Increased frequencies of micronucleated reticulocytes and T-cell receptor mutation in Aldh2 knockout mice exposed to acetaldehyde

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ABSTRACT — Aldehyde dehydrogenase-2 (ALDH2) metabolizes acetaldehyde produced from ethanol into acetate and plays a major role in the oxidation of acetaldehyde in vivo. About half of all Japanese people have inactive ALDH2. We generated homozygous Aldh2 null (Aldh2−/−) mice by gene targeting knockout as a model of ALDH2-deficient humans. To investigate the mutagenicity of acetaldehyde, a micronucleus assay and a T-cell receptor (TCR) gene mutation assay were performed in Aldh2−/− mice and wild-type (Aldh2+/+) mice exposed to acetaldehyde. The mice were continuously exposed to 125 and 500 ppm of acetaldehyde vapor for 2 weeks. Another group was orally administered 100 mg/kg once a day for 2 weeks continuously. The mice were killed after 2 weeks of exposure to acetaldehyde, and the frequency of micronucleated reticulocytes was measured by flow cytometry. We also observed the incidence of TCR gene mutations in T-lymphocytes by measuring the variant CD3−CD4+ expression by flow cytometry. The frequency of micronucleated reticulocytes induced by acetaldehyde was significantly increased in Aldh2−/− mice, but not in Aldh2+/+ mice. TCR mutant frequency was also associated with acetaldehyde exposure in Aldh2+/− mice, especially after oral administration; however, it was not associated with acetaldehyde exposure in Aldh2+/+ mice. In conclusion, Aldh2−/− mice showed high sensitivity in the micronuclei and TCR mutation assays compared with Aldh2+/+ mice after exposure to acetaldehyde.

Key words: Aldh2 knockout mouse; Acetaldehyde; Genetic damage; T-cell receptor gene; Micronuclei; Flow cytometry

INTRODUCTION

Ethanol is oxidized to acetaldehyde by alcohol dehydrogenase (ADH). Acetaldehyde is then metabolized to acetic acid by aldehyde dehydrogenase (ALDH). ALDH has been reported to have more than nine isozymes (Yoshida et al., 1998). Among them, ALDH2, which is called low Km ALDH, plays a major role in the metabolism of acetaldehyde to acetic acid. However, about half of all Japanese people lack ALDH2 activity because of a point mutation in the ALDH2 gene (Kawamoto et al., 1994, 1995). In ALDH2-deficient persons with a drinking habit, the body fails to metabolize acetaldehyde rapidly, leading to its excessive accumulation (Mizoi et al., 1994). These persons are reported to have a higher risk of developing head and neck cancers (e.g., esophageal, pharyngeal, and oral cavity) than do ALDH2-normal individuals (Yokoyama et al., 1996, 1998, 2001; Muto et al., 2002).
Recent genetic-epidemiological studies, however, have not been definitive on the role of ALDH2, because the activity levels of many metabolic enzymes show high interindividual variation that is due not only to the genetic polymorphisms but also to other factors, including the overall health status of the individual.

In the present study, we used Aldh2 knockout (Aldh2−/−) as a model of ALDH2-deficient humans to clarify the effects of ALDH2 polymorphism on the carcinogenicity of acetaldehyde. The Aldh2−/− mouse has null mRNA, null ALDH2 protein, and null mitochondrial aldehyde-oxidation activity in the liver, but maintains a normal level of cytosolic aldehyde-oxidation activity (Kitagawa et al., 2000).

Specific locus mutation, chromosomal aberrations, and loss of heterozygosity are the three types of mutations seen in human cancers, and the ability to quantify somatic mutations provides toxicological information that is relevant to the assessment of cancer risk (Heddle and Swiger, 1996). We had previously developed a T-cell receptor (TCR) somatic cell mutant assay (Kunugita et al., 1996, 2007).

Aldh2−/− and wild-type (C57BL/6, Aldh2+/+) mice were exposed to two different concentrations of acetaldehyde (125 and 500 ppm) for 2 weeks in exposure chambers or were orally administered acetaldehyde at a dosage of 100 mg/kg/day for 2 weeks, after which the micronucleated reticulocytes and TCR gene mutation in T-lymphocytes were measured to compare the genetic damage.

MATERIALS AND METHODS

Experimental animals

The Aldh2−/− mice were generated as described previously (Kitagawa et al., 2000). The mice were back-crossed with C57BL/6 mice for more than 10 generations. Male Aldh2++/+ and Aldh2−/− mice aged 12–16 weeks were used. The mice were housed in plastic cages under a 12-hr light/dark cycle condition, a constant temperature (23 ± 1°C), and humidity (55 ± 5%), and were given ad libitum water and food. The number of mice involved in the experiment is shown in each figure. All the animals were treated in accordance with the guidelines of the Animal Welfare and Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan.

