Original Article

The threshold dose for liver tumor promoting effects of dicyclanil in ICR mice

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ABSTRACT — To determine the threshold dose of dicyclanil (DC) that induces hepatocellular tumor-promoting effects associated with reactive oxygen species (ROS) generation via their metabolic pathways, partial hepatectomized ICR male mice were fed diets containing 0, 187.5, 375 or 750 ppm DC after an intraperitoneal injection of N-diethylnitrosamine (DEN) to initiate hepatocarcinogenesis. Immunohistochemically, the proliferating cell nuclear antigen (PCNA)-positive cell ratio was significantly increased in the DEN + 750 ppm DC group compared with the DEN alone group. However, significant increases in the number of γ -glutamyltranspeptidase (GGT)-positive cells and formation of microsomal ROS were not observed in the DEN + DC groups compared with the DEN alone group. Real-time polymerase chain reaction (RT-PCR) showed that the expression of *Cyp1a1*, *Cyp1a2*, and *OGG1* genes was significantly up-regulated in mice given diets containing 375 ppm DC or more, 187.5 ppm DC or more, and 750 ppm DC, respectively. These results suggest that the threshold dose of DC that induces ROS-mediated liver tumor promotion in mice is more than 750 ppm, although expression of the *Cyp1a2* gene, which is related to ROS generation, was up-regulated in the liver of mice, even at a DC dose of 187.5 ppm.

Key words: Dicyclanil, ROS generation, Threshold dose

INTRODUCTION

It has been shown that oxidative stress is an important factor that affects the pathogenesis of degenerative and inflammatory diseases, aging, and cancer (Wiseman and Halliwell, 1996; Trush and Kensler, 1991). Indeed, reactive oxygen species (ROS) are believed to play a pivotal role in the etiology of liver cancer, and ROS overproduction and subsequent oxidative DNA damage enhance the development of hepatocellular carcinomas caused by carcinogenic agents, such as the hepatitis C virus (Moriya *et al.*, 2001), metal (Asare *et al.*, 2006), insecticides (Lin *et al.*, 2007; Schilezinger *et al.*, 1999), and synthetic derivatives of naturally occurring flavonoids (Dewa *et al.*, 2008). In addition, it has been reported that upregulation of c-Myc resulting from the excessive ROS production may play an

important role on cellular proliferation in the liver tumor promotion (Kawai *et al.*, 2009).

Dicyclanil (DC), 4,6-diamino-2-cyclopropylamino-pyrimidine-5-carbonitrile, is a pyrimidine-derived insect growth regulator that inhibits the molting and development of insects and is used in veterinary medicine to prevent myiasis (fly strike) in sheep. It has been reported that the incidence of hepatocellular carcinomas was increased in mice fed a diet containing 1,500 ppm of DC for 18 months, but negative results were obtained in *in vitro* and *in vivo* genotoxicity studies of DC (WHO, 2000). Based on these results, this chemical substance is classified as a nongenotoxic rodent carcinogen (WHO, 2000). In our previous study, to clarify the possible mechanism of DC-induced liver tumor promoting effects, we performed short- and long-term two-stage hepatocarcinogenesis

