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A trial to find appropriate animal models of dichloropropane-induced cholangiocarcinoma based on the hepatic distribution of glutathione S-transferases

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Abstract: A trial to find appropriate animal models of dichloropropane-induced cholangiocarcinoma based on the hepatic distribution of glutathione S-transferases: Lingyi ZHANG, *et al*. Department of Occupational and Environmental Health, Faculty of Pharmaceutical Sciences, Tokyo University of Science—Objectives: It has been reported that 1,2-Dichloropropane (DCP) induced cholangiocarcinoma (CCA) in offset color proof-printing workers. However, exposure to DCP by inhalation or gavage for 2 year did not induce CCA in mice and rats. The present study mapped the hepatic distribution of GST, which is known to activate dihalogenated alkanes, and proliferative and fibrotic changes in bile ducts in various species to find the most appropriate animal model of DCP-induced CCA. **Methods:** First, 12 each of C57BL/6J mice, Balb/cA mice, F344 rats, Syrian hamsters, and guinea pigs were divided into four equal groups and exposed to DCP at 0, 300, 1,000, or 3,000 ppm 8 hours/day for 7 days. Second, 32 Balb/cA mice and 32 Syrian hamsters were each divided into four equal groups and exposed to DCP at 0, 200, 400, and 800 ppm 6 hours/day for 14 days. After the last exposure, the animals were decapitated, and the livers were dissected out for histopathological evaluation. Immunostaining was conducted to determine the distribution of GSTT1, GSTM1, and GSTPi, as well as the expression of proliferation marker Ki67. **Results:** GSTT1, GSTM1, and GSTPi were expressed in both hepatocytes and bile duct cells in all control and exposed animals. There was no clear difference in the expression of Ki67 between the exposed groups and the control. No fibrotic changes were observed in any species or strains examined. **Conclusions:** Expression of GSTT1 or other GST isozymes might not explain the difference in sensitivity of hepatocytes and the bile duct to DCP between humans and rodents.
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Key words: 1,2-Dichloropropane, Animal model, Cholangiocarcinoma, Glutathione S-transferase

1,2-Dichloropropane (DCP) is an organic solvent used in industrial processes as a raw material to produce propylene, carbon tetrachloride, tetrachloroethylene, and other chemicals. It is also used in paint removers and varnishes and as a chemical intermediate. Several cases of cholangiocarcinoma were reported in March 2013 in offset color proof-printing workers at factories using DCP and/or dichloromethane[1]. A cleaning solvent containing DCP was the most suspected cause of these cases of cholangiocarcinoma, and DCP has been classified recently by the International Agency for Research on Cancer (IARC) Monograph Working Group as a compound carcinogenic to humans[2]. However, animals exposed to DCP did not develop cholangiocarcinoma in previous studies[3–5]. Exposure to DCP by gavage for 103 weeks showed increased rates of adenomas and carcinomas of the liver in both male and female mice and an increased rate of adenocarcinomas of the mammary gland in female rats[5]. Interestingly, rats exposed to

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Abbreviations: CCA: cholangiocarcinoma. DCP: 1,2-dichloropropane. GSH: glutathione. GST: glutathione S-transferase.

DCP did not show increased rates of liver or bile duct carcinomas[5]. 2-year inhalation study reported that exposure to DCP increased the incidence of bronchioloalveolar adenomas and carcinomas in female mice and marginally increased Harderian gland adenoma in male mice[3], and it was also reported that it increased papilloma in the nasal cavity in male and female rats[4]. Mechanistic studies showed that 1,2-dihalogenated alkanes, such as dibromoethane and dichloroethane, are conjugated with glutathione (GSH) by glutathione S-transferase in the liver, producing highly reactive episulfonium ion, which forms DNA adducts, resulting in DNA mutation[6]. Previous studies also showed that halogenated hydrocarbons are the substrate of glutathione S-transferase (GST) theta 1 (GSTT1)[7–9], and another study showed that the glutathione conjugating activity of GSTT1 toward dibromoethane was highest among GST isozymes in rats[10]. Several other studies showed that GSTT1 activity enhances the genotoxicity of dihaloalkanes[11–13]. Thus, we focused on GSTT1, as it contributes to activation of dihaloalkanes that lead to DNA damage and carcinogenicity. However, we also examined other isozymes of GST, as they also contribute to the conjugation of dihaloalkanes and their expression levels are relatively higher than that of GSTT1 in humans[10]. Given the structural similarity of DCP to dibromoethane and dichloroethane, it is possible that DCP is activated through the GSH pathway, forming a DNA adduct that ultimately induces DNA mutation.

