served to be slight in the DC group and moderate in the DMN + DC + PH group. Lipofsucin is one of the age-associated pigments that have been regarded as cellular debris derived from lipid peroxides by the free radical-induced oxidative stress, and therefore, is considered as one of the indices of lipid peroxidation in tissues (Tsuchida et al., 1987). Our histopathological findings also support the possibility that treatment with DC at this dose results in the induction of oxidative stresses.

In the gene expression analyses of Exp. II, the up-regulation of oxidative stress-related genes, such as *Cyp1a1*, *Cyp1a2*, *Por*, *GST*s, and *Txnrd1*, was also observed in the both of DC-treated groups. *Pro* is the gene of P450 oxidoreductase which may independently act as a co-enzyme of P450. It is generally known that some chemicals induce the production of super oxide and/or lipid peroxidation by this enzyme reaction. Therefore, the formation of lipofuscin observed in the both of DC-treated groups might result from the activation of P450 oxidoreductase via lipid peroxidation. Thioredoxin reductase 1 is a subtype of thioredoxin reductase, which utilizes NADPH to catalyze the conversion of oxidized thioredoxin to reduced thioredoxin (Luthman and Holmgren, 1982). Reduced thioredoxin provides reducing equivalents such as: (i) thioredoxin peroxidase that brakes down H_2O_2 to water; and (ii) ribonucleotide reductase that reduces ribonucleotide to deoxyribonucleotide for DNA synthesis (Laurent et al., 1964; Chae et al., 1994). Additionally, it has been reported that thioredoxin reductase plays an important role in the regulation of cell growth, inhibition of apoptosis, and inflammation closely associated with NF-κB activation (Mustacich and Powis, 2000; Sakurai et al., 2004). In fact, the concentration of activated NF-κB, an indicator of NF-κB activity that indicates the inhibition of apoptosis and an increase in inflammation and necrosis, was highest in the DMN + DC + PH group in which significant up-regulation of the *Txnrd1* expression and some necrotic foci were observed. These results may thus suggest that DC treatment inhibits apoptosis via activation of NF-κB due to the increase in thioredoxin reductase 1 and cell damages such as inflammation originating from oxidative stresses; this pathway may partially be associated with the development of GGT-positive foci.

DNA damages originating from oxidative stresses was considered as another possible pathway of hepatocarcinogenesis induced by DC, because the up- or down-regulation of DNA damage-/repair-related genes, such as *Ercc5*, *Gadd45a*, and *Ogg1*, was observed in the DMN + DC + PH group. It is known that *Ercc5*, one of nucleotide excision repair (NER) genes, is involved in the transcription-coupled repair (TCR) of DNA lesions (Cheng et al., 2000). *Gadd45a* is a p53-effector and a stress-inducible gene; it is related to the maintenance of genomic stability and the positive regulation of apoptosis (Fornace et al., 1989; Hollander et al., 1999, 2001; Smith et al., 2000; Jeffrey et al., 2002; Jin et al., 2003). In fact, the tendency of down-regulation for *p53* was observed in the DC alone and the DMN + DC + PH groups that were shown in a similar tendency of *Gadd45a*, suggesting that the instability of *p53* might be induced by treatment with DC. However, no significant differences were observed in the expression level of *p53* as well as in the levels of the activated NF-κB. These changes might be resulted to the weak levels of the gene and protein in an early tumor promotion stage of hepatocarcinogenesis, but they are considered to be enhanced as a tumor promotion stage progresses. *Ogg1* is a repaired gene of 8-hydroxy-2′-deoxyguanosine (8-OHdG); it is known as an indicator of oxidative DNA damage and is potentially involved in the carcinogenesis in various experimental models (Nakae et al., 1997; Yoshida et al., 1999; Kinoshita et al., 2002, 2003). However, it has been reported that DC was negative in the in vitro genotoxicity tests using CHO and CHL cells under the conditions of presence and absence of S9 mix (WHO, 2000). In our previous study, a single oral administration of DC did not induce DNA damages in the in vivo comet assay (Moto et al., 2003). Furthermore, remarkable fluctuations of DNA damage-/repair-related genes were not observed in the liver of the mice used in Exp. I (Table 1). These results may suggest that the assumed DNA damages originating from oxidative stresses induced by DC will not result in point mutations or chromosomal aberrations in the liver of mice fed with 1500 ppm of DC for up to 7 weeks because of the up-regulation of these DNA repair genes. However, it is unclear whether DNA damages originating from oxidative stresses will result in the occurrence of gene mutations or chromosomal aberrations due to the prolonged administration of DC. Therefore, additional studies are now in progress to clarify whether DNA damages are induced by the generation of oxidative stresses due to treatment with DC for a long period of time.

In conclusion, the present study suggests the possibility that the inhibition of apoptosis and DNA damages originating from oxidative stresses are involved in the mechanism of hepatocarcinogenesis due to DC in mice. However, further investigations comprising measurements of the 8-OHdG formation by liver DNA extraction and the production of oxidative stress-inducible substances (ROS, H_2O_2) should be performed to elucidate the relationship among DC, oxidative stresses, and DNA damages.

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GENOTOXICITY AND CARCINOGENICITY

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Gene expression analysis in mice liver on hepatocarcinogenesis by flumequine

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Abstract mRNA expression profiles in the liver from mice treated with flumequine (FL) were analyzed in order to elucidate the mechanism of its tumor-promoting effect. The liver from a C3H/He mouse that received a diet containing 4,000 ppm of FL for 4 weeks was examined by cDNA microarray in comparison with an untreated mouse. Furthermore, to obtain a more comprehensive sequence, time-course changes in selected genes were determined by real-time RT-PCR. Microarray analysis revealed 15 upregulated and 9 downregulated genes in an FL-treated mouse. The upregulated genes included signal transducers and cell cycle regulators. In addition, the levels of stress response genes, particularly glutathione S-transferase (GST) α and GSTμ, were very high, indicating the generation of oxidative stress. On the other hand, the downregulated genes included phase I metabolic enzymes, such as cytochrome P450 (CYPs) enzymes, and apoptosis-associated proteins. These changes were confirmed by quantitative RT-PCR and were generally consistent with each other. Time-course observations revealed consistent results, particularly with regard to GSTα, GSTμ, ERK5, and CYP2E1. In addition, the expression of 8-oxoguanine DNA glycosylase 1 (OGG1) was increased in a time-dependent manner. These results suggest the possibility that responses against oxidative stress may play a major role in hepatocarcinogenesis by FL in mice.

Keywords Flumequine · Hepatocarcinogenesis · Oxidative stress · Tumor-promoting effect

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Introduction

Flumequine (FL), a quinolone-antibacterial agent, has been used for veterinary treatment of infections (Greenwood 1978). Although it was found to increase the incidence of hepatocellular tumors in a conventional 18-month carcinogenicity study in mice, the Food and Agriculture Organization (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA) (WHO 1998) considered that the carcinogenic effect was not due to DNA damage but rather due to a necrosis regeneration response. This consideration was based on the fact that there was no evidence of FL genotoxicity in in vitro bacterial and mammalian cell gene mutation assays and in an in vivo chromosomal aberration test that was performed in the bone marrow cells of rats (WHO 1998). However, recent studies revealed that a 13-week treatment with FL could induce hepatocellular foci in the livers of mice with or without any prior initiation treatment (Yoshida et al. 1999; Kashida et al. 2002). We have also demonstrated elevated incidences of hepatocellular foci even in the livers of mice that were treated with FL for 2 weeks at the initiation phase followed by phenobarbital as a tumor promoting treatment for 13 weeks (Kashida et al. 2002). Based on these results, FL is suspected to have both tumor initiation and promotion effects in the liver. In our previous study, using comet assay, we demonstrated that FL induces DNA degradation in regenerative livers of mice (Kashida et al. 2002). In addition, Yoshida et al. (1999) found that the intensity of immunostaining of 8-hydroxy-2′-deoxyguanosine (8-OHdG) was increased in the livers of mice that were treated with FL for 13 weeks. These results imply that oxidative

stress and/or topoisomerase II inhibition during the regeneration of liver cells are crucial factors for tumor initiation by FL. However, the tumor-promoting mechanism of FL continues to remain unclear.

Recently, global gene expression analysis techniques using cDNA microarray, oligonucleotide array, or GeneChip array have been widely used for defining the characteristics and specific patterns of gene expression elicited by a given toxicant. Tumor-promoting compounds including phenobarbital, clofibrate, di(2-ethylhexyl) phthalate, and oxazepam have already been investigated for determining their effects on the livers of rodents (de Longueville et al. 2002; Wong and Gill 2002). It is hoped that these gene expression analysis techniques will become a powerful tool for predicting the toxicological and carcinogenic potentials of newly developed drugs by accumulating a pool of gene profiling data for comparative purposes. In the present study, using cDNA microarray, we examined global mRNA expression changes in the livers of mice that were treated with FL for 4 weeks. In addition, we used real-time RT-PCR to perform a detailed investigation of the selected genes that were determined by this microarray analysis to elucidate the possible mechanism of tumor promotion by FL. In addition, we analyzed the gene expression of 8-oxoguanine DNA glycosylase 1 (OGG1) as a marker of DNA damage by oxidative stress (Boiteux and Radicella 1999; Kinoshita et al. 2002).