studies in which mice were given 1,500 ppm DC after initiating hepatocarcinogenesis with N-dimethylnitrosamine (DMN) (Moto *et al.*, 2005, 2006b). The results of these two-stage hepatocarcinogenesis studies showed up-regulation of cytochrome P450 1A1 and cytochrome P450 1A2 (*Cyp1a1* and *Cyp1a2*) and thioredoxin reductase 1 (*Txnrd1*), which are metabolism- and/or oxidative stress-related genes, 8-oxoguanine DNA glycosylase (*OGG1*) and excision repair cross-complementing rodent repair deficiency group 5 (*Ercc5*), which are DNA damage-/repair-related genes (Moto *et al.*, 2005). Moreover, there were significant increases in the number of altered foci that were positive for γ -glutamyltranspeptidase (GGT-positive foci) and in the liver DNA levels of 8-hydroxydeoxyguanosine (8-OHdG), a representative marker of oxidative DNA damage, in mice of the DMN + DC group. In addition, a dose-dependent increase in microsomal ROS production was observed in these mice (Moto *et al.*, 2005, 2006b). These results suggest that DC has the potential to generate ROS via metabolic pathways and induce oxidative stress, including oxidative DNA damage, resulting in the induction of hepatocellular tumors in mice. However, the threshold dose of the liver tumor-promoting effect of DC in mice has not yet been elucidated. Additionally, in our previous study, real-time RT-PCR analysis revealed increased expression of the *Cyp1a1* gene and of phase II enzyme genes such as *Txnrd1* and *OGG1* in mice administered DC after an intraperitoneal injection of DMN compared with the gene expression in the corresponding control group (Moto *et al.*, 2006b). *Cyp1a1* generates ROS as byproducts of microsomal oxidation, and phase II enzymes are involved in the protection against oxidative stress (Guengerich and Shimada, 1991; Puntarulo and Cederbaum, 1998). Thus ROS generation and protection against ROS by phase II enzymes probably occur concomitantly in mice administered DC after an intraperitoneal injection of DMN. Accordingly, it is considered that the dose increasing ROS generation, cell proliferative activity, GGT positive foci or cell and drug-metabolizing enzymes that protect against ROS is the threshold dose of liver tumor promotion in mice. However, the threshold dose of DC in mice is not known.

In our previous studies, the incidence of hepatocellular tumors (adenomas and carcinomas) significantly increased in partially hepatectomized male ICR mice that were intraperitoneally injected with N-diethylnitrosamine (DEN) and given a diet containing 1,500 ppm of DC for 20 weeks, and gene expression analysis on the micro-dissected liver tissues of the mice in this group showed the highest expression levels of oxidative stress-related genes, such as *Cyp1a1* and *Txnrd1*, in the hepa-

tocellular tumor areas (Moto *et al.*, 2006a). Therefore, in the present study, we selected DEN rather than DMN as an initiator, since it was confirmed that mice were more susceptible to DEN than DMN in our preliminary studies (personal observations). In addition, we used a two-stage hepatocarcinogenesis model in mice to identify the threshold dose of DC that induces liver tumor-promoting effects, microsomal ROS generation and drug-metabolizing enzymes that protect against ROS.

MATERIALS AND METHODS

Animals and chemicals

Four-week-old male ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were maintained on a powdered basal diet (MF; Oriental Yeast, Co., Ltd., Tokyo, Japan) and tap water until they were 5 weeks old. The mice were housed in polycarbonate cages with paper bedding and were maintained under standard conditions (room temperature, $22 \pm 2^\circ\text{C}$; relative humidity, $55 \pm 5\%$; light/dark cycle, 12 hr). Animal care and experiments were carried out in accordance with the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology.

DC (CAS No. 112636-83-6) was kindly provided by Novartis Animal Health Inc. (Basel, Switzerland) for the experiment. DEN was purchased from Nacalai Tesque, Inc. (Kyoto, Japan).

Experimental design

The two-stage liver carcinogenesis model of mice was used based on a modified protocol of Della Porta *et al.* (1987) and Lee *et al.* (1989) (Fig. 1). To initiate hepatocarcinogenesis, an intraperitoneal injection of DEN at a dose of 30 mg/kg body weight was administered to the animals (day 0). Twenty-four hours before the DEN injection, a two-thirds partial hepatectomy was performed on the mice to enhance the regeneration of the liver with DNA damage. One week after the DEN injection, mice were fed a powdered diet containing DC at a concentration of 0 (DEN alone), 187.5, 375 or 750 ppm for 10 weeks. For liver sampling, the survivors at 11 weeks were sacrificed by exsanguination from the abdominal aorta under ether anesthesia.

