別添 4

厚生労働科学研究費補助金（食品の安心・安全確保推進研究事業）
分担研究報告書

動物用医薬品等に関する畜水産食品の安全性確保に係る研究
－ 牛脊柱からの背根神経節の除去に関する研究－

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研究要旨　BSE（牛海綿状脳症）の特定危険部位である牛の背根神経節について、その完全除去がと畜場において可能かどうかを除去率の算定により検討した。また同時に、牛の品種別、牡牝別、及び月齢別に除去率に差があるか否かも検討した。今年度の調査結果では、平均して全背根神経節の８８％の除去がと畜場で可能となっているが、１００％の除去は現時点の技術では依然として困難であるといわざるを得ない。と畜場での背根神経節の完全除去を達成するためには、今後さらなる技術の改良が必要である。また、牛の品種別、牡牝別、及び月齢別の除去率に差は認められなかった。

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A. 研究目的

牛の背根（脊髄）神経節が BSE の特定危険部位に指定されて以来、背根神経節が脊柱内にあり、その脊柱からの分離が困難なことから、本来安全な脊柱も同時に廃棄されているのが現状である。本研究では、と畜場において背根神経節を脊柱から完全に分離する手法を確立し、牛の脊柱という資源の活用をはかることを目的としている。と畜場において脊髄除去後に脊柱に残る硬膜と付随する脊髄神経を、背根神経節ができるだけ脊柱に残らないようにナイフで引き剥がしたものから、背根神経節がどの程度硬膜に残存しているかを計測することによって、脊柱から背根神経節がどの程度除去されているか（除去率）を調べた。さらに、品種別、牡牝別、及び月齢別の除去率について比較し、除去率に差があるか否かについて検討を加えた。

B. 研究方法

１．牛の脊柱からの背根神経節の除去

牛の背根神経節は１頭あたり、頚椎部８対１６個、胸椎部１３対２６個、腰椎部６対１２個、及び仙骨部５対１０個の計３２対６４個（背割り後の枝肉［半頭分］では３２個）である（尾骨部はこれに含まれていない）。ここでは、第１頚神経から第５仙骨神経までの脊髄神経の背根神経節の、脊柱からの除去率を調べた。

硬膜周辺から脂肪を取り除いて、付随する背根神経節を明らかにし、頚椎部（C）、胸椎部（T）、腰椎部（L）、及び仙骨部（S）について、脊柱からの除去率を算出した。算出に用いた牛の硬膜は２００７年３月から２００８年２月までの計２７０検体である。算出方法は、背根神経節の全体が付随しているものを１とし、背根神経節の大部分が付随しているものを2/3、背根神経節の半分程度が付随しているものを1/2、背根神経節の一部が付随しているものを1/3、背根神経節が全く付随していないものを０として合計し、Ｃ１からＳ５までの背根神経節の数３２（枝肉当たり：半頭分）に対する割合を求めた。背根神経節の大きさの判定は、目視によるから必ずしも厳密なものではなく、また、約１

/3個分が除去率の百分率の１％分に相当する。したがって除去率は小数点以下の数値に意味はないと考え、有効数字は１の位までとした。

２．牛の品種別、牡牝別、及び月齢別の脊柱からの背根神経節の除去率

１．と同じ試料、方法を用いて、牛の品種別、牡牝別、及び月齢別の除去率を比較検討した。牛の品種別、及び牡牝別では、「ホルスタイン去勢牡」、「和牛（黒毛和種）去勢牡」、「交雑種（黒毛♂Ｘホルスタイン♀）牝」、及び「交雑種去勢牡」の４グループに区分した。「和牛牝」については、サンプル数が極端に少ないことから、評価の対象から除外した。また、月齢では、「ー１９ヶ月齢」、「２０ー２４ヶ月齢」、「２５ー２９ヶ月齢」、及び「３０ヶ月齢以上」に区分してそれぞれ比較した。

（倫理面への配慮）

本研究は動物実験ではなく、と畜場の協力を得て、作業過程で除去された牛の硬膜と背根神経節を研究材料として使用していることから、倫理面への配慮は特に必要としないと考える。

C. 研究結果と D. 考察

１．牛の脊柱からの背根神経節の除去

各月の除去率は文末、及び付表１ー１０に示した。なお、付表１ー１０（１２月、１月はデータなし）において、○は背根神経節の全体（１）が付随しているものを表し、□は背根神経節の大部分（2/3）が付随しているものを、△は背根神経節の半分程度（1/2）が付随しているものを、▲は背根神経節の一部（1/3）が付随しているものを、×は背根神経節が全く付随していない（０）ものを表す。表の最も左の欄の数字は検体番号を表す。また、除去率の月別推移を図１に、各神経節毎の除去率を図２に、牛の品種別、及び牡牝別の除去率の月別推移を図３に、牛の品種別、及び牡牝別の年間を通しての除去率を図４に示した。

背根神経節の脊柱からの除去率は、２００７年３月から２００８年２月の間で平均８８％であった（図１）。依然として第４、５，６腰神経、及び第１，２，３仙骨神経の背根神経節の除去率の低さ（３６ー６９％）が顕著であった。また、第１１胸神経の背根神経節の除去率も他の部位と比べると低い値（７７％）を示した（図２）。上記の７部位を除いた残りの２５部位は９０％以上の除去率を示した。

第１頚神経から第１０胸神経の背根神経節が除去されやすいのは、硬膜から背根神経節までの背根の長さが短いことと、背根神経節がある程度大きいことに起因すると思われる。

第４腰神経ー第３仙骨神経の背根神経節が除去されにくい点については依然として不明である。なお、第１仙骨神経とその前後の脊髄神経で背根神経節は大きいことから、第４腰神経ー第３仙骨神経の背根神経節が除去されにくいことと背根神経節の大きさとは無関係だと考えられる。

２．牛の品種別、牡牝別、及び月齢別の脊柱からの背根神経節の除去率

牛の品種別、及び牡牝別に脊柱からの背根神経節の除去率を調べた（図３、４）。「ホルスタイン去勢牡」、「和牛去勢牡」、「交雑種牝」、「交雑種去勢牡」のいずれの除去率も、概ね85%から90%の間にあり、わずかではあるが、和牛去勢牡の除去率が高いものの、明瞭な差は認められなかった。各グループ別の除去率は月毎にも調べた

が、どのグループも80%から95%の間で推移した。全体として、各グループ間で除去率に差は生じなかった。

牛の月齢については、19 ヶ月齢以下の牛はほとんどがホルスタインの去勢牡であり、和牛は大部分が25～29ヶ月齢、30ヶ月齢～の2つのカテゴリーに属し、交雑種は牝、去勢牡とも20～24ヶ月、25～29ヶ月の 2 通りに分類されるのがほとんどであった。各グループ間で除去率に差がないことから、当然ながら月齢による除去率も差は認められなかった。

E. 結論

1．では２００７年３月から２００８年２月までに得られた試料について、背根神経節の脊柱からの除去率を調べたところ、平均８８%であり、前年度に比べ、わずかではあるが向上している。除去率の極端に低い第４腰神経ー第３仙骨神経の背根神経節、それに次いで除去率の低い第１１胸神経の背根神経節において、何故除去が難しいのか、その理由は不明であり、これらの部位の除去率が向上しない限り、完全除去達成は困難である。

また、牛の品種別、牡牝別、及び月齢別の脊柱からの背根神経節の除去率を比較検討したが、いずれも除去率に差は見られなかった。

牛の脊柱をゼラチンや牛エキスの原材料として利用するためには、と畜場において背根神経節が完全に脊柱から分離されなければならないが、現在までのところ、除去率はその状況には達していない。今後、さらなる除去技術の改良が必要である。