Materials and methods

Chemicals and animals

The FL sample, a fine white crystalline powder, was kindly provided by Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). A total of eighteen 4-week-old male C3H/He mice were purchased from Japan SLC Inc. (Shizuoka, Japan) and maintained in an air-conditioned animal room with a 12-h light cycle and were given free access to powdered diet and tap water. After a 3-week acclimatization period, they were assigned to control or FL-treated groups. The mice were fed a basal diet containing 4,000 or 0 ppm of FL for 1, 4, or 8 weeks. This dose of FL was previously demonstrated to induce hepatocellular foci by a 13-week treatment (Kashida et al. 2002).

On completion of the treatment period, the mice were killed by exsanguination under ether anesthesia and necropsied. The livers were excised, macroscopically examined, weighed, and cut into small pieces that were frozen in liquid nitrogen and stored at –80°C for subsequent analysis.

cDNA microarray analysis

The liver samples from one control and one FL-treated mice were sent to BD Biosciences Clontech Japan (Tokyo, Japan) for cDNA microarray analysis. Using an Atlas Mouse Cancer 1.2 Nylon Array, a membrane hybridization analysis was performed in accordance with the standard protocol provided by Atlas. In brief, total RNA was isolated and DNase I was treated and labeled with [α–^{32}P]dATP using an Atlas Pure Total RNA Labeling Kit. Following probe purification with Atlas NucleoSpin Extraction Columns, overnight hybridizations were performed on microarray membranes in ExpressHyb hybridization solutions containing sheared salmon testes DNA at 68°C. Following hybridization, the microarray membranes were washed, sealed, and exposed to a phosphorimager. The array images were analyzed with AtlasImage, and the data were normalized to the total intensity of the array using the global normalization method. For each spot, the ratio of intensities between the control and FL-treated mice was analyzed.

Real-time RT-PCR

The genes of interest identified to be up- or downregulated by FL treatment were validated by quantitative real-time RT-PCR in the same liver that was used in the microarray analysis. Total RNA was extracted by using TRIzol Reagent (Invitrogen, CA, USA) in accordance with the manufacturer's instructions. After the concentration was measured by a spectrophotometer and the RNA quality was determined by electrophoresis, cDNA was synthesized from 1 μg of RNA in the presence of DTT, dNTPs, random primers, RNaseOUT (Invitrogen), and ThermoScript reverse transcriptase (Invitrogen) in a total of 20 μl reaction mix. Real-time RT-PCR was conducted by using ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, USA) and SYBR Green I chemistry (Applied Biosystems) in accordance with the manufacturer's instructions. Primers for the assay were designed by using the Primer Express 2.0 software (Applied Biosystems) to include the exon junction in at least one primer or amplicon in order to amplify only mRNA and not genomic DNA. The primer sequences are listed in Table 1. Each primer concentration was fully optimized between 300 and 900 nM of final concentration. The amount of target gene, normalized to an endogenous reference (ubiquitin C) and relative to a control, was given by a 2 $\Delta\Delta$CT method (Livak and Schmittgen 2001).

To investigate the time-course change in gene expression, additional real-time RT-PCR analyses were performed in mice that were treated with FL for 1, 4, or 8 weeks (3 mice each) and in corresponding control mice in the same manner as described above.

Statistical analysis

The parametric data from the 4- and 8-week treatment groups ($n = 3$) were analyzed using the F-test followed by t test in order to detect any significant differences from the corresponding control group values.

Table 1 Sequence of primers using real-time RT-PCR analysis

Gene name	Symbol	Forward primer	Reverse primer	Accession no.
Glutathione S-transferase, alpha 2	GSTα	ACATGAAGGAGAGAGCCCTGAT	GCAGTCTTGGCTTCTCTTTGGT	J03958
Glutathione S-transferase, mu2	GSTμ	TTCCCAATCTGCCCTACTTGA	TCTCCACACAGGTTGTGCTTTC	J04696
Cek 5 receptor protein tyrosine kinase ligand	CEK5R	AGCTTGGAGGGACTGGAGAAC	TGCCCAACCTTCATAACGATCT	U12983
Fibroblast growth factor 7	FGF7	AGGGACCCAGGAGATGAAGAA	TGATTGCCACAATTCCAACTG	Z22703
Extracellular signal-regulated kinase 5	ERK5	TGAACCCAGTGCCCGAAT	GGCGCAATCAGGCTCATC	U25278
B cell translocation gene 1	BTG1	TAAACATCACTGGTTCCCAGAGA	GATGCGAATACAACGGTAACCT	L16846
Protein kinase C, epsilon	PKCε	TCATAGGAAAACAGGGATATCAATGTC	GGGCATGTTGACGCTGAAC	AF028009
Cyclin-dependent kinase regulatory unit	CDK5R	TGAAGCGGCACTCCATCAT	ATGTTGCTCTGGTAGCTGCTGTT	S82819
Mitogen-activated protein kinase kinase 5	MEK5	GCC GCT GCA GAT ATT TCC AA	CAG CCC GTG TAT TCA CCT TCA	U25265
Extracellular signal-regulated kinase 6	ERK6	CAAGCGCGCCTACAGAGAGT	AACACATCCAGTAGCCCAATGA	Y13439
Large multifunctional protease 7	LMP7	GCCAAGGAGTGCAGGTTGTATT	TGGAAAGCAGCTTGGATGCT	X64449
Cytochrome P450 IIE1	CYP2E1	TCAAAAAGACCAAAGGCCAGC	TCCGCAATGACATTGCAGG	L11650
BCL2- and adenoviral E1B-interacting protein	NIP3	CAGCATGAGAAACACAAGCGT	CAATGTAGATCCCCCAAGCCA	AF041054
T cell death-associated gene 51	TDAG51	CACCCGGGCCACTCAAG	CAATGCACTCTTCCCACTTTCC	U44088
8-oxoguanine DNA-glycosylase 1	OGG1	GCCAACAAAGAACTGGGAAACT	CAGCATAAGGTCCCCACAGATT	NM010957
Ubiquitin C	UBC	ACCGCTGACGATGCAGATCTT	TGCCTTGACATTCTCAATGGTG	BC021837

Results

In the microarray analysis of a mouse liver treated with FL for 4 weeks, a total of 170 or 105 out of 1,176 genes were revealed to be over- or underexpressed, respectively. After omitting genes with weak signals to provide the most reliable results, 15 and 9 genes showed 2-fold or greater and smaller expression, respectively (Table 2). The real-time RT-PCR analysis for 14 selected target genes that were normalized to ubiquitin C as the internal control showed trends similar to those observed in the microarray analysis. In particular, the expressions of glutathione S-transferase (GST) α2 and GSTμ2 were extremely high in the FL-treated mice. For the other upregulated genes, Cek 5 receptor protein tyrosine kinase ligand (CEK5R), fibroblast growth factor 7 (FGF7), B cell translocation gene 1 (BTG1), and extracellular signal-related kinase 5 (ERK5) were also reproducible by real-time RT-PCR analysis, although fold changes were relatively lower than those in the microarray analysis. Increases in mitogen-activated protein kinase 5 (MEK5) and ERK6 were not apparent in the real-time RT-PCR analysis. The relative expressions of protein kinase C epsilon (PKCε) and cyclin-dependent kinase 5 regulatory unit (CDK5R) in the FL-treated mice against the control mice were inconsistent with the findings of the microarray analysis. The downregulated genes such as cytochrome P450 2E1 (CYP2E1), bcl-2 and adenoviral E1B-interacting protein (NIP3), T cell death-associated gene 51 (TDAG51), and large multifunctional protease 7 (LMP7) were examined and they showed a low expression.

The time-course changes of selected genes demonstrated by real-time RT-PCR are shown in Fig. 1. Consistent changes throughout the treatment period were evident in GSTα2 and GSTμ2, ERK5, and CYP2E1 with statistically significant differences ($P < 0.05$). The increases in GSTα and GSTμ were 55.9-fold and 9.4-fold higher than those in the control group, respectively; they were the highest at 4 weeks. ERK5 showed a time-dependent increase, with the highest values of 4.8-fold at 8 weeks. CEK5R was significantly increased after 4 weeks of treatment, while CDK5R and BTG1 were significantly increased after the 8-week treatment. ERK6, PKCε, FGF7, and MEK5 showed mild increases without any significant difference throughout the treatment period. The expressions of LMP7 and TDAG51 were different from those in the microarray analysis and fluctuated throughout the treatment period.

The expression of OGG1 was increased in a time-dependent manner with significant differences by the 4- and 8-week treatments ($P < 0.05$).

