Accumulation of Hepatic Acetaldehyde and Reduced Alcohol Drinking in Transgenic Mice Carrying the Oriental Variant of the Human Aldehyde Dehydrogenase 2 Gene

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Abstract

Mitochondrial aldehyde dehydrogenase (ALDH2) is the major ALDH isozyme involved in acetaldehyde metabolism. It is well established that a single nucleotide substitution (G to A) leads to the amino acid change (Glu487Lys) and dominant inactivation of ALDH2 activity. Inactivation of ALDH2 activity causes the aversive reactions associated with "flushing" response observed in many East Asian people following alcohol intake. Although the ALDH2^2 allele (Lys487) has been shown to have a protective role against the development of alcoholism, the physiological and behavioral consequences of altered aldehyde metabolism remain unclear. The purpose of this study was to investigate the role of ALDH2 in acetaldehyde accumulation and alcohol drinking behavior in transgenic mice carrying the human ALDH2^2 allele (Lys487), which would form heterotetramers with mouse ALDH2, thus inactivating it. We produced transgenic mice carrying the human ALDH2^2 variant (hALDH2^2) using a pcDNA3 vector containing the full-length cDNA for the hALDH2^2 coding region and the mitochondrial leader sequence under the control of cytomegalovirus promoter. Expression of hALDH2^2 in liver and brain of transgenic mice was detected by RT-PCR of mRNA, enzyme assays and immunoblot analyses using polyclonal antibodies directed against ALDH2. In transgenic mice, human ALDH2^2 protein was expressed at low levels in all tissues examined. Mouse ALDH2 enzyme activity was slightly inhibited in these animals. Although there was no significant difference in liver or brain pathology in mice treated with 20% ethanol (4 g/kg/day) for 2 weeks, hepatic acetaldehyde levels in transgenic mice were increased 50% at 2 h after ethanol injection ($p<0.03$) relative to control mice while hepatic alcohol concentrations were unchanged. Female transgenic mice, but not males, drank less alcohol than did control mice ($p<0.03$). The data indicates that transgenic mice carrying the hALDH2^2 might be able to be used as a model to study the role of ALDH in alcohol preference, acetaldehyde accumulation, and acetaldehyde-mediated tissue damage after long-term alcohol treatment.

Keywords: aldehyde dehydrogenase; human mutation; transgenic mice; acetaldehyde; drinking behavior

Introduction

The major enzymes involved in ethanol metabolism in mammalian liver are the alcohol dehydrogenases (ADH) and the mitochondrial aldehyde dehydrogenase (ALDH2). ADH isozymes oxidize ethanol into acetaldehyde and the mitochondrial, low K_m ALDH2 catalyzes the conversion of acetaldehyde to acetic acid. Both human ADH and ALDH2 utilize NAD^+ as a cofactor for the catalysis and both are polymorphic with alleles with different kinetic properties (Harada et al., 1982; Goedde et al., 1983). The most notable

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source of variation in alcohol metabolism in humans is Glu487Lys, the "Oriental Variant" of ALDH2 (Yoshida et al., 1984). The Glu487Lys substitution is due to a single nucleotide change (G to A) and leads to inactivation of the ALDH2 activity under physiological conditions (Farres et al., 1994, Wang et al., 1996, Zhou and Weiner, 2000). The ALDH2^2 (Lys487) allele is abundant in East Asian populations (30% to 50% carrier frequency) and has been extensively evaluated for its biochemical and clinical effects (Yoshida et al., 1984; Higuchi et al., 1996). Individuals who are either homozygous Lys487/Lys487 or heterozygous Glu487Lys exhibit a markedly diminished rate of acetaldehyde metabolism, demonstrating dominance of the action of the Lys487 allele in ALDH2 tetramers (Farres et al, 1994; Xiao et al., 1995). The latter point proved to occur with the human heterotetrameric form of the enzyme, but yet to be tested with enzyme from other species. Although Lys487 is a protective allele against alcohol drinking behavior and the development of alcoholism (Bosron et al., 1993; Crabb et al., 1995) and the increased tissue damage or carcinogenesis that occurs as a consequence of alcoholism (Day et al., 1991; Yokoyama et al., 1998), many questions remain unanswered, due to the difficulty of dissecting the effects of altered ALDH2 activity from other variables including social, cultural, gender, and other genetic influences on drinking behavior. The physiological and behavioral consequences of altered acetaldehyde metabolism remain unclear. Therefore, animal models with the same genetic background, that can be studied under defined experimental conditions, would be valuable to investigate the effects of reduced ALDH2 activity on drinking behavior, the tissue damage resulting from long-term ethanol consumption, and the metabolism of endogenous substrates. In this study, we investigated the role of ALDH2 in acetaldehyde accumulation and alcohol drinking preference in transgenic mice carrying the dominant negative human ALDH2^2 (Lys487) allele.