F. 健康危険情報

なし

G. 研究発表

なし

H. 知的財産権の出願・登録状況

なし

２００７年３月分　　４６検体（付表１）
最大　９６%
最小　７５%
平均　８８%　　標準偏差　５%

２００７年４月分　　４３検体（付表２）
最大　９８%
最小　７６%
平均　８８%　　標準偏差　５%

２００７年５月分　　２２検体（付表３）
最大　９７%
最小　８０%
平均　８９%　　標準偏差　５%

２００７年６月分　　２４検体（付表４）
最大　９６%
最小　８１%
平均　８８%　　標準偏差　４%

２００７年７月分　　２４検体（付表５）
最大　９６%
最小　８０%
平均　８９%　　標準偏差　５%

２００７年８月分　　１８検体（付表６）
最大　９４%
最小　７２%
平均　８７%　　標準偏差　６%

２００７年９月分　　２１検体（付表７）
最大　９９%
最小　７９%
平均　９０%　　標準偏差　５%

２００７年１０月分　２３検体（付表８）

最大　９６％
最小　８０％
平均　８８％　　標準偏差　４％

２００７年１１月分　５検体（付表９）
最大　９４％
最小　８０％
平均　８８％　　標準偏差　５％

２００７年１２月分　０検体
最大
最小
平均　　標準偏差

２００８年１月分　　０検体
最大
最小
平均　　標準偏差

２００８年２月分　４４検体（付表１０）
最大　９７％
最小　７４％
平均　８７％　　標準偏差　６％

２００７年３月ー２００８年２月
２７０検体
最大　９９％
最小　７２％
平均　８８％

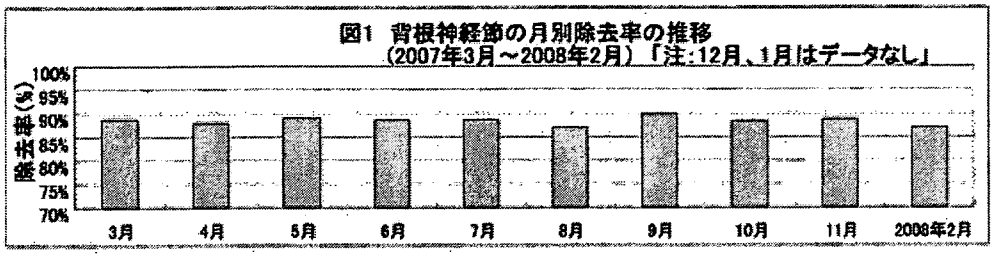
図1 背根神経節の月別除去率の推移
(2007年3月～2008年2月)「注:12月、1月はデータなし」
除去率(%)
100%
95%
90%
85%
80%
75%
70%
3月
4月
5月
6月
7月
8月
9月
10月
11月
2008年2月

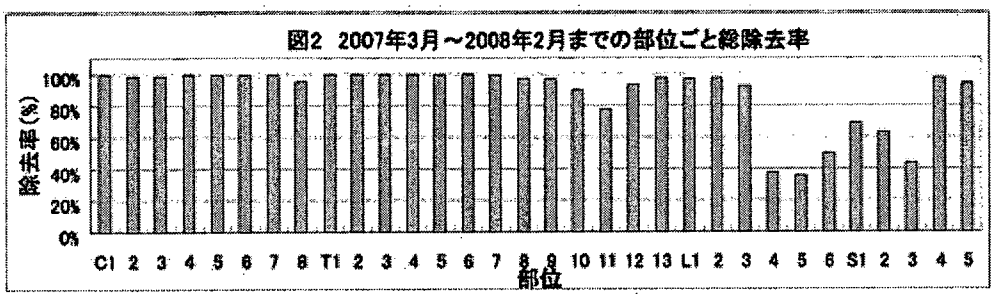
図2 2007年3月～2008年2月までの部位ごと総除去率
除去率(%)
100%
80%
60%
40%
20%
0%
C1 2 3 4 5 6 7 8 T1 2 3 4 5 6 7 8 9 10 11 12 13 L1 2 3 4 5 6 S1 2 3 4 5
部位

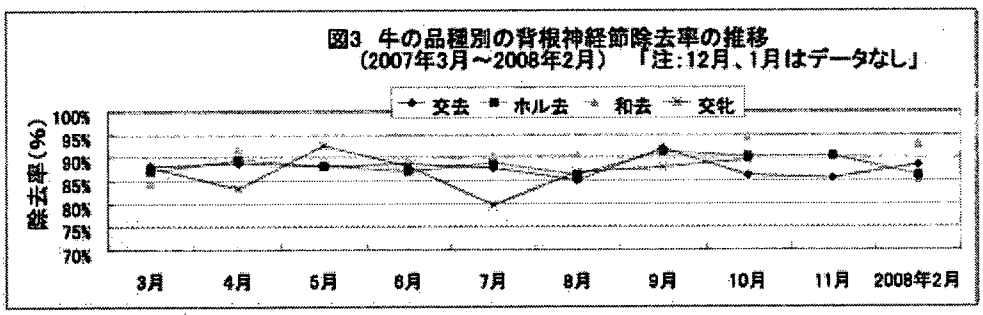
図3 牛の品種別の背根神経節除去率の推移
(2007年3月～2008年2月) 「注:12月、1月はデータなし」
交去
ホル去
和去
交牝
除去率(%)
100%
95%
90%
85%
80%
75%
70%
3月
4月
5月
6月
7月
8月
9月
10月
11月
2008年2月

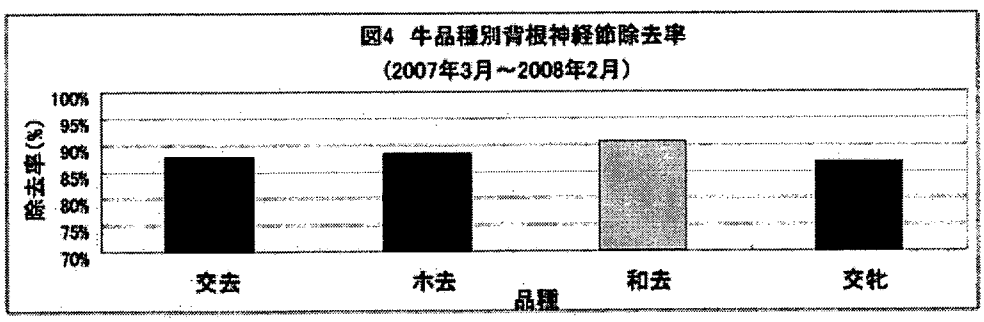
図4 牛品種別背根神経節除去率
(2007年3月～2008年2月)
除去率(%)
100%
95%
90%
85%
80%
75%
70%
交去
ホ去
和去
交牝
品種

別添 5

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Dewa, Y., Nishimura, J., Muguruma, M., Matsumoto, S., Takahashi, M., Jin, M., Mitsumori, K.	Gene expression analysis of the liver in rats treated with oxfendazole.	Arch. Toxicol.	81	647-654	2007
Umemura, T., Kuroiwa, Y., Tasaki, M., Okamura, T., Ishii, Y., Kodama, Y., Nohmi, T., Mitsumori, K., Nishikawa, A., and Hirose, M.	Detection of oxidative DNA damage, cell proliferation and *in vivo* mutagenicity induced by dicyclanil, a nongenotoxic carcinogen, using *gpt* delta mice.	Mutat. Res.	63	46-54	2007

Arch Toxicol (2007) 81:647–654
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ORGAN TOXICITY AND MECHANISMS

Gene expression analyses of the liver in rats treated with oxfendazole

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Abstract The effect of oxfendazole (OX), a benzimidazole anthelmintic, on hepatic gene expression was investigated in the liver of rats as a preliminary study to elucidate the possible mechanism of its non-genotoxic hepatocarcinogenesis. The liver from a male F344/N rat given a diet containing 500 ppm of OX for 3 weeks was examined by global gene expression analysis in comparison with an untreated rat. Microarray analysis revealed that phase I and phase II detoxifying enzymes were up-regulated in an OX-treated rat. In addition to these genes, the expressions of several upregulated genes related to xenobiotic metabolism and oxidative stress [e.g. *Cyp1a1*; NAD(P)H dehydrogenase, quinone 1 (*Nqo1*); glutathione peroxidase 2 (*Gpx2*); glutathione *S*-transferase Yc2 subunit (*Yc2*)], were confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR). Furthermore, rats were administered 500 or 1,000 ppm of OX for 9 weeks, and the effect of OX on oxidative stress responses was evaluated by real-time RT-PCR along with conventional toxicological assays, including lipid peroxidation (thiobarbituric acid-reactive substance; TBARS). A longer treatment period and/or a higher dose of OX tended to increase the gene expressions of not only phase I (*Cyp1a1* and *Cyp1a2*) but also phase II (*Nqo1*, *Gpx2*, *Yc2*, and *Akr7a3*) drug metabolizing enzymes. Toxicological parameters, such as TBARS, serum aspartate aminotransferase (AST), and serum alkaline phosphatase (ALP), showed slight but significant increases after treatment with OX for 9 weeks. These results indicate that OX elicits adaptive responses against oxidative stress in the liver and suggest that the imbalance in redox status might be one of the factors triggering the initial step of OX-induced non-genotoxic carcinogenesis in the liver of rats.

