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Aldehyde dehydrogenase (ALDH) 2 associates with oxidation of methoxyacetaldehyde; in vitro analysis with liver subcellular fraction derived from human and *Aldh2* gene targeting mouse

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Abstract A principal pathway of 2-methoxyethanol (ME) metabolism is to the toxic oxidative product, methoxyacetaldehyde (MALD). To assess the role of aldehyde dehydrogenase (ALDH) in MALD metabolism, in vitro MALD oxidation was examined with liver subcellular fractions from Japanese subjects who carried three different *ALDH2* genotypes and *Aldh2* knockout mice, which were generated in this study. The activity was distributed in mitochondrial fractions of *ALDH2*1/*1* and wild type (*Aldh2+/+*) mice but not *ALDH2*1/*2*, **2/*2* subjects or *Aldh2* homozygous mutant (*Aldh2−/−*) mice. These data suggest that ALDH2 is a key enzyme for MALD oxidation and ME susceptibility may be influenced by the *ALDH2* genotype.

Key words: Aldehyde dehydrogenase 2; 2-Methoxyethanol; Methoxyacetaldehyde; Gene targeting mouse

1. Introduction

2-Methoxyethanol (ME), or ethylene glycol monomethyl ether, is a water-miscible solvent used extensively in the chemical industry. Encephalopathy, central nervous system symptoms, erythropenia and granulocytopenia have been reported in workers exposed to ME [1,2]. Moreover, human reproductive toxicity, including oligospermia, azoospermia, sperm count reduction and ovarian luteal cell toxicity, has also been reported [2–6].

The biotransformation of ME plays an important role in the appearance of its toxicity [7–9]. Moss et al. [10] and Miller et al. [7] have both reported that urinary methoxyacetic acid (MAA) excretion was a major metabolic pathway in rat. It has been speculated that the main pathway of ME metabolism is the oxidation to methoxyacetaldehyde (MALD) by alcohol dehydrogenase (ADH), and the successive oxidation of MALD to MAA by aldehyde dehydrogenase (ALDH) [8–11]. The ADH inhibitor pyrazole reduces the conversion of ME to MAA and concomitantly reduced the ME testicular toxicity in rat [7,8,10]. On the other hand, an ALDH inhibitor, disulfiram, did not suppress the conversion of ME to MAA or reduce the testicular toxicity of ME [7,8,10] although it is not clear whether the concentration of disulfiram was sufficient to inhibit ALDH completely. MALD is also known to have genotoxic, immunotoxic and reproductive effects, at the level of in vitro exposure [12,13]. Therefore, it has remained unclear whether MALD besides MAA has a toxic function in vivo.

ALDH comprises more than nine families in humans, ALDH1, ALDH3 and ALDH9 are in liver cytosol, and ALDH2, ALDH4, ALDH5 and ALDH6 are in liver mitochondria. ALDH7 and ALDH8 are extrahepatic [14–19]. It has not been determined which family has a key function for MALD metabolism. It has been reported that ALDH2 metabolizes shorter chain aliphatic aldehydes like acetaldehyde [20,21]. As MALD is a short chain aliphatic aldehyde (C_3), it is considered a candidate for oxidation by ALDH2.

The genetic polymorphism of *ALDH2* has been characterized as *ALDH2*2* in relation to the wild type, *ALDH2*1*. ALDH2*2 is encoded with a lysine for glutamate substitution at residue 487 in the mature enzyme resulting in a loss of enzymatic activity [22,23]. In addition, the ALDH2*2 phenotype is dominant over ALDH2*1 [24,25]. If ALDH2 has a major role in MALD oxidation, concentrations of ME metabolites, including levels of MALD, may be expected to vary in humans in relation to the *ALDH2* genotype.

To investigate whether decreases in ALDH2 activity influence ME metabolism, liver subcellular fractions were prepared from Japanese surgical patients who carried *ALDH2*1/*1, *1/*2* or **2/*2* alleles, and examined in vitro for MALD oxidation activity. This approach, however, would not be definitive on the role of ALDH2 because activity levels of many metabolic enzymes show high interindividual variation that is due not only to genetic polymorphisms but also to other factors including the overall state of health of the individual.

