RESEARCH COMMUNICATION

Lack of Initiation Activity of 4-oxo-2-hexenal, a Peroxidation Product Generated from ω-3 Polyunsaturated Fatty Acids, in an *In Vivo* Five-Week Liver Assay

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Abstract

Peroxidation products formed from polyunsaturated lipids have DNA damaging potential. 4-oxo-2-hexenal (4-OHE), generated by the oxidation of ω-3 fatty acids, has been demonstrated to be mutagenic *in vitro* as assessed in the Ames test. To examine the carcinogenic risk of 4-OHE *in vivo*, initiation activity was investigated in a five-week liver assay, established to be effective for screening of carcinogenic potential of mutagens. Seven-week-old male F344 rats underwent two-thirds partial hepatectomy (PH) and were administered 4-OHE intragastrically at doses of 128, 80, 64, 40, 32, 20, or 0 mg/kg body weight (b.w.) at 18 hours thereafter, then being fed on diet containing 0.015% 2-acetylaminofluorene from weeks 2 to 4. All rats were given with 0.8 ml/kg b.w. CCl_4 at week 3. At week 5, all survivors were sacrificed and initiation activity was assessed with reference to induction of glutathione S-transferase placental form (GST-P) positive foci in the liver. Mortality was significantly increased to 72.7% in the 128 mg/kg b.w. dose group compared with 0.9% in the control group. However, the average number of GST-P positive foci in the "128" dose group was 3.26±1.66 foci/cm^2, not significantly different from the control value (2.78±1.33). Areas of GST-P positive foci were also similar (1.11±0.5 and 1.53±1.33 mm^2/cm^2 in "128" and the control groups, respectively). These results showed 4-OHE to have no significant initiation activity *in vivo*.

Key Words: 4-oxo-2-hexenal - ω-3 fatty acids - initiation assay - rat - GST-P form positive foci

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Introduction

Peroxidation of polyunsaturated lipids generates a range of substances that possess DNA damaging potential (Burcham, 1998; Esterbauer et al., 1991; Chung et al., 2000). 4-oxo-2-hexenal (4-OHE) is generated by oxidation of ω-3 polyunsaturated fatty acids, which are commonly found in dietary fish oil, perilla oil, rapeseed oil, and soybean oil (Kasai et al., 2005), and has in fact been detected in foodstuffs, such as cooked fish and perilla oil itself (Kawai et al., 2006). Recently, it was reported that 4-OHE is mutagenic to *Salmonella typhimurium* strains TA100 and TA104 without S9 mix (Maekawa et al., 2006), and forms dG, 4-OHE-dC- and 4-OHE-5-methyl-dC adducts in DNA of esophagus, stomach, and intestine tissues in mice after oral administration, pointing to carcinogenic potential in man (Kasai et al., 2005). However, since no carcinogenic tests using rodents have hitherto been performed, it has remained unclear whether 4-OHE can cause lesions *in vivo*.

An *in vivo* five-week initiation assay model has been established which features induction of cell proliferation by partial hepatectomy (PH) after intragastric administration of test chemicals to F344 rats. Initiation activity of test chemicals is estimated by induction of glutathione S-transferase placental form (GST-P) positive foci in the liver, which are considered as preneoplastic lesions and are utilized as surrogate biomarkers in carcinogenicity assays (Chasseaud, 1979; Tsuda et al., 1980). In this model, it may be possible to determine whether mutagens have carcinogenicity regardless of their normal target organs, so that it is a powerful method for screening for carcinogenic potential (Tsuda et al., 1980; Ito et al., 1998; Sakai et al., 2002).

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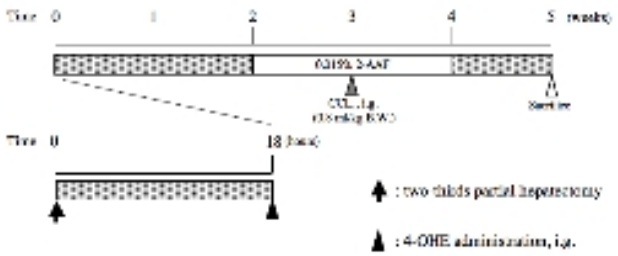

Figure 1. Experimental Protocol for Detection of Initiation Activity of 4-Oxo-2-Hexenal.