Acetaldehyde exposure

Acetaldehyde (SUPELCO, Bellefonte, PA) with 99.9% purity was used. Acetaldehyde vapor, generated by a diffusion tube, was introduced into the top of an exposure chamber (volume 100 l) made of stainless steel (Ogawa et al., 2006). Acetaldehyde in the chambers was sampled in a Sep-Pak DNPH-Silica cartridge (Waters Co., Milford, MA). Its level was determined by high-performance liquid chromatography. It was also monitored by an acetaldehyde detector tube (GASTEC) twice a day. The average level of acetaldehyde to which the mice were exposed in the exposure experiment was 126.3 ppm (expressed as 125 ppm) and 510.5 ppm (expressed as 500 ppm). The Aldh2++/+ and Aldh2−/− mice were placed in an exposure chamber and continuously exposed to 125 ppm (225 mg/m3) and 500 ppm (900 mg/m3) of acetaldehyde for 14 days, and the control group was exposed to normal clean air. In another group, mice were orally administered 100 mg/kg of acetaldehyde once a day for 14 days.

Micronucleus assay

The micronuclei in reticulocytes (MN-Reti) were observed by flow cytometry as described in detail previously (Dertinger et al., 1996). Briefly, 20 μl of blood was collected into tubes containing 100 μl of anticoagulant solution (500 units of heparin/ml saline) just after cessation of continuous inhalation or 24 hr after the last oral injection. Each blood sample was fixed by 1.3 ml of methanol at −80°C for at least 48 hr. A malaria-infected blood sample was prepared as a positive control. Fixed blood cells were washed with bicarbonate saline, simultaneously treated with RNase A (1.2 mg/ml) and fluorescein-isothiocyanate (FITC)-anti-CD71 antibody (Beckman Coulter, Inc, Fullerton CA), and finally resuspended with 0.5 ml propidium iodide (PI, 3 μg/ml) solution. These cells were analyzed using an EPICS-XL flow cytometer (Beckman Coulter, Inc). The frequency of MN-Reti was calculated as the number of events in the PI-positive and CD71+ window divided by the total number of events in the CD71+ cells.

TCR Assay

The TCR assay was performed as described previously (Kunugita et al., 1996). Briefly, the spleens from mice in all groups were resected just after the cessation of continuous inhalation or 24 hr after the last oral injection. The spleen cells were gently dissociated and filtered through a stainless steel mesh. After hemolysis using modified Gey’s solution containing NH4Cl in place of NaCl, the T-cells were then stained with phycoerythrin-anti-CD4 and FITC-anti-CD3 antibodies (Phar single, San Diego, CA,) and were analyzed using an EPICS-XL flow cytometer. Lymphocytes were examined by arranging bit map gates on a plot of the forward and the 90° light scatter. The CD3−CD4+ mutant T-cells were defined as those having a sur-
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face CD3 level less than 1/25th of that of the normal CD4+ cells. The TCR mutant frequencies (MFs) were calculated as the number of events in the CD3+CD4+ T-cell windows divided by the total number of events in the CD3+CD4+ T-cells.

**Statistical analysis**

All data are expressed as mean ± standard deviation (SD). At first, a two-way analysis of variance (ANOVA) test was used to evaluate the effects of genotype and doses of acetaldehyde. Next, comparisons among multiple groups were made using the one-way ANOVA test, followed by Dunnett’s post hoc test, to determine significant differences among the means of the data groups compared with the control mice (Dunnett, 1964). The threshold for statistical significant was p < 0.05. All statistical analyses were performed using StatView 5.0 (SAS Institute, Cary, NC).

**RESULTS AND DISCUSSION**

**Micronucleus frequency in reticulocytes after 2 weeks of inhalation exposure to acetaldehyde**

The effect of acetaldehyde exposure to Aldh2+/+ and Aldh2−/− mice on MN-Reti is shown in Fig. 1. Two-way ANOVA indicated that there was a significant difference in induction of MN-Reti between Aldh2+/+ and Aldh2−/− mice after acetaldehyde exposure (genotype: p < 0.005; acetaldehyde doses: p < 0.05). We performed a one-way ANOVA test followed by Dunnett’s post hoc test to evaluate acetaldehyde toxicity among groups. Although there was no difference in the frequency of MN-Reti in Aldh2+/+ mice between the 125-ppm, 500-ppm, and control groups, a significant increase of approximately 1.8- to 1.9-fold in the frequency of MN-Reti was observed in the 125-ppm (p < 0.05) and 500-ppm (p < 0.01) Aldh2−/− mice compared with the Aldh2+/+ control mice after 2 weeks of exposure to acetaldehyde. The frequency of MN in Aldh2−/− mice exposed to 500 ppm acetaldehyde also significantly increased compared with Aldh2−/− control mice (p < 0.05).

