a better understanding of interaction with parasitoids such as Tachinid flies, whose larval development is closely correlated with that of the host[16].

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Developmental changes of aldehyde dehydrogenase isozymes in human livers: Mitochondrial $ALDH_2$ isozyme is expressed in fetal livers

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Summary. Previous reports suggested that the major cytosolic aldehyde dehydrogenase ($ALDH_1$) was present in fetal and infant livers, but the major mitochondrial isozyme ($ALDH_2$) was absent or severely diminished. Re-examination by means of starch gel electrophoresis followed by enzyme activity staining, and by means of dot blot immuno-hybridization of liver samples with known genotypes of the $ALDH_2$ locus, indicated that both $ALDH_1$ and $ALDH_2$ genes are expressed in fetal and infant livers. In addition, $ALDH_4$ isozyme was also observed. The results imply that a fetus with the 'usual' homozygous $ALDH_2^1/ALDH_2^1$ genotype, but not one with the atypical $ALDH_2^1/ALDH_2^2$ or $ALDH_2^2/ALDH_2^2$, is capable of detoxifying acetaldehyde transferred from the mother.
Key words. Aldehyde dehydrogenase; developmental changes; gene expression.

Developmental changes in human alcohol dehydrogenase (alcohol: NAD^+ oxidoreductase, EC 1.1.1.1, abbreviation ADH) and aldehyde dehydrogenase (aldehyde: NAD^+ oxidoreductase, EC 1.2.1.3, abbreviation ALDH) have previously been observed. At the embryonic and fetal stages, liver ADH activity is much lower than at the adult stage. Among the three non-allelic genes for the class I ADH isozymes, the ADH_1 gene (for the α subunit) is expressed during the early stages of embryonic development, and the expression of the ADH_2 gene (for the β subunit) follows. The ADH_3 gene (for the γ subunit) starts to be expressed in infants[1]. In parallel with the isozyme activities, the quantity and quality of the mRNA components for the individual subunits also change during development[2, 3].

Relatively little is known about the developmental changes of ALDH isozymes. It was reported that the activity of cytosolic isozyme ($ALDH_1$) was detected, while the activities of other ALDH isozymes, i.e. mitochondrial $ALDH_2$, $ALDH_3$ and $ALDH_4$, were undetectable in fetal and infant livers[4, 5]. Acetaldehyde is far more toxic than ethanol, and the mitochondrial $ALDH_2$, which has a low K_m value for acetaldehyde, is considered to play a major role in aldehyde detoxification. In order to understand the background of fetal alcoholic syndrome, developmental changes of alcohol metabolizing enzymes, particularly $ALDH_2$, need to be examined. We found that, contrary to the previous reports, both $ALDH_1$ and $ALDH_2$ activities are expressed even in early embryonic stages.

Materials and methods

Liver samples: Liver samples (all Japanese) were from fetuses obtained after stillbirths and therapeutic abortions, and from neonates and infants who died due to various complications. Six samples (25 weeks of gestation to 45 days after birth), which were used for the preliminary study, were from the Department of Pathology, Kitasato University Hospital, Japan. 37 samples (32 samples of 15–42 weeks of gestation, and 5 infants) were

from Osaka Medical Center and Research Institute for Maternal and Child Health, Osaka, Japan. These 37 samples were prepared and preserved in the following manner. Liver materials were taken within 8 h post mortem, and, after cutting into small pieces, the samples were quickly frozen by isopentane-dry ice, and stored at −80 °C. In compliance with the Helsinki declaration, necessary consents and institutional approval were obtained.

Starch gel electrophoresis and enzyme activity staining: Liver samples were homogenized with an equal volume of cold deionized water containing 1 mM mercaptoethanol and 1 mM EDTA by a micro homogenizer (Virtis Hi-Speed −23) for 45–60 s and centrifuged (48,000 × g 20 min) at 4 °C. NAD and NADP were added to the extracts to a final concentration of 20 μM each. The extracts were immediately subjected to starch gel (12 % Electrostarch) electrophoresis. Buffer systems used were: Tris-EDTA-borate buffer, pH 8.6, containing NAD and NADP for ADH and glucose-6-phosphate dehydrogenase (abbreviation G6PD), and phosphate buffer, pH 7.2, containing NAD for ALDH. The gels were stained for ADH (ethanol as substrate), ALDH (propionaldehyde as substrate) and G6PD (glucose-6-phosphate as substrate) activities. Details of electrophoresis and enzyme activity staining have been reported previously[6].