Keywords Oxfendazole · Cytochrome P450 induction · Oxidative stress · Rat

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Introduction

Oxfendazole [methyl 5-(phenylsulfinyl)-2-benzimidazolecarbamate] (OX) is a benzimidazole anthelmintic that has been widely used for the treatment and prevention of gastrointestinal parasites in livestock animals, including cattle, pigs, and sheep (Jacobs and Taylor 2001; Velik et al. 2004). The pharmacological effects of OX depend on its inhibition of glucose uptake and of tubulin polymerization in the parasitic worm (Delatour and Parish 1986). Because humans are exposed to

OX and residues of its metabolites through meat or milk derived from food-producing animals, it poses a public safety concern.

Fenbendazole (FEN), which is first metabolized to its sulphoxide form (OX) in vivo, has been reported to cause a slight increase in the incidence of hepatocellular carcinomas in female rats treated orally with a high dose of FEN (WHO 1991). On the contrary, OX has not been found to elicit any carcinogenic response in rats or mice; however, the 38th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) indicated that the lack of carcinogenic potential of OX could be attributed to its administration at lower dose levels (WHO 1991).

On the other hand, the tumor-promoting potential of OX has been suggested by using a two-stage hepatocarcinogenesis model in rats (Mitsumori et al. 1997). Results showed that the number of hepatocellular foci positive for the antibody to the placental form of glutathione *S*-transferase, a putative preneoplastic marker enzyme for chemical hepatocarcinogenesis (Kitahara et al. 1984), was increased in rats treated with OX for 8 weeks following an intraperitoneal injection of diethylnitrosamine. In addition, OX increased cytochrome P450 enzyme proteins (CYP1A1/2 and CYP2B1) and decreased the immunohistochemically positive spots of connexin 32 in a dose-dependent manner. Each change is known to be a biological hallmark for characterizing a chemical as a tumor promoter (Ito et al. 1996; Klaunig and Ruch 1990; Diwan et al. 1988). Moreover, the same results were obtained in the two-stage carcinogenesis study of FEN in the liver of rats (Shoda et al. 1999). Considering that OX as well as FEN has no mutagenic activity in short-term genotoxicity assays (WHO 1991), these results suggest that OX would exert its tumor-promoting activity in the rat liver in a nongenotoxic or an indirect genotoxic manner; however, its molecular mechanism has not yet been fully elucidated.

In the present study, the effects of OX treatment on hepatic gene expression in the liver of rats was investigated by using DNA microarrays and real-time reverse transcription polymerase chain reaction (RT-PCR) analyses to obtain information for the possible hepatocarcinogenesis of OX.

Materials and methods

Animals and experimental design

Five-week-old male F344/N rats were purchased from Japan SLC, Inc. (Shizuoka, Japan) and maintained in an air-conditioned room with a 12-h light/dark cycle and given free access to a powdered diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water. After a 1-week acclimatization period, the rats (five or six rats per dose group) were fed a diet containing 0 or 500 ppm of OX (99.7% purity; Hayashi Pure Chemical Industries, Osaka, Japan) for 3 or 9 weeks. For the 9-week treatment, an additional dose of 1,000 ppm of OX was administered to investigate whether OX induces dose-related changes in hepatic gene expression. At necropsy, the blood was sampled from the abdominal aorta under ether anesthesia. The livers were excised, macroscopically examined, weighed, cut into small pieces that were frozen in dry ice, and stored at −80°C until analysis. The serum samples obtained were sent to SRL, Inc. (Tokyo, Japan) to assess for alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP). Animals received humane care in accordance with the Guide for Animal Experimentation by Tokyo University of Agriculture and Technology.

DNA microarray analysis

Total RNA was extracted from a control and an OX-treated rat by using an RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and shipped to KURABO Industries (Osaka, Japan) for oligonucleotide microarray analysis. By using a CodeLink Bioarray (Rat Whole Genome Bioarray; GE Healthcare) consisting of more than 34,000 genes, the differentially expressed genes were analyzed in a control and an OX-treated liver. More than a twofold increase or less than a halffold decrease was regarded as a significant change, and gene information (molecular activity and biological function) was extracted from the Gene Ontology website (http://www.geneontology.org). The gene accession numbers and the UniGene symbols of each gene listed in Table 1 were retrieved from the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Real-time RT-PCR analysis

To confirm the results of microarray analysis, the expression of several genes identified to be up-regulated by the OX treatment and their related genes were quantified by real-time RT-PCR. Briefly, the total RNA from three rats per dose group was extracted using TRIzol reagent (Invitrogen, CA, USA), according to the manufacturer's instructions. These RNA

Table 1 Genes related to xenobiotic metabolism and oxidative stress selected from microarray in the liver of a rat administered OX for 3 weeks

Accession number	Fold change	Gene description	Gene symbol	UniGene symbol	Molecular functions	Biological pathways
NM_013215	2.5	Aldo-keto reductase family 7, member A3	*Akr7a3*	Rn.6043	Aldehyde reductase	Aldehyde metabolism
X00469	2.0	Cytochrome P450, family 1, subfamily a, polypeptide 1	*Cyp1a1*	Rn.10352	Monooxygenase	Electron transport
NM_183403	3.6	Glutathione peroxidase 2	*Gpx2*	Rn.3503	Glutathione peroxidase	Oxidative stress
X78847	4.8	Glutathione S-transferase Yc2 subunit	*Yc2*	Rn.120929	Glutathione transferase	Metabolism
NM_012600	2.9	Malic enzyme 1	*Me1*	Rn.3519	Electron transporter	Malate metabolism
NM_017000	2.5	NAD(P)H dehydrogenase, quinone 1	*Nqo1*	Rn.11234	Oxidoreductase	Xenobiotic metabolism

samples included those of the liver used in the microarray analysis. The total RNA was reverse transcribed by using ThermoScript reverse transcriptase (SuperScript III First-Strand Synthesis System; Invitrogen). All PCR reactions performed using SYBR Green I chemistry (Applied Biosystems, CA, USA) were carried out under the following conditions: one cycle at 50°C for 2 min followed by 95°C for 10 min and 45 cycles at 95°C (15 sec) and 60°C (1 min) in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The forward and reverse primers listed in Table 2 were designed using the Primer Express 2.0 software following Applied Biosystems' instructions for optimal primer design. For *Cyp1a1*, the primers were obtained from SuperArray Biosciences Corp. (MD, USA). The relative differences in gene expression were calculated using cycle time (Ct) values that were first normalized with hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), i.e. the endogenous control in the same sample, and then relative to a control Ct value by a 2-ddCt method described in Applied Biosystems User Bulletin #2: Relative quantification of gene expression. The data represents the average fold changes with standard deviation. To investigate the time- and dose-related changes in gene expression, additional real-time RT-PCR analysis was performed in rats treated with OX at doses of 0, 500, or 1,000 ppm for 9 weeks (five rats per dose group) in the same manner as described above.

Histopathological examination

Livers from the rats of all groups were fixed in 10% buffered formalin, dehydrated in graded ethanol, embedded in paraffin, sectioned, and stained with hematoxylin/eosin (H/E) for histopathological examination.

Measurement of thiobarbituric acid-reactive substances

Lipid peroxidation in the livers was assessed as the formation of thiobarbituric acid-reactive substances (TBARS) (Ohkawa et al. 1979). Briefly, 0.2 ml of liver homogenate in 1.15% KCl, 0.2 ml of 8.1% sodium dodecyl sulphate (SDS), and 3.0 ml of 0.4% thiobarbituric acid in 10% acetic acid (pH 3.5) were mixed; heated at 95°C for 60 min; and then cooled. The reaction mixture was centrifuged at 4,000 rpm for 10 min after adding 1.0 ml of distilled water and 5.0 ml *n*-butanol and pyridine (15:1 v/v). The absorbance of the resulting solution was determined spectrophotometrically at 532 nm by using the Synergy HT Multi-Detection Microplate Reader (BioTek, VT, USA).

