanol because NADH production via two enzymes, ADH and low-Km ALDH, seems to be more prominent in this region.

High-Km ALDH has been known to exist mainly in cytoplasm and microsomes, however, its precise role in liver has not yet been fully understood. Since millimolar range of Km value for acetaldehyde is physiologically insignificant, this enzyme has been thought to be isozymes which involve oxidation of other substrates such as aromatic or long chain aldehydes rather than acetaldehyde. In the liver acinus, the ratio of high-Km ALDH activity to total ALDH activity was the highest in zone 3 (0.54) and the lowest in zone 1 (0.50). This values were somewhat lower than the data (0.64) measured by biochemical method in our previous study.[28] A considerable amount of high-Km ALDH activity in pericentral zone may be mainly due to microsomal ALDH since pericentral hepatocytes have more abundant endoplasmic reticulum.[29]

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