The difference between previous animal experiments using mice and rats and the human cases is hypothesized to be due to species differences in the localization of glutathione S-transferase in the liver and bile duct. A study showed that the enzymatic activity of GSTT1 in catalyzing the conjugation of GST theta-specific substrates methyl chloride (MC), DCM, and 1,2-epoxy-3-(p-nitro-phenoxy)propane (EPNP) is higher in mice than the enzymatic activity of GSTT1 in humans[14]. Furthermore, GSTT1 has been reported to be overexpressed in the nuclei of mouse hepatocytes and is not expressed in the bile duct, while in humans, GSTT1 is expressed in the epithelial cells of the bile duct and some hepatocytes, in both the nucleus and cytoplasm[15]. Although DCP did not induce cholangiocarcinoma (CCA) after exposure of mice and rats for 2 years, it was thought to be the most probable cause of CCA in a case series in Japanese offset-color-proof printing workers[16]. These findings were attributed to the possible differences in GSTT1 distribution between rats/mice and humans as mentioned above. These studies suggest that the mouse and rat are probably not suitable models for study of the carcinogenicity of DCP in the human bile duct.

Although GSTT1 is mainly responsible for glutathione conjugation with haloalkanes such as dibromoethane, other isozymes of GST can also, at least in part, contribute to this reaction[10]. For this reason, the present study investigated not only GSTT1 but also other isozymes of GST. Human cases of DCP toxicity showed sclerosis of the bile duct with variable degrees of inflammatory cell proliferation, injury of the biliary epithelium, focal loss of the bile duct, and hyperplasia of the biliary epithelium at various sites of the bile ducts in noncancerous hepatic tissues[17]. Based on these histopathological effects, the study also investigated the extent of proliferative or fibrotic changes in the liver in a group of animals to find a suitable animal model of DCP toxicity. Five types of animals were investigated for the expression of GST isozymes and subacute toxicity in the liver, and bile duct after inhalation exposure to DCP. Based on the findings, we aimed to propose the most suitable animal model for DCP-induced CCA.

Methods

Animals and exposure to DCP

The study consisted of two parts. In the first series of experiments, 12 of each of C57BL/6J mice, Balb/cA mice, F344 rats, Syrian hamsters, and guinea pigs were each divided into four equal groups and exposed to DCP (≥90.0% GC, Fulka Analytical, Sigma-Aldrich Corporation, St. Louis, MO, USA) vapor at 0, 300, 1,000, or 3,000 ppm, using an inhalation system of 8 h/day from 10:00 a.m. to 6:00 p.m. for 7 days. In the second series of experiments, 32 each of Balb/cA mice and hamsters were divided into four equal groups and exposed to DCP at 0, 200, 400, and 800 ppm for 6 h/day from 10:00 a.m. to 4:00 p.m. for 14 days. Before exposure, the animals were acclimated to a room where the temperature and humidity were maintained at 23–25°C and 57–60%, respectively, and the light cycle was controlled with lights on at 9:00 a.m. and off at 9:00 p.m. The animals had free access to food and water. The inhalation exposure system was described in detail in a previous study (Ichihara *et al*. 2000). Briefly, a regulated volume of DCP was evaporated at room temperature and mixed with a larger volume of filtered fresh air to achieve the target concentrations. The vapor concentration of DCP in the chamber was measured every 10 sec by gas chromatography and electronically controlled to within ± 5% of the target dose. The mean concentration measured every 10 sec for 8 hours was considered the value for that day.