At necropsy, tissue samples were collected from the remaining lobes of the liver. One-third of these samples were fixed with natural-buffered formalin for histopathological examinations, and one-third was embedded in OCT compound (Tissue-Tek; Miles Inc., Elkhart, IN, USA) and frozen for GGT staining. The remaining liver samples were weighed, frozen in dry ice and stored at -80°C until

Fig. 1. Experimental design

further analysis.

Histopathological, histochemical and immunohistochemistry evaluations

Formalin-fixed liver tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE) for the histological examinations. Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) antibody (PC10; DakoCytomation, Glostrup, Denmark) was performed by the avidin-biotin-peroxidase complex method. Histochemical staining of GGT was performed by modifying the methods proposed by Rutenberg *et al.* (1969). The frozen tissues were cryosectioned and fixed using acetone. After air-drying, the freshly prepared solution containing the substrate L-glutamic acid- γ -(4-methoxy- β -naphthylamide) (Sigma-Aldrich, St. Louis, MO, USA) and fast blue BBN (Wako Pure Chemical Industries, Osaka, Japan) in 0.1 M Tris-buffered saline (pH 7.4) was coated onto the section. After incubation, the slides were transferred to 0.1 M cupric sulfate solution. The sections were then stained with hematoxylin, and mounted in 10% glycerol. The number of PCNA-positive cells per 400-500 cells in each slide was counted from ten different areas to determine the PCNA positive index (PCNA PI). The number of GGT-positive cells per area was calculated from the number of positive cells in all lobes on the slide and from the total area in all lobes measured using computer-assisted image analyzer (NIH image).

RNA isolation and real-time RT-PCR

Total RNA was isolated from the liver samples using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA). Reverse transcription (RT) was carried out using ThermoScript RT-PCR System (Invitrogen Corp.), and the cDNA aliquots were used in quantitative real-time RT-PCR with SYBR Green using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR reaction was repeated twice in triplicate for each gene. The PCR primers for the genes of cytochrome P450 1A1 (*Cyp1a1*, accession number NM_009992), cytochrome P450 1A2 (*Cyp1a2*, accession number NM_009993), 8-oxoguanine DNA-glycosylase 1 (*OGG1*, accession number NM_010957), thioredoxin reductase 1 (*Txnrd1*, accession number NM_015762), aldehyde dehydrogenase family 1 subfamily A1 (*Aldh1a1*, accession number NM_013467), NADPH P450 reductase (*Por* or NADPH, accession number NM_008898), Excision repair cross-complementing rodent repair deficiency complementation group 5 (*Ercc5*, accession number NM_011729), superoxide dismutase 1 (*Sod1*, accession number NM_128337) and Glutathione peroxidase 2 (*Gpx2*, accession number MN_030677) in the present study were prepared as previously reported (Moto *et al.*, 2005, 2006a and 2006b). The primers used in this PCR are summarized in Table 1. The expression levels of each gene were corrected based on the expression of β -actin in the same cDNA sample.

Table 1. Primers of genes used for real-time RT-PCR

Accession number	Abbreviation of gene name	Primer (upper: forward primer, lower: reverse primer)
NM_009992	<i>Cyp1a1</i>	AGGATGTGTCTGGTTACTTG AGAAACATGGACATGCAAG
NM_009993	<i>Cyp1a2</i>	GCTACTTGTGACATGGCCTA AAGCCATTCACTGAGGTGTC
NM_010957	<i>Ogg1</i>	CAGCATAAGGTCCCCACAGATT GCCAACAAGAACTGGGAAACT
NM_015762	<i>Txnrd1</i>	GGTTGCATACCTAAGAAGCTGATG CCATAGTTGCGCGAGTCTTTC
NM_013467	<i>Aldh1a1</i>	GACTTGAAGATTCAACATACC TCACAGCTTTGTCAACATCA
NM_008898	<i>Por</i> or <i>NADPH</i>	GCCTGCCTGAGATCGACAAG GGGTCGCCTTCTCCGTATGT
NM_011729	<i>Ercc5</i>	TCAACTAGGACTGGACCGTAACAA AGTTGGTATCCCTTCCGTATAGTCA
NM_128337	<i>Sod1</i>	TGATTGGGATTGCGCAGTAA TGGTTTGAGGGTAGCAGATGAGT
NM_030677	<i>Gpx2</i>	GCTGCCCTACCCTTATGATGAC CGCACGGGACTCCATATGAT