Statistical analysis

All data were expressed as the means with standard deviation. The statistical significance of the difference between the control and the OX-treated group(s) was determined by Student's *t* test or the Aspin-Welch *t* test (for the 3-week study) or one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison test (for the 9-week study). *P* values of less than 0.05 and less than 0.01 were regarded as statistically significant.

Results

Irrespective of the treatment period, none of the administered OX doses affected body weight or food consumption.

Firstly, changes in hepatic gene expression in a rat treated with 500 or 0 ppm OX for 3 weeks were examined

Table 2 Sequences of primers used for real-time RT-PCR analysis

Gene name	Gene symbol	Forward primer	Reverse primer
Cytochrome P450, family 1, subfamily a, polypeptide 2	*Cyp1a2*	5′-AAGCGCCGGTGCATTG-3′	5′-TGCAGGAGGATGGCTAAGAAG-3′
NAD(P)H dehydrogenase, quinone 1	*Nqo1*	5′-TCCGCCCCCAACTTCTG-3′	5′-TCTGCGTGGGCCAATACA-3′
Glutathione peroxidase 2	*Gpx2*	5′-ACCGATCCCAAGCTCATCAT-3′	5′-TCTCAAAGTTCCAGGACACATCTG-3′
Glutathione S-transferase Yc2 subunit	*Yc2*	5′-AAGCTGAGCAGGGCTGATGT-3′	5′-ACAATGCCTGGGTCCATCTC-3′
Aldo-keto reductase family 7, member A3	*Akr7a3*	5′-CCGCTTCTTTGGGAATCCAT-3′	5′-GGCGATGCCATTGAAGTGT-3′
Malic enzyme 1	*Me1*	5′-CGACCAG-CAAAGCTGAGTGTT-3′	5′-CTGCCGCTGGCAAAGATC-3′
Nuclear factor erythroid 2-related factor 2	*Nrf2*	5′-TGCCCCTGGAAGTGTCAAA-3′	5′-GGCTGTACTGTATCCCCAGAAGA-3′
Hypoxanthine-guanine phosphoribosyltransferase	*Hprt*	5′-GCCGACCGGTTCTGTCAT-3′	5′-TCATAACCTGGTTCATCATCACTAATC-3′

using an oligonucleotide microarray. OX induced a slight but significant increase in the relative liver weight (130%) and very slight hypertrophy and vacuolation of hepatocytes (data not shown). Of the approximately 34,000 genes in the CodeLink Bioarray, 109 or 48 genes showed more than a twofold increase or a less than halffold decrease, respectively, in their expression in an OX-treated rat compared to a control animal. Among these, we focused on six genes related to xenobiotic metabolism and oxidative stress; *Cyp1a1*, *Nqo1*, *Gpx2*, *Yc2*, *Akr7a3*, *Me1* (Table 1), and the increased expressions of these genes were confirmed by real-time RT-PCR (Fig. 1).

To evaluate the effect of OX treatment on hepatic gene expression of xenobiotic and oxidative stress-related molecules, rats were administered 500 or 1,000 ppm of OX for 9 weeks, and real-time RT-PCR analysis was performed (Fig. 1). Generally, a longer treatment period and/or a higher dose of OX increased these mRNA expressions. In particular, the expression of *Cyp1a1* was drastically increased (>100 fold) in the 9-week treatment period. In addition, *Cyp1a2*, glutathione peroxidase *Gpx2*, and the NAD(P)H-generating enzyme *Me1* were up-regulated in a time- and dose-dependent manner. The expression of phase II enzyme genes such as *Nqo1*, *Yc2*, and *Akr7a3* was also significantly increased by a longer treatment regimen. The gene expression of *Nrf2* showed a slight but significant increase in rats administered a dose of 500 ppm of OX for 9 weeks.

Other toxicological parameters that were examined in the 9-week treatment of OX are shown in Fig. 2 and Table 3. OX induced slight hypertrophy and vacuolation of hepatocytes with a significant increase in the liver weight. In addition, not only serum AST and ALP but also hepatic TBARS showed slight but significant increases after treatment with OX for 9 weeks.

Discussion

OX, FEN, and their prodrug febantel (FB) have been widely utilized as anthelmintics in veterinary fields. FEN is metabolically interconvertible to OX which is its most effective pharmacological form. FB is not a benzimidazole but is converted in vivo by cyclization to FEN and subsequent oxidation at the sulfur atom to OX; thus, both FEN and FB are metabolized to OX. These three anthelmintics showed no apparent mutagenicity in numerous tests, including the Ames/Salmonella test, in vivo cytogenetics assays, and micronucleus assays (WHO 1991). With regard to carcinogenicity, the 50th meeting of JECFA finally concluded

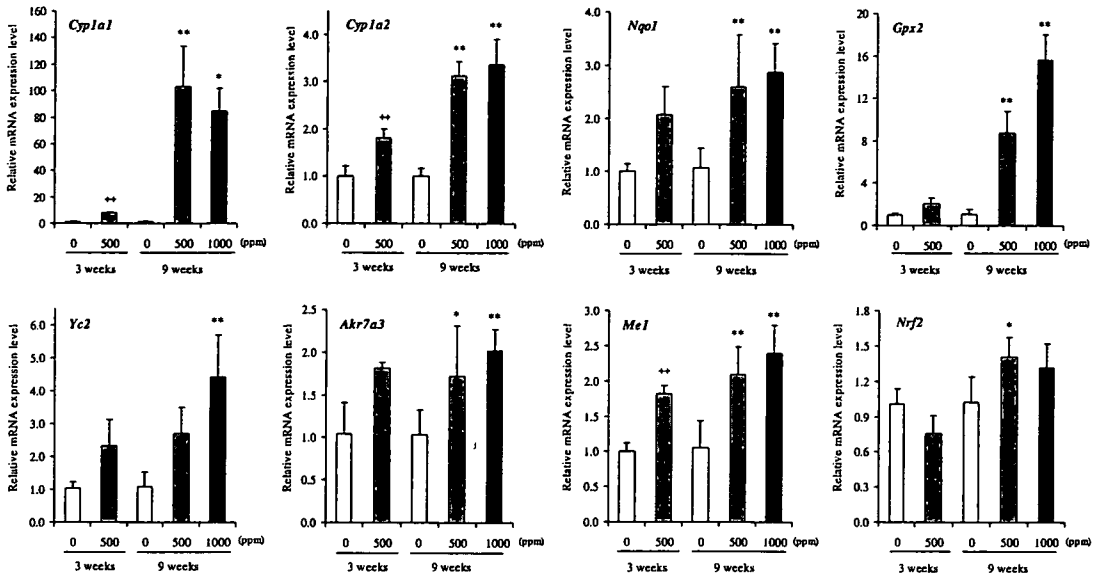

Fig. 1 The mRNA expression levels of *phase I* and *phase II* xenobiotic enzymes and *Nrf2*-related molecules in OX-treated rats quantified by real-time RT-PCR. Each column represents the mean + SD of three (3-week treatment) or five (9-week treatment) animals. ++, *, ** indicate significant differences from the time-matched control (++$P < 0.01$ by Student's t test; *$P < 0.05$ or **$P < 0.01$ by Dunnett's multiple comparison test)

Table 3 Body weight, organ weight, lipid peroxidation (TBARS), and serum chemistry in rats administered OX for 9 weeks

Groups	9-week treatment		
	Control	Oxfendazole	
		500 ppm	1,000 ppm
Body weight	290 ± 12 (6)	293 ± 15 (5)	281 ± 15 (6)
Liver weight			
Absolute (g)	7.2 ± 0.3	8.4 ± 0.5**	8.2 ± 0.3**
Relative (g/100 g bw)	2.5 ± 0.0	2.9 ± 0.1**	2.9 ± 0.1**
TBARS (nmol/g liver)	24.92 ± 3.69	32.75 ± 5.12*	34.40 ± 6.34**
ALT (U/l)	42 ± 3	48 ± 3	58 ± 6
AST (U/l)	78 ± 8	90 ± 3*	112 ± 10**
ALP (U/l)	608 ± 44	661 ± 12*	699 ± 38**

Each parenthesis represents the number of animals per group
*, **: significantly different from control ($P < 0.05$, $P < 0.01$, respectively; Dunnett's multiple comparison test)

that OX has no carcinogenic potential in rats and mice although OX might have tumor-promoting potential in rats; thus, the acceptable daily intake (ADI) of 0–7 μg/kg/day was set as a group ADI for OX, FEN, and FB in JECFA (WHO 1999). However, the tumor promotion mechanisms of OX in rats at the molecular basis have still not been clarified.