Dissection and tissue preparation

The animals were decapitated 15 to 18 hours after termination of the last exposure in the 7-day experiment and 17 to 20 hours after termination of the last

exposure in the 14-day experiment. Blood samples were collected using a heparinized funnel. The liver was dissected out carefully and fixed in 4% paraformaldehyde. Protection and control of animals during the entire experiment were in accordance with the Japanese Act on Welfare and Management of Animals and the Guide of Animal Experimentation of Nagoya University School of Medicine. The experimental protocol was approved by the Animal Care Ethics Committee of Nagoya University School of Medicine.

Histopathological examination

Liver paraffin sections (5 μm thick) were cut and mounted on slides. Histopathological changes were checked after hematoxylin-eosin (H&E) staining and immunostaining using various antibodies including anti-GSTM1 (Lifespan Biosciences, Seattle, WA, USA), anti-GST T1 (Proteintech Group, Chicago, IL, USA), anti-GST pi (Abcam, Cambridge, MA, USA) and anti-Ki67 (Spring Bioscience, Pleasanton, CA, USA).

Statistical analysis

Data were expressed as the mean ± SEM. One-way analysis of variance (ANOVA) was used for comparison of group data, followed by Dunnett's multiple comparison. The significance level was set at $p<0.05$. Data were analyzed using the JMP8 software.

Results

DCP exposure outcome

In the 7-day experiment, one rat in the 3,000 ppm group died at day 6 of exposure. All guinea pigs in the 3,000 ppm group died at day 5 of exposure. All hamsters in the 1,000 and 3,000 ppm groups died at day 2 of exposure. One of the Balb/cA mice in the control group, one of the Balb/cA mice in the 300 ppm group, and all the Balb/cA mice in the 1,000 and 3,000 ppm groups died at day 2 of exposure. One of the C57BL/6J mice in the 300 ppm group died at day 3, two of the C57BL/6J mice in the 1,000 ppm group died at day 2, the last C57BL/6J mouse in the 1,000 ppm group died at day 3, and all three of C57BL/6J mice in the 3,000 ppm group died at day 2.

In the 14-day experiment, all hamsters in the 800 ppm group died at day 2 of exposure. All mice exposed to 400 and 800 ppm DCP died after the first day of exposure. After 14 days of exposure, the body weights of the hamsters in the 200 and 400 ppm groups were lower than that of the control group. Also, the liver wet weights were lower in all hamster exposure groups compared with the control. The ratios of the liver weight to body weight of the hamsters in the 200 and 400 ppm groups were also lower than that of the control. No significant change in body or organ weight was seen in mice after two weeks of exposure.

Histopathological changes

In the 7-day experiment, the livers of rats exposed to DCP at 3,000 ppm were pale brown in color macroscopically, and H&E stained liver sections showed fat-like droplets under optical microscopy. Inflammatory cell infiltration was also observed both in the exposed groups and the control. No major changes were observed in the control, 300 ppm, and 1,000 ppm groups. Fat-like droplets were observed in the control, 300 ppm, and 1,000 ppm groups of guinea pigs, but no major differences were detected. No differences were observed in hamsters between the control and 300 ppm group. In both Balb/cA and C57BL/6J mice, vacuoles were found in hepatocytes in the 300 ppm group.

In the 14-day experiments, slight dilatation of hepatic sinusoids was noted in the 400 ppm group of hamsters, but no necrosis or fibrosis was detected. Furthermore, vacuoles were observed in hepatocytes in the 200 ppm group of mice.

Immunohistochemistry

Table 1 summarizes the results of immunochemistry from the 7-day experiment.