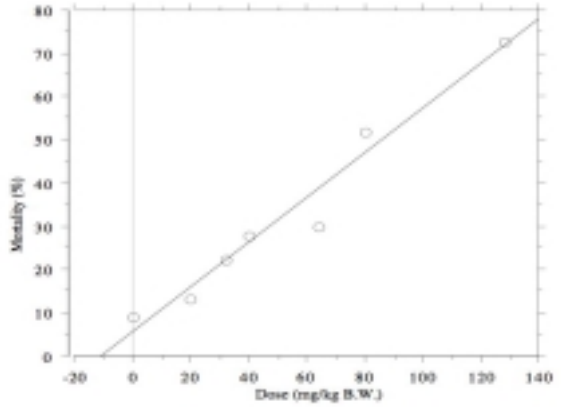

Figure 2. Mortality at the End of Experiment. y=5.6 + 0.516x, when x=dose and y=mortality; R2 = 0.96; P<0.05 (Spearman's rank correlation).

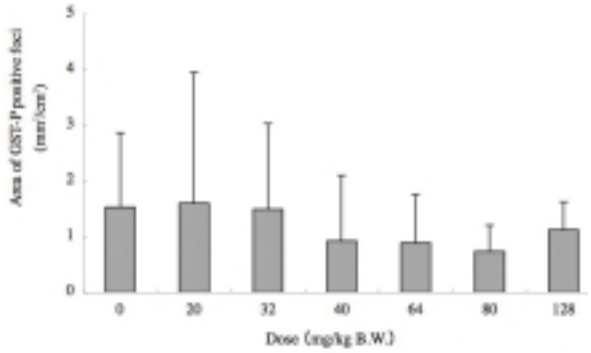

Figure 3. Areas of GST-P positive cell foci > 0.5 mm in diameter. Data are mean ± SD values.

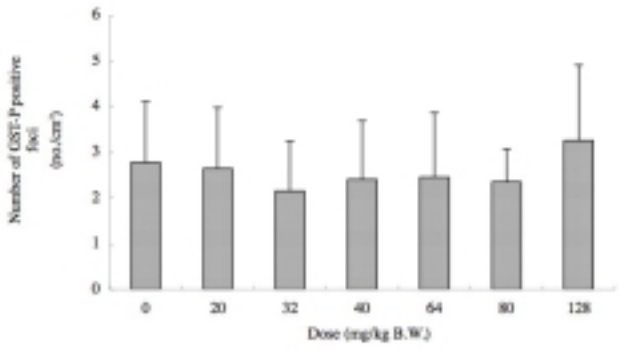

Figure 4. Numbers of GST-P positive liver foci > 0.5 mm in diameter. Data are mean ± SD values.

In the present study, to examine the initiation potential of 4-OHE, an assay was performed taking advantage of the *in vivo* induction of GST-P positive foci as end point lesions (Tatematsu et al., 1987; Tsuda et al., 1980; Tatematsu et al., 1988).

Materials and Methods

Animals

Male Fisher 344 rats (Charles River Japan Inc., Atsugi, Japan) housed 5 per plastic cage on softwood chip bedding, were maintained under constant conditions (12 hours light/dark cycle, 60% humidity at 22 °C ±2 °C), and fed CLEA Rodent diet CA-1 (CLEA Japan Inc., Tokyo, Japan) and tap water ad libitum. Following 1-week acclimatization, the weights of 7-week-old rats were in the range 120-150g.

Chemicals

4-OHE was synthesized as described previously (Kawai et al., 2006; Maekawa et al., 2006). Carbon tetrachloride (CCl_4) was purchased from Wako Pure Chemical Industries Ltd (Osaka, Japan) and 2-acetylaminofluorene (2-AAF) from Tokyo Chemical Industrials Ltd (Tokyo, Japan).