In this present study, we investigated the effects of OX on hepatic gene expression in rats by DNA microarray and real-time RT-PCR analyses. Firstly, to select genes involved in oxfendazole-induced hepatocarcinogenesis, global gene expression analysis was performed in the liver of a rat treated with 500 ppm OX for 3 weeks. In this treatment regimen, OX induced vacuolation of hepatocytes which was the earliest sign of the compound-related effect in rats (WHO 1991). Microarray analysis confirmed that phase I (*Cyp1a1*) and phase II (*Nqo1*, *Gpx2*, *Yc2*, and *Akr7a3*) enzymes were up-regulated in an OX-treated animal, which was confirmed in the OX treatment group (five rats) by real-time RT-PCR. The longer period (9 weeks) and/or higher dose (1,000 ppm) treatment of OX significantly increased the mRNA expressions of these genes and their related genes (*Cyp1a2*, *Me1*, and *Nrf2*).

The induction of *Cyp1a1* and *Cyp1a2* observed in this study was in agreement with previous reports on several benzimidazole class compounds. OX induced the CYP1A2 protein in the rabbit liver (Gleizes et al. 1991); FEN, albendazole, and mebendazole induced the CYP1A1 and CYP1A2 protein in primary rat hepatocytes and HepG2 cells (Baliharova et al. 2003); thiabendazole, an anthelmintic and fungicide, induced *Cyp1a1* in rabbit hepatocytes in in vitro conditions (Aix et al. 1994); omeprazole, the gastric pump inhibitor, induced *Cyp1a1* and *Cyp1a2* in primary human hepatocytes (Diaz et al. 1990) and rat hepatocytes (Lemaire et al. 2004). In the [^{3}H]-2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) displacement study (Daujat et al. 1992; Aix et al. 1994), it was demonstrated that the binding of TCDD to the aryl hydrocarbon receptor (AhR) was not required for the activation of *Cyp1a1*

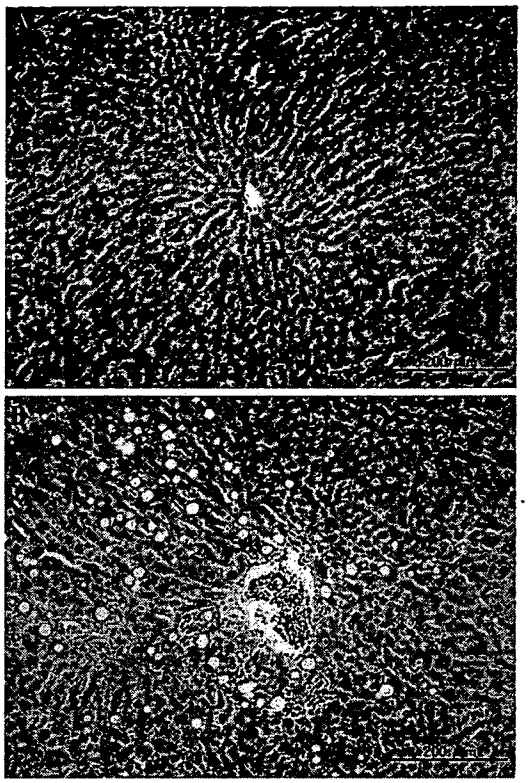

Fig. 2 Representative histopathological changes of H/E-stained liver sections of rats are given **a** basal diet, and **b** 1,000 ppm OX for 9 weeks. OX-induced slight hypertrophy and vacuolation of hepatocytes

gene expression by omeprazole and thiabendazole; the induction is dependent on a protein tyrosine kinase-mediated signal transduction pathway in HepG2 cells (Kikuchi et al. 1998) and rat hepatocytes (Lemaire et al. 2004). CYP1A1 induction has also been linked with the production of reactive oxygen species (ROS) in not only classical AhR ligands, such as TCDD (Park et al. 1996; Knerr et al. 2006), and coplanar polychlorinated biphenyl congeners (Schlezinger et al. 2006) but also the non-AhR ligand dicyclanil that is an insecticide (Moto et al. 2005). Furthermore, it has been suggested that oxidative stress responses were induced in the livers of rats treated with albendazole for up to 10 days (Locatelli et al. 2004).

The present study also showed that OX induced the phase II enzyme genes *Gpx2*, *Nqo1*, *Yc2*, and *Akr7a3* and their related gene *Me1*. Interestingly, all these enzymes are regulated under the transcriptional factor Nrf2 (Thimmulappa et al. 2002; Kwak et al. 2003). Nrf2 binds to the antioxidant response element (ARE) which is a cis-acting sequence, found in the 5′-flanking region of the genes encoding a group of detoxification and antioxidant enzymes (McMahon et al. 2001). Glutathione peroxidase is the antioxidant enzyme that scavenges hydrogen peroxide or organic hydroperoxides and thus protects cellular components against oxidative stress (Brigelius-Flohe 1999). Gpx2 is known as gastrointestinal GPx which is located in a cytosolic fraction expressed dominantly in the epithelium of the gastrointestinal tract (Chu et al. 1993). Nqo1, a NAD(P)H dehydrogenase, catalyses the obligatory two-electron reduction and detoxification of endogenous and environmental quinones (Riley and Workman 1992; Talalay et al. 1995). Glutathione *S*-transferase Yc2 (Yc2) catalyses the conjugation of glutathione to a variety of endogenous and xenobiotic electrophils (Hayes and Pulford 1995) and represents an important cellular defense by acting as scavengers of ROS (Hayes and McLellan 1999). Akr7a3, known as aflatoxin B1-aldehyde reductase, converts aflatoxin B1-dihydrodiol to the less toxic dialcohol metabolite and plays an important role in the detoxification of AFB1 by protecting against the formation of protein adducts (Hayes et al. 1993; Judah et al. 1993). Me1 is a NAD(P)H-regenerating enzyme, and its increased expression may be beneficial for the function of detoxifying enzymes directly (NQO1, Akr7a3) or indirectly (Gpx2, Yc2) (Thimmulappa et al. 2002).

A significant increase in the mRNA expression of *Nrf2* itself was observed only in the liver of rats treated with 500 ppm OX for 9 weeks. The reason why treatment period- and/or dose-related increases of OX on *Nrf2* mRNA expression were not observed is unclear, but it has been reported that Nrf2 expression control occurs at the translational level rather than at the transcriptional level (Kwak et al. 2002). Recently, the important roles of Nrf2 in maintaining redox balance have been proven in Nrf2-knockout mice treated with diesel exhaust (Aoki et al. 2001), acetaminophen (Enomoto et al. 2001), and pentachlorophenol (Umemura et al. 2006); thus, the Nrf2-driven phase II inducers were ineffective, and consequently, the reactive phase I metabolites are not conjugated for excretion but form electrophiles that may attack intracellular macromolecules, including DNA and protein (Noda et al. 2003). In this study, OX induced the gene expression of phase I and phase II enzymes with a small increase in TBARS. The present results indicate that adaptive responses against oxidative stress are elicited in the liver of OX-treated rats and suggest that a longer treatment period of 2 years and the relatively high dose of OX used in this study might induce neoplastic lesions in the liver of rats due to such oxidative stresses overwhelming the detoxifying systems.

In conclusion, we have demonstrated the up-regulation of phase I and phase II enzyme gene expression in rats treated with OX by microarray and real-time RT-

PCR analyses. It is necessary to clarify whether altered expression of such oxidative stress-related genes is also responsible for the OX-induced preneoplastic and neoplastic lesions in the liver of rats, and therefore further mechanistic studies are now in progress.