Discussion

In the present microarray analysis, when compared with the control group, 15 and 9 genes were identified to be

Table 2 Differentially expressed genes from cDNA microarray in the liver after FL treatment

Accession no.	Gene name	Function	Fold change	
			Microarray	Real-time RT-PCR
J03958	Glutathione S-transferase alpha 2	Xenobiotic metabolism, stress response	8.8	22.47
J04696	Glutathione S-transferase mu 2	Xenobiotic metabolism, stress response	4.3	3.69
U12983	Cek 5 receptor protein tyrosine kinase ligand	Signal transduction	3.7	1.95
Z22703	Fibroblast growth factor 7	Signal transduction	3.1	1.71
U25278	Extracellular signal-regulated kinase 5	Signal transduction	2.9	2.17
L16846	B cell translocation gene 1, anti-proliferative	Cell cycle regulation	2.8	1.22
AF028009	Protein kinase C epsilon	Signal transduction	2.4	0.79
S82819	Cyclin-dependent kinase 5 regulatory subunit	Cell cycle regulation	2.4	0.90
M93275	Adipose differentiation related protein	Extracellular transport/carrier protein	2.2	–
U43205	Frizzled homolog 3	Signal transduction	2.2	–
U25265	Mitogen-activated protein kinase kinase 5	Signal transduction	2.1	1.07
Y13439	Extracellular signal-regulated kinase 6	Signal transduction	2.1	1.06
M81483	Protein phosphatase 3 catalytic subunit beta isoform	Signal transduction	2.0	–
X55023	Heat shock 60-kDa protein	Protein processing, stress response	2.0	–
M13926	Granulocyte colony-stimulating factor	Signal transduction	2.0	–
X64449	Large multifunctional protease 7	Protein turnover	0.29	0.57
L11650	Cytochrome P450 IIE1	Xenobiotic metabolism, stress response	0.37	0.62
Z49086	Eph-related tyrosine-protein kinase receptor	Signal transduction	0.38	–
AF041054	NIP3; BCL2- and adenoviral E1B-interacting protein	Apoptosis	0.43	0.52
D00725	Serine protease inhibitor 2	Protein turnover	0.43	–
M21019	Harvey rat sarcoma oncogene subgroup R protein	Oncogene	0.45	–
U60473	CD59 antigen	Extracellular transport/carrier protein	0.48	–
U44088	T cell death-associated gene 51	Apoptosis	0.50	0.26
AF092734	Growth differentiation factor 11	Signal transduction	0.50	–

–, not determined by real-time RT-PCR

upregulated and downregulated, respectively, in an FL-treated mouse. The dominantly upregulated genes included signal transducers and cell cycle regulators, which were indicative of cell proliferation. In addition, stress response molecules such as GSTs and heat shock proteins were included in the upregulated genes. On the other hand, phase I metabolic enzymes (CYPs) and apoptosis-associated proteins such as TDAG51 and NIP3 were mainly downregulated. Some of these changes were confirmed by quantitative real-time RT-PCR, and the findings obtained by the two techniques were generally consistent. Furthermore, time-course observations revealed a consistent result, particularly the upregulation of GSTα2, GSTμ2, and ERK5 and the downregulation of CYP2E1 were prominent after 1-, 4-, and 8-week treatment with FL. Here, we will comment on the small number of contradictory results obtained on comparison of the microarray and real-time RT-PCR findings. Genes with very low control expression, such as FGF7 and ERK6, showed relatively large deviations that might influence the accuracy of the analysis. Furthermore, selection of the internal control for normalization might affect the outcome with real-time RT-PCR analysis since commonly used internal controls can quantitatively vary in response to various factors (Thellin et al. 1999). Here, we used ubiquitin C for this purpose, since it was the only unchanged gene among the nine housekeeping genes (ubiquitin C, phospholipase A2, hypoxanthine-guanine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase, myosin Iα, ornithine decarboxylase, (β-actin, 45-kDa calcium-binding protein precursor, and 40S ribosomal protein S29) that were analyzed within the microarray employed in the present study.

Of the genes identified, the expression levels of GSTα2 and GSTμ2 were very high in both the microarray and real-time RT-PCR analyses throughout the treatment period, with the highest values obtained after 4 weeks. GSTs catalyze the conjugation of glutathione to electrophilic substrates, which can react with cellular components like DNA. Thus far, many isoforms of GSTs, including alpha, mu, pi, theta, omega, and sigma, have been identified in mammals (Hayes and Pulford 1995; Landi 2000), and each isoform is considered to play a role in stress responses and metabolism in various ways. Complex pathways regulate the mRNA expression of GSTs, and many xenobiotics, classified as bifunctional inducers, are capable of directly or indirectly inducing both phase I and phase II enzymes such as CYPs and GSTs, respectively, via the "xenobiotic response element" (XRE). On the other hand, monofunctional inducers such as butylated hydroxyanisole (BHA) or isothiocyanates act via the "antioxidant/electrophile response element" (ARE/EpRE) without activation of CYPs. In the present microarray analysis, the mRNA of any phase I enzymes did not show significant increase. Although FL metabolism has not been studied in detail, our data strongly suggest that FL should be classified as one of the monofunctional GST inducers. These inducers are known to mediate the transcriptional

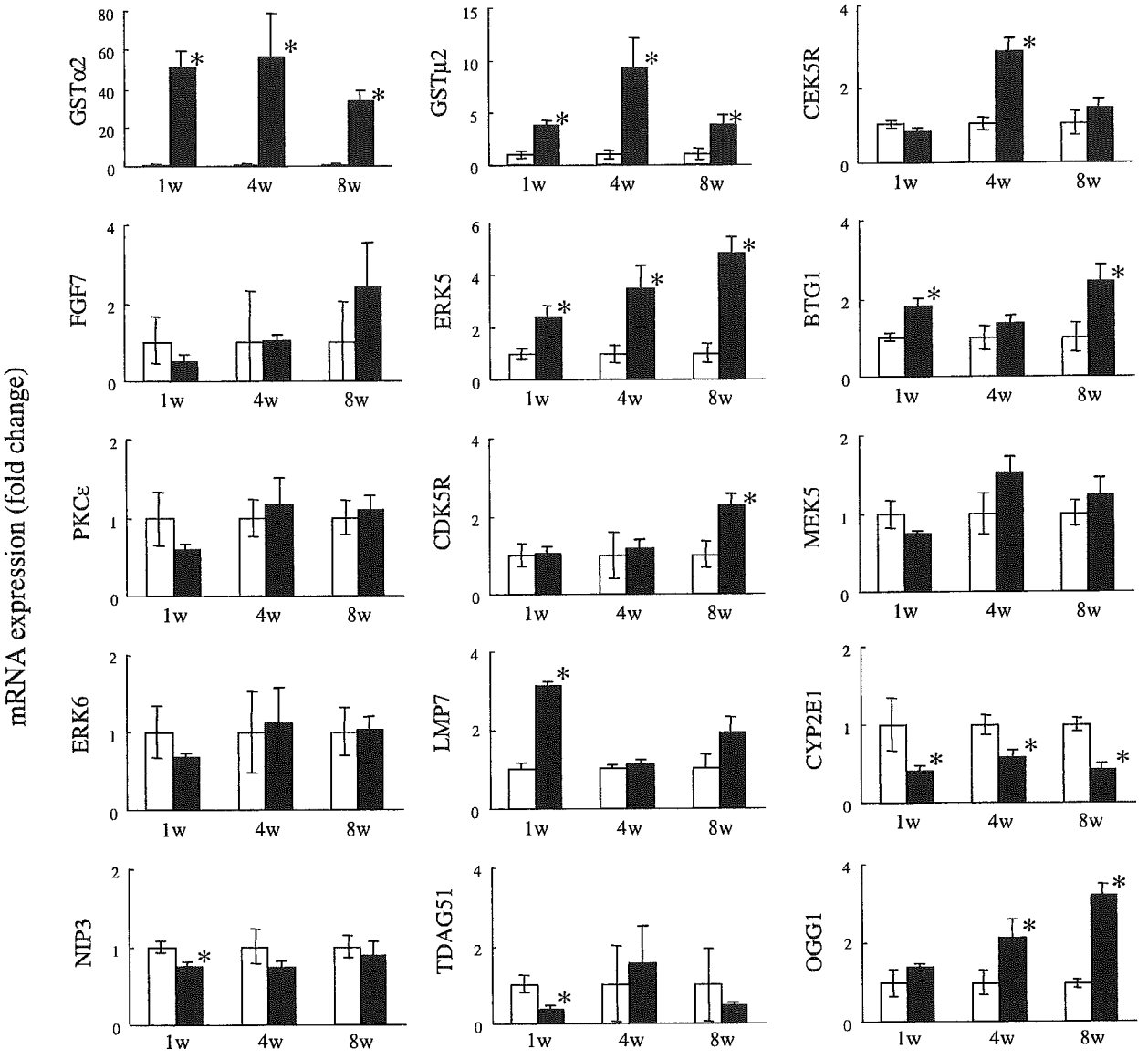

Fig. 1 Real-time RT-PCR analysis of mRNA expression in livers of mice treated with FL (*closed column*) for 1, 4, and 8 weeks and corresponding controls (*open column*). Individual gene expression levels were normalized by using ubiquitin C. The presented values are expressed as group mean fold change over control. * Significantly different from control at $P<0.05$. $n=3$