To confirm the in vivo significance of *ALDH2* polymorphism, mice lacking *Aldh2* were generated with the use of gene targeting in embryonic stem (ES) cells, and MALD oxidation activity was assayed in their liver subcellular fractions.

2. Materials and methods

2.1. Subjects

Tissues from one hemangiosarcoma and six carcinomas were ob-

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tained during hepatic surgery from seven patients aged 40–79 years. The non-pathological tissues peripheral to the tumors were dissected from the pathological tissues and stored at −80°C until analysis. All the subjects gave their informed consent.

2.2. Chemicals

Acetaldehyde (AALD), benzaldehyde (BALD), propionaldehyde (PALD), decylaldehyde (DALD), methanol and pyrazole analytical grade, purity 85–98%, were purchased from Wako, Japan. MALD was kindly supplied by Tokyo Kasei, Japan; the purity of this preparation could not be determined because of polymerization during storage but was estimated to be less than 87%.

2.3. Genotyping of human ALDH2

Genomic DNA was extracted from a small part of the liver tissue. The genotype of human *ALDH2* was checked by PCR with a common forward primer (Kb1F; 5′-CAAATTACAGGGTCAACTGCTAT-3′) and *ALDH2*1* specific reverse primer (Kb2R; 5′-CCACACTCACAGTTTTCACTTC-3′), or Kb1F and *ALDH2*2* specific reverse primer (Kb3R; 5′-CCACACTCACAGTTTTCACTTT-3′). The annealing site of each primer is shown in Fig. 1A. Because *ALDH2*2* has a base substitution at exon 12, the region around exon 12 was selected as the PCR target. The reaction mixture was composed of approximately 100 ng genomic DNA, 2 μM of each primer, 0.2 mM of each dNTP, 1 mM $MgCl_2$, 1 U of rTaq polymerase (Toyobo, Japan) in 50 μl of 1×reaction buffer (Toyobo, Japan). Amplification was performed by denaturing at 94°C for 30 s, annealing at 56°C for 30 s and extending at 72°C for 30 s for 30 cycles using the program temperature control system PC-701 (Astec, Japan). Each primer pair yielded a single 135 bp product (Fig. 1B).

2.4. Liver subcellular fractionation

Mitochondrial, cytosolic and microsomal fractions were prepared from human and mouse liver as described by Kishimoto et al. [26] with a minor modification.

2.5. In vitro assay of ALDH activity toward aldehydes

Oxidation activities of ALDH toward various aldehydes were measured by the change in absorbance at 340 nm due to formation of NADH as described by Lebsack et al. [27]. Protein concentration of the liver fraction was determined by the Bio-Rad Protein Assay. All aldehydes were dissolved in water except DALD, which was dissolved in methanol. When added to the assay mixture, the final concentration of each substrate was 50 μM, and the methanol concentration did not exceed 1%. Pyrazole was added to the reaction mixture at a concentration of 50 μM to inhibit NAD activity, and methanol was shown not to affect the enzymatic reaction at that concentration.

2.6. Generation of Aldh2−/− mice

The targeting vector was constructed as described [28]. The maintenance, transfection, and selection of embryonic day 14 ES cells were performed as described [29,30]. G418 and gancyclovir resistant clones were screened for homologous recombinants by subjecting cell lysates to PCR with an *Aldh2* specific forward primer (5′-CCTTACGGGTGCTCGGTAGAGC-3′) and a phosphoglycerate kinase (PGK) promoter specific reverse primer (5′-TGCTAAAGCGCATGCTCCAGACTG-3′), the annealing sites of which are shown in Fig. 2A. The results of PCR screening were confirmed by Southern blot analysis; DNA prepared from PCR positive ES clones was digested with *Xba*I, fractionated by electrophoresis, transferred to a nylon membrane (Biodyen B; Paul), and then hybridized with the 0.6 kb internal probe and 0.6 kb external probe. The expected sizes of hybridizing fragments for wild type and recombinant alleles were 4.5 and 5.5 kb, respectively (Fig. 2B,C). ES cells heterozygous for the targeted mutation were microinjected into C57BL/6 blastocysts, which were then implanted into pseudopregnant ICR females. The resulting male chimeras were mated with female C57BL/6 mice. The germ-line transmission of injected ES cells was confirmed by the inheritance of agouti coat color in the F1 animals, and heterozygous offspring (*Aldh2*+/−) were intercrossed to produce homozygous mutant (*Aldh2*−/−) animals (F2). All offspring were tested for the presence of the mutated and/or wild type *Aldh2* alleles by PCR with a *Aldh2* specific forward primer (5′-CCGTACTGACTGTCCCATGCAGTGCT-3′) in the 5′ homology region, a PGK promoter specific reverse primer (5′-GGTGGATGTGGAATGTGTGCGAGGC-3′) and *Aldh2* specific reverse primer (5′-CCGTACTGACTGTCCCATGCAGTGCT-3′), where was replaced by a PGK-neo-poly(A) cassette in the mutant allele (Fig. 2D). F2 male mice (33–55 weeks) were used for the following examination.