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Detection of oxidative DNA damage, cell proliferation and *in vivo* mutagenicity induced by dicyclanil, a non-genotoxic carcinogen, using *gpt* delta mice

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Abstract

To ascertain whether measurement of possible contributing factors to carcinogenesis concurrently with the transgenic mutation assay is useful to understand the mode of action underlying tumorigenesis of non-genotoxic carcinogens, male and female *gpt* delta mice were given dicyclanil (DC), a mouse hepatocarcinogen showing all negative results in various genotoxicity tests, at a carcinogenic dose for 13 weeks. Together with *gpt* and Spi$^-$ mutations, thiobarbituric acid-reactive substances (TBARS), 8-hydroxydeoxyguanosine (8-OHdG) and bromodeoxyuridine labeling indices (BrdU-LIs) in the livers were examined. Whereas there were no changes in TBARS levels among the groups, significant increases in 8-OHdG levels and centrilobular hepatocyte hypertrophy were observed in the treated mice of both genders. In contrast, BrdU-LIs and liver weights for the treated females, but not the males were significantly higher than those for the controls. Likewise, the *gpt* mutant frequencies (MFs) in the treated females were significantly elevated, GC:TA transversion mutations being predominant. No significant alterations were found in the *gpt* MFs of the males and the Spi$^-$ MFs of both sexes. The results for the transgenic mutation assays were consistent with DC carcinogenicity in terms of the sex specificity for females. Considering that 8-OHdG induces GC:TA transversion mutations by mispairing with A bases, it is likely that cells with high proliferation rates and a large amounts of 8-OHdG come to harbor mutations at high incidence. This is the first report demonstrating DC-induced genotoxicity, the results implying that examination of carcinogenic parameters concomitantly with reporter gene mutation assays is able to provide crucial information to comprehend the underlying mechanisms of so-called non-genotoxic carcinogenicity.

Keywords: 8-Hydroxydeoxyguanosine; Cell proliferation; *gpt* delta mice; Dicyclanil

1. Introduction

The standard battery of genotoxicity tests consisting of an *in vitro* test for gene mutations in bacteria, an *in vitro* test for chromosomal damage and/or gene

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mutations in mammalian cells and an *in vivo* test for chromosomal damage in rodent hematopoietic cells is usually applied in order to identify genotoxicity of environmental chemicals such as pesticides, food additives and pharmaceuticals [1]. However, the existence of discrepancies between genotoxicity and *in vivo* long-term carcinogenicity is well known [2]. There are several reasons which may explain the occurrence of false negative or positive results. For instance, although most carcinogens require biotransformation to DNA reactive species for the purpose of exerting genotoxic effects, the enzyme systems to metabolize xenobiotics in both bacteria and mammalian cells using *in vitro* assays are lacking or are expressed to only a limited extent [3]. Likewise, in *in vivo* short-term assays, it is doubtful whether target cells are exposed to test chemicals at adequate doses for a sufficient period of time, partly because of test chemical toxicity and/or a low biotransformation capacity in hematopoietic cells [3]. Thus, it is a natural consequence that alternative batteries of *in vitro* and/or *in vivo* genotoxicity tests do not fully make up the gap [4], which means we must focus our attention on the mode of action in terms of the risk assessment for environmental agents.

In this respect, reporter gene-transgenic rodents may be useful tools to predict carcinogenicity because studies can be performed with similar protocols as for the long-term bioassay [5]. Transgenic mutation assays also have the advantage of allowing a battery of other *in vivo* mutation assays such as micronucleus tests in the same animals [6]. Additionally, various proposed mechanisms underlying the actions of direct genotoxic carcinogens (e g. generation of DNA adducts) [7], indirect genotoxic carcinogens (e.g. aneugenicity or oxidative DNA damage) [8] and non-genotoxic carcinogens (e.g. methylation, mitogenicity or cytotoxicity-associated cell proliferation) [9–11] are able to be investigated concurrently with transgenic mutation assays. In fact, we have reported that simultaneous analysis of glutathione *S*-transferase placental form (GST-P) immunohistochemistry in the livers of *gpt* delta rats provided crucial information for understanding the chemical carcinogenesis of 2-amino-3-methylimidazo[4,5-*f*]quinoline, *N*-nitrosopyrrolidine and di(2-ethylhexyl)phthalate [12]. Also, finding of increases in hepatocyte proliferation together with a lack of the transgene mutations in *gpt* delta mice given flumequine, an anti-bacterial quinolone agent, helped us to define this mouse liver carcinogen as a genuine promoter [13].

Dicyclanil (4,6-diamino-2-cyclopropylaminopyrimidine-5-carbonaitrite; DC), a pyrimidine-derived insect growth regulator, has given all negative results for *in vitro* reverse mutations, gene mutations, chromosomal aberrations, unscheduled DNA synthesis, *in vivo* micronucleus formation [14] and alkaline single cell electrophoretic change [15]. However, DC has been reported to be a hepatocarcinogen in female mice [14] and recent studies revealed a possible involvement of oxidative stress [16]. In the present study, to explore the mode of action underlying DC hepatocarcinogenesis, lipid peroxidation, 8-hydroxydeoxyguanosine (8-OHdG) and hepatocyte proliferation in the livers of male and female *gpt* delta rats given DC at a carcinogenic dose were examined along with the transgenic mutation assay.

2. Materials and methods

2.1. Chemicals

Dicyclanil was kindly provided by Novartis Animal Health Co., Ltd. (Basel, Switzerland) (Fig. 1). Alkaline phosphatase and bromodeoxyuridine (BrdU) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and nuclease P1 from Yamasa Co. (Chiba, Japan).

2.2. Animals and treatments

The protocol for this study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences. Male and female B6C3F1 *gpt* delta mice carrying 80 tandem copies of the transgene lambda EG10 in a haploid genome status were raised by mating of C57BL/6 *gpt* delta and non-transgenic C3H/He mice (Japan SLC, Inc., Shizuoka, Japan). Ten male and 10 female B6C3F1 *gpt* delta mice were each randomized by weight into two groups. They were housed in a room with a barrier system, and maintained under the following constant conditions: temperature of 23 ± 2 °C, relative humidity of 55 ± 5%, ventilation frequency of 18 times/h and a 12-h light:12-h dark cycle, with free access to CRF-1 basal diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water. Starting at 8 weeks of age the mice were

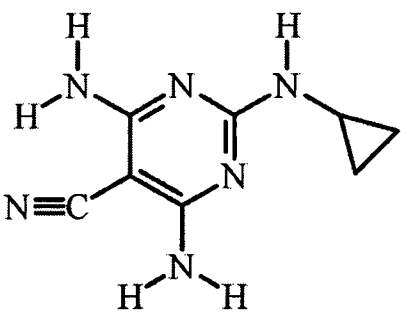

Fig. 1. Chemical structure of dicyclanil (DC).

fed diet containing 0.15% DC or maintained as non-treatment controls for 13 weeks. The dose of DC was a reported carcinogenic dose in a 18-month carcinogenicity study [14]. All mice received BrdU (100 mg/kg) by i.p. injection once a day for the final 2 days of exposure and once on the final day, 2 h before killing, as previous described [17]. All mice were killed at week 13 by exsanguination under ether anesthesia and the livers were immediately removed and weighed; slices were fixed in buffered formalin for hematoxylin and eosin (H&E) staining or BrdU immunohistochemistry. Remaining pieces of liver were frozen with liquid nitrogen and stored at −80 °C until measurement of 8-OHdG in nuclear DNA, and levels of thiobarbituric acid-reactive substances (TBARS) and performance of mutation assays.

2.3. Measurement of nuclear 8-OHdG

In order to prevent 8-OHdG formation as a byproduct during DNA isolation [18], liver DNA was extracted by a slight modification of the method of Nakae et al. [19]. Briefly, nuclear DNA was extracted with a commercially available DNA Extracter WB Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing an antioxidant NaI solution to dissolve cellular components. For further prevention of autooxidation in the cell lysis step, deferoxamine mesylate (Sigma Chemical Co.) was added to the lysis buffer [20]. DNA was digested to deoxynucleotides with nuclease P1 and alkaline phosphatase and levels of 8-OHdG (8-OHdG/10^5 deoxyguanosine) were assessed by high-performance liquid chromatography (HPLC) with an electrochemical detection system (Coulochem II, ESA, Bedford, MA, U.S.A.).