activation of ARE/EpRE via mitogen-activated protein kinase (ERK1/2, ERK5, JNK, and p38) cascades, followed by the activation of Nrf2 as a transcription factor that heterodimerizes with small Maf and binds the ARE/EpRE sequences (Venugopal and Jaiswal 1996; Itoh et al. 1997; Owuor and Kong 2002). The activation of MAP kinases is characteristic for particular stimuli. For example, EGF strongly activates ERK1/2 and ERK5, but weakly activates JNK/SAPK and p38 (Cano et al. 1994). Hyperosmolar stress is a strong stimulus for p38 (Han et al. 1994). Oxidative stress activates ERK1/2 when the agent is superoxide and not H_2O_2 (Baas and Berk 1995). On the other hand, stimulation by H_2O_2 can result in ERK5 activation by MEK5 phosphorylation of ERK5 (Abe et al. 1996). In contrast with the other members of the MAP kinase family, the mechanism of activation of MEK5 is not clear, but specific stimulations such as EGF and H_2O_2 can induce the cascade. In the present study, the upregulation of ERK5 was pronounced, which strongly indicated cell responses to the oxidative stress via specific pathway. Yoshida et al. (1999) reported a marked increase of 8-OHdG in hepatocytes treated with FL. It is known that oxidative stress can induce both cell proliferation and deletion by either apoptosis or necrosis with varying exposure to oxidative stress (Dypbukt et al. 1994). The modulation

of apoptosis-associated proteins might also be in response to the oxidative stresses. Therefore, the fluctuations in mRNA levels of GSTα2, GSTμ2, and ERK5 observed in the FL-treated mice suggest the induction of responses related to the oxidative stress. In addition, the significant elevation of OGG1 expression observed in a time-dependent manner in the FL-treated mice indicates the possibility that continuous formation of 8-OHdG is caused by oxidative stress following DNA repairs, as demonstrated in other experiments on the formation of 8-OHdG in rat liver via generation of oxidative stress (Boiteux and Radicella 1999; Kinoshita et al. 2002).

Of the downregulated genes, the decreases in CYP2E1 and NIP3 were confirmed by quantitative RT-PCR. CYP2E1, one of the major constitutive CYPs in mouse liver, is capable of enhancing the toxic effects by metabolizing and activating many toxicologically important substrates, including ethanol, carbon tetrachloride, and acetaminophen (Johnson and Klaassen 2002; Cederbaum 2003). The induction of CYP2E1 by ethanol is the central pathway by which ethanol generates oxidative stress. The mechanisms of the downregulation of this enzyme are currently unclear. However, time-dependent decreases in CYP2E1 might indicate an adaptive reaction in order to reduce excess oxidative stress in the hepatocytes. NIP3 is known as a proapoptotic mitochondrial protein classified in the Bcl-2 family. It functions by heterodimerizing with Bcl-2/Bcl-XL, resulting in apoptotic cell death (Chen et al. 1999). Further, it has been reported that NIP3 is strongly induced in response to hypoxia (Salnikow et al. 2003; Bruick 2000). On the other hand, the downregulation of NIP3 was reported in several proliferative diseases (Chung et al. 2002; Sironen et al. 2002; Sayah et al. 1999). Since NIP3 is considered to be a relatively sensitive marker of apoptosis, its decrease in the present study might reflect the suppression of apoptosis in the FL-treated liver.

It has been reported that FL is hepatotoxic and causes hepatocellular fatty degeneration and focal necrosis in male and female mice (WHO 1998). Taking into account the present results and previous observations (Yoshida et al. 1999; Kashida et al. 2002), the available data indicate that oxidative stress is likely to be involved in multistage hepatocarcinogenicity by FL in mice. We speculate that FL induces DNA damage by oxidative stress, double strand breaks related to topoisomerase II inhibition, or both at the initiation phase (Kashida et al. 2002). At the same time, it is considered that hepatocytes undergo fatty degeneration and necrosis on repeated administration of FL and subsequently proliferate as a regenerative response in the promotion phase. This degeneration–regeneration cycle and overall oxidative stress may result in tumor formation with long-term FL treatment. It has been suggested that iron-induced reactive oxygen species have the capacity to cause not only lipid peroxidation resulting in DNA and protein damage but also activation of a variety of signal transduction pathways, leading to altered expression of genes and cancer development (Toyokuni 1996; Galaris and Evangelou 2002). Beddowes et al. (2003) also suggested that carbon tetrachloride, which is generally regarded as a nongenotoxic carcinogen, induces DNA damage that results in hepatic tumors at high concentration in association with cytotoxicity, oxidative stress, or reactive hyperplasia. Although carcinogens have been conventionally classified as genotoxic or nongenotoxic on the basis of their mutagenic potential, chemicals generating reactive oxygen species that also result in secondary DNA damage are classified as nongenotoxic carcinogens. Generally, genotoxic carcinogens damage DNA directly or indirectly and result in tumor suppressor gene activation and regulation such as cell cycle arrest, apopotosis and DNA repair (Meyer et al. 2003; van Delft et al. 2004). On the other hand, nongenotoxic carcinogens show more varied and complicated responses including the modulation of metabolizing enzymes, the induction of peroxisome proliferation and stimulation of oxidative stress, suppression of apoptosis and the stimulation of regenerative cell growth (Wong and Gill 2002; Kiyosawa et al. 2003; Meyer et al. 2003; van Delft et al. 2004). Taken together, FL is considered to be a nongenotoxic carcinogen that generates reactive oxygen species followed by secondary DNA damage. However, further investigation is needed to determine FL as a nongenotoxic carcinogen, since positive results were obtained in in vivo comet and 2-stage liver initiation assays in mice (Kashida et al. 2002).

In conclusion, in the present study, microarray analysis revealed gene expression changes in the liver of a mouse that was treated with FL. These changes were generally reproducible by the application of real-time RT-PCR. Although the number of genes identified by the present microarray analysis was not numerous when compared with other reports dealing with global gene expression analysis using prototype liver tumor promoters, we could successfully determine specific gene changes in the livers of mice treated with FL. The present results suggest the possibility that responses against oxidative stress may play a major role in the hepatocarcinogenesis by FL in mice. Further mechanistic studies are required to clarify whether altered expression of such oxidative stress-related genes is also responsible for the FL-induced development of preneoplastic and neoplastic lesions in mice liver.

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ORGAN TOXICITY AND MECHANISMS

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Possible involvement of oxidative stress in dicyclanil-induced hepatocarcinogenesis in mice

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Abstract Our previous study suggested the possibilities that dicyclanil (DC), a nongenotoxic carcinogen, produces oxidative stress in the liver of the two-stage hepatocarcinogenesis model of mice and the stress induced probably causes secondary oxidative DNA damage. However, clear evidences demonstrating the relationship between DC-induced hepatocarcinogenesis, oxidative stress, and oxidative DNA damage have not been obtained. To clarify the relationship, further investigations were performed in the liver of the partially hepatectomized (PH) mice maintained on diet containing 1,500 ppm of DC for 13 and 26 weeks after intraperitoneal injection of dimethylnitrosamine (DMN). Significant increases in mRNA expressions of some metabolism- and oxidative stress-related genes with a formation of γ-glutamyltranspeptidase (GGT) positive foci were observed in the DMN + DC + PH group by the treatment of DC for 13 and 26 weeks. The levels of 8-hydroxy-deoxyguanosine (8-OHdG) in the liver DNA also significantly increased in mice of the DMN + DC + PH group at weeks 13 and 26 and mice given DC alone for 26 weeks. The in vitro measurement of reactive oxygen species (ROS) generation from the mouse liver microsomes showed a significant increase of ROS production in the presence of DC. These results suggest that DC induces oxidative stress which is probably derived from its metabolic pathway, partly, and support our previous speculation that oxidative stress plays one of the important roles in the DC-induced hepatocarcinogenesis in mice.

Keywords Dicyclanil · Hepatocarcinogenesis · Oxidative stress · Nongenotoxic carcinogen · Mouse

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Introduction

Dicyclanil (DC), 4,6-diamino-2-cyclopropylamino-pyrimidine-5-carbonitrile (CAS number 112636-83-6), is a pyrimidine-derived insect growth regulator that inhibits the molting and development of insects and is used in the field of veterinary medicine to prevent myiasis (fly-strike) in sheep. As a result, minute amounts of the parent drug and its metabolites are occasionally detected as residues in the edible tissues of sheep such as the muscle, liver and fat (WHO 2000). In the 54th meeting of the Joint Food and Agriculture Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA), it was reported that the incidence of hepatocellular carcinomas increased in the mice that were fed diet containing 1,500 ppm of DC for 18 months. Negative results were obtained from the in vivo and in vitro genotoxicity studies of DC. Based on these results, the 54th meeting of JECFA concluded that DC is a nongenotoxic carcinogen (WHO 2000). However, detailed molecular analyses of the mechanism of the DC-induced hepatocarcinogenesis in mice had not been performed.