Materials and Methods

Materials

A kit for RT-PCR was from Stratagene (La Jolla, CA). Restriction enzymes were from New England Biolabs (Beverly, MA). Dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) standards were from Research Biochemicals International (Natick, CA).

Preparation of human ALDH 2^2 transgene construct and production of transgenic mice

The cDNA coding for the mature human ALDH2^2 (hALDH2^2) was obtained, as previously described (Farres et al., 1994). The cDNA coding for the mitochondrial leader peptide of ALDH2 was prepared by PCR amplification of human liver cDNA. The mitochondrial leader sequence was ligated to the mature ALDH2 coding fragment to generate the precursor form of ALDH2 (pALDH2). The resulting full-length cDNA for the hALDH2^2 protein coding region was subcloned into a mammalian expression vector, pcDNA3 (Invitrogene) under the control of cytomegalovirus (CMV) promoter with a polyadenylation signal and transcription termination sequences from bovine growth hormone to enhance RNA stability. The non-essential part of the plasmid was removed by *Nru*I digestion followed by partial digestion with *Pvu*II. The 2633 bp DNA fragment was purified using a commercial plasmid purification kit (Qiagen) and then microinjected into protonuclei from one-day old fertilized eggs of FVB/N mice. The microinjected eggs were then implanted into oviducts of pseudopregnant C57BL/6 foster females at Bioqual, Inc. (Rockville, MD), and under our supervision.

Screening of positive hALDH2^2 transgenic founders

Genomic incorporation of hALDH2^2 was monitored by PCR and/or Southern blot analyses of DNA isolated from the tail of every potential transgenic offspring. Each positive transgenic founder was maintained in a specific pathogen-free environment and bred to produce potential heterozygous F1 offspring to establish a transgenic line. Selected F1s from each line were sacrificed and the expression of the transduced hALDH2^2 in various tissues was determined by RT-PCR, as described (Jeng et al, 1998). Eighty potential transgenic mice were screened and ten positive founders carrying the hALDH2^2 (Lys487) were identified. Two out of the ten transgenic lines (#493 and #9868) contained hALDH2^2 mRNA, however, the #493 line had diminished breeding success. F1 offspring from the #9868 line were mated to produce homozygous F2 transgenic mice carrying the hALDH2^2 gene. The results presented in this report were obtained from the #9868 line.

Detection of hALDH2^2 expression in transgenic mice

A) mRNA detection by RT-PCR: Cytosolic total RNA was isolated from brain, testis, kidney, and liver of F1 mice carrying the hALDH2^2 transgene using RNAzol^{TM} (Friendswood, TX). Expression of hALDH2^2 was monitored by RT-PCR using two sets of primers. One primer pair was specific for the mouse ALDH2 (mALDH2) sequence: AL8 sense primer, 5'-CGCTGGCTGGGCTGACAAGTACCAT-3' and AL15 anti-sense primer, 5'-TGCTCCGTCCGGCTGTCGAAGGGGT-3'. The second pair was specific for the human hALDH2^2 transgene sequence, as indicated in Fig. 1A: T7 promoter sense primer, 5'-TAATACGACTCACTATAGGGCG–3' and AL3 anti–sense primer, 5'-GGAGATGACATAGGGCTTGCCATTG-3'.

