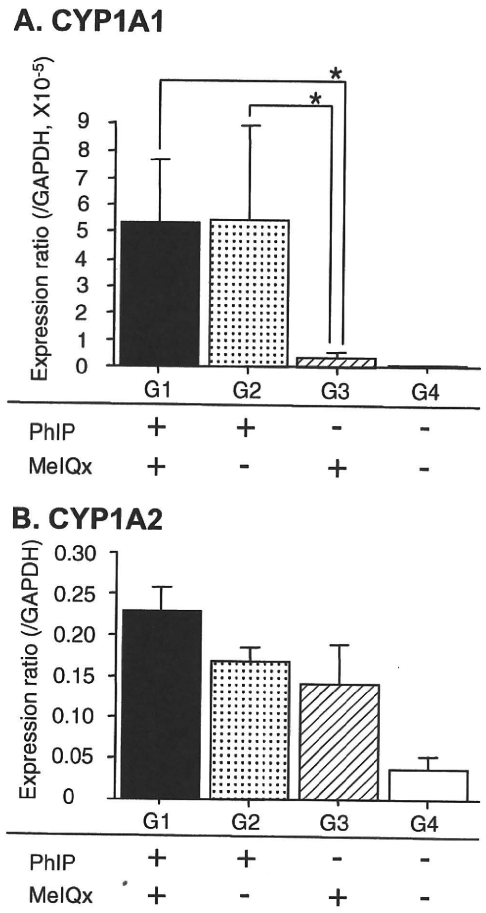

Fig. 4. Expression levels of mRNA of CYP 1A1 (A) and 1A2 (B) in the livers of hepatectomized and HCAs-administered rats by quantitative RT-PCR. Data are mean ± SD values for four animals in each group. *Significant difference between groups, $P < 0.05$. With both CYP 1A1 and 1A2, each group treated with HCAs (groups 1–3) significant alteration from the control group (group 4) was apparent ($P < 0.05$).

30 and 36 h after PH (approximately 16–24% in BrdU labeling indices) than between 12 and 18 h after PH (approximately 0–34%) and the effect on foci formations was also limited (Sakai et al., 2001). Thus, the shortening of time for MeIQx metabolism was unlikely to have a significant effect on the formation of GST-P positive foci in Exp. I-B.

In conclusion, the present data clearly showed that our *in vivo* 5-week initiation assay is useful to analyze carcinogenic potential of HCAs including combined risks of multiple HCAs. It is noteworthy that complex exposure to multiple HCAs is not always associated with increased risk of carcinogenesis given that they are simultaneously and continuously ingested under normal lifestyle circumstances.

Conflict of interest statement

The authors declare that there are no conflict of interest.

Acknowledgements

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Application of a New Bioassay Technique Using Goldfish for Assessment of Water Toxicity

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ABSTRACT: There are a variety of chemicals in aquatic environment, so it is important to assess the toxicity. The biomarkers such as induction of DNA damage, micronuclei, vitellogenin, and hepatic P450 in fish are known to be effective for monitoring genotoxic and/or estrogenic chemicals. However, there is little study to use these biomarkers in same fish. Goldfish (*Carassius auratus*) is widely used and is suitable in size to collect blood or organs. In this study, validity of multiple-biomarkers in goldfish was checked using standard chemicals and applied in the river water. Ho River, which flows through the textile dyeing factory in Shizuoka Prefecture, Japan, was reported to show genotoxicity toward *Salmonella typhimurium* TA98 and YG1024. When the goldfish were exposed to Ho River, DNA damage, estrogenic activity, and CYP1A induction were observed. Through the study, it was assumed that not only mutagens/carcinogens but also endocrine disrupting chemicals and poly aromatic hydrocarbons were present in Ho River. Therefore, chemical identification should be required. We could evaluate both genotoxicity and estrogenic activity simultaneously, so goldfish might be a good experimental model for estimation of chemical contamination levels in aquatic environment. © 2008 Wiley Periodicals, Inc. Environ Toxicol 23: 720–727, 2008.

Keywords: goldfish; genotoxicity; estrogenic activity; P450 enzyme activity; biomonitoring

INTRODUCTION

Many kinds of chemicals showing mutagenic/carcinogenic potency are present in aquatic environment. Ohe et al. (2004) reviewed that 7–15% of river water around

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the world showed genotoxicity toward *Salmonella typhimurium* TA98 and TA100 strain which detect frameshift point mutations and base substitution, respectively. Genotoxicity studies of river water have employed the *Salmonella*/genotoxicity test mainly, but it is important to evaluate the river water using aquatic animals. Micronucleus (MN) test has been used in genotoxicological study with fish (Al-Sabti and Metcalfe, 1995; Hayashi et al., 1998), and it detects MN resulting from either chromosomal breakage during cell division or chromosome loss events in anaphase damages (Kirsch-Volders et al., 2003). MN test is often applied with comet assay. Comet assay detects DNA damage including single-strand breakage or other lesions such as alkali-labile sites and DNA cross-link by measuring the migration of DNA from immobilized nuclear DNA (Singh et al., 1988). Comet assay has been widely used for studies on genotoxicity of environmental chemicals with fish (Lee and Steinert, 2003). Induction of hepatic cytochrome P450 enzymes in fish is also known to be sensitive biomarkers for monitoring polychlorinated biphenyl (PCB) or poly aromatic hydrocarbons (PAHs) (Ahokas et al., 1976). The determination of ethoxy resorufin-*O*-dealkylase (EROD) activity in fish has been used as an indicator of PAHs (Wassenberg et al., 2005). Methoxy resorufin-*O*-dealkylase (MROD), penthoxy resorufin-*O*-dealkylase (PROD), and bezyloxy resorufin-*O*-dealkylase (BROD) activities are known, and Russell et al. (2004) evaluated PCB using these enzyme activities.

It is well known that not only mutagens/carcinogens but also endocrine disrupting chemicals (EDCs) are present in aquatic environment (Whaley et al., 2001). Vitellogenin (VTG) is known to be an effective biomarker for monitoring EDCs (Li et al., 2005). VTG is a protein synthesized in female liver and delivered into blood, then taken up by oocytes to be processed into egg yolk. However, VTG production can be induced in males by administration of EDCs. So VTG has been used as an indicator of EDCs. They are known to be effective and sensitive biomarkers, but there is little study to determine genotoxicity and estrogenic activity simultaneously using fish. Among all kinds of aquatic organisms, goldfish (*Carassius auratus*) is suitable in size to collect any organs. We thought that goldfish is a good model for evaluation of aquatic environment. In this study, we checked the validity of multiple-biomarkers in goldfish using standard chemicals and applied the river water. Recently, Watanabe et al. (2006) reported that Ho River, which flows through the textile dyeing factory in Shizuoka Prefecture, showed genotoxicity toward TA98 and YG1024 (*O*-acetyltransferase overexpressing strain) with metabolic activation (+S9 mix). So we evaluate the toxicity of Ho River not only genotoxicity but also estrogenic activity and P450 enzyme activity using goldfish.

MATERIALS AND METHODS

Chemicals

β-naphthoflavone (β-NF), low-melting point agarose, *O*-phenylenediamine, 7-ethoxyresorufin, 7-methoxyresorufin, 7-penthoxyresorufin, 7-benzyloxyresorufin, and resorufin were purchased from SIGMA (St. Louis, MO). HRP-conjugated-anti-rabbit IgG goat antibody was purchased from Seikagaku Corporation (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Fish

We obtained goldfish weighing 20–25 g from a local dealer in Hamamatsu City, Japan. Before the experiments, goldfish were acclimatized for 2 weeks in a well-aerated aquarium maintaining at 20°C ± 2°C under 12 h light/12 h dark photoperiod. For estrogenic activity assay, a low estrogenic diet which does not contain any soybean meal was developed with Kyorin Corporation (Himeji, Japan).

Genotoxicity Assay

Genotoxicity was assessed by comet assay and MN test according to Deguchi et al. (2007). In both assay, methyl methanesulfonate (MMS) and mitomycin C (MMC) were used as positive control. MMS and MMC were dissolved in dimethylsulfoxide (DMSO) and injected intraperitoneally (i.p.) once at the following doses: 10, 50 mg/kg body weight for MMS and 2, 4 mg/kg body weight for MMC. In the negative control group, DMSO was injected i.p. into fish. Five fish were used in each group, and kept in a 10-L glass tank. In comet assay, blood was collected 3, 6, and 24 h after injection. The comet images were analyzed using a fluorescence microscope (magnification 200×) equipped with CCD camera. Two slides were prepared for each fish, and 50 cells per slide were examined. The tail moment of DNA was measured by using Komet 4.0 image analyzed program (Kinetic Imaging, Liverpool, UK) or Comet Analyzer (Youworks, Tokyo, Japan). In MN test, blood was collected 48, 96, and 144 h after injection. Two slides were prepared for each fish. At least 1000 erythrocytes per slide were observed by a fluorescence microscope (magnification 400×), and the number of micronucleated cells were recorded. MN frequency was calculated as the number of micronucleated cells per 1000 cells.

Estrogenic Activity Assay

Male goldfish were exposed to 10-L that contained 1, 10, 100 μg/L of 17β-estradiol (E2), bisphenol A (BPA), and 4-nonylphenol (NP) in glass tank in static condition. Negative control group was only exposed to solvent carrier (DMSO

100 μL/L). Five fish were used in each group, and test solutions were exchanged every day. Blood was collected after 9 days and centrifuged at 3000 rpm for 20 min, then the plasma was divided into aliquots and stored at −20°C until use. The concentrations of VTG in plasma were determined by enzyme-linked immunosorbent assay (ELISA) using monoclonal mouse antibody against goldfish lipovitellin (Lv). The goldfish VTG and Lv were purified from sexually mature female goldfish plasma and oocyte, respectively, as described by Hara et al. (1993). Purified VTG was used as a standard, and diluted samples were used for measurement of VTG in duplicate. Visualization was accomplished using HRP-conjugated-anti-rabbit IgG goat antibody and *O*-phenylenediamine as a HRP substrate. After 10 min, the reaction was stopped by adding 2 M H_2SO_4 and absorbance was measured with a microplate reader (Thermo Fisher Scientific, MA) at 492 nm. These assays were performed at room temperature. The concentrations of VTG were calculated from the linear part of the log-transformed standard curve. The detection limit of VTG in present study was 0.02 μg/mL.