Measurement of microsomal ROS production in the liver

Liver microsomes were obtained from four mice from each group treated with 0, 187.5, 375 or 750 ppm DC for 10 weeks and which were subjected to the two-stage hepatocarcinogenesis model. Their liver microsomes were extracted using the following method. The liver samples were rinsed with ice-cold homogenate buffer (1.15% KCl, 0.2 mM ethylene diamine tetra-acetic acid disodium (EDTA-2Na), 0.1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 20% glycerin at pH 7.4) and homogenized in three volumes of the homogenate buffer using a glass pestle. The homogenate was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was ultracentrifuged at $105,000 \times g$ for 90 min. The microsomal pellet was resuspended in the homogenate buffer, and the protein content of the homogenate was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Pierce, IL, USA) with BSA as a standard. For the measurement of ROS, the nonfluorescent probe DCFH-DA was used as to

detect ROS formation during cellular metabolism; in the presence of ROS, this probe oxidizes to a highly fluorescent dichlorofluorescein (DCF) (LeBel *et al.*, 1990; Serron *et al.*, 2000; Szejda *et al.*, 1984). The microsomes (0.05 mg protein) were incubated in the dark at 37°C in 40 mM Tris buffer (pH 7.4) and DCFH-DA (5 μ M) for 30 min. At the end of the incubation period, 2.5 mM β -NADPH was added and the sample was incubated at 37°C for 30 min in the dark. ROS formation was detected by measuring the fluorescent product by a microplate reader (excitation, 485 nm and emission, 528 nm).

Statistical evaluation

Statistical analyses were performed using the statistical software StatLight (Yukms Co., Ltd., Tokyo, Japan), and all results are presented as means $\pm$ S.D. The significance of the differences between the DEN alone group and each DC group was determined by Dunnett's test after one-way ANOVA. A p-value less than 0.05 was considered statistically significant in both analyses.

RESULTS

General observations and histological evaluation

During the experimental period, death and remarkable treatment-related clinical signs were not observed in the DEN alone or the DEN + DC groups. No significant differences in the final body weight, and absolute and relative liver weights of mice were observed in any of the treated groups (Table 2, Fig. 2).

No remarkable histopathological changes were observed in the liver of mice treated with 187.5, 375 or 750 ppm DC. In immunohistochemical examinations, GGT-positive cells were observed in the liver of mice treated with DEN alone or DEN + DC, but GGT-positive foci consisting of the cluster of GGT-positive cells were not observed in these groups. The number of GGT-positive cells was not significantly increased in the DEN +

DC groups compared with that in the DEN alone group (Fig. 3a). However, the PCNA-positive cell ratio was significantly increased in the 750 ppm DC group compared with that in the DEN alone group (Fig. 3b).

mRNA expression in liver tissues

The results of the analyses are summarized in Fig. 4. A significant up-regulation of stress response-related genes such as *Cyp1a1* and *Cyp1a2* was observed in mice administered 375 ppm DC or more and mice in the DC-treated groups as compared with the control group, respectively. The expression of *OGG1*, a DNA related gene, was significantly up-regulated only in the 750 ppm DC group compared with the control group. No significant differences were found in the expression of Heme oxygenase 1 (*Hmox1*, MN_010442), *Ercc5*, *Por* (NADPH), *Txnrd1*, *Sod1*, *Gpx2*, and oxidative stress-related genes between the DEN alone and DEN + DC groups.