**Micronucleus frequency in reticulocytes after oral administration of acetaldehyde for 2 weeks**

To compare the influence of administration route on the genetic effects of acetaldehyde in each genotype, the mice were orally administered 100 mg/kg acetaldehyde for 2 weeks. Even after the oral administration, there were no differences in the frequency of MN-Reti in Aldh2+/+ mice between the 100 mg/kg and control groups (Fig. 2). On the other hand, the frequency of MN-Reti in Aldh2−/− mice administered acetaldehyde was significantly greater, by 1.7- and 2-fold, than that in the Aldh2−/− (p < 0.05) and

![Fig. 1. Comparison of micronucleus (MN) frequencies in reticulocytes between Aldh2+/+ and Aldh2−/− mice exposed to 125 or 500 ppm acetaldehyde for 2 weeks. Error bars represent the standard deviation. Mean values and standard deviations for each group are shown above the bar graphs. *p < 0.05, **p < 0.01 vs Aldh2+/+ control mice. *p < 0.05 vs Aldh2−/− control mice.](image)
Aldh2+/+ (p < 0.01) control mice, respectively.

**TCR gene mutant frequency after 2 weeks exposure to acetaldehyde**

To evaluate another target of genetic damage by acetaldehyde, we compared TCR MFs in the T-lymphocytes of Aldh2+/+ and Aldh2−/− mice after 2 weeks of inhalation of 500 ppm of acetaldehyde or oral administration of 100 mg/kg acetaldehyde. Although there was no difference in the TCR MFs between the 500-ppm, 100-mg/kg, and control Aldh2+/+ mice, a 1.6-fold significant increase in the TCR MFs was observed in the 100-mg/kg Aldh2−/− mice (p < 0.05) compared with the Aldh2−/− control mice. Furthermore, a 1.7- and 2.4-fold significant increase in TCR MFs was observed in the 500-ppm (p < 0.01) and 100-mg/kg (p < 0.01) in Aldh2−/− mice compared with Aldh2+/+ control mice, respectively.

A significant increase in the frequencies of sister chromatid exchanges was observed in human lymphocytes from ALDH2-deficient habitual drinkers compared with those from ALDH2-proficient individuals (Morimoto and Takeshita, 1996). The results of an MN assay in human lymphocytes conducted by Ishikawa et al. strongly suggested that ALDH2-deficient habitual drinkers show a higher frequency of MN than do ALDH2-proficient individuals (Ishikawa et al., 2003, 2006). These recent genetic-epidemiological studies, however, were limited by the presence of many confounding factors stated above, such as metabolic enzyme activities and the overall health status of the subjects, which did not allow determination of the definitive role of ALDH2.

Aldh2−/− mice have null mitochondrial aldehyde oxidation activity and exhibit alcohol avoidance. After unlimited exposure to ethanol and water, the brain and liver acetaldehyde concentrations of Aldh2−/− mice were almost equal to those of wild Aldh2+/+ mice, although the Aldh2−/− mice drank less ethanol than did the Aldh2+/+ mice (Isse et al., 2002). After gavage of ethanol at a dose of 1 g/kg body weight, the blood acetaldehyde concentration of Aldh2−/− mice was 9.3 times that of Aldh2+/+ mice (Isse et al., 2005). We previously reported that the mean blood acetaldehyde concentration of Aldh2−/− mice was significantly higher than that of Aldh2+/+ mice after exposure to 125 and 500 ppm of acetaldehyde. The mean blood acetaldehyde concentrations of Aldh2−/− mice and Aldh2+/+ mice were 2.39 vs. 1.65 and 8.90 vs. 1.72 μM in the 125 and 500 ppm exposure groups, respectively. Furthermore, pathological changes in the epithelium of the nose and upper airway were greater in Aldh2−/− mice than in Aldh2+/+ mice (Oyama et al., 2007). Also, urinary 8-hydroxydeoxyguanosine levels were slightly increased in Aldh2−/− mice but not in Aldh2+/+ mice after exposure to 125 ppm of acetal-
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Acetaldehyde (Ogawa et al., 2006). These results show that Aldh2−/− mice maintained a high blood acetaldehyde concentration after acetaldehyde exposure, which caused increased DNA damage and a susceptibility to acetaldehyde. These may cause alcohol drinkers, who have no ALDH activity, to have an increased risk of carcinogenesis.

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