P450 Enzyme Activity Assay

P450 enzyme activity was measured using β-NF and phenobarbital (PB) as positive control. β-NF and PB were dissolved in DMSO and injected i.p. once at the following doses: 1, 10 mg/kg body weight for β-NF and 50, 100 mg/kg body weight for PB. In the negative control group, DMSO was injected i.p. into fish. Five fish were used in each group, and kept in a 10-L glass tank. Liver was collected 24, 48, and 96 h after injection and rinsed in 1.15% KCl then homogenized with three volumes of homogenization buffer (100 mM Tris-HCl containing 1 mM EDTA-2Na, 100 mM KCl, pH 7.4) in a Potter-Elvehjem homogenizer, then centrifuged at 9000 × *g* for 20 min at 4°C. The supernatant was ultracentrifuged at 105 000 × *g* for 60 min at 4°C. The microsome pellet was resuspended and homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA-2Na and 20% glycerol. Microsome was divided into aliquots and stored at −80°C until use. The protein content was determined according to BCA protein assay (PIERCE, USA) using bovine serum albumin as a standard. EROD, MROD, PROD, and BROD activities of liver microsome were measured using the method described by Burke et al. (1994) with some modifications. Microsome (50 μg) and 25 μL of NADPH generation system (100 mM $MgCl_2$, 10 mM NADP, 100 mM G-6-P, and 1000 U/mL G-6-P DH) were preincubated for 5 min at 30°C (total volume: 200 μL), and then the 50 μL of each substrates (25 μM of 7-ethoxyresorufin, 25 μM of 7-methoxyresorufin, 25 μM of 7-pethoxyresorufin, or 25 μM of 7-benzyloxyresorufin) were added to start the reaction. After 5 min, the reaction was stopped by adding 250 μL of ice-cold ethanol. The mixture was centrifuged at 15 000 rpm for 3 min at 4°C. The fluorescence of the supernatant was measured on a fluorescence microplate reader (Thermo Fisher Scientific, MA) with an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each enzyme activities were expressed as the rate of resorufin formation, and were calculated based on the fluorescence of a standard curve of resorufin standards.

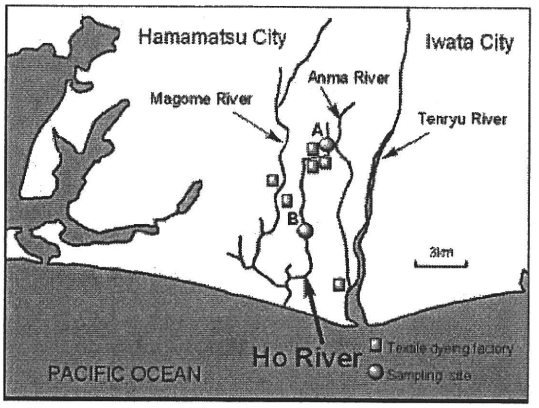

Fig. 1. Geographic locations of the sampling points, the textile dyeing factories, and the Ho River in Shizuoka Prefecture.

Exposure to River Water

We collected river water from upstream (point A) and downstream (point B). The sampling points were shown in Figure 1. At first, temperature, pH, and COD_{Mn} of Ho River were measured. Male goldfish were exposed to 10-L river water in glass tank in static condition. Negative control group was tap water. Five fish were used in each group, and a half volume of the river water was exchanged with same river water every day. After exposure for 1 and 2 weeks, the goldfish were removed from each tank and we examined genotoxicity, estrogenic activity, and P450 enzyme activity.

Statistical Analysis

Dunnett's test after one-way ANOVA was used to evaluate the significance of the differences in each test between the control group and the treated group; p values lower than 0.05 were considered to indicate statistical significance.

RESULTS

Genotoxicity

DNA damage and MN induction were evaluated using MMS and MMC. Figure 2 showed the mean values of

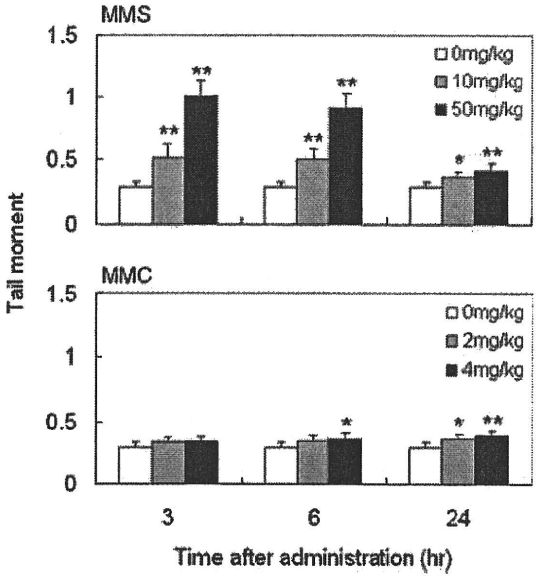

Fig. 2. DNA damage in peripheral blood cells of goldfish administered MMS (upper graph) and MMC (lower graph). One hundred cells were counted per fish. The mean values were obtained from 500 cells. The bars represented the SEM values. Tail moment = DNA migration × Tail intensity (using Komet 4.0 image analyzed program). Significant difference: $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 mg/kg).

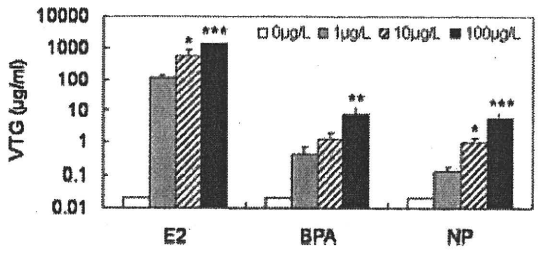

Fig. 4. Induction of VTG in plasma of male goldfish exposed to E2, BPA, and NP for 9 days. The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 g/L).

DNA tail moment in blood. MMS caused stronger DNA damage compared to MMC. In MMS, DNA damage was increased until 3 or 6 h after injection and then decreased. In MMC, DNA damage was not observed at 3 h after injection, but then showed weak DNA damage after 24 h. Figure 3 showed the frequencies of MN in blood. MMC caused stronger chromosomal aberration compared to MMS, and the frequencies of MN significantly increased until 96 h after injection and then decreased. The average of frequencies for 48, 96, and 144 h (MMC 4 mg/kg) were 1.18‰ (=12/10 204, $p < 0.05$), 2.79‰ (=28/10 028, $p < 0.01$), and 1.18‰ (=12/10 186, $p < 0.05$), respectively.

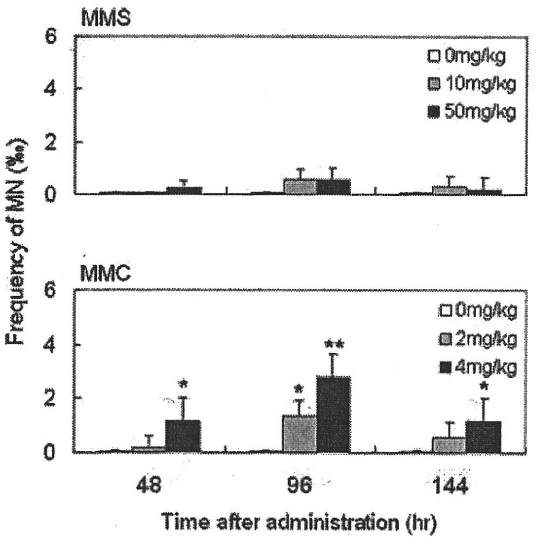

Fig. 3. The frequency of MN in peripheral blood cells of goldfish administered MMS (upper graph) and MMC (lower graph). The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 mg/kg).

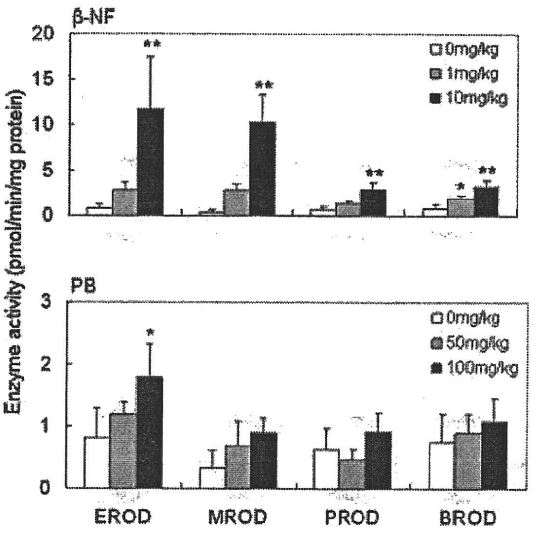

Fig. 5. Activity of four liver enzymes (EROD, MROD, PROD, and BROD) of goldfish administered β-NF (upper graph: after 48 h) and PB (lower graph: after 24 h). The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 mg/kg).

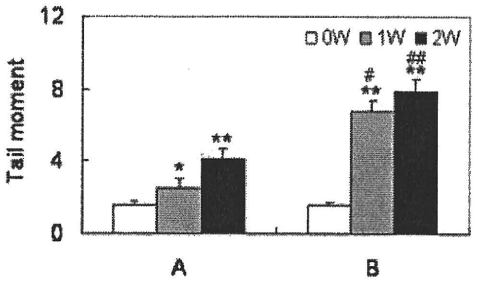

Fig. 6. DNA damage in peripheral blood cells of goldfish exposed to Ho River for 2 weeks. One hundred cells were counted per fish. The mean values were obtained from 500 cells. The bars represented the SEM values. Tail moment = Tail distance × Ratio (using Comet Analyzer). Significant difference: $^{**}p < 0.01$, $^{*}p < 0.05$ (vs. control; 0 W), $\#p < 0.05$ (vs. point A; 1 W), $\#\#p < 0.05$ (vs. point A; 2 W).