B) Protein detection by immunoblot analysis: Brain and liver mitochondria from background and transgenic mice were prepared as described (Jeng et al., 1998). Mitochondrial matrix proteins (100 μg protein/lane) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblot analysis with a polyclonal antibody directed against human ALDH2, which was purified to apparent homogeneity by using 4–hydroxyacetophenone-based affinity column (Ghenbot and Weiner, 1992). This antibody detected one intense band of an apparent molecular mass of 54 kDa ALDH2 protein separated by SDS-PAGE. The immunoreactive bands were visualized by enhanced chemiluminescence (ECL) and the density of each band was determined by computerized densitometry (Molecular Dynamics, Sunnyvale, CA).

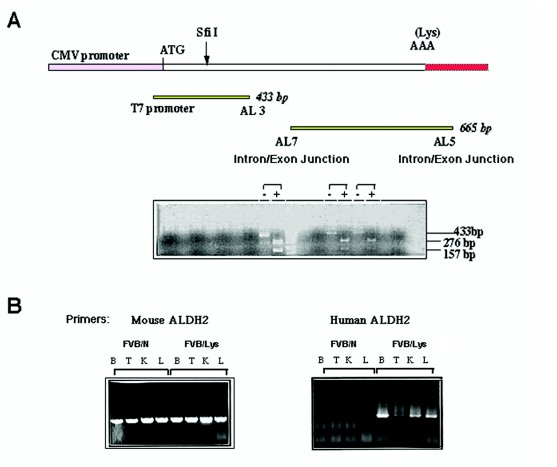

Figure 1. Construction of the full-length cDNA for pALDH2^2 transgene. The recombinant construct containing the full length cDNA for pALDH2^2 was prepared as described in Materials and Methods. A pair of primers specific for the hALDH2^2 construct was used to identify positive founders. Primers AL7 and AL5 contain sequences at the junction points between two exons (exon 8/9 for AL7 and exon 12/13 for AL5). The T7 promoter primer sequence present only in the construct and a restriction enzyme site specific for the hALDH2^2 sequence (SfiI) were used to confirm the fidelity of the amplified DNA fragment as the hALDH2^2 transgene. (B) Expression of hALDH2^2 mRNA in transgenic mice. Positive transgenic F1s were sacrificed and total RNA isolated from brain (B), testis (T), kidney (K), and liver (L). The levels of mALDH2 and hALDH2^2 transgene in various mouse tissues were determined by RT-PCR using specific primers for mALDH2 (left panel) and hALDH2^2 (right panel).

In vitro ALDH2 assay using dopamine as a substrate

Mitochondrial proteins (100 μg/assay) were used for ALDH2 enzyme assay by the method previously described (Tottmar, 1986), with the following minor modifications. The reaction mixture contained 100 mg of mitochondrial protein and 0.8 mM NAD^+ in 50 μM sodium pyrophosphate buffer (pH 9.0). The reaction was initiated by addition of dopamine (10 μM) and allowed to proceed at 37° C for 15 min. The reaction was stopped by the addition of 0.1 ml of 0.2 M HCl, followed by centrifugation at 40,000 × *g* for 10 min. The formation of 3,4–dihydroxyphenylacetic acid (DOPAC) in the supernatant fraction was determined by high-performance liquid chromatography (HPLC) with electrochemical detection (ESA Coulochem II, Chelmsford, MA).

Determination of hepatic acetaldehyde and ethanol concentrations

Transgenic mice (FVB/Lys) and background mice (FVB/N) were singly housed during the experiments. Ethanol (4g/kg/day) or saline was administered i.p. for 14 consecutive days. On day 14, mice were sacrificed by cervical dislocation and the livers were rapidly excised and freeze clamped within 17 seconds after killing using aluminum disks pre-chilled in liquid nitrogen. Frozen liver samples were coded for double-blind analysis and shipped under dry ice to Dr. Eriksson's laboratory. Hepatic acetaldehyde and ethanol levels were determined using headspace gas

chromatography (GC), following methods previously described (Eriksson et al., 1977).