2.4. Measurement of TBARS

Malondialdehyde (MDA, nmol/g) was assessed as an index of lipid peroxidation by the method of Uchiyama and Mihara [21]. In brief, a 0.15 g portion of liver was homogenized with 1.35 mL of 1.15% KCl solution. To 0.05 mL of this homogenate, 0.2 mL 8.1% SDS and 3.0 mL 0.4% 2-thiobarbituric acid in 10% acetic acid solution (pH 3.5) were added, followed by heating in a water bath at 95 °C for 60 min. After cooling, 5.0 mL of *n*-butanol and pyridine (15:1, v/v) and 1.0 mL distilled water were added and the mixture was centrifuged at 1870 × *g* for 10 min. TBARS were measured with a Hitachi F-2500 fluorescence spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan) at 515 nm (excitation) and 553 nm (emission) in the butanol/pyridine phase.

2.5. Immunohistochemical procedures

For immunohistochemical staining of BrdU, sections were treated sequentially with normal horse serum, monoclonal mouse anti-BrdU (1:80), biotin-labeled horse anti-mouse IgG (1:400) and avidin–biotin–peroxidase complex (ABC) after denaturation of DNA with 4N HCl. The sites of peroxidase binding were demonstrated by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.). The immunostained sections were lightly counterstained with hematoxylin for microscopic examination.

2.6. Cell proliferation quantification

For each animal at least 3000 hepatocytes were counted. The labeling index (BrdU-LI) was calculated as a percentage value derived from the number of labeled cells divided by the total number of cells counted.

2.7. In vivo mutation assays

6-TG and Spi$^-$ selections were performed as previously described [5]. Briefly, genomic DNA was extracted from the livers, and lambda EG10 DNA (48 kb) was rescued as the lambda phage by *in vitro* packaging. For 6-TG selection, the packaged phage was incubated with *Escherichia coli* YG6020, which expresses Cre recombinase, and converted to a plasmid carrying *gpt* and chloramphenicol acetyltransferase. Infected cells were mixed with molten soft agar and poured onto agar plates containing chloramphenicol and 6-TG. In order to determine the total number of rescued plasmids, 3000-fold diluted phages were used to infect YG6020, and poured on the plates containing chloramphenicol without 6-TG. The plates were then incubated at 37 °C for selection of 6-TG-resistant colonies. Positively selected colonies were counted on day 3 and collected on day 4. The mutant frequency (MF) was calculated by dividing the number of *gpt* mutants by the number of rescued phages.

For the Spi$^-$ selection, the packaged phage was incubated with *E. coli* XL-1 Blue MRA for survival titration and *E. coli* XL-1 Blue MRA P2 for mutant selection. Infected cells were mixed with molten lambda-trypticase agar plates. Next day, plaques (Spi$^-$ candidates) were punched out with sterilized glass pipetters and the agar plugs were suspended in SM buffer. In order to confirm the Spi$^-$ phenotype of candidates, the suspensions were spotted on three types of plates where XL-1 Blue MRA, XL-1 Blue MRA P2 or WL95 P2 strains were spread with soft agar. Real Spi$^-$ mutants, which made clear plaques on every plate, were counted.

For characterizing the mutation spectra of *gpt* mutants, a 739 bp DNA fragment containing the 456 bp coding region of the *gpt* gene was amplified by PCR as described previously [5]. DNA sequencing was performed with Big Dye™ Terminater Cycle Sequencing Ready Reaction (Applied Biosystems, Foster City, CA, USA) on an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

2.8. Statistical evaluation

For statistical analysis, the Student's *t*-test was used to compare body and liver weights, as well as quantitative data for BrdU-LIs, TBARS, 8-OHdG and MFs between groups.

3. Results

3.1. Body and liver weights

Data for final body and liver weights in male and female *gpt* delta mice given DC are shown in Table 1. Although all of the values in DC-treated male mice were significantly lower than those in the controls, liver and relative liver weights in the treated female mice were significantly increased as compared with the controls.

3.2. In vivo mutation assays

Data for *gpt* MFs analyzed by 6-TG selection are summarized in Table 2. While there were no significant differences in the MFs between the male groups, the MF in the DC-treated females was 2.23 ± 0.55, which was significantly higher than the control value (0.48 ± 0.29). To characterize *gpt* mutations due to DC exposure, they were analyzed by DNA sequencing (Table 3). In the DC-treated female mice, G:C pairs were the preferred bases for mutation, accounting for 67.3% of the mutations (70/104). In the base substitutions, the predominant type was GC:TA (34/104, 32.7%) followed by GC:AT (26/104, 25.0%) and GC:CG (10/104, 9.6%). In addition, 16.3% (17/104) of mutant colonies were identified as carrying single- or multiple deletions. As shown in Table 4, Spi^- MFs in the treated male and female mice were not significantly different from those in the relevant controls.

Table 1
Body liver and relative liver weights of *gpt* delta mice given DC

Sex	Treatment	No. of mice	BW (g)	Liver (g)	Liver/BW (%)
Male	Control	5	32.6 ± 1.6	1.66 ± 0.21	5.07 ± 0.54
	Dicyclanil	5	29.0 ± 1.1**	1.42 ± 0.08*	4.89 ± 0.21*
Female	Control	5	25.0 ± 0.6	1.06 ± 0.05	4.25 ± 0.16
	Dicyclanil	5	24.0 ± 0.8	1.27 ± 0.07**	5.29 ± 0.12**

* $p < 0.05$ vs. Control.
** $p < 0.01$ vs. Control.

Table 2
gpt MFs in the livers of *gpt* delta mice given DC

Sex	Treatment	Animal No.	Cm^R colonies ($\times 10^5$)	6-TG^R and Cm^R colonies		Mutant frequency ($\times 10^{-6}$)	Mean ± S.D.
				Total	Independent		
Male	Control	1	9.0	6	5	0.56	0.42 ± 0.20
		2	10.7	7	7	0.66	
		3	6.9	4	3	0.44	
		4	9.5	4	3	0.31	
		5	12.4	2	2	0.16	
		6	11.5	6	5	0.43	
		7	10.7	1	1	0.09	
	Dicyclanil	8	8.9	8	8	0.90	0.48 ± 0.31
		9	6.1	4	4	0.66	
		10	9.7	3	3	0.31	
Female	Control	11	8.8	4	4	0.45	$0.48 + 0.29$
		12	6.5	5	2	0.31	
		13	12.6	2	2	0.16	
		14	7.6	7	7	0.93	
		15	8.7	5	5	0.57	
		16	7.0	31	19	2.72	
		17	13.0	29	26	2.01	
	Dicyclanil	18	11.1	51	25	2.26	2.23 ± 0.55*
		19	7.0	11	10	1.42	
		20	8.7	34	24	2.75	

* $p < 0.01$ vs. Control.

Table 3
Mutation spectra of *gpt* mutant colonies

Sex	Male				Female			
Treatment	Control		Dicyclanil		Control		Dicyclanil	
	Number (%)	MF ($\times 10^{-6}$)	Number (%)	MF ($\times 10^{-6}$)	Number (%)	MF ($\times 10^{-6}$)	Number (%)	MF ($\times 10^{-6}$)
Base substitutions								
Transversions								
GC:TA	1[a] (5.0)	0.02	1 (4.8)	0.02	1 (5.0)	0.02	34 (32.7)	0.73
GC:CG	3 (15.0)	0.06	3 (14.3)	0.07	1 (5.0)	0.02	10 (9.6)	0.21
AT:TA	1 (5.0)	0.02	3 (14.3)	0.07	1 (5.0)	0.02	10 (9.6)	0.21
AT:CG	0	0	0	0	2 (10.0)	0.05	2 (1.9)	0.04
Transitions								
GC:AT	6 (30.0)	0.13	11 (52.4)	0.25	5 (25.0)	0.12	26 (25.0)	0.56
AT:GC	3 (15.0)	0.06	1 (4.8)	0.02	3 (15.0)	0.07	0	0
Deletions								
Single bp	5 (25.0)	0.11	2 (9.5)	0.05	6 (30.0)	0.14	15 (14.4)	0.32
Over 2 bp	0	0	0	0	0	0	2 (1.9)	0.04
Insertions	1 (5.0)	0.02	0	0	1 (5.0)	0.02	1 (1.0)	0.02
Complexes	0	0	0	0	0	0	4 (3.8)	0.08
Total	20	0.42 ± 0.20	21	0.48 ± 0.31	20	0.48 ± 0.29	104	2.23 ± 0.55*

[a] The number of colonies with independent mutations.
* $p < 0.01$ vs. Control.