Recently, to clarify the possible mechanism of DC-induced hepatocarcinogenesis at the gene and molecular levels, we performed two different experiments consisting of a 2-week feeding study of DC in mice and a short-term study using dimethylnitrosamine (DMN)-initiated two-stage hepatocarcinogenesis model of mice having partial hepatectomy in which DC was administered to mice 7 weeks. The results showed that

the mRNA expression was up-regulated in several metabolism- and/or oxidative stress-related genes, such as cytochrome P450 1A (CYP1A), thioredoxin reductase 1 (TXNRD1), and NADPH P450 reductase (POR) in the liver of mice that were fed diet containing 1,500 ppm DC for 2 weeks. In addition to the genes belonging to the same category, fluctuations in the mRNA expression of DNA damage/repair genes, such as 8-oxoguanine DNA glycosylase (OGG1) and growth arrest/DNA-damage-inducible alpha (GADD45A), were observed in the liver of the two-stage hepatocarcinogenesis model of mice given DC at the same doses for 7 weeks. Furthermore, the number of γ-glutamyltranspeptidase (GGT) positive cells, a preneoplastic marker in the liver of mice, significantly increased in the liver of mice administered DC. These results suggest the possibility that secondary, but not direct DNA damage via oxidative stress is probably involved in the mechanism of DC-induced hepatocarcinogenesis in mice (Moto et al. 2005). However, investigations on the measurement of 8-hydroxy-deoxyguanosine (8-OHdG) in the liver DNA, a representative marker of DNA damage (Dybdahl et al. 2003; Kinoshita et al. 2002; Nakae et al. 1997; Umemura et al. 1998; Yoshida et al. 1999), and the measurement of oxidative stress-inducing substances have not yet been performed to elucidate the relationship between DC, oxidative stress, and DNA damage.

Oxidative stress has been implicated in the etiology of a variety of pathological lesions, including cancer (Fortini et al. 2003). It is generally recognized that oxidative stress occurs in cells when the equilibrium between prooxidant and antioxidant species favors the prooxidant stress; this occurs due to the reactive oxygen species (ROS) generated by exogenous and endogenous factors, cellular metabolism, etc. (Grishko et al. 2005). For example, it has already been reported that dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) and polychlorinated biphenyls, which act as hepatocarcinogens in rodents, induced CYP1A1/2 and 1B1 and increased the ROS production in the rodent liver, and that ROS may play an important role in a variety of diseases (Coumoul et al. 2001; Hatanaka et al. 2001; Santostefano et al. 1999; Shimada et al. 1996; Van Birgelen et al. 1995; Walker et al. 1999). In addition, it is assumed that carcinogenic potentials of polycyclic aromatic hydrocarbons have a relationship with the potencies with which these compounds induce the expression of CYP1A1 and CYP1B1, and that these CYPs have a relationship with carcinogenesis in mice (Shimada et al. 2002). Therefore, it is postulated that the assumed oxidative stress induced by DC is generated via the metabolic pathway because the results of our previous study showed up-regulation of the metabolism-related gene mRNA expression including CYPs (Moto et al. 2005).

The aim of the present study was to investigate the relationship between DC, oxidative stress, oxidative DNA damage, and the induction of preneoplastic lesions. By using the two-stage hepatocarcinogenesis model of mice with partial hepatectomy, we performed histopathological examinations, mRNA expression analyses of genes related to the metabolism or oxidative stress, and measurements of the level of 8-OHdG in the liver DNA which is one of the marker of oxidative DNA damage. Additionally, to measure the ROS production through the metabolic pathway of DC, microsomal ROS products were measured in in vitro.

Materials and methods

Animals and chemicals

Four-week-old male ICR mice (for in vivo experiment) or nine-week-old male ICR mice (for in vitro experiment) (Japan SLC, Inc., Hamamatsu, Japan) were maintained on powdered basal diet (MF; Oriental Yeast, Co., Tokyo, Japan) and tap water until they were 5 or 10 weeks of age, respectively; the study was started at these ages. The mice were housed in polycarbonate cages with paper beddings and were maintained under standard conditions (room temperature 22±2°C; relative humidity 55±5%; light/dark cycle 12 h). The animal care and experiments were carried out in accordance with the Guide for Animal Experimentation of the Tokyo University of Agriculture and Technology.

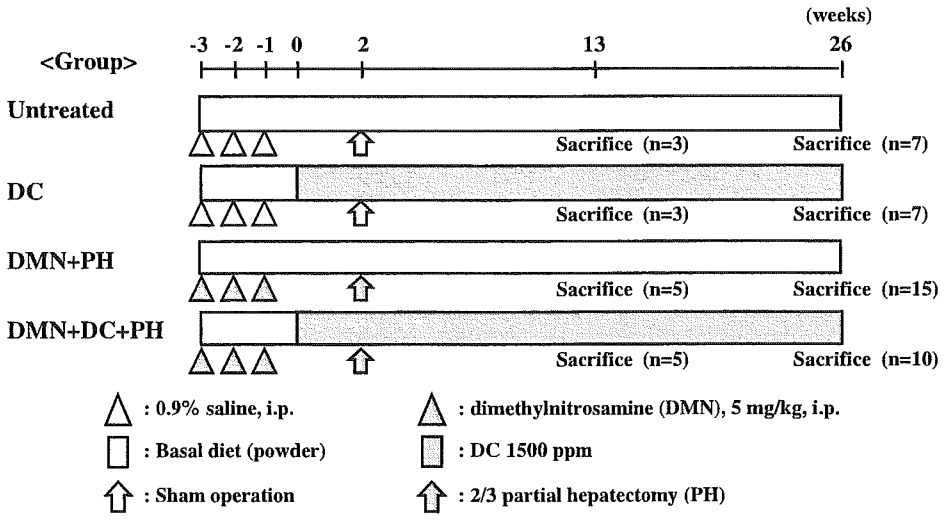
Dicyclanil was kindly provided from Novartis Animal Health Inc. (Basel, Switzerland) for the experiment. *N*-dimethylnitrosamine (DMN) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and dimethylsulfoxide (DMSO) was obtained from Wako Pure Chemicals, Inc. (Osaka, Japan).

Experimental design (in vivo)

A two-stage liver carcinogenesis model of mice was employed (Fig. 1). To initiate hepatocarcinogenesis, DMN at a dose of 0 (vehicle, 0.9% saline) or 5 mg/kg body weight was intraperitoneally injected into the animals for three times (once a week). One week after the final injection, the mice were fed with the powdered diet containing DC at a concentration of 0 or 1,500 ppm for 13 and 26 weeks. To enhance the hepatocellular proliferation in the liver, these mice were subjected to two-thirds partial hepatectomy (Tsuda et al. 1979) or a sham operation at experimental week 5. For sampling of the liver at weeks 13 and 26, the survivors were killed under anesthesia with ether by exsanguination from the abdominal aorta.

At necropsy, tissue samples for all analyses were collected from the right lateral lobe of the liver. One section obtained from each liver sample was fixed with natural-buffered formalin for the histochemical analysis, and another section was embedded in the OCT compound (Tissue-Tek; Miles Inc., Elkhart, USA) to freeze it for evaluation of the γ-glutamyltransferase (GGT) positive foci—a marker of preneoplastic foci—in mice

Fig. 1 Experimental design



livers (Cater et al. 1985). The remaining liver samples were weighed, frozen in liquid nitrogen, and stored at −80°C until subsequent gene expression analyses and the measurements of 8-OHdG.

Histological and histochemical examination

Formalin-fixed liver tissues were embedded in paraffin, sectioned and stained with hematoxylin and eosin (H–E) for histological examinations. The histochemical staining of GGT was accomplished as described previously (Moto et al. 2005). In GGT evaluation, the number and area of positive focus (> 0.05 mm) were calculated from the number or area from the total area of the tissue sections by measurement with a computer-assisted image analyzer (NIH image).

mRNA expressions in the liver

The total RNA from the liver was isolated 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 the quantitative real-time RT-PCR with SYBR Green using ABI Prism 7000 Sequence Detection System (Applied Biosystems, CA, USA). The PCR reaction was repeated twice in triplicate for each gene. The PCR primers for the genes of CYP1A1 (accession number NM_009992), P450 oxidoreductase (POR, accession number NM_008898), TXNRD1 (accession number NM_015762), superoxide dismutase 1 (SOD1, accession number XM_128337), and OGG1 (accession number NM_010957) in the present study were prepared as reported previously (Moto et al. 2005). The expression levels of each gene were corrected based on the expression level of β-actin in the same cDNA sample.