Ethanol Drinking Preference

Both male and female homozygous transgenic (FVB/Lys) and background (FVB/N) mice were evaluated for ethanol drinking preference. Singly housed mice were preconditioned with 5% and 10% ethanol (v/v) for 10 days before they were given a two-bottle choice between water and 10% ethanol, as described (Phillips et al., 1998). The positions of the two bottles were switched daily to avoid position preference. Average ethanol intake was calculated as volume (ml) of 10% ethanol consumed per gram of body weight or the amount of ethanol intake over the total liquid intake during the 4-week testing period.

Results

Confirmation of hALDH2^2 transgene expression

The hALDH2^2 (E487K) vector we prepared contained a strong CMV promoter, the mitochondrial leader sequence, the coding sequence of hALDH2^2, and the 3' untranslated sequence of bovine growth hormone to stabilize the ALDH2 mRNA (Fig. 1A, top). To specifically detect the expression of the hALDH2^2 transgene, two sets of oligonucleotide primers were prepared to detect either mouse ALDH2 (mALDH2) or hALDH2^2 mRNA by RT-PCR analysis with *Sfi*I restriction enzyme digestion (Fig. 1A and B). For instance, transgenic mice with hALDH2^2 allele was confirmed by the production of 276 and 157 bp DNA fragments from the 433 bp amplicon after *Sfi*I restriction enzyme digestion (Fig. 1A, bottom). Expression of *hALDH2^2* transgene was also confirmed by the amplification of 665 bp DNA fragment with another pair (AL7 and AL5 in Fig. 1A) specific for the *hALDH2^2* allele.

Production of transgenic mice carrying the hALDH2^2 transgene

Two lines of mice expressing the *hALDH2^2* transgene were produced. Line 493 expressed hALDH2^2 at 10-fold higher levels than Line 9868, but showed breeding problems. Line 9868 was bred successively until homozygous transgenic mice were produced. Fig. 1B presents the results of RT-PCR analyses for RNA samples isolated from four tissues (brain, testis, kidney and liver) of background mice and transgenic mice homozygous for the hALDH2^2 transgene. Murine ALDH2 mRNA was detected with the specific primers (AL8 and AL15) designed for mALDH2 in all four tissues in background and transgenic mice (left panel, Fig. 1B). Expression of hALDH2^2 transgene was also detectable but only in transgenic mice (right panel, Fig. 1B) and not in mice from the same genetic background, confirming primer specificity and selective expression of hALDH2^2 in transgenic mice.

Expression of hALDH2^2 protein

Relative expression of ALDH2 protein in liver and brain mitochondria was determined by immunoblot and densitometric analysis of background and transgenic mice. In transgenic mice, ALDH2 protein level was elevated approximately 30% in brain and 10% in liver, relative to expression in background mice (n=4 for each group) (Fig. 2A). ALDH2 enzyme activity in background and transgenic mice was compared using the rate of synthesis of DOPAC from dopamine, as determined by HPLC. In transgenic mice, ALDH2 enzyme activity was inhibited approximately 13% in brain and 3% in liver, relative to expression in background mice (Fig. 2B). The small degree of inhibition of ALDH2 activity in the transgenic mice may result from the relatively low level of that of the hALDH2^2 transgene, particularly in liver where the ALDH2 protein seems to represent more than 0.59% of the total matrix proteins of hepatic mitochondria (Ikawa et al., 1983). Alternatively, the stability of mouse/human ALDH2 heterotetramers may be diminished (as discussed later). A third possibility is that that the human E487K subunit might not be dominant as it is in the human ALDH heterotetramer (Zhou and Weiner, 2000).