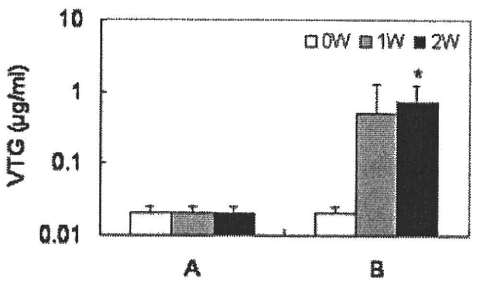

Fig. 7. Induction of VTG in plasma of male goldfish exposed to Ho River for 2 weeks. The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{*}p < 0.05$ (vs. control; 0 W).

Estrogenic Activity

VTG synthesis was evaluated using E2, BPA, and NP. Figure 4 showed the concentrations of plasma VTG in male goldfish exposed to E2, BPA, and NP for 9 days. VTG were significantly increased by treating with 10 μg/L of E2 and NP ($p < 0.05$), 100 μg/L of BPA ($p < 0.01$), and 100 μg/L of E2 and NP ($p < 0.001$).

P450 Enzyme Activity

EROD, MROD, PROD, and BROD activities in liver were measured at 24, 48, and 96 h after injection of β-NF and PB. Figure 5 showed the enzyme activities at 48 h after injection of β-NF and at 24 h after injection of PB. In β-NF, all activities were gradually increased until 48 h after injection, and then decrease at 96 h (data not shown). All activities were significantly increased by β-NF at 48 h after administration. In PB, only EROD activity was significantly increased at 24 h after injection, and then decrease. Other activities were not increased until 96 h (data not shown).

Evaluation of Ho River

The temperature of point A and point B were 22.2 and 23.5°C, respectively. The pH of point A and point B were 7.42 and 7.39, respectively. The COD_{Mn} of point A and point B were 4.95 and 13.86, respectively. Ten male goldfish were exposed to Ho River for 1 or 2 weeks. The survival ratio of goldfish was 100% in both the points. In comet assay, Ho River showed DNA damage in both the points, however DNA damage was stronger in point B than that of point A (Fig. 6). On the other hand, MN was not significantly increased in both the points (data not shown). The plasma VTG synthesis was not observed in point A, however the plasma VTG levels was significantly increased in point B (Fig. 7). Although all hepatic P450 activities were not significantly increased in point A, significant induction of MROD activity was found in point B (Fig. 8).

DISCUSSION

A lot of bioassay techniques using aquatic animals have been developed so far. We evaluated genotoxicity, estrogenic activity, and P450 enzyme activity using goldfish. In

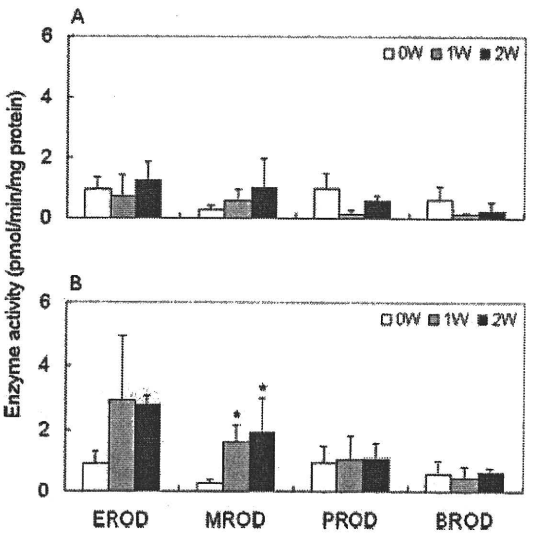

Fig. 8. Activity of four liver enzymes (EROD, MROD, PROD, and BROD) of goldfish exposed to point A (upper graph) and point B (lower graph) for 2 weeks. The mean values were obtained from five fish. The bars represented the SD values. Significant difference: $^{*}p < 0.05$ (vs. control; 0 W).

comet assay and MN test, we used MMS (alkylating agent) and MMC (DNA crosslinker). In comet assay, MMS showed stronger DNA damage than that of MMC, but in MN test, MMC showed higher frequency of MN than that of MMS. The numerous biomarkers such as DNA strand breaks, DNA adducts, chromosomal aberrations, sister chromatid exchange, and MN were used to monitor mutagens/carcinogens in aquatic environment. Mitchelmore and Chipman (1998) commented that DNA strand breaks, particularly as measured by the comet assay, act as a biomarker of genotoxicity in fish and other aquatic species. They also emphasized that this approach should be combined with the use of other biomarkers. Thus, a combination of the comet assay and the MN test is a useful tool for monitoring mutagens/carcinogens.

Next, we measured VTG as a biomarker for estrogenic activity. Although VTG is regarded as female specific protein, male fish can also produce VTG in the liver if exposed to EDCs. Therefore the measurement of plasma VTG in male fish has been used as a sensitive biomarker of EDCs in aquatic environment. It is known that normal fish diets contain the phytoestrogens such as genistein, daidzein, equol, and coumestrol. Ishibashi et al. (2002) reported that VTG was detected by feeding normal fish diets, but VTG was not detected by feeding casein-based formulated fish diet which does not contain soybean and fish meal. So we developed a low estrogenic fish diet which does not contain soybean. The concentrations of VTG of male goldfish were decreased by feeding this fish diet (data not shown). When male goldfish were exposed to E2, BPA, and NP for 9 days, the concentrations of VTG were significantly increased by treating with 10 μg/L of E2 and NP ($p < 0.05$), 100 μg/L of BPA ($p < 0.01$), and 100 μg/L of E2 and NP ($p < 0.001$). The concentration of 1 μg/L was much higher than those detected in river water in Japan. In this study, VTG were detected by treating with 1 μg/L of each substance, so the measurement of plasma VTG in male goldfish was useful to monitor EDCs. Jobling et al. (1996) reported that VTG synthesis was found in rainbow trout (*Oncorhynchus mykiss*) after treating with 10 μg/L of NP. So goldfish seemed to have similar sensitivity to NP as rainbow trout.

We also determined EROD, MROD, PROD, and BROD activities in liver microsome. Many carcinogens are known to be metabolically activated by drug-metabolizing enzymes including CYP enzymes. CYP1A enzymes catalyzed PAHs and heterocyclic amine (HCA). CYP2B enzymes catalyzed the dichloro diphenyl trichloroethane (DDT) and PB. In this study, CYP1A and CYP2B activities were determined from EROD and MROD activities, PROD and BROD activities, respectively. EROD and MROD activities of goldfish were rose by β-NF (as a CYP1A inducer). In rats, EROD and MROD activities are related to CYP1A1 and CYP1A2, respectively. In fish, EROD activity is related to CYP1A1 (Stegeman et al., 1997), and Smeets et al. (2002) found a high correlation between EROD and MROD activities in rainbow trout (*Oncorhynchus mykiss*), dab (*Limanda limanda*), European flounder (*Platychthis flesus*), and lemon sole (*Microstomus kitt*). Therefore, the basic metabolic machinery in goldfish is similar to that of rats. PB was known to be a CYP2B inducer in rats, but neither PROD nor BROD activities rose when we administrated PB to goldfish. Sadar et al. (1996) reported that PROD activity did not rise by PB, but EROD activity rose in primary culture of rainbow trout hepatocytes. In this study, PROD activity did not rise, but EROD activity rose in goldfish. So it was strongly suggested that PB is metabolized by CYP1A in goldfish. On the other hand, Ruus et al. (2002) found high correlations between EROD and PROD activities in cod (*Gadus morhua*) and bullrout (*Myoxocephalus scorpius*). Like this, PROD and BROD activities in fish were unclear, so further investigations are required to elucidate these activities.

Through these studies, we could construct several biomarkers, such as genotoxicity, estrogenic activity, and P450 enzyme activity. So next, we applied these biomarkers to evaluate the river water. Recently, Watanabe et al. (2006) reported that Ho River, which flows through the textile dyeing factory in Shizuoka Prefecture, Japan, showed genotoxicity toward TA98 and YG1024 with S9 mix. We collected river water from upstream (point A) and downstream (point B) of textile dyeing factory, and we compared each point. It was assumed that mutagens/carcinogens, EDCs, and PAHs were existed in point B. Watanabe et al. (2006) also collected from same point and non-Cl phenylbenzotriazole (PBTA)-2, -3, and -7 were detected from this point. Several PBTAs were detected from river water in Japan (Morisawa et al., 2003; Ohe et al., 2006), and PBTAs were reported to show strong genotoxicity toward *Salmonella typhimurium* YG1024 in the presence of S9 mix (Ohe et al., 1999; Shiozawa et al., 2000; Nukaya et al., 2001; Watanabe et al., 2001, 2002). Masuda et al. (2004) reported that PBTA-6 showed genotoxicity toward goldfish. Although other PBTAs including non-Cl PBTAs were not tested *in vivo*, it was suggested that the non-Cl PBTAs might be contributed to their genotoxicity. Ho River also showed estrogenic activity and CYP1A induction. There is no data about PBTAs on VTG synthesis or P450 enzyme activity. So we must examine the effects of PBTAs on VTG synthesis and P450 enzyme activity in the future. Point A also showed DNA damage to goldfish. So we need to monitor Ho River including upstream in different season. Through these studies, we could evaluate both genotoxicity and estrogenic activity simultaneously, so goldfish might be a good experimental model for estimation of chemical contamination levels in aquatic environment.