In order to assess the condition of preservation of liver samples, G6PD activity and protein concentration of the extracts were also determined as previously described[7,8]. For starch gel electrophoresis, samples were adjusted with cold deionized water containing 1 mM mercaptoethanol and 1 mM EDTA to contain approximately the same G6PD activity.

Dot blot immuno-hybridization: One μl of serial dilutions of the extracts was blotted on a nitrocellulose filter. The filter was hybridized with an anti-$ALDH_1$ antibody (1:10,000 dilution) or with an anti-$ALDH_2$ antibody (1:5000 dilution). The filter was washed and subsequently hybridized with peroxidase-conjugated goat anti-serum against rabbit IgG (Bio-Rad. Lab.), and stained for peroxidase activity. Details of immuno-hybridization, peroxidase staining, and the origin of rabbit anti-$ALDH_1$ and anti-$ALDH_2$ were previously described[9]. The antibodies are specific for $ALDH_1$ and $ALDH_2$ respectively at the dilutions specified, and do not cross-react with other ALDH isozymes. The common atypical Oriental type $ALDH_2^2$ variant enzyme reacts with the anti-$ALDH_2$[6]. However, the quantity of the variant enzyme is severely diminished (~ 10 %) in adult livers with the atypical Oriental phenotype[6].

Genotype determination: Genomic DNAs were prepared from the liver samples according to the method described by Lacy et al.[10]. Genotypes of the $ALDH_2$ locus, i.e. homozygous usual $ALDH_2^1/ALDH_2^1$, heterozygous $ALDH_2^1/ALDH_2^2$ or homozygous atypical $ALDH_2^2/ALDH_2^2$, were determined by the use of allele specific oligonucleotide probes as previously described[11].

Results

In the preliminary study of six liver samples (25 weeks of gestation to 45 days after birth), $ALDH_2$ activity was virtually null, but $ALDH_1$ and $ALDH_4$ activities were demonstrated (photograph is not shown). However, immuno-staining demonstrated the existence of both $ALDH_1$ and $ALDH_2$ proteins in fetal and infant livers (photograph is not shown). These results suggest that the absence of $ALDH_2$ activity might be due to enzyme inactivation during sample storage, not due to diminished expression of the $ALDH_2$ gene in early developmental stages.

Subsequently, 37 fetal and infant liver samples, which were more quickly obtained and carefully preserved as described in 'Materials and methods', were subjected to electrophoresis-enzyme activity staining and immuno-staining. Activity of G6PD, which is a 'house-keeping' enzyme and is expressed in early stages of development, served as an internal reference for the liver extracts.

All Caucasians thus far examined have the 'usual' enzyme pattern (homozygous $ALDH_2^1/ALDH_2^1$), and their livers exhibit $ALDH_2$ activity. By contrast, approximately 50 % of Orientals are 'atypical' and lack $ALDH_2$ activity in their livers at the adult stage[12,13]. These subjects are either heterozygous atypical $ALDH_2^1/ALDH_2^2$ or homozygous atypical $ALDH_2^2/ALDH_2^2$[14]. Thus, genotypes of the $ALDH_2$ locus should be determined in order to ascertain the developmental-dependent expression of $ALDH_2$.

Starch gel electrophoresis followed by enzyme activity staining demonstrated that some samples exhibited $ALDH_2$ activity while others lacked the activity (table and fig. 1). Genotype determination indicated that all

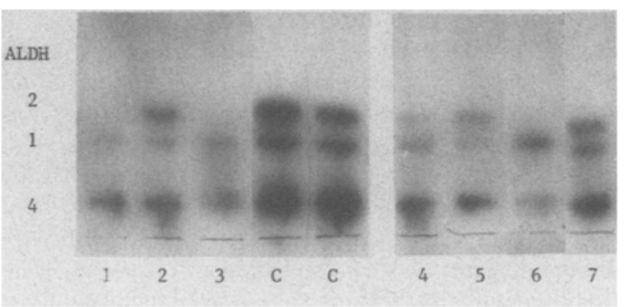

Figure 1. Starch gel electrophoretic patterns of liver extracts. The gel was stained for ALDH activity using propionaldehyde as substrate. Samples are:
1 (88-42 in table): 23 weeks of gestation, genotype $ALDH_2^2/ALDH_2^2$
2 (88-52 in table): 20 weeks of gestation, genotype $ALDH_2^1/ALDH_2^1$
3 (88-54 in table): 29 weeks of gestation, genotype $ALDH_2^1/ALDH_2^2$
4 (87-33 in table): 19 weeks of gestation, genotype $ALDH_2^1/ALDH_2^1$
5 (87-34 in table): 19 weeks of gestation, genotype not determined
6 (87-35 in table): 35 weeks of gestation, genotype $ALDH_2^2/ALDH_2^2$
7 (88-8 in table): premature newborn, genotype $ALDH_2^1/ALDH_2^1$
C: Control adult, genotype $ALDH_2^1/ALDH_2^1$
Samples were adjusted to contain approximately the same G6PD activity.