Levels of hepatic alcohol and acetaldehyde

We studied potential liver damage by daily ethanol administration (4 g/kg/day, intraperitoneal injection) for 14 consecutive days. In addition, the levels of hepatic alcohol and acetaldehyde accumulated in transgenic mice and background mice were determined 2 hours after the last ethanol injection in a double blind manner. Acute ethanol injections for 14 consecutive days did not cause any detectable damage to livers and brains of transgenic mice relative to background mice as determined by histopathological examination of the formalin-fixed tissues stained with hematoxylin and eosin. As shown in Fig. 3A, hepatic ethanol concentrations in male

and female background mice were 42.6 ± 4.3 and 43.1 ± 10.6 mM, respectively, whereas those in male and female transgenic mice were 45.9 ± 3.1 and 41.3 ± 5.4 mM, respectively. The differences in the hepatic ethanol concentrations between background mice and transgenic mice were statistically insignificant. In contrast, hepatic acetaldehyde levels in male and female background mice (n=5 for male and n=6 for female in Fig. 3B) were 37.9 ± 2.8 and 32.7 ± 2.1 μM, respectively. Acetaldehyde levels were elevated 40% in male transgenic mice ($p<0.03$) and 50% in female transgenic mice ($p<0.01$). The greater accumulation of acetaldehyde without significant changes in ethanol levels in the liver in both male and female transgenic mice strongly indicates that mouse ALDH2 activity is inhibited *in vivo* by the hALDH2[2] transgene, despite its low levels of expression.

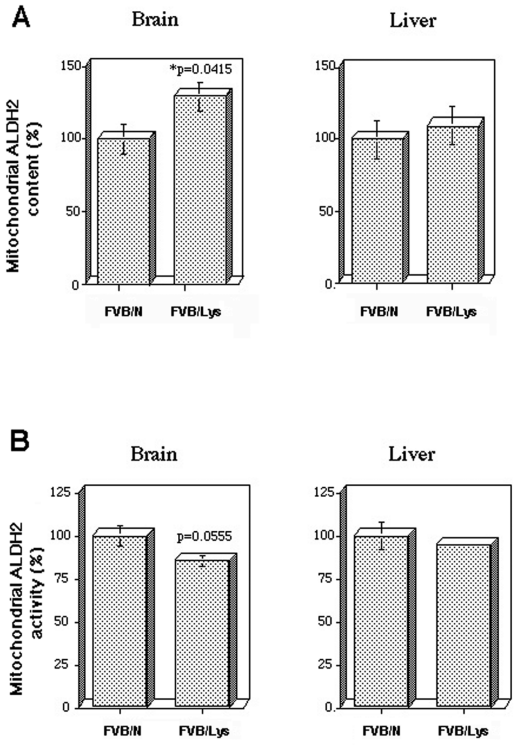

Figure 2. Comparison of the ALDH2 protein contacts and activities in the liver and brains of background and transgenic mice. (A) Brain and liver mitochondria were isolated from background and transgenic mice (FVB/Lys). Mitochondrial proteins (100 μg/well) of brain (left panel) and liver (right panel) were subjected to immunoblot analysis using a polyclonal antibody against hALDH2. The average density of immunoreactive signals for each group is presented. (B) Average ALDH2 activities in brain (left panel) and liver (right panel) mitochondria from background and transgenic mice (FVB/Lys) are presented.

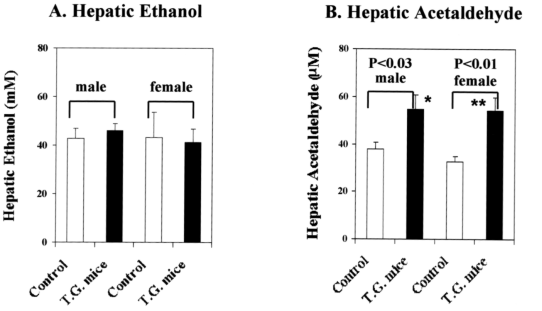

Figure 3. Comparison of hepatic ethanol and acetaldehyde concentrations in background and transgenic mice. Hepatic acetaldehyde concentrations in transgenic (FVB/Lys) and background mice (FVB/N) were analyzed by headspace gas chromatography in a double blind fashion, as described in Materials and Methods.