2.7. Immunoblot analysis of ALDH2 protein in liver of Aldh2 knockout mice

To prepare recombinant mouse ALDH2 protein, complementary DNA encoding mouse ALDH2 (mALDH2) was generated by RT-PCR with high fidelity DNA polymerase KOD (Toyobo), sequenced, and subcloned into pGEX-6P-3 (Pharmacia). Glutathione *S*-transferase fused mALDH2 protein was produced in *Escherichia coli* cells, adsorbed to glutathione-Sepharose CL4B (Pharmacia), and cleaved by PreScission Protease (Pharmacia). The recombinant mALDH2 protein and liver mitochondrial fractions derived from knockout mice were subjected to immunoblot analysis with anti-mALDH2 antibody (Fig. 2E). Anti-mALDH2 polyclonal antibody was raised against N-terminal peptides of mALDH2, which was provided by Iwaki Glass Co., Japan. Antiserum obtained from an immunized rabbit was purified using Ampure PA kit (Amersham).

3. Results

3.1. Genotyping of human ALDH2

The genotyping was done with genomic DNA from the seven Japanese surgical patients and resulted in three *ALDH2*1/*1*, three *ALDH2*1/*2* and one *ALDH2*2/*2*.

3.2. MALD oxidation activity in human liver subcellular fractions

The ALDH activities toward MALD in mitochondrial, cytosolic and microsomal fractions were determined in comparison with various aldehydes. It is reported that AALD and PALD are efficient substrates of ALDH2, DALD of ALDH1, and BALD of ALDH3.

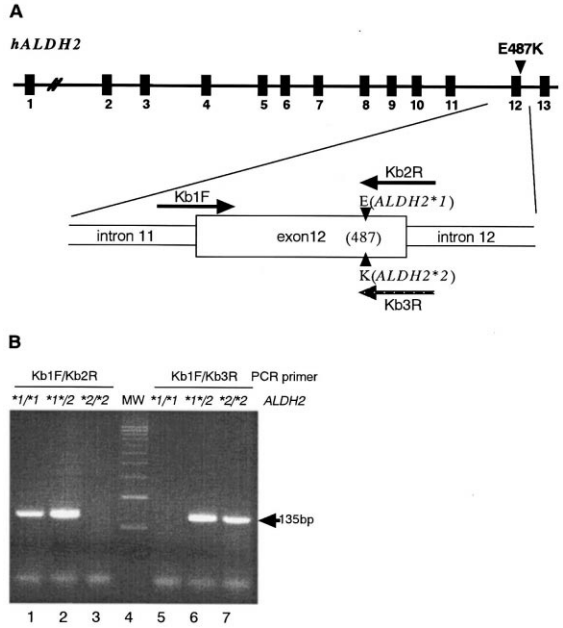

Fig. 1. Genotyping of human *ALDH2*. A: The position of primers (Kb1F, Kb2R and Kb3R) used for the genotyping of ALDH2 is indicated. B: PCR analysis of *ALDH2* genotype. Lanes 1 and 5 are derived from an *ALDH2*1/*1* individual. Lanes 2 and 6 are derived from an *ALDH2*1/*2* individual. Lanes 3 and 7 are derived from an *ALDH2*2/*2* individual. Lane 4 indicates molecular weight markers.