Table 2. Body and liver weights of mice given DC after DEN initiation

Group	DEN alone	DEN+DC 187.5 ppm	DEN+DC 375 ppm	DEN+DC 750 ppm
Animal No.	7	12	12	10
Body weight (g)	43.8 ± 6.5 ^{a)}	43.5 ± 4.0	42.2 ± 4.0	41.4 ± 3.2
Absolute liver weight (g)	2.0 ± 0.2	2.2 ± 0.9	1.9 ± 0.2	2.0 ± 0.4
Relative liver weight (%)	4.7 ± 0.2	5.0 ± 1.7	4.7 ± 0.5	4.8 ± 0.8

^{a)}: Mean ± S.D.

Fig. 2. Body weight changes of mice given DC for 10 weeks after DEN initiation

Fig. 3. The number of GGT positive cells and the PCNA positive ratio in mice given DC for 10 weeks after DEN initiation. Each graph shows the number of GGT-positive cells (a) and the PCNA-positive cell ratio (b) in the livers of mice given DC for 10 weeks after DEN initiation. Column represents means $\pm$ S.D. *Significantly different from the 0 ppm DC group at $p < 0.05$ (Dunnett's test).

Formation of microsomal ROS in liver

As shown in Fig. 5, there was a tendency for increased formation of microsomal ROS in the liver of the DC-treated groups, but there were no significant differences between the DEN alone and DEN + DC groups.

DISCUSSION

DC has been reported to potentially exert hepatocarcinogenic and hepatocellular tumor-promoting effects in mice (WHO, 2000; Moto *et al.*, 2005, 2006a and 2006b). In our previous study, we performed a two-stage liver carcinogenesis experiment in which partially hepatectomized mice were given 1,500 ppm DC for 8, 13 or 20 weeks after initiation of hepatocarcinogenesis by DMN or DEN, and the number and area of GGT-positive foci and PCNA-positive ratio were significantly increased in mice of the DMN + DC or DEN + DC group compared with those in

the DMN or DEN alone group (Moto *et al.*, 2005, 2006a and 2006b). GGT is used as a marker for preneoplastic lesions in chemically induced hepatocarcinogenesis of mice (Peraino *et al.*, 1983). In the present study, there was no increase in the number of GGT-positive cells in the livers of mice administered DC compared with that in the DEN alone group. However, immunohistochemical findings revealed a significant increase in the PCNA-positive ratio in the 750 ppm DC group compared with that in the DEN alone group. It has been reported that the amount of PCNA positive cells were corresponding with proliferative condition of the hepatic proliferative lesions (Chen *et al.*, 1998). Furthermore, there are many reports that PCNA positive cell significantly increased in the liver of mice with the increased number of GGT positive foci or cells (Moto *et al.*, 2005, 2006a and 2006b; Kenmochi *et al.*, 2007). These results suggested the possibility that the present concentration of DC is not enough to the forma-

Fig. 4. Real time RT-PCR analysis of mRNA expression in the livers of mice given DC for 10 weeks after DEN initiation. The expression of target genes was normalized by β -actin. Values are expressed as the group mean fold change over control. Columns represents means $\pm$ S.D. *, ** and *** significantly different from the 0 ppm DC group at $p < 0.05$, $p < 0.01$ or $p < 0.001$, respectively (Dunnett's test).

tion of preneoplastic lesions, although an increase in cell proliferation activity was observed in the liver of mice given 750 ppm DC. Accordingly, the practical threshold level of DC that induces hepatocellular tumor-promoting effects was considered to be more than 750 ppm in the present experimental condition.