3.3. Oxidative DNA damage and lipid peroxidation

The results for 8-OHdG and TBARS in the livers of *gpt* delta mice given DC are illustrated in Figs. 2 and 3, respectively. 8-OHdG levels in the males and females (males; 0.62 ± 0.06, $p < 0.01$, females; 0.65 ± 0.13 8-OHdG/10^5dG, $p < 0.01$) treated with DC were elevated compared with the relevant control values (male;

Table 4
Spi$^-$ MFs in the livers of *gpt* delta mice given DC

Sex	Treatment	Animal No.	Plaques within XL-1 Blue MRA ($\times 10^5$)	Plaques within XL-1 Blue MRA (P2) (Spi$^-$)	Mutant frequency (10^{-5})	Mean ± S.D.
Male	Control	1	10.4	4	0.39	0.27 ± 0.17
		2	13.3	2	0.15	
		3	19.4	3	0.16	
		4	14.2	2	0.14	
		5	11.7	6	0.51	
		6	20.4	10	0.49	
		7	17.1	4	0.23	
	Dicyclanil	8	10.1	4	0.40	0.42 ± 0.12
		9	12.5	7	0.56	
		10	11.6	5	0.43	
Female	Control	11	16.7	15	0.90	0.68 ± 0.29
		12	10.9	10	0.92	
		13	33.9	10	0.29	
		14	ND	ND	ND	
		15	19.4	12	0.62	
		16	18.9	14	0.74	
		17	22.6	29	1.28	
	Dicyclanil	18	17.4	7	0.40	0.83 ± 0.35
		19	15.4	10	0.65	
		20	16.0	17	1.06	

ND, not detected.

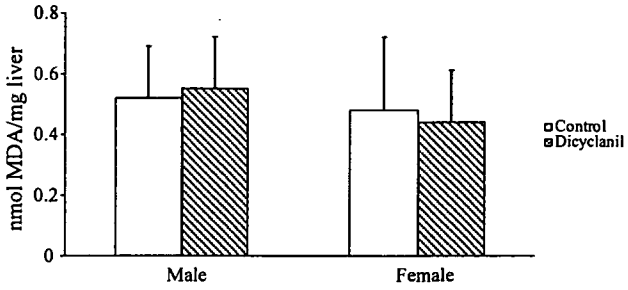

Fig. 2. Changes of TBARS levels in livers of male and female *gpt* delta mice fed DC in the diet at concentrations of 0 (Control) or 0.15% for 13 weeks. The values are means ± S.D.s of data for five animals.

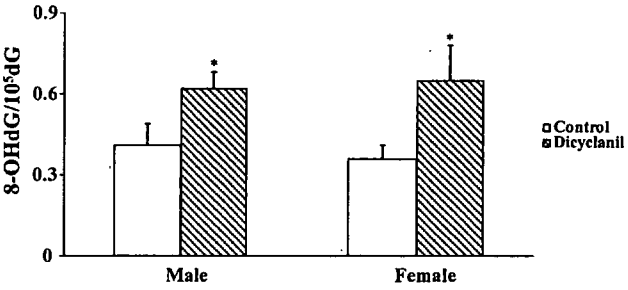

Fig. 3. Changes of 8-OHdG levels in liver nuclear DNA of male and female *gpt* delta mice fed DC in the diet at concentrations of 0 (Control) or 0.15% for 13 weeks. The values are means ± S.D.s of data for five animals. Significant differences from the relevant control are shown by $^{*}p < 0.01$.

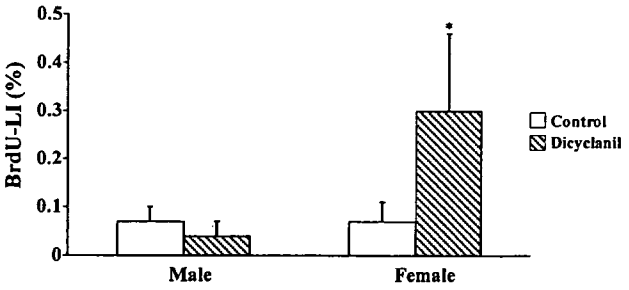

Fig. 5. Changes of BrdU-LIs in hepatocytes of male and female *gpt* delta mice fed DC at concentrations of 0 (Control) or 0.15% for 13 weeks. The values are means ± S.D.s of data for five animals. Significant differences from the relevant control are shown by $^{*}p < 0.05$.

0.41 ± 0.08, female; 0.36 ± 0.05 8-OHdG/10^5dG) with statistical significance. In contrast, there were no significant differences in TBARS levels among the groups.

3.4. Histopathology and immunohistochemical analysis of BrdU

Histopathologically, swelling of centrilobular hepatocytes was observed in the treated mice of both sexes without overt hepatocyte necrosis, the extents being almost equal in both genders (Fig. 4a and b). Fig. 5 summarizes changes in BrdU-LI for hepatocytes in male

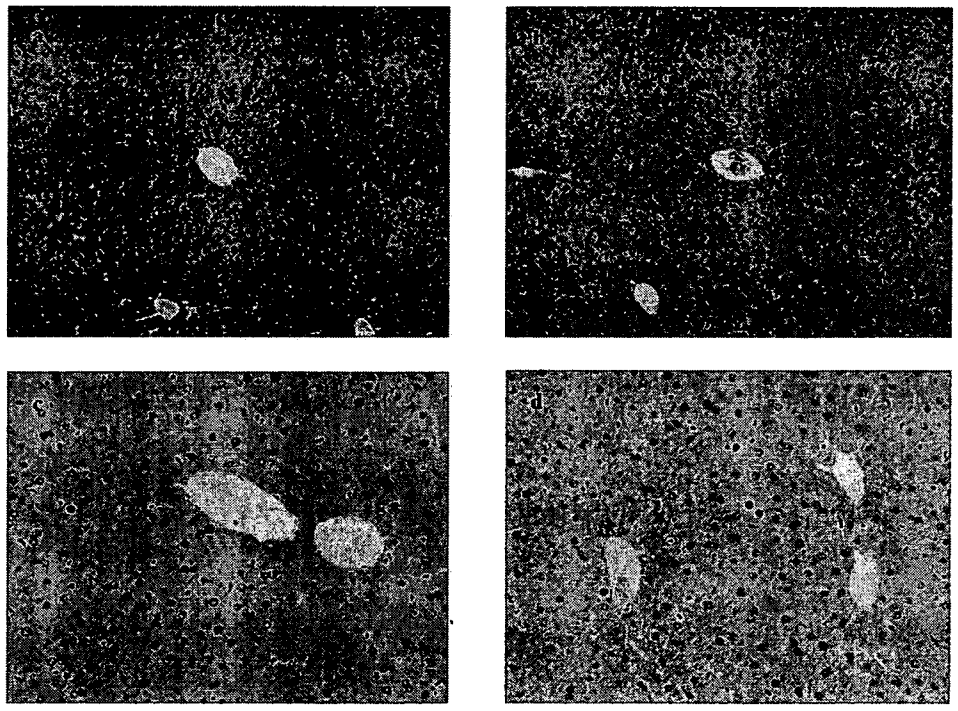

Fig. 4. Photomicrographs of livers of male (a and c) and female (b and d) *gpt* delta mice fed DC at a concentration of 0.15% for 13 weeks. Centrilobular hepatocyte hypertrophy is evident in both sexes (a and b). H&E staining at ×100 original magnification. In contrast to few BrdU-positive hepatocytes in a male (c), an appreciable number of the positive hepatocytes is evident in a female (d). BrdU immunohistochemical staining at ×200 original magnification.