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Evidence of a Threshold-Effect for 2-Amino-3,8-dimethylimidazo-[4,5-f]quinoxaline Liver Carcinogenicity in F344/DuCrj Rats

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ABSTRACT

To estimate potential human risk of exposure to a food-derived, genotoxic hepatocarcinogen, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), a 2-year carcinogenicity test was conducted using male F344 rats administered MeIQx-containing diet at doses of 0 (control), 0.001, 1, and 100 ppm. The lowest dose 0.001 ppm was established as equivalent to the daily intake of this carcinogen in humans (0.2 to 2.6 μg/man/day). Significant decreases of survival rate and body weight gain were observed in rats treated with 100 ppm MeIQx. Histopathological examination revealed significant induction of hepatocellular carcinomas, adenomas, and development of glutathione *S*-transferase placental form–positive foci with MeIQx at 100 ppm. Moreover, the incidences of Zymbal's glands carcinoma, mammary fibroadenoma, and subcutaneous fibroma were found significantly increased in a 100 ppm MeIQx group. However, no significant induction of altered preneoplastic hepatocellular foci was observed in 0.001 and 1 ppm groups as compared to the controls. 8-Hydroxy-2'-deoxyguanosine levels in the rat liver DNA of the 100 ppm-treated group were not elevated, but MeIQx-DNA adduct formation increased as compared with the 1 ppm case, albeit without significance. No significant induction of any other neoplastic lesions related to the carcinogen administration was found in MeIQx-administered groups except for 100 ppm. These results imply that 1 ppm may be a no-effect level for MeIQx carcinogenesis.

Keywords: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; MeIQx; heterocyclic amine; low dose carcinogenicity; threshold.

INTRODUCTION

Heterocyclic amines (HCAs) detected in cooked or heated meat or fish are known to have mutagenic potential (Sugimura et al., 2004) and show carcinogenicity not only in rodents but also in monkeys (IARC, 1992; Adamson et al., 1995). It can be surmised that carcinogenicity is also exerted in man and this group of agents is designated as possible carcinogens in the IARC carcinogen classification (IARC, 1992). Therefore, for cancer prevention, avoidance of exposure to HCAs is highly recommended (Sugimura et al., 2004).

2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) is one of the HCAs being first reported as a potent mutagen in fried beef (Kasai et al., 1981). The mutagenicity of MeIQx in *Salmonella typhimurium* TA98 and TA100 with S9 mix was reported to be the strongest of all HCAs examined (Sugimura et al., 2004). MeIQx is metabolized in vivo to DNA-reactive metabolites, which form DNA adducts considered important for HCA carcinogenesis in various organs (Langouet et al., 2001; Turteltaub et al., 1997; IARC, 1992). Actually, 1.8 to 18 DNA adducts per 10^{10} nucleotides have been detected in human organs such as the colon (Totsuka et al., 1996). In rats, MeIQx was reported to induce tumors of liver, lung, Zymbal's gland, clitoral gland, large intestine, oral cavity, mammary gland, and skin. Furthermore, tumorigenicity of MeIQx after its administration at relatively high dose levels (≥100 ppm) was observed regarding mice liver, lung, forestomach, and large intestine, as well as induction of lymphomas and leukemias (Sugimura et al., 2004; IARC, 1992). Chronic administration of MeIQx is reported to result primarily in carcinomas of the liver in rodents (Kato et al., 1988).

It has been generally accepted that genotoxic carcinogens have no threshold in exerting their potential for cancer induction. However, the nonthreshold theory can be challenged for cancer risk assessment in humans. For example, a low-exposure study of diethylnitrosamine questioned whether the effects of carcinogens at high doses can be quantitatively extrapolated to those at low doses (Williams et al., 1993). Recently we have found that DNA adduct formation is elevated linearly in proportion to a rise in the MeIQx dose (Fukushima et al., 2002). However, no increase of formation of 8-hydroxy-2'-deoxyguanosine

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Abbreviations: MeIQx, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline; HCAs, heterocyclic amines; GST-P positive foci, glutathione *S*-transferase placental positive foci; AHF, altered hepatocellular foci.

(8-OHdG), which is the most abundant DNA adduct associated with oxidative stress and resulting in specific types of mutation, as well as induction of glutathione *S*-transferase placental form (GST-P)–positive foci, considered the rat liver preneoplastic lesions, was observed at (≤10 ppm) of MeIQx (Fukushima, 1999; Fukushima et al., 2002, 2003). Although the number of GST-P-positive foci at 1 ppm was not different from control, it was increased at 10 ppm, albeit without statistical significance. Thus, in the case of exposure to genotoxic carcinogens at low doses, no-effect levels may exist for parameters relevant to carcinogenicity. Therefore, we hypothesize that a no-effect level should exist for cancer induction by MeIQx. Since the carcinogenicity tests of MeIQx at low-dose levels corresponding to human daily intake level have hitherto not been carried out, the present investigation was conducted with F344/DuCrj rats, which are commonly used in carcinogenicity studies. The lowest level, 0.001 ppm, of MeIQx was established as equivalent to the daily intake of this carcinogen in humans (0.2 to 2.6 μg/man/day) (IARC, 1992).

Materials and Methods

Animals

The animals used in the present study were handled in accordance with recommendations of the Guide for the Care and Use of Animals (Institute of Laboratory Animal Resources, 1996). Four-week-old male F344/DuCrj rats were purchased from Charles River Japan, Inc (Hino, Japan) and housed 3 rats to a plastic cage (RT type, Charles River Japan, Inc) with paper chips for bedding in a room maintained under a 12-h (07:00–19:00) light-dark cycle, at a constant temperature of 25°C ± 1°C and a relative humidity of 55% ± 10%. All rats were given pelleted MF diet (Oriental Yeast Co, Tokyo, Japan) and water ad libitum up to 6 weeks of age, and then were used for the experiments. Mortality and general condition were checked daily.

Chemical

The carcinogen, MeIQx (purity, 99.9%), was purchased from the NARD Institute, Ltd (Nishinomiya, Japan).

Experimental Design

Two-hundred-four rats were randomly divided into 4 groups, receiving MeIQx at doses of 0 (group 1, a control), 0.001 (group 2), 1 (group 3), and 100 ppm (group 4) in pelleted MF diet, continuously. The lowest level, 0.001 ppm, of MeIQx was established as equivalent to the daily intake of this carcinogen in humans (0.2 to 2.6 μg/man/day) (IARC, 1992). Pelleted MF diets containing MeIQx were prepared by Oriental Yeast Co. MeIQx concentration in each diet was confirmed by high-performance liquid chromatography (HPLC), and the actual measurement value of MeIQx concentration in food was within ±5% as compared with nominal concentration. The total observation period was 104 weeks. Body weights were measured monthly along with the amount of food consumed on a per-cage basis. At the end of the experiment, all rats were euthanized under anesthesia with diethyl ether for the pathological examination.

Pathological Examination

At necropsy, after macroscopic observation, the livers were removed, weighed, processed for fixation with 10% phosphate-buffered formalin (pH 7.4), routinely embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for light microscopic examination. All blocks included the left lobe and median lobe as well as nodules observed macroscopically in order to carry out quantitative analysis of altered hepatocellular foci (AHF) including basophilic, eosinophilic, clear, vacuolated, and mixed-cell foci according to the criteria reported previously (Harada et al., 1989). Nodules/masses in other organs including the Zymbal's gland, subcutis, and colon, in which tumors are reported to be induced by MeIQX (Kato et al., 1988; Sugimura et al., 2004; IARC, 1992), were recorded macroscopically and examined histopathologically.

Assessment of GST-P-Positive Foci, 8-OHdG, and MeIQx-DNA Adduct Levels in the Livers

As described previously (Fukushima et al., 2002), liver sections of the left and median lobes were routinely processed for immunohistochemical staining of GST-P (with anti-rabbit GST-P antibody; MBL Co, Ltd, Nagoya, Japan) by the avidin-biotin peroxidase complex (ABC) method using Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Numbers of GST-P-positive foci comprising 2 or more positive hepatocytes were counted under a light microscope. Total areas of sections were measured using a color image processor (IPAP Sumika Technos, Osaka, Japan), and numbers of foci per square centimeter of liver tissue were calculated.

The 8-OHdG formation in liver specimens was measured by a previously described method (Nakae et al., 1995) in macroscopically normal tissue. The number of samples/group assayed was 5. 8-OHdG levels were determined using the calibration from HPLC runs of standard samples containing known amounts of authentic 8-OHdG and dG, and expressed as the number of 8-OHdG per 10^5 dG.

The levels of MeIQx-DNA adducts in groups 3 and 4 were measured by the ^{32}P-postlabeling method under modified adduct intensification conditions using frozen samples, as previously reported (Totsuka et al., 1996). The number of samples/group assayed was 5. Data are mean ± SD values for 3 samples per group and 3 independent experiments.

Data Evaluation

Statistical analysis of the incidence of histopathological lesions was performed with the Fisher's exact probability test (program by Shionogi & Co, Ltd, Osaka, Japan). The rest of the data were evaluated with the Dunnett's *t* test (program by Shionogi Co, Ltd).

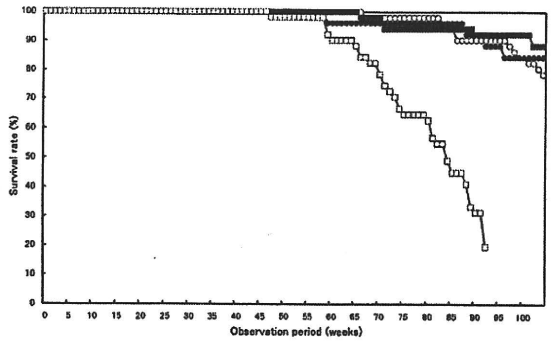

FIGURE 1.—Survival curves of F344/DuCrj rats. (|*ccircle*|), control (group 1); (|*wcirc*|), 0.001 ppm (group 2); (|*wtriup*|), 1 ppm (group 3); and (|*opensq*|), 100 ppm (group 4).