Experimental protocol

The experimental protocol is shown in Figure 1. Rats in Groups 1 to 6 were administered 4-OHE by oral gavage at doses of 128, 80, 64, 40, 32 and 20mg/kg body weight (b.w.) respectively, at 18 hours after two-thirds partial hepatectomy. Following the 4-OHE administration, rats were fed on basal diet for 2 weeks, and then diet containing 0.015% 2-AAF for the following 2 weeks. At week 3, all rats were received a single dose of CCl_4 by oral gavage at a dose of 0.8ml/kg b.w. The control group (Group 7) was treated as groups 1 to 6 but without 4-OHE administration. At the end of week 5, all the survivors were sacrificed and the livers were excised. Liver slices were fixed in 10% neutral buffered formalin for immunohistochemical examination of GST-P positive foci.

Immunohistochemical staining and analysis of sections The avidin-biotin complex method was used to demonstrate GST-P positive foci using anti-GST-P IgG (MBL, Nagoya, Japan). The numbers and areas of foci >0.5 mm in diameter per total areas of liver sections examined were measured as described in previous reports (Kobayashi et al., 1997; Tatematsu et al., 1988; Sakai et al., 2000), the values being expressed in foci/cm² and mm²/cm², respectively. The significances of differences in the quantitative data of experiment were statistically evaluated using ANOVA.

Results

Mortality

Data for mortality are shown in Figure 2. In the dosed groups, the mortality was increased in a dose dependent manner, to as high as 72.7% in group 1.

Induction of GST-P positive foci (areas)

Data for areas of GST-P positive foci per unit area of

liver section are summarized in Fig. 3. The value for the control group (Group 7) was 1.53±1.37 mm²/cm². In the dosed groups, the values were 1.12±0.62, 0.73±0.49, 0.89±0.94, 0.92±1.23, 1.49±1.67, and 1.60±2.45 mm²/cm² in groups 1 to 6, respectively. Statistically, there were no significant differences between the 4-OHE administered and control groups.

Induction of GST-P positive foci (numbers)

Data for the numbers of GST-P positive foci per unit area of liver section are summarized in Fig. 4. The value for the control group (Group 7) was 2.78±1.37 foci/cm². In the dosed groups, the values were 3.26±2.03, 2.35±0.75, 2.46±1.53, 2.40±1.34, 2.15±1.18, and 2.64±1.39 foci/cm² in groups 1 to 6, respectively. Statistically, no significant differences were observed.

Discussion

In the in vivo five-week initiation assay model used in the present study, mutagenic carcinogens independent of whether they are hepatocarcinogens have been demonstrated to have initiation activity. On the other hand, non-carcinogen mutagens do not give rise to an increase in the number of GST-P positive foci. Similarly, carcinogens without mutagenic potential do not significantly induce GST-P positive foci (Sakai et al., 2002). The present results showed that orally administered 4-OHE at doses up to 128 mg/kg lacked initiation activity in the assay, despite marked toxicity. Although the cytotoxic mechanism of 4-OHE remains to be clarified, it has been reported to induce apoptosis in human leukemia HL-60 cells (Kasai et al., 2005) and may also react with arginine, histidine, and lysine residues in proteins (Maekawa et al., 2006).

The induction of GST-P positive foci with direct acting carcinogens not requiring metabolic activation is well known to depend on the timing of administration after PH (Kobayashi et al., 1997). The first peaks of DNA synthesis and division of hepatocytes are observed at 18-24h and 18h after PH, respectively (Kobayashi et al., 1997; Sakai et al., 2000). Therefore, considering the previous report of mutagenicity of 4-OHE without S9 mix (Maekawa et al., 2006), predicting it to be a direct carcinogen, we adopted administration of 4-OHE by oral gavage at 18 hr after PH, expecting high sensitivity to any initiation activity. However, the results were negative.

It would be premature to conclude that 4-OHE has no carcinogenic potential and species differences between mice and rats may be important, so that more studies using mice may be required. Nevertheless, we can conclude from the present study that 4-OHE has no significant carcinogenic potential *in vivo* under the conditions of the model used.

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