Measurement of 8-OHdG in the liver DNA

The measurement of 8-OHdG levels in liver DNA was performed according to the method of Nakae et al. (1995). Nuclear DNA was extracted with a DNA Extracter WB Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan). During the extract operation, an iron chelator was used to avoid oxidation of DNA (Kasai 2002). The DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase and levels of 8-OHdG (8-oxodG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II; ESA Biosciences, Inc., MA, USA).

Measurement of microsomal ROS production of the liver (in vitro)

The nonfluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes, Inc., OR, USA) has been used as a sensitive intracellular probe for detecting ROS formation during cellular metabolism, and in the presence of ROS it is oxidized to a highly fluorescent DCF (LeBel and Bondy 1990; Serron et al. 2000; Szejda et al. 1984). Assays were performed in our study by using this probe.

The mice liver tissue was homogenized in ice-cold 10 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose. Hepatic microsomes obtained from the homogenates were routinely purified by differential centrifugation method (Sequeira et al. 1992), suspended in 10 mM Tris–HCl (pH 7.4), and stored at −80°C until evaluation. The measurement of ROS was carried out by partially modifying the method of Dumple and Yamamoto (1995). Microsomes (0.2 mg protein) were incubated in the dark at 37°C in 40 mM Tris buffer (pH 7.4) and DCFH-DA (5 μM). At the end of the incubation period, DC (0.01–3 mM), H_2O_2 (1 mM; positive

control) or the vehicle (6% DMSO/40 mM Tris–HCl) and 0.6 mM NADPH were added and incubated at 37°C for 30 min under dark condition. The rate of ROS formation as a fluorescent product was measured by a microplate reader (excitation 485 nm; emission 528 nm). These reactions were repeated three times in duplicate. The data was normalized to control values and the control was assigned a value of 100%.

Statistical evaluation

Statistical analyses were performed using statistical software (JMP 4.0.5J; SAS Institute, Inc., NC, USA). The data obtained from in vivo experiments were compared between two corresponding groups by using the Student's *t* test after the one-way ANOVA. Additionally, Dunnett's test was used to isolate the group(s) that differed significantly from the untreated group. The data from the in vitro experiments were also compared using Dunnett's test. A *P* value of less than 0.05 was considered to be statistically significant.

Results

General observations and histopathological findings in the liver

During the experimental period, neither death nor treatment-related clinical signs were observed in any of the groups. However, significant reduction in body weight gain as well as a slight decrease in food consumption was found in the DC and DMN+ DC + PH groups. Macroscopically, all mice of these two groups killed at weeks 13 and 26 showed discoloration of the liver (data not shown).

On histopathological examinations, there were no remarkable changes in the liver of the untreated and DMN groups at weeks 13 and 26. On the other hand, all mice of the DC and DMN + DC + PH groups at weeks 13 and 26 had centrilobular hypertrophy of hepatocytes. Slight and moderate deposition of lipofuscin was observed in the DC and DMN + DC + PH groups, respectively. Other findings included small necrotic foci in both of these groups treated with DC (Fig. 2).

On histochemical stainings for GGT, no GGT-positive reaction on hepatocytes was observed in the untreated group at weeks 13 and 26. In the DMN + PH group, there was no formation of GGT-positive focus at weeks 13 and 26. On the other hand, the DC group at week 26 showed the formation of GGT-positive foci in the liver, although such a reaction at week 13 was detected at only single cell level. The number and area of GGT-positive foci significantly increased only in the DMN + DC + PH group in comparison with that in the untreated or DMN + PH group at week 13. This group showed the highest value of them at week 26, but the value was not significantly different (Fig. 3, Table 1).

mRNA expression in the liver

Findings of the mRNA expression levels of CYP1A1, POR, TXNRD1, SOD1, and OGG1 in the liver are shown in Fig. 4. The expression levels of almost all these mRNAs significantly increased in the DC and DMN + DC + PH groups as compared to the untreated or DMN + PH group, and their highest levels were observed in the DMN + DC + PH group. However, the time-dependent increases of all these genes were not evident in these two groups.

Formation of 8-OHdG in the liver DNA

The data of 8-OHdG are shown in Fig. 5. On HPLC analysis, the levels of 8-OHdG of all groups at week 26 were approximately twice as high as those at week 13, and the time-dependent increasing tendency was found. In the DMN + DC + PH group, the level of 8-OHdG significantly increased in comparison with that of the DMN + PH group and indicated the highest level at weeks 13 and 26. In addition to this group, the DC group also showed significant increases in 8-OHdG as compared to the untreated group at week 26.

Generation of ROS

As shown in Fig. 6, in the presence of H_2O_2 as the positive control, significant increases were found at 0.1 mM of H_2O_2, and the maximum percentage of increase (approximately 120%) was observed at 1 mM in this condition. In the case of DC, a concentration-dependent increase in ROS production was observed, a significant increase being observed at 0.3 mM or greater concentration of DC. At high concentrations of DC (>1 mM), the percentage of increase was higher than that of H_2O_2. On the other hand, the ROS production was depressed to about 40% of the vehicle control (0 mM) because of a high concentration of DMSO.

Discussion

Oxidative stress is believed to be a result of ROS generation occurring in cells due to exogenous factors or cellular metabolism (Grishko et al. 2005). For example, the active oxygen species can be generated by uncoupling of the microsomal monooxygenase reactions. Superoxide anion and H_2O_2 can be formed during P450 turnover. Superoxide can be produced during the autooxidation of the oxycytochrome P450 complex (Kuthan and Ullrich 1982; Sligar et al. 1974). In the

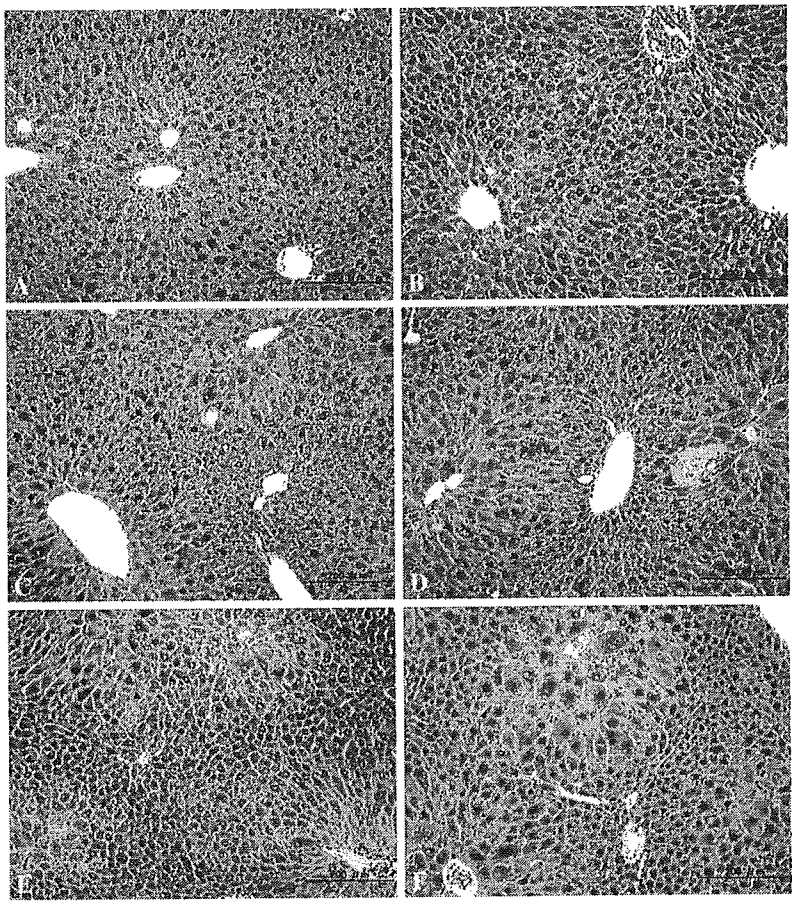


Fig. 2 H–E staining in the liver of hepatectomized mice treated with DC for 13 and 26 weeks after DMN initiation. No remarkable change is observed in the untreated (**a**) and DMN + PH (**b**) group at week 26. Slight centrilobular hypertrophy and some necrotic cells are observed in the DC group at weeks 13 (**c**) and 26 (**d**). Slight to moderate centrilobular hypertrophy and some necrotic cells are observed in the DMN + DC + PH group in weeks 13 (**e**) and 26 (**f**)

present study, the production of ROS in microsomes significantly increased when DC was present at a concentration of 0.3 mM or more in vitro. Although we have no toxicokinetic data such as accurate blood concentrations and systemic distributions of DC achieved by dietary administration in mice, it was previously reported that a single oral administration of 50 mg/kg bw of DC in rats would result in a maximum concentration of 0.1 mM DC (20 μg/ml) in plasma (WHO 2000). The dose level of DC used in the present study is calculated to be equivalent to approximately 150–200 mg/kg bw of DC, and is 3–4 fold higher than that administered to rats in the earlier study. Therefore, it can be expected that since DC was administered to mice by mixing with the diet, the mice are exposed to a limited amount of DC, which is able to produce ROS in hepatocytes for an extended period and approximately for the whole day. Actually, the expressions of mRNAs of metabolism- or oxidative stress-related genes, such as CYP1A1, POR, TXNRD1, and SOD1, in both the DC-treated groups were also significantly up-regulated. These data support our speculation that the oxidative stress was probably induced by ROS generated via the metabolic pathway of DC. However, it is well known that other cellular sources such as mitochondria and peroxisomes also have a potential to generate ROS by various stimulation. Therefore, although measurements of ROS production of these organelles were not performed in our study, it should be considered that the ROS generated in hepatocytes of the DC-treated group are derived from these organelles as well as the metabolic pathway of DC.