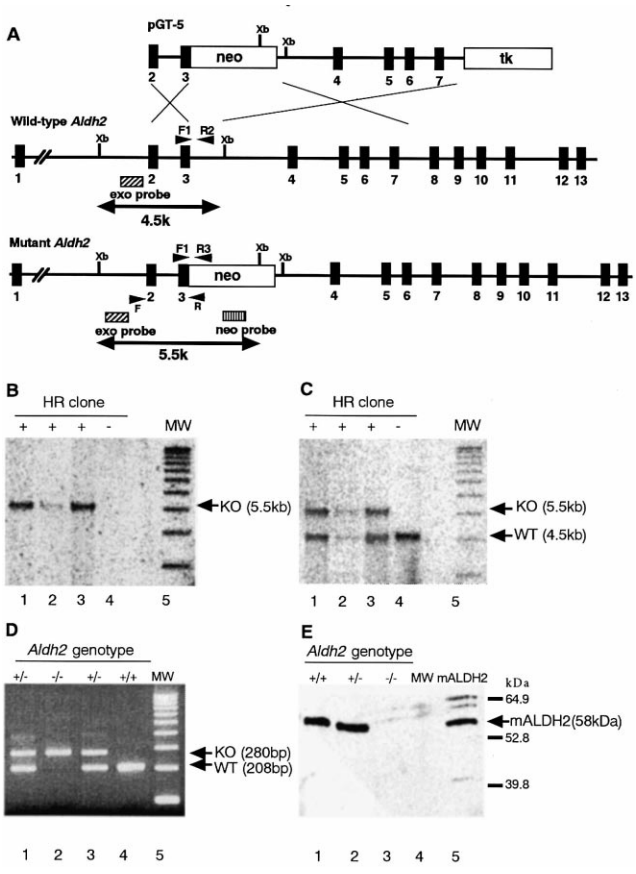

Fig. 2. Targeted disruption of mouse *Aldh2*. A: Structures of the targeting vector (pGT-5) of the mouse *Aldh2* locus, and of the mutant allele resulting from homologous recombination. Filled boxes depict exons of *Aldh2*. The position of a set of primers (F and R) used for the screening of ES clones is indicated. The genomic fragments (neo probe and exo probe) used as probes for Southern blot analysis are shown as a striped box, and the expected sizes of the *Xba*I fragments that hybridize with the probe are indicated. The position of two sets of primers (F1, R2 and R3) used for genotyping of targeting mice is also indicated. Abbreviations: neo, the neomycin transferase gene linked to the PGK promoter; tk, thymidine kinase gene derived from herpes simplex virus linked to the PGK promoter. The orientations of both neo and tk are the same as that of Aldh2. Restriction sites: Xb, *Xba*I; not all restriction sites are shown in the figure. B, C: Southern blot analysis of genomic DNA extracted from ES cells for the screening of homologous recombinants. The DNA was digested with *Xba*I and subjected to hybridization with the neo and exo probes in B and C, respectively. The sizes of hybridizing fragments derived from wild type (WT) and knockout (KO) alleles are shown on the right, lane number is correlated in B and C. All subjects (lanes 1–4) except four are homologous recombinants. Lane 5 indicates molecular weight markers. D, PCR analysis of *Aldh2* DNA extracted from F2 mouse tails. Both lanes 1 and 3 indicate *Aldh2*+/−, lanes 2 and 4 indicate *Aldh2*−/− and +/+, respectively. Lane 5 indicates molecular weight markers. E: Western blot analysis of mALDH2 expression. The targeting mouse liver mitochondrial fractions were subjected to immunoblot analysis with anti-mALDH2 antibody. Lanes 1–3 are *Aldh2*+/+, +/− and −/−, respectively. Lane 4 contains protein weight markers. Lane 5 is recombinant mALDH2 protein as a size marker. Each signal was estimated at about 58 kDa.