GSTM1: In the 7-day experiment, rats showed expression of GSTM1 in both hepatocytes and bile ducts. In guinea pigs, hamsters, and Balb/cA and C57BL/6J mice, GSTM1 antigen was expressed in hepatocytes and the bile duct, but exposure to DCP did not induce an obvious change in immunostaining pattern. In the 14-day experiment, hamsters expressed GSTM1 in both hepatocytes and the bile duct. There was a slight increase in GSTM1 expression level after 14-day exposure to DCP. However, there was no significant change in GSTM1 expression level in Balb/cA mice.

GSTT1: In the 7-day experiment, rats and Balb/cA and C57BL/6J mice expressed GSTT1 in both hepatocytes and the bile duct. In guinea pigs, immunohistochemistry was negative for all antigens, probably because of lack of cross-reactivity of the used antibodies with GSTT1 of the guinea pig. In hamsters, GSTT1 was expressed in some hepatocytes and bile duct cells, although no apparent difference was identified between the control and exposure groups (Fig. 1). The 14-day experiment showed similar results in Balb/cA mice and hamsters.

GSTPi: In the 7-day experiment, all animals showed higher expression levels of GSTPi in bile ducts compared with hepatocytes, but there was no obvious difference between the control and exposure groups. Similar results were found in Balb/cA mice and

Table 1. Summary of immunochemistry staining of the livers of animals exposed to 1,2-dichloropropane for 7 days

		GSTM1			GSTT1			GSTPi			Ki67		
		HE	Liver cell			Liver cell							
		(fatty droplet)	Nucleus	Cytoplasm	Bile duct	Nucleus	Cytoplasm	Bile duct	Nucleus	Cytoplasm	Bile duct	Nucleus of liver cell	Bile duct
Rat	0	–	+	+	+	+–	+	++	+	+	+	+	+–
	300	–	+	+	+	+–	+	++	+	+	++	++	++
	1,000	+–	+	+	+	+–	+	++	+	+	++	+	++
	3,000	+	+–	+	+	+–	+–	++	+	++	++	–	++
Guinea pig	0	–	+	+	++	–	–	–	+	+	++		
	300	–	+–	++	++	–	–	–	+–	+	++		
	1,000	+–	+	++	++	–	–	–	+	++	++		
Hamster	0	–	+	+	+	+–	+	+	+	+	++	+–	–
	300	–	+	+–	++	+–	+–	+	+	+	++	+–	–
Mice (B6)	0	–	+–	+–	++	+–	+–	+	++	+	++	+	+
	300	+	+–	+	+–	+	+	++	++	++	++	+	+
Mice (Balb)	0	–	+–	+	++	+	+	++	++	++	++	+	+
	300	+	+–	+	++	++	+	+	++	+	++	++	++

Symbols: –, no expression; +–, positive expression in some cells but not all; +, positive expression in all the target cells; ++, strong expression.

hamsters in the 14-day experiment.

Ki67: In the 7-day experiment, apart from the guinea pig whose Ki67 was not recognized by the antibody, Ki67-positive cells were detected in both hepatocytes and bile ducts of rats, hamsters, and Balb/cA and C57BL/6J mice. However, there were no obvious differences between the exposed groups and the control. Similar results were found in the 14-day experiment.

Discussion

A previous study showed different distributions of GSTT1 (which is thought to play an important role in metabolism of haloalkane) in humans and rodents; GSTT1 was expressed only in mouse hepatocytes, but was expressed in both hepatocytes and bile duct cells in humans[15]. This difference in GSTT1 expression seemed to explain the difference in the outcome resulting from exposure to DCP between human workers and animal experiments. However, the present study showed GSTT1 expression in bile duct cells in nonexposed rats and two strains of mice. This finding is in accordance with the results of another recent study that investigated the expression of GSTT1 in nonexposed rats and mice[18]. This difference between the study of Sherratt *et al*. and two recent studies, including the present study, might have been caused by the difference in strains or antibodies used. Sherratt *et al*. used B6C3F1 mice[15], while we used C57BL/6J and Balb/cA mice and Sato *et al*. used ICR mice[18]. Also in the study of Sherratt *et al*.[15], the authors made the GSTT1 antibody themselves, but our study and that of Sato *et al*. used a commercially available antibody provided by the same supplier[18].