The cytochrome P-450 family is known to generate ROS as byproducts of microsomal oxidation. Among the CYPs, CYP1A1 has been reported to be the most active CYP enzyme that catalyzes procarcinogens (Guengerrich and Shimada, 1991; Puntarulo and Cederbaum, 1998), and the up-regulation of the CYP1A1 and/or CYP1A2 isoform(s) indirectly results in the production of very large amounts of ROS compared with that of other CYPs (Paolini *et al.*, 1996; Putarulo and Cederbaum,

1998; Valgimigli *et al.*, 2001). In our previous studies in which mice were given diet containing 1,500 ppm DC after DMN/DEN initiation, a dose-dependent increase in microsomal ROS production was observed in these treated mice (Moto *et al.*, 2005, 2006b). In the present study, the expressions of *Cyp1a1* in mice given 375 ppm DC or more and *Cyp1a2* in mice from all of the DC-treated groups were significantly and higher than that in the DEN alone group, although there were no significant differences in the formation of microsomal ROS between the DEN alone and DEN + DC groups. These findings may suggest a possible generation of ROS through P450-mediated metabolism of DC and that the ROS produced in the liver of mice given DC is derived from the microsomes. On the other hand, it is also well known that ROS gen-

Fig. 5. Effect of DC on hepatic microsomal ROS production. Column represents mean $\pm$ S.D.

erates from other cellular organelle in hepatocytes, such as mitochondria and peroxisomes (Parola and Robino, 2001), and macrophages (Kupffer cells) (Loguercio and Federico, 2003). However, no morphological changes were observed in Kupffer cells as well as mitochondria and peroxisomes of hepatocytes in DC-treated mice in our previous and present studies (Moto, *et al.*, 2005, 2006a and 2006b). Therefore, the possibility that ROS generated from these organelles and Kupffer cells in DC-treated mice can be eliminated.

In the preset study, the formation of microsomal ROS in the liver tended to increase in the DC-treated groups, but there were no significant differences between the DEN alone and DC-treated groups. This finding may suggest that no oxidative DNA damage occurs in mice given 750 ppm DC or lower. On the other hand, the expression of *OGG1* was significantly increased only in the 750 ppm DC group compared with that in the control group. *OGG1* is a repair gene of 8-OHdG (Kinoshita *et al.*, 2002, 2003). 8-OHdG, a marker of oxidative DNA damage, is known as one of the causes for DNA point mutations such as G-T transversion (Kasai, 1997; Shibutani *et al.*, 1991), and is potentially involved in the carcinogenesis in various experimental models (Nakae *et al.*, 1997; Yoshida *et al.*, 1999; Kinoshita *et al.*, 2002, 2003). The up-regulation of the *OGG1* gene in the present study indicates that oxidative DNA damage in mice given 750 ppm DC may be prevented by this DNA repair gene. On the

other hand, the mRNA expression of metabolism-, oxidative stress- or DNA damage-/repair-related genes, such as *Txnrd1*, *Por*, *Aldh1a1*, *Sod1*, *Gpx2* and *Ercc5*, in the DC-treated groups was not significantly higher than that in the DEN alone group. These findings may indicate that neither oxidative stress nor DNA damage is induced in mice given DC at concentrations of 750 ppm or lower. Taking into account our results, we speculate that *Cyp1a1* and *Cyp1a2* are the most sensitive molecular markers of the treatment-related metabolic changes of DC, but the up-regulation of these genes at a concentration of DC of 750 ppm or lower is not enough to produce ROS.

In conclusion, in the present study, an increased number of PCNA-positive cells, and enhanced expression of oxidative stress- or DNA damage-/repair-related genes such as *Cyp1a1*, *Cyp1a2* and *OGG1* were observed in the livers of mice given 750 ppm DC compared with the DEN alone group, but the number of GGT-positive cells and formation of microsomal ROS were not increased in this group. Therefore, the results of our study suggest that the threshold dose for ROS-mediated liver tumor promotion of DC is more than 750 ppm. Accordingly, the possibility that hepatocellular proliferative lesions may be induced in mice given 750 ppm DC or lower is extremely low.

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