ADH and ALDH isozyme patterns and genotypes of $ALDH_2$ locus

Sample code	Gestation (week)	ADH[a]	$ALDH_1$[b]	$ALDH_2$[b]	$ALDH_2$ genotype[c]
A86-26	15	αα	+	−	
87-33	19	αα	+	+	1/1
87-34	19	αα	+	+	
88-52	20	αα, αβ	+	+	1/1
A85-31	22	αα, αβ	+	−	1/2
87-20	23	not determined	+	−	
88-42	23	αα, αβ	+	−	2/2
87-63	25	αα	±	−	
87-21	24 + 3	αα, αβ, ββ	+	−	
A84-22	25 + 4	αα, αβ	+	−	
88-54	29	αα, αβ	+	−	1/2
88-36	31	not determined	+	−	1/2
A84-8	31	not determined	+	−	
A81-3	32	αβ, ββ	+	−	
A82-58	32	not determined	+	+	
A83-13	32	αβ, ββ	+	−	
A85-7	34	αβ, ββ	+	−	
A85-59	31 + 3	αβ, ββ	+	−	
A82-59	34	not determined	d		
A84-44	35	αα, αβ	+	−	
87-17	35	αα, αβ	+	−	2/2
A85-33	36	αα, αβ	+	+	1/1
A85-39	36	αα, αβ	+	−	
A83-12	37	αα, αβ, ββ	+	−	
A83-63	37	αβ, ββ	+	−	
A83-10	38	not determined	d		
A85-18	39	not determined	d		
A83-8	40	not determined	+	−	
88-8	38 + 2	αα, αβ	+	+	1/1
A83-67	41	αβ, ββ	+	+	
A84-48	41	αβ, ββ	+	−	2/2
A86-51	42	αβ, ββ	+	−	
A86-56	35 + 4	not determined	d		
A86-45	38 + 3mo	not determined	d		
A88-39	Infant 4mo	αβ, ββ	+	+	
A87-35	Infant 8mo	αβ, ββ	+	−	2/2
A88-55	Infant 4y	αβ, ββ	+	−	

a: Major ADH isozymes observed in starch gel electrophoresis; b: $ALDH_1$ and $ALDH_2$ observed in starch gel electrophoresis (see fig. 1); c: Genotypes of the $ALDH_2$ locus determined by allele specific oligonucleotide probes; d: Enzymes were severely inactivated. Isozyme patterns were not determined. These subjects died of arthrogryposis, cystic fibrosis, herpes infection, congenital cirrhosis or hansenula infection. It is not clear whether or not the low activity of alcohol-metabolizing enzymes in these livers is related to these diseases.

livers without $ALDH_2$ activity were either heterozygous $ALDH_2^1/ALDH_2^2$ or homozygous atypical $ALDH_2^2/ALDH_2^2$, while livers with $ALDH_2$ activity were homozygous usual $ALDH_2^1/ALDH_2^1$. Therefore, the absence of $ALDH_2$ activity in some fetal and infant livers is not due to the developmental dependence of the expression of the $ALDH_2$ gene, but it is due to the mutant $ALDH_2^2$ gene which is common (gene frequency 0.35) among Japanese and other Orientals[14]. All livers examined had $ALDH_1$ activity and $ALDH_4$ activity, although the activities were somewhat lower (< 50 %) than that of adult livers (fig. 1). Immuno-staining results confirmed the existence of $ALDH_1$ and $ALDH_2$ proteins in fetal and infant livers (fig. 2). Judging from the staining intensity, the quantities of $ALDH_1$ and $ALDH_2$ existing in fetal and infant livers seem to be approximately 50 % of those in adult livers. It can be concluded that all $ALDH_1$, $ALDH_2$ and $ALDH_4$ genes are expressed and produce catalytically active enzymes from the early fetal period (15 weeks of gestation or earlier).