Reactive oxygen species can interact with multiple macromolecules, including lipids, nucleic acids, and proteins (Grishko et al. 2005). 8-OHdG, a marker of oxidative DNA damage, is potentially involved in the carcinogenesis in various experimental models, and is known as one of the causes for DNA point mutations such as G–T transversion (Kasai 1997; Nakae et al. 1997; Shibutani et al. 1991). Significant induction and steady elevation of 8-OHdG are considered to play an important role in chemically induced carcinogenesis (Shibutani et al. 1991). In the present study, a significant increase of 8-OHdG level was observed in the DMN + DC + PH and DC alone groups, which showed the formation of GGT-positive foci. These results suggest that the long-term treatment of DC at this dose has the potential to cause oxidative DNA damage. It has been reported that 8-OHdG DNA adducts are repaired by an enzyme OGG1, and the inactivation of OGG1 mRNA in yeast and mice leads to an elevated frequency of spontaneous mutation (Dybdahl et al. 2003; Shinmura and Yokota 2001). In fact, Kinoshita

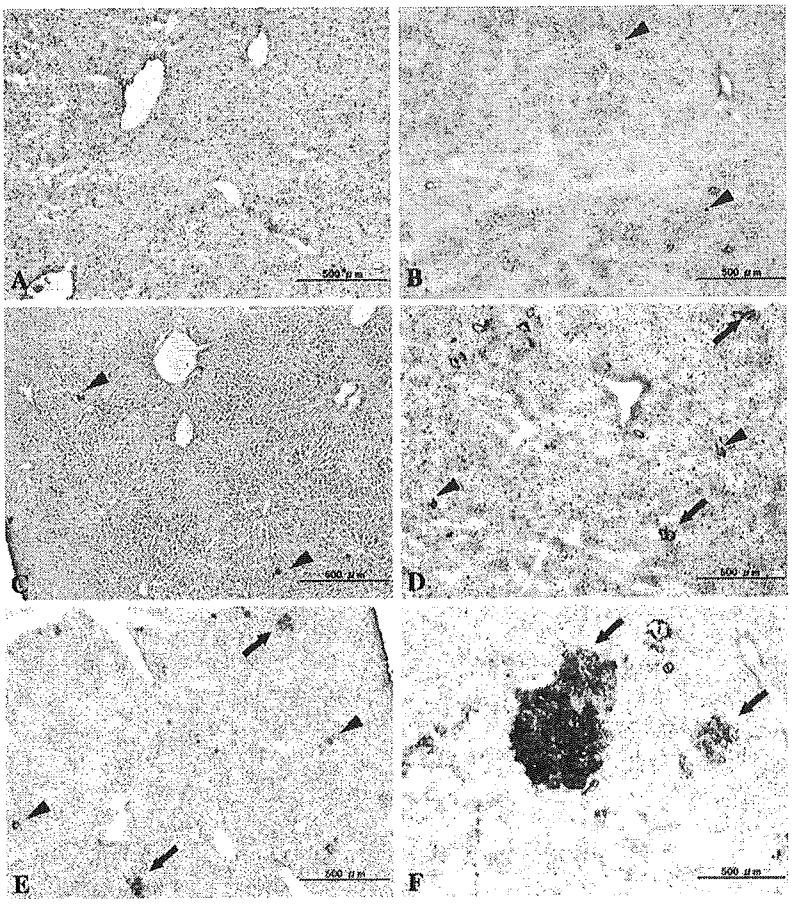


Fig. 3 Histochemical staining of GGT in the liver of hepatectomized mice treated with DC for 13 and 26 weeks after DMN initiation. No positive reaction is observed in the untreated group at week 26 (**a**). GGT-positive reactions at a single cell level (*arrow head*) are detected in the DMN + PH at week 26 (**b**) and the DC group at week 13 (**c**). In addition to GGT-positive cells, the formation of GGT-positive foci (*arrow*) is observed in the DC group at week 26 (**d**). The formation of GGT-positive foci is evident in the DMN + DC + PH group at weeks 13 (**e**) and 26 (**f**)

et al. (2002) reported that the depression of a temporal increase of 8-OHdG induced by a single administration of phenobarbital was due to the reaction following the increase in OGG1. The mRNA expression analyses of the present study showed that the expressions of OGG1 mRNA significantly elevated in both of the groups treated with DC, and the highest level of expression was observed in the DMN + DC + PH group that showed a significant elevation of 8-OHdG. However, a time-dependent remarkable increase of the expression level of OGG1 mRNA was not observed in this group. Therefore, it can be considered that the formation of 8-OHdG in our study was the outcome of the accumulation of imbalance between the DNA repair and DNA damages resulting from the excessive oxidative stress caused by the prolonged DC treatment, although the response of OGG1 mRNA expression was normal.

In many in vivo and in vitro reports on the elevations of 8-OHdG level in mammalian cells exposed to genotoxic or nongenotoxic carcinogens, it is speculated that the formation of 8-OHdG may be one of the causes of point mutations that contribute to the activation of oncogenes or the inactivation of suppressor genes leading to tumorigenesis (Cheng et al. 1992; Kamiya

Table 1 Quantitative data of GGT positive cells and foci in the liver of hepatectomized mice treated with DC for 13 weeks after DMN initiation

Group	Number of animals	GGT positive focus (> 0.05 mm)	
		Number (No./cm^2)	Total area (mm^2/cm^2)
13 weeks			
Untreated	3	0[a]	0
DC	3	0	0
DMN + PH	5	0	0
DMN + DC + PH	5	13.89 ± 11.22*,†	0.072 ± 0.059*,†
26 weeks			
Untreated	7	0	0
DC	7	1.19 ± 3.15	0.004 ± 0.012
DMN + PH	15	0	0
DMN + DC + PH	10	7.32 ± 13.41	0.234 ± 0.640

* Significantly different from the DMN + PH group at $P < 0.05$ (t test)
† Significantly different from the untreated group at $P < 0.05$ (Dunnett's test)
[a]Data are mean ± SD

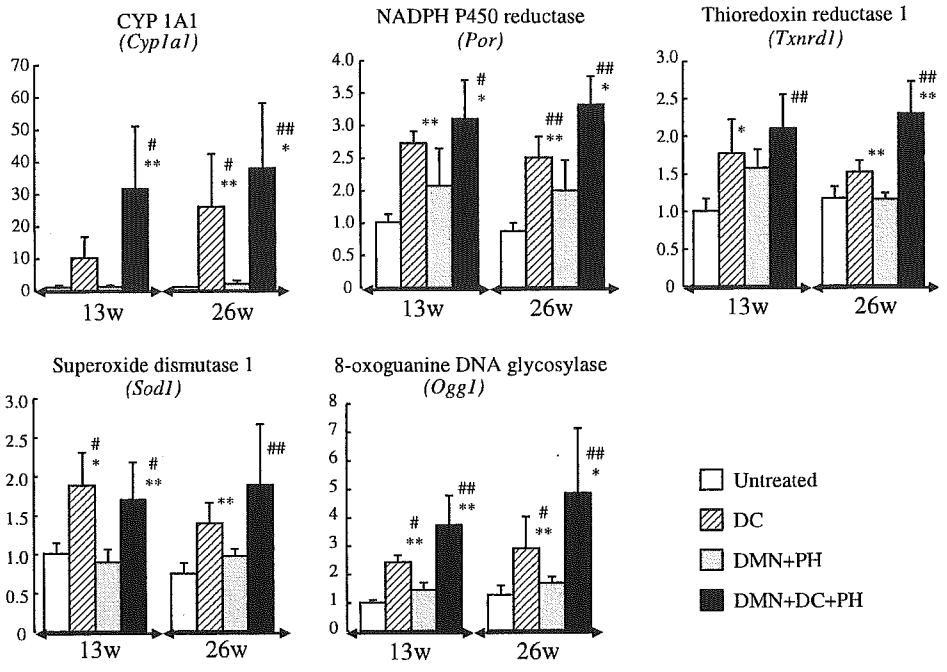


Fig. 4 mRNA expression in the liver of hepatectomized mice treated with DC for 13 and 26 weeks after DMN initiation. *Columns* represent the mean ± SD. * or ** represents the significant difference from the untreated group or DMN + PH group at $P < 0.05$ or 0.01, respectively (t test/ ANOVA). # or ## represents the significant difference from the untreated group at $P < 0.05$ or 0.01, respectively (Dunnett's test)

et al. 1992; Umemura et al. 1998). Klaunig et al. (1995, 1998) reported the possibility that subchronic exposure of ROS resulted in the modulation of the expressions of genes such as growth regulatory genes and cell communication genes which are associated with the hepatocarcinogenesis caused by oxidative stress in mice. However, it is unclear whether the gene mutations and modulations that are induced by the treatment with DC or by DNA damages originating from the oxidative stress result in the formation of DC-induced hepatocellular tumors. Recently, *gpt* delta transgenic mice and rats in which it is possible to detect mutations, including point mutations and long deletions of genes in in vivo, have been developed, and many data on carcinogens have been accumulated (Kanki et al. 2005; Masumura et al. 1999; Nishikawa et al. 2001; Nohmi et al. 1996). The study using *gpt* transgenic mice is useful to clarify the location and the amount of mutations that occur when the 8-OHdG formation is detected in the DNA of the liver of DC-treated mice.