RESULTS

General Observations

Survival curves and rates are presented in Figure 1 and Table 1. Survival in the high-dose group (group 4) deteriorated from week 59 due to tumor development, and the survival rate was only 27% at week 92. Therefore, all survivors in this group were killed at this time point. Mean survival periods for control (group 1), low (group 2), and middle (group 3) dose groups did not differ. Data for rat body weights, liver weights, and intakes of food and MeIQx are summarized in Table 2. Mean body weights of the high-dose group at week 92 were approximately 21% lower than that of the control group (Table 2). At the scheduled sacrifice, mean body weights of rats in the other 3 groups (groups 1–3) did not differ statistically. Relative liver weights in the middle- and high-dose groups were significantly higher than in the control. The first unscheduled necropsy was performed with a rat of group 4 at week 59, and a Zymbal's gland tumor was found. All remaining F344 rats that survived beyond the 59-week time point were included as the effective numbers. MeIQx intake increased in line with the dose.

Development of GST-P-Positive Foci, AHF, and Liver Tumors

Table 3 summarizes data for GST-P-positive foci and the incidences of liver tumors. The numbers of GST-P-positive foci per unit liver area in group 4 was significantly higher than that of groups 1 to 3. In group 4, larger GST-P-positive foci could be easily recognized macroscopically. Furthermore, hepatocellular carcinomas and adenomas were observed in rats given 100 ppm (group 4). The incidence of AHF in group 4 was significantly different from that of groups 1 to 3, independent of whether eosinophilic, clear, vacuolated, or mixed cell foci were counted (Table 4). Namely, the incidences of eosinophilic and vacuolated foci significantly increased, and the incidences of clear and mixed foci decreased.

TABLE 1.—Data for survival of animals treated with MeIQx for 104 weeks.

Group	Dose (ppm)	Initial number of animals	Survivors (%)[a] Week 66	Week 92[b]	Week 104	Mean survival period (weeks)
1	0	51	51 (100)	46 (90)	41 (80)	101 ± 8
2	0.001	51	50 (98)	46 (90)	44 (86)	100 ± 10
3	1	51	51 (100)	47 (92)	45 (88)	101 ± 9
4	100	51	45 (88)	14 (27)[c]	0	80 ± 13

[a]Survivors (%): Number of rats that survived at the end / Number of rats at the start of experiment.
[b]All survivors in group 4 were killed at experimental week 92.
[c]Significantly different at $P < 0.01$ (vs. group 1, Fisher's exact probability test).

Rat Liver 8-OHdG Levels

The values for 8-OHdG in the rat liver (mean ± SD) of groups 1, 2, 3, and 4 were 0.92 ± 0.14, 0.81 ± 0.18, 0.94 ± 0.30, and 0.78 ± 0.16, respectively. No significant differences in 8-OHdG formation were observed among the groups.

MeIQx Adduct Formation

The MeIQx-DNA adduct levels in the rat liver DNA (mean ± SD) of groups 3 and 4 were $10.8 \times 10^{-7} \pm 0.38 \times 10^{-7}$ and $37.9 \times 10^{-7} \pm 31.1 \times 10^{-7}$, respectively, with no significant differences between the 2 groups.

Results of Histopathological Examination in Other Organs

Nodules/masses considered to be related to the influence of MeIQx were noted in the Zymbal's glands of 0, 1, 1, and 15 rats of groups 1–4, respectively, and in the subcutis of 10, 10, 9, and 30 rats. In group 4, most nodules developed on the dorsal thoracic and abdominal skin. The Zymbal's gland masses were carcinomas, whereas the majority of masses in the subcutis were mammary fibroadenomas and fibromas. One colon mass observed in a group 4 rat was an adenoma. The other neoplastic lesion noted was leukemia, which was associated with splenomegaly and liver infiltration by malignant large granular lymphocytes in 3, 3, 5, and 8 rats of groups 1–4, respectively.

DISCUSSION

The lowest level, 0.001 ppm, of MeIQx was established as equivalent to the daily intake of this carcinogen by humans (0.2 to 2.6 μg/man/day) (IARC, 1992). This human dose level is 0.003 to 0.043 μg/kg/day if the body weight of man for calculation is considered to be 60 kg. In this study, the mean MeIQx intake in rats of the lowest dose group was 0.040 μg/kg/day. Since this MeIQx intake level was approximately equivalent to the maximum daily intake in humans, we consider that the lowest dose of 0.001 ppm is appropriate to estimate potential human risk of MeIQx.

The present study demonstrated that low doses of MeIQx lack evident hepatocarcinogenic activity in F344 rats, in clear

TABLE 2.—Data for average body weight, intakes of food, and MeIQx and organ weights in animals treated with MeIQx for 104 weeks.

Group	Dose (ppm)	Initial number of animals	Body weight (g)[a]			Food intake (g/kg/day)[a]	MeIQx intake (μg/kg/day)[a]	Relative liver weight (g/100g bw)[a]
			Initial	Week 92[b]	Final			
1	0	51	125 ± 6	470 ± 40	458 ± 39	39 ± 16	—	3.0 ± 0.3 [41[c]]
2	0.001	51	125 ± 5	467 ± 28	458 ± 31	38 ± 13	0.04 ± 0.01	3.0 ± 0.2 [44]
3	1	51	126 ± 5	461 ± 29	447 ± 40	38 ± 13	38.30 ± 13.38	3.2 ± 0.6[d] [45]
4	100	51	126 ± 5	373 ± 50[e]	—	40 ± 14	4015.39 ± 1391.07	4.2 ± 0.8[e] [14]

[a]Mean ± SD.
[b]All survivors in group 4 were killed at experimental week 92.
[c]Number of animals examined.
[d]Significantly different at $P < 0.05$ (vs. group 1, Dunnett's multiple comparison test).
[e]Significantly different at $P < 0.01$ (vs. group 1, Dunnett's multiple comparison test).

TABLE 3.—GST-P-positive foci and tumors in the liver of animals treated with MeIQx for 104 weeks.

Group	Dose (ppm)	Effective number of animals	Incidence of proliferative and neoplastic lesions			
			Liver			
			GST-P-positive foci (No./cm^2)[a]	Adenomas (%)	Hepatocellular carcinomas (%)	Zymbal's gland Carcinoma (%)
1	0	51	22.9 ± 4.68	0	0	0
2	0.001	51	22.2 ± 5.94	0	0	1 (2)
3	1	51	21.7 ± 5.62	0	0	1 (2)
4	100	51	85.7 ± 39.5[b]	14 (27)[c]	6 (12)[c]	15 (29)[c]

[a]Mean ± SD.
[b]Significantly different at $P < 0.05$ (vs. group 1, Dunnett's multiple comparison test).
[c]Significantly different at $P < 0.01$ (vs. groups 1 to 3, Fisher's exact probability test).

TABLE 4.—Incidence of rats with altered hepatocellular foci in 104 weeks treatment of MeIQx.

Group	Dose (ppm)	Number of animals examined[a]	Incidence (%) of rats with each type of focus					
			Any type	Basophilic	Eosinophilic	Clear	Vacuolated	Mixed
1	0	44	100	41	23	91	16	0
2	0.001	42	98	38	38	76	17	7
3	1	41	98	46	39	80	29	5
4	100	25	100	56	100[b]	12[b]	68[b]	12[c]

[a]The samples examined were the liver of rats that survived beyond 72 experimental weeks and were free of mononuclear cell leukemia or other extensive lesions that might influence the occurrence or appearance of altered hepatocellular foci.
[b]Significantly different at $P < 0.01$ (vs. groups 1 to 3, Fisher's exact probability test).
[c]Significantly different at $P < 0.05$ (vs. group 1, Fisher's exact probability test).

contrast to the high dose of 100 ppm. A previous report indicated that in case of exposure to genotoxic carcinogens at low doses, different no-effect levels may exist for different parameters relevant to carcinogenicity (Fukushima et al., 2002). Thus, while formation of MeIQx-DNA adducts was increased even at extremely low dose levels (<0.01 ppm), significant generation of 8-OHdG was only observed at 0.01 ppm and significant induction of GST-P-positive foci only at 100 ppm in the livers of rats given MeIQx for 16 weeks (Fukushima et al., 2002). Subsequently, Hoshi et al. showed the no-effect level for in vivo liver mutagenicity of MeIQx in the diet for 16 weeks to be 1 ppm in Big Blue rats (Hoshi et al., 2004). The dose causing in vivo genotoxicity was thus lower than the level for GST-P-positive preneoplastic lesion induction (100 ppm or perhaps 10 ppm) (Fukushima et al., 2002; Hoshi et al., 2004). Moreover, no initiation activity was noted at doses of 0.001–1 ppm when using an in vivo rat medium-term bioassay for detection of initiating activity featuring application of phenobarbital, a well-known hepatopromoter (Fukushima et al., 2003). Therefore, we proposed that no-effect levels should exist for cancer induction by MeIQx (Fukushima et al., 2002). The present study showed no induction of any preneoplastic or neoplastic

lesions in any organs including liver of rats treated with MeIQx at 0.001 and 1 ppm.

The present value of number and area GST-P-positive foci of the control group was relatively high and close to the data observed after exposure to MeIQx at 100 ppm after 16 weeks exposure (Fukushima et al., 2002). The incidence of GST-P-positive foci in untreated rats tends to increase depending on observation period (week of age) (Fukushima et al., 2002). Similarly, it was reported that AHF increased with age in untreated aging rats of the F344 strain (Harada et al., 1989). In this study, the number of GST-P foci and the occurrence of eosionophilic type of AHF in the 100 ppm group were significantly higher than those in other groups, in line with the tumor induction. Indeed, it was reported that GST-P-positive foci are available for identification of eosinophilic and clear cell foci in the literature (Narama et al., 2003). Stepwise accumulation of alterations in cancer-related genes leading to malignant neoplasia is considered responsible for carcinogenesis. While it is considered that DNA adduct formation is a good marker for exposure to several carcinogens (Kang et al., 2006), in the present experiment, the results did not show significant difference between 1 and 100 ppm, although the value for MeIQx adducts with 100 ppm had a tendency for increase as compared to the 1 ppm level. The reason why DNA adduct levels were not significantly elevated might be the small number of samples (3 samples/group) examined, because a wide standard deviation, which indicates a higher degree of variability, was observed in the 100 ppm dose group. In the 16-week study, MeIQx-DNA adduct formation level in the livers of rats increased with linear relationship among the various doses (0.01 to 100 ppm) of MeIQx (Fukushima et al., 2002). Furthermore, in the short-term experiment, the difference between 1 and 100 ppm was about 100 times and the data of 100 ppm in the 16-week study did not differ greatly from that of the present experiment. In our study, 4 times higher mean value for DNA adducts was observed in the 100 ppm group as compared to the 1 ppm group. This small difference might imply that the data of 100 ppm indicates the saturation of exposure and the accumulation of adduct formation in the lower dose.