Gender–Specific Alcohol Drinking Preference

It has been shown that acetaldehyde accumulation, whether it is due to the inherited Lys 487allele or from ALDH2 inhibition by disulfiram, metronidazole, or daidzin (Keung et al., 1997), deters alcohol consumption. Low drinking as a consequence of high acetaldehyde levels has also been demonstrated in rats selected for non-drinking (Eriksson, 1973). We therefore evaluated alcohol preference of transgenic mice using a two bottle choice model (10% ethanol and water bottles). Both male and female background mice drank about 0.40 ± 0.02 ml of 10% ethanol/day and 0.25 ± 0.02 ml of tap water. There was no significant difference in the amounts of alcohol consumed between male background and transgenic mice. However, female transgenic mice drank significantly less alcohol (about 17%, $p<0.004$, n=14) than did background mice (n=12) (Fig. 4). This small but significant reduction in alcohol consumption by the transgenic mice may result from elevated levels of acetaldehyde in these animals, as recently demonstrated by the increased acetaldehyde concentration per ethanol consumption in ALDH2$^{-/-}$ deficient mice (Isse et al., 2002). The reason for the apparent gender differences in alcohol preference of the transgenic mice is unknown. However, gender differences in alcohol preference have been previously observed and quantitative trait loci for alcohol preference in mice

has been proposed to act in a gender specific fashion (Melo et al., 1996).

Discussion

The data presented show that mRNA for hALDH2^2 allele in the transgenic mice is expressed in all the tissues examined and that ALDH2 activities in brain and liver are slightly inhibited by the inactive hALDH2^2 transgene. We do not know the reason for finding just a low level of inhibition of ALDH2 activity in the transgenic mice. It could be a result of a low level of expression of hALDH2^2 protein, despite the presence of its mRNA in the transgenic mice. It was difficult to accurately determine the expressed level of hALDH2^2 protein in the transgenic mice, due to lack of specific antibody which only recognizes the hALDH2^2 variant protein. Alternatively, the ALDH2 proteins from different species (human versus mice) might assemble into weakly-associated heterotetramers. Furthermore, it is possible that the rodent heterotetramers are not as inhibited as the human counterpart (92%, Wang et al., 1996, Zhou and Weiner, 2000) because the rat homotetrameric E487K enzyme is highly active compared to the human enzyme (52% versus 8%, respectively, Farres et al., 1994).

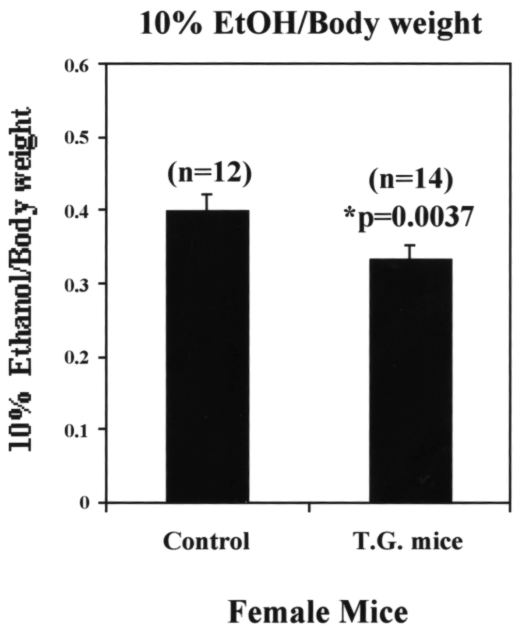

Figure 4. Comparison of alcohol drinking preference in background and transgenic mice. Homozygous transgenic mice and background mice were used for the ethanol drinking preference study, as described in the Materials and Methods. The ethanol intake was calculated as volume (ml) of 10% ethanol consumed per gram of body weight.