The present study investigated the expression of not only GSTT1 but also other GST isozymes in DCP-exposed rats, mice, hamsters, and guinea pigs, and identified the expression of GSTs in both hepatocytes and bile ducts. Furthermore, the study also showed that exposure to DCP did not change the distribution of any GST isozymes in rats, but rather tended to increase the expression of GSTT1 in the bile duct in one strain of mice. It is difficult to explain how the previous experimental studies in mice or rats failed to show cholangiocarcinoma based on the distribution of GSTT1 or other isozymes of GST.

The mutagenicity of dihaloalkanes (e.g., dibromoethane and dichloroethane) is attributed to the episulfonium ion formed by reaction with GSH[6]. Although one can hypothesize that DCP could also be activated by GSTT1 in a manner similar to dibromoethane and dichloroethane, a study on the metabolism of DCP in F344 rats showed no episulfonium ion formation in rats administered DCP[19]. Thus, factors other than episulfonium ion formation should be considered in the mechanism of DCP-induced liver damage and cholangiocarcinoma. In this regard, a previous study showed that DCP-induced GSH depletion was

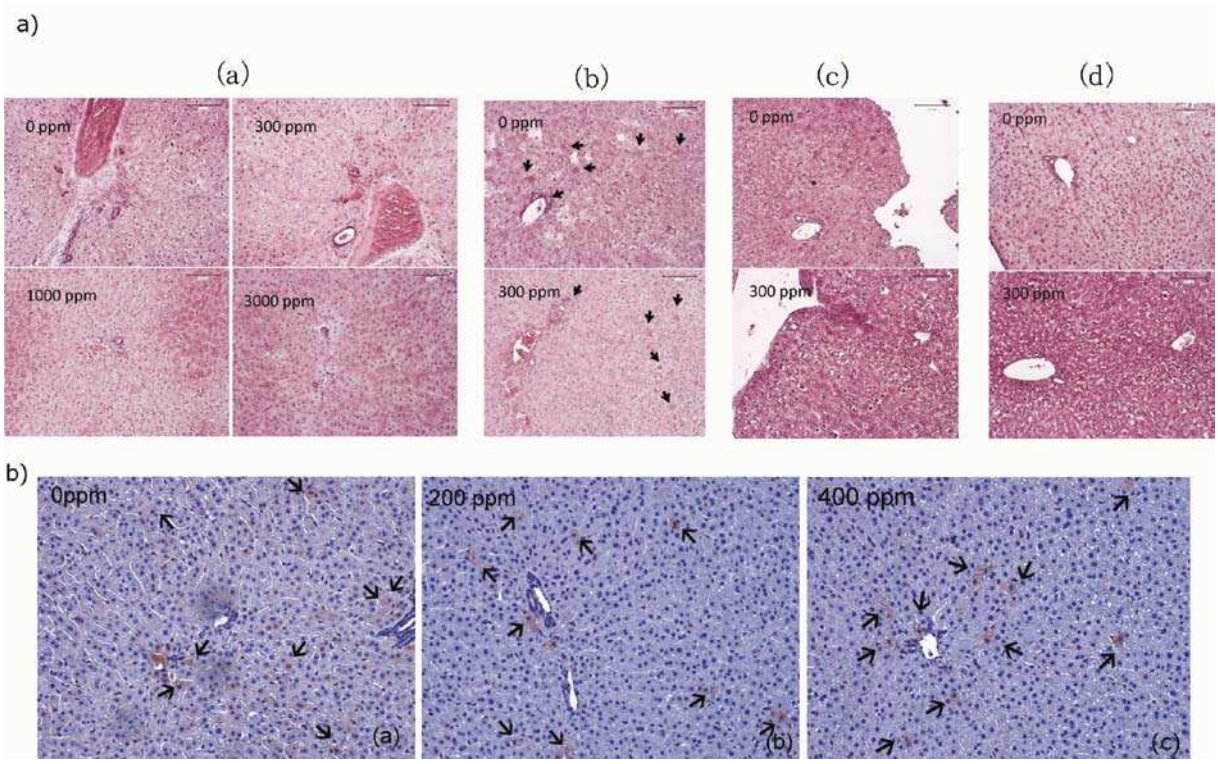

Fig. 1. a) Anti-GSTT1 immunostaining of paraffin-embedded liver sections from animals exposed to DCP for 7 days. (a) F344 rats. (b) Golden hamster. Arrows: GSTT1-positive cells in the Syrian hamster liver showing a random distribution. (c) C57BL/6J mice. (d) Balb/cA mice. Magnification: ×20, for all sections. b) Anti-GSTT1 immunostaining of paraffin-embedded liver sections from Syrian hamsters exposed to different concentrations of DCP for 14 days. (a) Liver section of a Syrian hamster from the control group, (b) liver section of a Syrian hamster exposed to 200 ppm DCP, (c) liver section of Syrian hamster exposed to 400 ppm DCP, Arrows: GSTT1-positive cells in the Syrian hamster liver showing a random distribution. Magnification: ×20, for all three sections.

prevented by CO, an inhibitor of P450 in renal cortical slices, and that loss of the anion gap induced by DCP was prevented by inhibitors of γ-glutamyl transferase and β-lyase. These results suggest that DCP nephrotoxicity is probably related to mercapturic acid metabolism after oxidation by P450[20]. Another more recent study also argued for the role of cytochrome P450 2E1 in the initiation of DCP-induced liver damage[21]. The present study showed that the distribution of GSTT1 in hamsters was similar to that in humans; that is, GSTT1 was