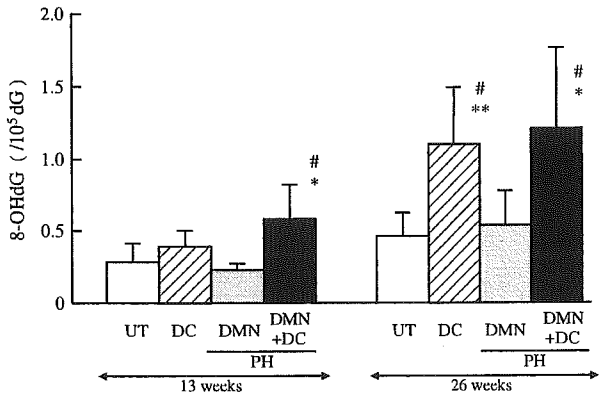


Fig. 5 Levels of 8-OHdG in the liver DNA of hepatectomized mice treated with DC for 13 and 26 weeks after DMN initiation. *Columns* represent mean ± SD. *, ** represents a significant difference from the each control (untreated or DMN + PH) group at $P < 0.05$ or 0.01 (t test). # represents a significant difference from the untreated group at $P < 0.05$ (Dunnett's test)

In the present study, during the stage that showed formation of preneoplastic foci, remarkable deposition of lipofuscin was histologically observed in the liver of the DMN + DC + PH group. Lipofuscin is one of the age-associated pigments that have been regarded as cellular debris derived from lipid peroxides formed by oxidative stress, including free radicals (Tsuchida et al. 1987). Therefore, it can be inferred that the increase in the oxidative markers level was induced by oxidative stress, suggesting the high possibility that treatment with DC at 1,500 ppm induces oxidative stress. Furthermore, the formation of GGT-positive foci was observed in the DC alone group at week 26 as well as the DMN + DC + PH group at weeks 13 and 26. In our previous 7-week study with DC, GGT-positive reaction was also observed as a single cell but not as a focus in the DC alone group, while a significant increase of such a focus was found in the liver of mice treated with DC after initiation treatment of DMN (Moto et al. 2005). These findings may suggest that

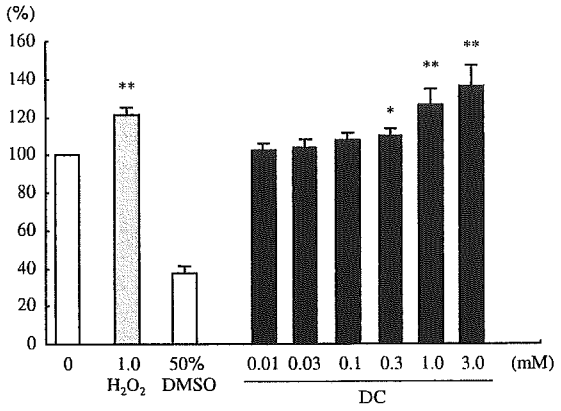


Fig. 6 Effects of DC on hepatic-microsomal ROS production. Microsomes were prepared from the liver of male mice and the formation of DCF as a reactive marker of ROS was measured at excitation 485 nm/emission 528 nm. *Columns* represent the relative value of fluorescent strength estimated at 100% in the absence of DC (0 mM). Values are mean ± SD of three times assays performed in duplicate. * or ** represents significant differences from the vehicle (0 mM) at $P < 0.05$ or 0.01, respectively (Dunnett's test)

cellular altered foci, which are probably derived from gene mutations induced by DNA damages attributable to the oxidative stress, are developed by the treatment of DC. However, the possibility that cellular altered foci originating from spontaneous gene mutations are grown up by the prolonged stimulation of cell proliferation due to the tumor promoting effect of DC cannot be ruled out. Further studies are needed to clarify why such altered foci were induced in the DC alone group by the prolonged treatment of DC for more than 26 weeks.

In conclusion, our data in the present study indicate that DC has potentials to generate ROS via its metabolic pathway and to induce oxidative stress including oxidative DNA damage. These results suggest the possibility that DNA damage originating from the oxidative stress induced by DC results in the induction of hepatocellular tumors in mice, but a long-term treatment with high doses of DC is necessary for the induction of oxidative DNA damage via oxidative stress. In general, it is well recognized that genotoxic carcinogens have direct effects on DNA or chromosomes and there is no threshold on these effects. However, it has been reported that genotoxicity of DC was negative in in vitro studies in the presence and absence of S9 mix (WHO 2000) and a single oral administration of DC did not induce any in vivo DNA damage in the liver of mice (Moto et al. 2003). In addition, the enhancement of hepatocarcinogenesis in a carcinogenicity study using mice was observed in the high dose group (> 500 ppm DC in diet) but not in middle and low dose groups (WHO 2000). Therefore, it can be considered that DC is not a genotoxic carcinogen but a nongenotoxic carcinogen, because there is no evidence suggestive of a direct DNA damage of DC but DNA damage secondary to the oxidative stress induced by DC. However, we have to reconsider the appropriateness of the terminology that such a carcinogen inducing secondary DNA damage is categorized into one of the nongenotoxic carcinogens. Further investigations are now in progress to clarify whether the treatment with DC for a prolonged period results in DNA damage via oxidative stresses and, finally, the induction of hepatocellular tumors in mice.

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Methacarn fixation—effects of tissue processing and storage conditions on detection of mRNAs and proteins in paraffin-embedded tissues

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Abstract

In this study, we examined suitable conditions for tissue fixation with methacarn and ethanol dehydration and storage of paraffin-embedded tissues (PETs) on gene expression analysis. With fixation and dehydration of rat liver tissues for up to 16 h (overnight) and 1 week, respectively, at 4 °C, integrity of extracted total RNAs and polypeptides did not vary, the former integrity being constantly lower than that with unfixed frozen tissue, while protein yield was slightly reduced with increasing dehydration. Retained expression levels of mRNAs and proteins were mostly unaffected by the period of fixation but slightly fluctuated with the length of dehydration. When PETs were stored for up to 12 months, integrity of both total RNAs and polypeptides was retained at 4 °C but reduced at room temperature. Reduced expression levels of mRNAs and proteins were also noted by storage at room temperature after 12 and 3 months, respectively. However, neither tissue processing nor storage affected variability in either mRNA or protein levels among samples. Thus, the results suggest that, for gene expression analysis, tissues can be fixed with methacarn and dehydrated for at least 1 day and 1 week, respectively, and PETs can be stored for at least 12 months, but a temperature of 4 °C is preferable.

Keywords: Methacarn; Molecular integrity; Paraffin-embedded tissue; Expression analysis

Molecular analysis of pathological specimens has provided insights into mechanisms underlying disease, facilitating development of diagnostic/therapeutic agents. To elucidate pathological processes in target cells involving alterations in cellular functions, application of microdissection techniques allows high performance even with a complex tissue architecture [1–3]. For molecular analysis, tissue samples should preferably be kept biochemically unmodified, and therefore unfixed frozen tissue (UFT)[2] has become the gold standard for analysis by microdissection and microbiochemical techniques. However, as compared with paraffin-embedded tissues (PETs), UFTs are inconvenient with regard to storage and skills required for preparation and subsequent microdissection itself. Therefore tissue embedding after fixation is preferable if extraction of molecules with high quality and yield can be guaranteed.

We have recently found that methacarn, an organic-solvent-based noncross-linking fixative [4], retains advantages for analysis of expression levels of mRNAs and proteins and for analysis of mutations of target genes in microdissected tissue samples from PETs, with performance close to that possible with UFTs [5–10]. Another group confirmed the suitability of methacarn among a series of fixatives for differential mRNA expression analysis using microdissected PET specimens [11]. For these earlier studies, tissue fixation with methacarn and ethanol dehydration was performed at 4 °C, default time settings for each step were

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[2] *Abbreviations used:* UFT, unfixed frozen tissue; PET, paraffin-embedded tissue; PB, sodium phenobarbital; PCNA, proliferating cell nuclear antigen; EGFR, epidermal growth factor receptor.

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