Mitochondrial fractions from *ALDH2*1/*1* individuals exhibited high dehydrogenase activities toward AALD, PALD and MALD (Fig. 3A), whereas those derived from *ALDH2*1/*2* individuals had very low activities toward all three aldehydes, about one tenth the activities of those from *ALDH2*1/*1* individuals. No ALDH activity was detected toward all examined aldehydes in the mitochondrial fraction from the *ALDH2*2/*2* individual. This result suggests that mitochondrial ALDH activities toward these aldehydes are derived from ALDH2. Compared with mitochondrial fractions, cytosolic fractions from *ALDH2*1/*1* exhibited lower activities toward both AALD and MALD (Fig. 3B). The cytosolic fraction from *ALDH2*2/*2*, like the mitochondrial fractions, exhibited no ALDH activity toward AALD and MALD. Small amounts of ALDH2 activity may be included in the cytosolic fraction, because human liver ALDH2 activity is known to be distributed in both mitochondria and cytosol [31]. Three genotypes exhibited almost the same ALDH activities toward DALD in their cytosolic fractions. On the other hand, none of the fractions examined had ALDH activities toward BALD. It is thus reasoned that ALDH1 is expressed in all, and ALDH3 in none of the subjects examined. None of the microsomal fractions examined had any ALDH activity toward any of five aldehydes (data not shown). The patterns of enzymatic activities toward MALD were almost same as those toward AALD and PALD, in all three fractions, in all subjects examined. These results suggest strongly that ALDH2 is the key enzyme in the oxidation of MALD.

3.3. Generation of *Aldh2*−/− mice

The mouse *Aldh2* genomic locus comprises 13 exons spanning ~26 kb [32]. The targeting construct was designed to generate the termination signal in the PGK promoter sequence, which was connected with exon 3. Of the 52 G418 and gancyclovir resistant clones screened for homologous recombination events by PCR, four clones (7.7%) were identified as positive. All four of these clones were confirmed to contain the mutant allele by Southern blot analysis (Fig. 2B,C). Two of these were injected into C57BL/6 blastocysts, and chimeric males were obtained, in which a large proportion of cells contained the mutant allele (as revealed by coat color) and in which the mutant allele was transmitted to the germ line. Heterozygous offspring of these chimeras appeared normal and fertile. Mating of heterozygotes yielded wild type (*Aldh2*+/+), heterozygous (*Aldh2*+/−) and homozygous (*Aldh2*−/−) offspring (Fig. 2D). As the offspring were approximately in the expected sex and Mendelian ratio, no substantial embryonic lethality was suspected. Both males and females were fertile and no developmental problems were observed in the *Aldh2*−/− homozygotes. Therefore, ALDH2 would be not essential for mouse development. This observation is consistent with the phenotype of *ALDH2*2/*2* in that no adverse developmental or physiological problems are apparent.

The expression of mALDH2 protein in liver was studied by Western blot analysis using anti-mALDH2 polyclonal antibody. A single band was detected in both *Aldh2*+/+ and +/− mouse mitochondrial fractions at the same size as the recombinant mALDH2 and no such band was present in *Aldh2*−/− mitochondrial fraction (Fig. 2E). Although mALDH1, which is localized in the cytosol, has four identical amino acid sequences to mALDH2 at the antibody recognition site, no reaction bands were recognized in cytosolic fractions derived from either strain (data not shown). These results suggest strongly that anti-mALDH2 antibody recognized only mALDH2 and confirm that *Aldh2*−/− mice produced no mALDH2 protein.