It has been well established that reactive acetaldehyde or hydroxyethyl radical produced during ethanol metabolism is toxic to cells and often immunogenic due to reaction with various cellular proteins (Lieber, 1988; Jennett et al., 1990; Clot et al., 1997). Despite accumulation of acetaldehyde in the livers of transgenic mice, daily injections of ethanol for 14 consecutive days did not cause any detectable damage to livers and brains of transgenic mice relative to background mice. Absence of alcohol-mediated tissue damage in this study can be explained by the short duration and relatively low level of exposure to alcohol and subsequently acetaldehyde. Furthermore, nutrient-rich diet available *ad libitum* may contribute to the protection from alcohol induced tissue damage, as recently suggested (Lindros and Järveläinen, 1998).

Xiao et al. (1996) reported that the human ALDH2 heterotetramers composed of subunits with a glutamate at position 487 and others with a lysine at that position were relatively unstable as compared to the active homotetramer, so that very low levels of ALDH2 activity and protein were detected when the two alleles were co-expressed in HeLa cells. In addition, the immunoreactive ALDH2 content in autopsy human liver samples (Xiao et al., 1996) is lower in ALDH2^2 Lys487/Glu487 heterozygotes as compared to individuals with the Glu487/Glu487 genotype. This result was supported by Yin and colleagues (2000) who found that heterozygous individuals with low levels of hepatic ALDH2 activity (<13%) also have a lower content of immunoreactive ALDH2 protein, about 30–44% the level of immunoreactive protein seen in Glu487/Glu487 homozygotes. These data (Xiao et al., 1996) are consistent with earlier reports of low levels of ALDH2 protein in ALDH2^2 heterozygotes by Yoshida and co-workers (Impraim et al., 1982; Yoshida et al., 1983). Taken together, these data strongly indicate that ALDH2 heterotetramers are relatively unstable than homotetramers of ALDH2. However, work with pure recombinantly expressed heterotetramers did not reveal that the heterotetramers were less stable than were the homotetramers (Zhou and Weiner, 2000). From the structure it is known that the Glu487 forms an inter-subunit salt bond with Arg475. Though the homotetrameric Oriental variant of hALDH2^2 is thermally as stable as

is the active form of the enzyme (Wei et al., 2000), it is possible that the loss of the salt bonds contributes to the possible *in vivo* decreased stability of the heterotetramer. The data presented show for the first time that hybridization can occur between ALDH2 monomers from different species. This should not be unexpected as the isozymes share more than 95% sequence identity (Chang and Yoshida, 1994) but is a point that should be verified by co-expressing the subunits in *E. coli*. Further, it shows again that the Oriental variant subunit is dominant and caused the inactivation of its partner independent of whether the partner was an active mouse or human enzyme.

Another possible explanation for the low expression of hALDH2^2 protein and the relatively small inhibition of ALDH2 enzyme activity in the transgenic mice is that the cross-species heterotetramers containing the inactive E487K allele may be relatively less stable, as they might be in human (Xiao et al, 1996; Impraim et al., 1982; Yoshida et al., 1983), leading to more rapid degradation of the hALDH2^2 Lys487 transgene. Despite its possible diminished stability, the Lys487 transgene is able to meaningfully inhibit murine ALDH2 activity, as evidenced by enzyme activity measurement and the significant elevation of acetaldehyde accumulation 2 hours after acute ethanol injection, in both male and female transgenic mice. Reduced alcohol consumption in female transgenic mice could be a consequence of diminished acetaldehyde metabolism. It is not possible to speculate on why the male transgenic animals did not decrease their alcohol preference. It is also intriguing to speculate why the brain enzyme was not greatly inhibited. Independent of the detailed reasons, these data indicate that the transgenic mice carrying the hALDH2^2 transgene can be used as a model to study some biological roles of ALDH2 and alcohol-mediated organ damage after long-term alcohol treatment. Unfortunately, since completing the work reported in this study the transgenic mouse colony was destroyed due to low expression of the hALDH2^2 transgene and the availability of ALDH2 knock-out mice (Isse et al., 2002). Thus it will be necessary to recreate the line to continue the study. It is suggested that the mouse E487K be used or that it is shown that the heterotetramer between the mouse and human alleles are stable and have decreased activity compared to the native mouse ALDH2.

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