The dose threshold for induction of 8-OHdG in the rat liver DNA was earlier found to become lower in accordance with the length of treatment (Fukushima et al., 2002), so with prolonged exposure to MeIQx, no-effect levels for various parameters relevant to carcinogenicity might be lowered. Unexpectedly, the mean 8-OHdG formation values in all groups were slightly elevated as compared with the level of the untreated group in the 16-week experiment and did not differ among groups. Moreover, in animals of the 100 ppm group, the existence of liver tumors was not dependent on the level of 8-OHdG. However, the 8-OHdG level was reported not to be permanently elevated during the continuous treatment with carcinogens (Kinoshita et al., 2002). Since DNA repair is reported to be enhanced as a cellular response to chronic oxidative stress (Grishko et al., 2005), adaptation during long exposure to MeIQx might have occurred. Moreover, as indicated in the previous 28-week MeIQx treatment study, older animals might show higher background levels of 8-OHdG and low sensitivity to MeIQx (Kang et al., 2006).

Previously, strain differences were observed in susceptibility to hepatocarcinogens and the WS/Shi strain of rats was found the most susceptible to their exposure (Murai et al., 2000). In the MeIQx carcinogenicity test using WS/Shi rats, earlier occurrence and higher incidence of liver tumors was observed in the 100 ppm group (data not shown). Furthermore, gender differences in susceptibility to liver carcinogens may also be an important factor in assessing hepatocarcinogenicity. For instance, female rats are less susceptible to hepatocarcinogenicity of MeIQx than males (Kato et al., 1988), so that it is unlikely that MeIQx may induce liver neoplastic lesions with significant incidence at doses less than or equal to 1 ppm in carcinogenicity tests in females. Moreover, incidences of other tumors, including Zymbal's gland, clitoral gland, and skin lesions, observed in female rats are lower than the incidence of liver tumor in males (Kato et al., 1988).

In the present study, MeIQx at 100 ppm induced various nodules/masses of Zymbal's gland, subcutis, and colon, as previously reported (Sugimura et al., 2004; IARC, 1992). However, no significant induction of those tumor types was evident in the lower dose groups of MeIQx. Certainly, the evaluation of Zymbal's gland tumor induction is important for the confirmation of the threshold for MeIQx carcinogenicity, since this tumor was observed at low dose, but not in the control group. However, the incidence in the lower dose groups was within the NTP historical control range (0 to 4%) (National Toxicology Program, 1992). Under our experimental conditions, it appears that the Zymbal's gland is the most sensitive organ regarding MeIQx carcinogenesis except for liver; nevertheless, in a previous report using 400 ppm of MeIQx, the incidence of Zymbal's gland tumors was 75%, whereas the incidence of liver tumors reached 100% (Kato et al., 1988). Therefore, from our results, it might be considered that the sensitivity of liver and Zymbal's gland for MeIQx carcinogenesis is approximately equivalent. It was also found in another 2-year MeIQx carcinogenicity test using the WS/Shi strain that Zymbal's gland tumors were developed in the control group and the incidence was not significantly increased in lower dose groups (data not shown).

In conclusion, the present study supports our argument that a threshold, at least a practical threshold, exists for carcinogenicity of the genotoxic carcinogen MeIQx. This is of direct significance to cancer risk assessment in humans. However, further studies are necessary to establish the general concept of a threshold for carcinogenicity by genotoxic chemicals, since other genotoxic carcinogens including 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine, diethylnitrosamine, and dimethylnitrosamine have also shown no-effect levels in medium term carcinogenicity tests (Fukushima et al., 2002, 2004, 2005).

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Detection of endogenous DNA adducts, O^6-carboxymethyl-2'-deoxyguanosine and 3-ethanesulfonic acid-2'-deoxycytidine, in the rat stomach after duodenal reflux

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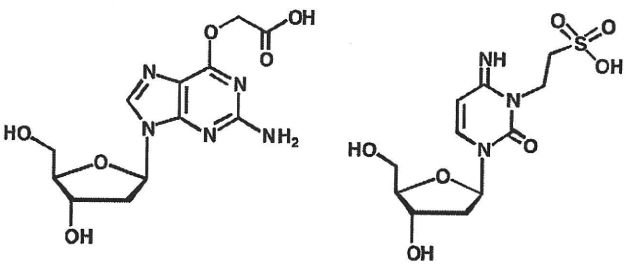
The endogenous DNA adducts O^6-carboxymethyl-deoxyguanosine (O^6-CM-dG) and 3-ethanesulfonic acid-deoxycytidine (3-ESA-dC) are produced from *N*-nitroso bile acid conjugates, such as *N*-nitrosoglycocholic acid (NO-GCA) and *N*-nitrosotaurocholic acid (NO-TCA), respectively. Formation of these DNA adducts *in vivo* was here analyzed by ^{32}P-postlabeling in the glandular stomach of rats subjected to duodenal content reflux surgery. In this model, all duodenal contents, including bile acid conjugates, flow back from the jejunum into the gastric corpus. The levels of O^6-CM-dG found at 4 and 8 weeks after surgery were 40.9 ± 9.4 and 56.3 ± 3.2 per 10^8 nucleotides, respectively, whereas the sham operation groups had values of 5.8 ± 2.3 and 5.9 ± 0.5 per 10^8 nucleotides. Moreover, adduct spots corresponding to 3-ESA-dC were detected in both duodenal reflux and sham operation groups and levels in the duodenal reflux groups were around four-fold elevated at 11.2 ± 1.0 and 8.9 ± 1.0 per 10^8 nucleotides after 4 and 8 weeks, respectively. When the duodenal reflux animals were treated with a nitrite trapping agent, thiazolidine-4-carboxylic acid (thioproline, TPRO), the levels of O^6-CM-dG and 3-ESA-dC were reduced to the same levels as in the sham operation animals. These observations suggest that NO-TCA and NO-GCA are formed by nitrosation of glycocholic acid and taurocholic acid, respectively, and these nitroso compounds produce DNA adducts in the glandular stomach of rats subjected to duodenal content reflux surgery. (*Cancer Sci* 2008; 99: 1741–1746)

Many reports have indicated associations between bile acids and human cancer development. Patients undergoing distal gastrectomy have been reported to be at increased risk of gastric carcinoma,[1,2] especially after Billroth II resection resulting in duodenogastric reflux.[1,3,4] Because intragastric formation of nitrosamides could be mediated by acid-catalyzed reaction of amides with nitrite, *N*-nitroso compounds have been suggested as plausible etiological factors in the development of gastric cancer in humans.[5,6] Nitrosation may also be mediated by activated macrophages in infected and inflamed organs.[7,8] In addition, Barrett's esophagus, a major risk factor for esophageal adenocarcinoma, could be associated with prolonged reflux of stomach and duodenal contents.[9] Refluxed bile acids appear to play a significant role in cancer development under these conditions.[10–13] In rats subjected to surgery in order to induce duodenal reflux into the stomach or the esophagus, the resultant chronic exposure to a mixture of duodenal and gastric juice for 50 weeks was found to induce adenocarcinomas at incidences of 31–41% in the stomach and 39% in the esophagus.[14–16] Furthermore, thiazolidine-4-carboxylic acid (thioproline, TPRO), an effective nitrite-trapping agent, inhibited the development of gastric and esophageal adenocarcinoma induced by gastro-duodenal reflux.[16,17] Therefore, it is likely that nitrosated bile acid conjugates could contribute to cancer development as endogenous mutagens/carcinogens.

N-Nitroso bile acid conjugates, such as *N*-nitrosoglycocholic acid (NO-GCA) and *N*-nitrosotaurocholic acid (NO-TCA), have already been demonstrated to exert mutagenic activity.[18,19] Moreover, these compounds also can induce liver and stomach cancers in F344 rats.[20] It is also reported that O^6-carboxymethyl-deoxyguanosine (O^6-CM-dG) and 3-ethanesulfonic acid-2'-deoxycytidine (3-ESA-dC) are the major products when NO-GCA or NO-TCA are incubated with calf thymus DNA *in vitro*[21,22] (Fig. 1). As mentioned above, nitrosated bile acid conjugates might contribute to some types of human cancer. However, the roles of *N*-nitroso bile acid conjugates have not been fully elucidated. Moreover, little is known about the formation of major DNA adducts derived from *N*-nitroso bile acid conjugates in different organs.

In the present study, we therefore investigated the generation of O^6-CM-dG and 3-ESA-dC in the glandular stomach of rats subjected to duodenogastric reflux surgery. Moreover, the effects of TPRO in the same animal model were also examined. The biological significance of nitrosated bile acid conjugates for gastric cancer development is discussed in the light of our observations.



O^6-Carboxymethyl-2'-deoxyguanosine (O^6-CM-dG)

3-Ethanesulfonic acid 2'-deoxycytidine (3-ESA-dC)

Fig. 1. Chemical structures of O^6-CM-dG and 3-ESA-dC.

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Materials and Methods

Materials. *N*-Nitroso bile acid conjugates (NO-GCA and NO-TCA) were obtained from the NARD Institute (Osaka, Japan) and 2'-deoxycytidine 3'-monophosphate (dCp), 2'-deoxyguanosine 3'-monophosphate (dGp), 2'-deoxycytidine (dC), and 2'-deoxyguanosine (dG) were purchased from Sigma Chemicals (St Louis, MO, USA). Micrococcal nuclease and phosphodiesterase II (bovine spleen) were from Worthington Biochemicals (Freehold, NJ, USA), and [γ-^{32}P]ATP, T4-polynucleotidekinase and nuclease P1 were obtained from ICN Biochemicals (Irvine, CA, USA), Nippon Gene (Toyama, Japan), and Yamasa Shoyu (Choshi, Japan), respectively. Polyethyleneimine (PEI)–cellulose TLC sheets (Polygram CEL 300 PEI) were from Macherey-Nagel (Duren, Germany). All other chemicals used were of analytical grade.