expressed in both the nucleus and cytoplasm of bile duct epithelial cells and some hepatocytes, although our hamsters did not show any specific susceptibility to DCP exposure. It seems that the difference in the distribution of GSTT1 might not entirely explain how DCP exposure induces CCA in offset color proof-printing workers in Japan, and it did not entirely explain how DCP exposure fails to induce CCA in animal experiments.

The present study also investigated the effects of DCP on cell proliferation and fibrotic changes in the liver and bile ducts. The results showed that DCP exposure did not induce cell proliferation or fibrosis. On the other hand, analysis of the survival rate showed that the order of species with respect to toleration of DCP exposure was rats > guinea pigs > hamsters > mice, but the mechanism explaining the difference in susceptibility between the species was not investigated in the present study.

The first report on cholangiocarcinoma among offset printing workers showed that DCP was the common exposure agent among all workers[1]. Furthermore, there were three other small printing plants with two cases of cholangiocarcinoma each, and all the six cases were exposed to DCP for a long term[22]. These findings suggest that DCP may contribute to development of cholangiocarcinoma in humans. However, there are no similar case reports in industries other than offset printing yet. Admittedly, data from only the offset printing factories do not categorically isolate DCP as the sole agent responsible. Other studies need to be conducted to investigate the possible involvement of other chemicals or factors in offset printing and to establish the relation between DCP

exposure and CCA, if any. In particular, experimental studies to understand the mechanism of DCP toxicity would provide a solid relationship between DCP exposure and the hepato- and cholangiotoxicity reported in epidemiological studies.

In conclusion, expression of GSTT1 or other GST isozymes might not explain the difference in sensitivity of hepatocytes and the bile duct to DCP between humans and rodents. The use of GSTT1 as a marker might be inappropriate for finding a suitable animal model. Other possible routes or factors that can explain the species differences with regard to the carcinogenic potential of DCP should be explored.

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Conflicts of interest: All authors declare that they have no conflicts of interest with regard to the present study.

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