Development-dependent expression of ADH isozyme components was confirmed by the present study (table). Early fetal livers (before 20 weeks of gestation) have only αα homodimer enzyme. The αβ heterodimer enzyme appeared in later fetal livers, and eventually ββ homodimer enzyme becomes predominant in infants and adults (starch gel electrophoretic patterns are not shown). These findings agree with the previous observation of developmental change of ADH isozyme patterns[1].

Discussion

Two groups reported that $ALDH_2$ activity was diminished or null in embryonic livers and other tissues[4,5]. In these studies, racial origin and $ALDH_2$ genotype of the samples examined were not disclosed, but the samples were most likely Caucasians with the 'usual' homozygous $ALDH_2^1/ALDH_2^1$, since the studies were carried out in Europe. Bauman et al.[15] briefly reported that the content of $ALDH_2$ mRNA in fetal livers was not severely diminished, but that the fetal mRNA differed from the adult mRNA in the 3′ region, suggesting the control of gene expression through a post-transcriptional mechanism. By contrast, Braun et al.[5] reported that the amount of $ALDH_2$ mRNA in fetal livers was only 20 %

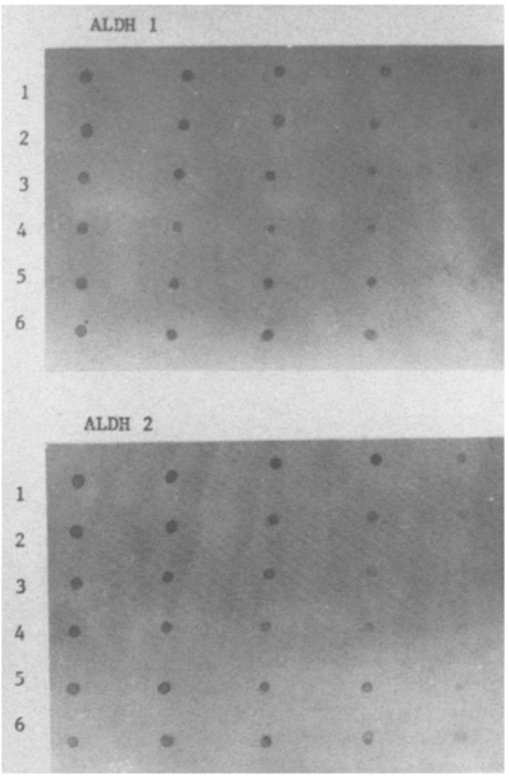

Figure 2. Dot blot immuno-hybridization. 1-µl samples of two-fold serial dilutions of the extracts were blotted on a nitrocellulose filter. Dots were visualized by immuno-hybridization with anti-$ALDH_1$ antibody or with anti-$ALDH_2$ antibody, followed by peroxidase activity stain. All samples are genotype $ALDH_2^1/ALDH_2^1$. 1: Caucasian adult; 2: Japanese adult; 3: 36 weeks of gestation; 4: 19 weeks of gestation; 5: premature newborn, same as sample 7 in fig. 1; 6: 20 weeks of gestation, same as sample 2 in fig. 1.

of that in the adult liver. Since the rate of inactivation and degradation of the enzymes and mRNAs in vivo and in vitro might differ between adult and fetal tissues, one should be cautious in interpreting these results.

The absence of $ALDH_2$ activity, observed in previous studies[4, 5], and in some samples used for our preliminary study, was most likely due to enzyme inactivation that occurred during the postmortem period, not due to the intrinsic lack of gene expression. As described in 'Results', some fetal livers without (or with severely diminished) $ALDH_2$ activity contained immunologically cross-reactive protein. In comparison to $ALDH_1$, other ALDH isozymes appear to be more labile in vitro[16–18]. Fetal and infant livers with the genotype $ALDH_2^1/ALDH_2^1$ have $ALDH_2$ as well as $ALDH_4$ and $ALDH_1$ activities (fig. 1). This fact implies that the early fetus has its own detoxification system against acetaldehyde which is transferred from the mother's body. Due to the genetic deficiency of $ALDH_2$ activity, fetuses with the genotype $ALDH_2^1/ALDH_2^2$ might be more vulnerable to fetal alcoholic syndrome than those with the genotype $ALDH_2^1/ALDH_2^1$ under the same environmental conditions. The possibility can be tested by determining the genotypes of fetal alcoholic patients and their relatives.

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