Preparation of authentic O^6-CM-dG-3'-monophosphate and 3-ESA-dC-3'-monophosphate. O^6-CM-dG-3'-monophosphate (O^6-CM-dGp) and 3-ESA-dC-3'-monophosphate (3-ESA-dCp) were prepared by the same procedures as described previously.[22–24] The chemical structures of these authentic compounds were confirmed by UV and LC-ESI/MS analyses (data not shown).

Preparation of O^6-CM-dG-3',5'-diphosphate (O^6-CM-pdGp) and 3-ESA-dC-3',5'-diphosphate (3-ESA-pdCp) as standard compounds. Phosphorylation of the 5'-terminal of the hydroxyl group of O^6-CM-dGp and 3-ESA-dCp was conducted by enzymatic reaction with T4-polynucleotide kinase under the same conditions as for ^{32}P-postlabeling methods.[22] Briefly, 20 μg aliquots of O^6-CM-dGp or 3-ESA-dCp were incubated with 3 μM ATP and T4-polynucleotide kinase (10 units) in 30 mM Tris-HCl buffer containing 10 mM DTT, 10 mM $MgCl_2$, and 1 mM spermidine at 37°C for 1 h. The mixtures were injected into analytical grade Amide-80 column (5 μm, 4.6 × 250 mm; Tosoh, Japan) with a gradient system of acetonitrile (from 75 to 63%) in 10 mM sodium phosphate buffer (pH 3.0) over 20 min. O^6-CM-pdGp and 3-ESA-pdCp were eluted at retention times of 16.2 min and 19 min, respectively, at a flow rate of 1 mL/min. To confirm the adduct structures, the peak fractions containing O^6-CM-3',5'-pdGp or 3-ESA-3',5'-pdCp were dephosphorylated with 0.2 unit of alkaline phosphatase in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM $MgCl_2$, as described previously.[25] O^6-CM-dG or 3-ESA-dC were clearly detected by LC-ESI/MS in both peak fractions. Therefore, these compounds were concluded to be O^6-CM-3', 5'-pdGp, and 3-ESA-3', 5'-pdCp.

Rat duodenogastric reflux model. The duodenogastric reflux model was prepared by a surgical procedure using male Wister rats as described previously.[14,17] For the sham operation group, animals underwent laparotomy and a gastrotomy incision.[14] Animals were allowed access to water 12 h postoperatively and to food 36 h later. No treatment with any known carcinogens was given. Postoperatively, surviving animals of the reflux group were divided into two groups: one given a normal diet (CRF-1) and the other given a diet containing 0.5% TPRO. The experiments were conducted according to the Guidelines for Animal Experimentation of Shiga University of Medical Science.

Analysis of endogenous DNA adducts in the glandular stomachs of rats subjected to duodenogastric reflux. Animals were sacrificed using diethyl ether at 4 ($n = 3$) and 8 ($n = 3$) weeks after reflux or sham surgery, and the glandular stomachs were excised. DNA was then prepared by a standard procedure involving enzymatic digestion of protein and RNA followed by extraction with phenol and chloroform/isoamyl alcohol (24:1, v/v), and stored at −20°C until adduct formation analysis. The DNA was digested with micrococcal nuclease and phosphodiesterase II, and then 50 μg of the enzymatic digests was injected into an analytical grade TSKgel ODS-80Ts column (5 μm particle size, 4.6 mm × 250 mm; Tosoh, Japan) for fractionation of O^6-CM-dGp and 3-ESA-dCp. The applied material was eluted at a flow rate of 1 mL/min with a linear gradient of methanol (from 2 to 20%) in 10 mM ammonium acetate over 20 min. O^6-CM-dGp and 3-ESA-dCp were eluted at retention times of 14–16 min and 3–5 min, respectively. After lyophilization of the fractionated samples, residues were dissolved in 5 μL of distilled water. The fractions were then analyzed by the ^{32}P-postlabeling method under adduct intensification conditions for O^6-CM-dGp, and nuclease P1 enrichment conditions for 3-ESA-dCp, as reported previously.[22,26]

Subsequently, the ^{32}P-postlabeled aliquots were applied to PEI-cellulose TLC sheets and developed with 0.1 M lithium chloride, 3 M urea, and 3 M acetic acid in water, as reported previously.[22] After [^{32}P]phosphate and [γ-^{32}P]ATP were removed from the ^{32}P-labeled samples by TLC, the spots corresponding to O^6-CM-dG-3', 5'-diphosphate (O^6-CM-pdGp) and 3-ESA-dC-3', 5'-diphosphate (3-ESA-pdCp) were cut out to remove the background radioactivity found at the same positions with authentic O^6-CM-pdGp or 3-ESA-pdCp, contact-transferred to other PEI-cellulose sheets, and then developed with 0.23 M sodium phosphate buffer (pH 6.0). To confirm correspondence with O^6-CM-pdGp and 3-ESA-pdCp, adduct spots on the TLC sheets were cut out again and extracted twice with 750 μL of 4 M pyridinium formate (pH 4.5) solution with shaking for 45 min. The extracts were centrifuged at 9 000*g* for 20 min, then the supernatants were passed through a 0.2-μm filter (Nihon Millipore Kogyo, Yonezawa, Japan), and evaporated. The residues were dissolved in an appropriate volume of distilled water, and then added to a standard O^6-CM-pdGp or 3-ESA-pdCp sample. The mixtures were injected into an analytical grade Amide-80 column and separated with the same solvent systems described above. Each fraction collected at 1-min intervals was evaporated, resolved into water, and then these fractions were applied on TLC and radioactivity was measured with a Bio-Image Analyzer (BAS-2000; Fuji Photo Film, Tokyo, Japan). Relative adduct labeling (RAL) was determined by the method of Gupta *et al.*[27] and Reddy *et al.*[28] and the values were calculated as averages of results from the three assays.

Results

Efficiency of the method for detection of O^6-CM-pdGp and 3-ESA-pdCp. To assess the capacity for detecting low levels of endogenous DNA adducts formed *in vivo*, first we tested the method's sensitivity. Authentic O^6-CM-dGp or 3-ESA-dCp were diluted with 3'-dGp or 3'-dCp to prepare various levels of standard samples, and these were subjected to HPLC. The eluates were collected at retention times of 14–16 min for O^6-CM-dGp and 3–5 min for 3-ESA-dCp, and lyophilized and analyzed by the ^{32}P-postlabeling method. After development with 0.1 M lithium chloride, 3 M urea, and 3 M acetic acid in water, adduct spots showing Rf values of 0.44 (O^6-CM-pdGp) and 0.28 (3-ESA-pdCp) were cut out (Fig. 2), contact-transferred to another PEI-cellulose sheet, and then developed with 0.23 M sodium phosphate buffer. Adduct spots for O^6-CM-pdGp and 3-ESA-pdCp on the TLC sheet (Fig. 3), were cut out again and extracted with 4 M pyridinium formate. Then, the extracts were subjected to HPLC together with standard O^6-CM-pdGp or 3-ESA-pdCp synthesized enzymatically and fractions collected at 1-min intervals were applied for TLC. As shown in Figs 4 and 5, the elution positions of these major radioactivities coincided with those of the UV absorption peaks of standard O^6-CM-pdGp and 3-ESA-pdCp, respectively. Using this method, the detection limit for O^6-CM-pdGp and 3-ESA-pdCp was found to be 1 adduct per 10^9 nucleotides.

Analysis of O^6-CM-pdGp and 3-ESA-pdCp in the glandular stomach of rats receiving duodenogastric reflux or sham surgery. Fifty μg aliquots of DNA digests derived from glandular stomachs of rats with duodenogastric or sham surgery were subjected to HPLC. Fractions corresponding to O^6-CM-dGp and 3-ESA-dCp were

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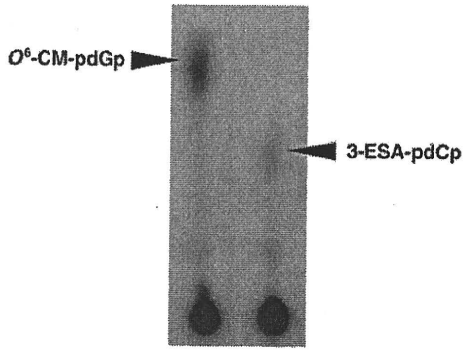

Fig. 2. Autoradiogram of O^6-CM-pdGp and 3-ESA-pdCp after the first development. Authentic O^6-CM-dGp and 3-ESA-dCp were analyzed by ^{32}P-postlabeling methods and developed with 0.1 M lithium chloride, 3 M urea, and 3 M acetic acid in water. The adduct levels were 1×10^{-8} for O^6-CM-dGp and 1×10^{-9} for 3-ESA-dCp.

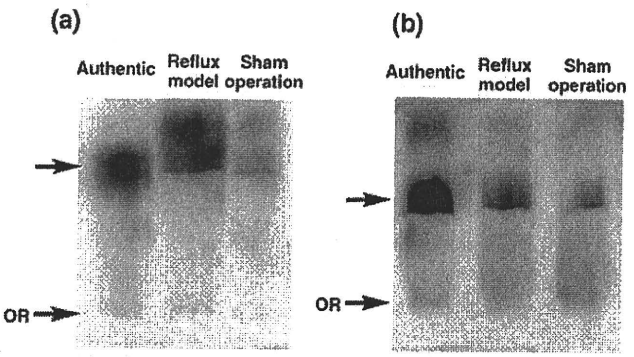

Fig. 3. Autoradiograms of O^6-CM-pdGp (a) and 3-ESA-pdCp (b) after the second development. Adduct spots showing the same Rf values of O^6-CM-pdGp or 3-ESA-pdCp on the first development were cut out, contact-transferred to another PEI-cellulose sheet, and then developed with 0.23 M sodium phosphate buffer. Authentic O^6-CM-pdGp or 3-ESA-pdCp are indicated by arrows. OR indicates the origin of sample application.