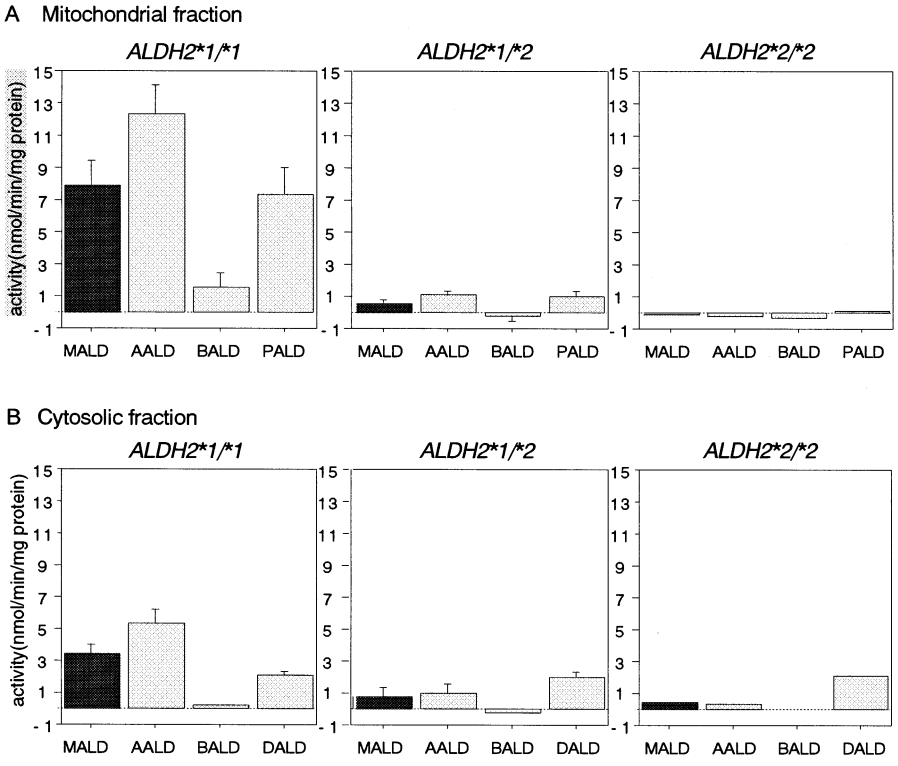

Fig. 3. Comparison of substrate specific activities of human liver ALDH derived from three *ALDH2* genotypes. A: Mitochondrial fractions prepared from *ALDH2*1/*1*, **1/*2* and **2/*2* individuals. B: Cytosolic fractions, prepared when mitochondrial fractions (A) were prepared. Values are mean ± S.E.M. of three and two subjects for *ALDH2*1/*1* and **1/*2*, respectively.

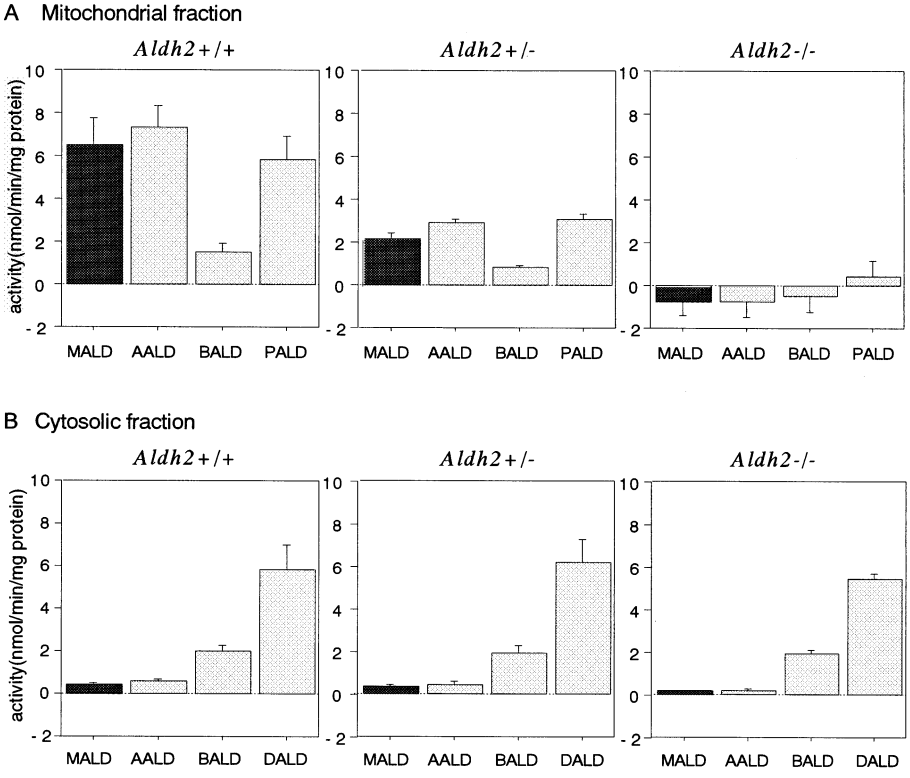

Fig. 4. Comparison of substrate specific activities of targeting mouse liver ALDH. A: Mitochondrial fractions prepared from *Aldh2*+/+, +/− and −/− mice. B: Cytosolic fractions, prepared when mitochondrial fractions (A) were prepared. Values are mean ± S.E.M. of three mice.