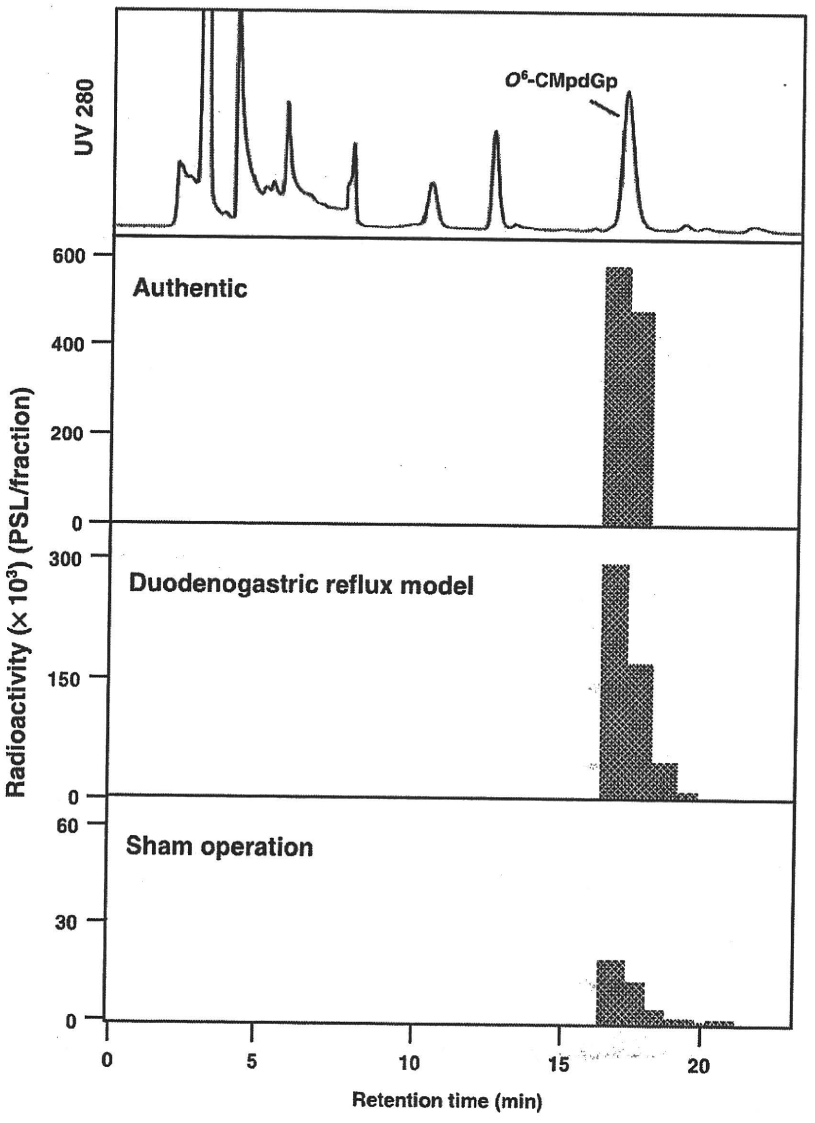

Fig. 4. HPLC chromatograms of extracts of spots corresponding to O^6-CM-pdGp. Samples derived from the authentic O^6-CM-pdGp and glandular stomachs of rats undergoing duodenogastric reflux or sham operations were applied to HPLC on an analytical grade Amide-80 column with a linear gradient of acetonitrile/10 mM sodium phosphate buffer (pH 3.0), as described in 'Materials and Methods', and the eluate was monitored by UV absorbance at 280 nm. The radioactivity of each fraction collected at 1-min intervals was measured by a Bio-Imaging Analyzer. Photo-stimulated luminescence (PSL) is the unit of radioactivity for the Bio-Imaging Analyzer system.

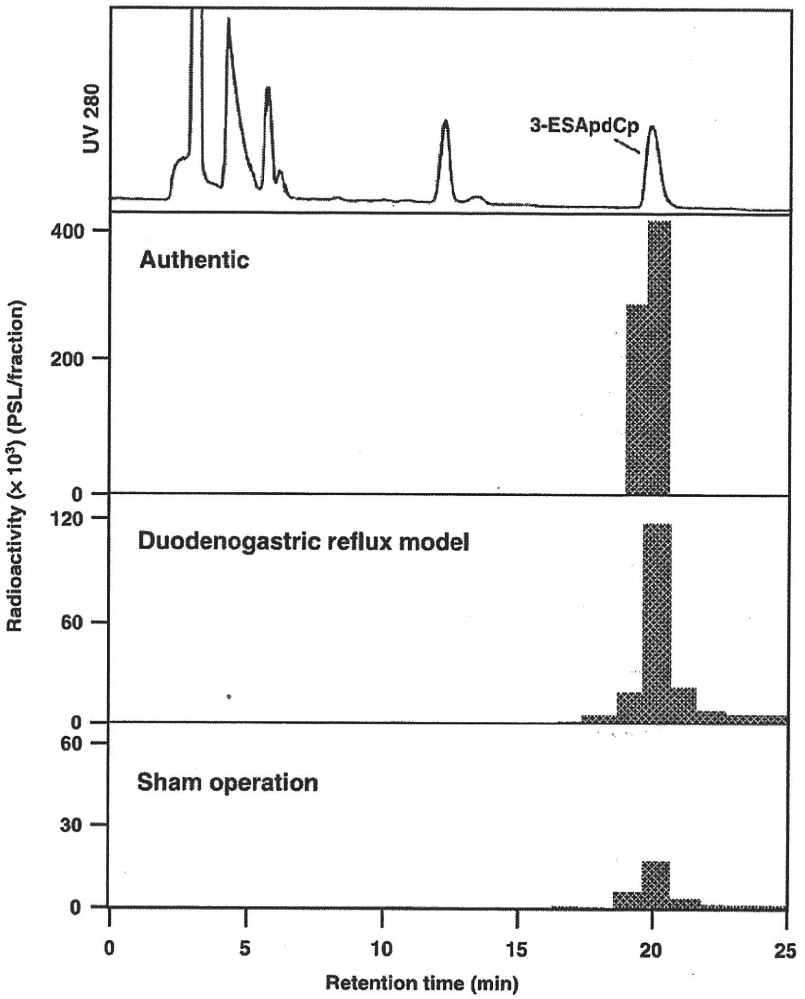

Fig. 5. Analyses of samples from authentic 3-ESA-pdCp and glandular stomachs of rats undergoing duodenogastric reflux or sham operations. The samples were subjected to HPLC with standard compound 3-ESA-pdCp, synthesized by enzymatic reactions. Conditions used for HPLC were the same as for Fig. 4.

collected, and then analyzed by the ^{32}P-postlabeling method as described above. At 4 weeks after surgery, two spots for O^6-CM-pdGp were clearly observed on TLC developed with 0.23 M sodium phosphate buffer, whereas only indistinct spots were observed with samples obtained after sham operations (Fig. 3a). The same TLC pattern was also observed with samples obtained with the reflux model at 8 weeks after surgery (data not shown). The lower spot detected in the rat reflux group was in the same position as authentic O^6-CM-pdGp on TLC. For further characterization, this adduct spot was extracted with pyridinium formate and subsequently subjected to HPLC together with standard O^6-CM-pdGp. As shown in Fig. 4, the elution position of the major radioactivity coincided with that of the UV absorption peak of standard O^6-CM-pdGp. The same spot was apparent after sham operations but the intensity of the radioactivity was 10–15 times lower than with reflux animals (Fig. 4).

A single spot corresponding to 3-ESA-pdCp was also detected in both reflux and sham operation animals; however, the level was estimated to be about four-fold elevated at 4 weeks after reflux surgery (Fig. 3b). Moreover, when extracts of the adduct spots were subjected to HPLC, radioactivity was mainly observed in a peak fraction coinciding to standard 3-ESA-pdCp (Fig. 5).

The levels of O^6-CM-pdGp found in the duodenal reflux group after 4 and 8 weeks surgery were 40.9 ± 9.4 and 56.3 ± 3.2 per 10^8 nucleotides, respectively, whereas the values were 5.8 ± 2.3 and 5.9 ± 0.5 per 10^8 nucleotides for the sham operation group, respectively. Similarly, the levels of 3-ESA-pdCp found in the duodenal reflux group after 4 and 8 weeks surgery were about four-times higher than those of sham operation group. Data for the adduct levels of O^6-CM-pdGp and 3-ESA-pdCp are summarized in Table 1.

Effects of TPRO treatment on formation of O^6-CM-dG and 3-ESA-dC. The development of gastric adenocarcinoma induced by duodenogastric reflux in rats is prevented by TPRO treatment.[16] Thus, formation of the endogenous DNA adducts related to duodenogastric reflux was expected to be inhibited by TPRO treatment. To confirm this, O^6-CM-pdGp and 3-ESA-pdCp were analyzed in DNA samples obtained from gastric reflux animals given a diet containing TPRO for 8 weeks. Values were estimated to be 3.3 ± 3.4 and 1.0 ± 0.3 per 10^8 nucleotides for O^6-CM-pdGp and 3-ESA-pdCp, respectively, in clear contrast to the 56.3 ± 3.2 and 8.9 ± 1.0 without TPRO treatment (Table 1). The adduct levels observed in rats with TPRO treatment were equivalent to 6% (O^6-CM-pdGp) and 11% (3-ESA-pdCp) of those without TPRO treatment.

Discussion

In the present study, endogenous formation of O^6-CM-dG and 3-ESA-dC DNA adducts could be clearly demonstrated in the glandular stomachs of rats receiving gastroduodenal reflux surgery, with much lower levels in sham operated animals.

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