3.4. MALD oxidation activities in knockout mouse liver subcellular fractions

Liver mitochondrial, cytosolic and microsomal fractions derived from *Aldh2*+/+, +/− and −/− male mice were examined for aldehyde oxidation activities. No oxidation activity was detected in mitochondrial fractions from *Aldh2*−/− mice (Fig. 4A). On the other hand, mitochondrial fractions derived from *Aldh2*+/+ mice exhibited oxidation activity toward AALD, PALD and MALD. The mitochondrial fractions from *Aldh2*+/− showed one half the activity of those from *Aldh2*+/+ mice. BALD oxidation activities were low in all examined mitochondrial fractions of three mouse genotypes. All cytosolic fractions had very low catalyzing activity toward AALD and MALD, whereas BALD and DALD oxidation activities were roughly equal among the three genotypes (Fig. 4B). Although hALDH3 is induced only in some neoplastic states in human liver [33], mALDH3 was expressed in all mouse livers examined. This result suggests that ALDH3 does not play a substantial role in MALD oxidation. None of the microsomal fractions examined had activities with any of the substrates examined (data not shown). The characteristics of ALDH activity toward MALD were the same as those toward AALD; that is, MALD dehydrogenase activity was deficient in mouse mitochondria without ALDH2. Those results also provide evidence that in mouse ALDH2 is the key enzyme in the oxidation of MALD.

4. Discussion

The mouse *Aldh2* gene encodes a protein of 519 amino acid residues which shares 95.8% identity with hALDH2 [32]. The high expression of mALDH2 is observed in liver mitochondria and mALDH2 shows high affinity with short chain aliphatic aldehydes including AALD and PALD [34]. These characteristics indicate that the phenotype of function of mALDH2 corresponds highly with that of hALDH2. It is known that hALDH2*1 exhibits its activity after taking a tetramer [24]. In the heterozygote (*ALDH2*1/*2*), ALDH2*2, a mutant subunit, reduces ALDH2 activity to 13% of the native activity, apparently because the heterotetramers of ALDH2*1 and ALDH2*2 subunits do not function properly [25]. This is also the case for the homotetramer of ALDH2*2. In the present study, the oxidation activities toward AALD in mitochondrial fractions from *ALDH2*1/*2* individuals were dramatically reduced, almost to the level of the *ALDH2*2/*2* individual. On the other hand, no ALDH2 enzyme is expressed from the *Aldh2* knockout gene due to the stop codon in the inserted PGK promoter gene. *Aldh2*−/− mitochondrial fractions completely lacked activity. Therefore, ALDH2 activity in homozygous knockout (*Aldh2*−/−) mice closely corresponds with that in human *ALDH2*2/*1* and *ALDH2*2/*2* subjects, and *Aldh2*−/− mice appear to be a valid animal model for both *ALDH2*1/*2* and *ALDH2*2/*2* individuals.

Aldehydes such as formaldehyde and AALD are toxic in the human body. Their toxicity is influenced with the various levels of the specific metabolic enzymes, which are, in turn, influenced by genetic polymorphisms and life-styles of individuals. In contrast, gene targeted mice have a congenic background after completion of back crossing. They are considered to have equal levels of all other physiological and biochemical enzyme activities except the gene targeted enzyme. Therefore, it is expected that the *Aldh2* knockout mouse generated in this study would be suitable to ascertain the effect of *Aldh2* polymorphism on the chemical toxicity at the level of the whole body.

It is likely that *ALDH2*1/*2* and *2/*2* individuals are defective in ME metabolic capacity at the whole body level, too. *ALDH2*1/*2* or *2/*2* carrying individuals are seen in Mongoloids and native Americans, and in about 50% of Japanese [35]. Moreover, disulfiram (Antabuse), an inhibitor of ALDH, is used by many alcoholic patients, and some antibiotics also decrease ALDH activity [36]. Individuals who are deficient in ALDH2 activity may be especially susceptible to the effects of ME. The differences of reproductive and hematological toxicity of ME due to ALDH2 polymorphism especially should be clarified at the in vivo level. Congenic *Aldh2* targeted mice would be an instrument in investigating the effects of *ALDH2* genetic polymorphism, including susceptibility to ME.

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