な機能のエピジェネティックなメカニズムによる変調が、PBOによる発がんに寄与している可能性が示唆された。

9. 動物用医薬品の発がん過程における酸化的ストレスの関与（梅村）

DCを雌の*nrf2*欠損マウスならびにその野生型マウスに投与したところ、肝DNA中の8-OHdGレベルはいずれの遺伝子型においても用量依存的に有意な上昇が認められたが、その程度はNrf2蛋白量に逆相関した。一方、glutathione（GSH）の律速酵素でNrf2の転写制御を受けるglutathione-cystein ligase活性に遺伝子型間で変化は認められなかった。つぎに、PBOを雄の*nrf2*欠損マウスならびにその野生型マウスに投与したところ、肝DNA中の8-OHdGレベルは*nrf2*欠損マウスでのみ低用量から有意な上昇を示したが、高用量での値を遺伝子型間で比較すると野生型が高く、明らかな遺伝子型間での差異は認められなかった。また、ラットで報告されている*NQO1*のmRNAレベルの上昇もすべての投与群で認められなかった。PBOの遺伝子障害性を*p53*欠損*gpt* deltaマウスならびにその野生型マウスに投与して、*gpt*変異頻度を検索したところ、いずれの投与群においても*gpt*変異頻度に変化はなかった。また、8-OHdGレベルにも群間ならびに遺伝子型間で差異はなかった。DCの投与により、酸化的DNA損傷の程度がNrf2蛋白量に逆相関したことから、DCが引き起こす酸化的ストレスに対する生体内防御機構にNrf2の関与が示唆された。また、GSH以外のNrf2関連酵素群がその防御に関与していると考えられた。一方、PBOによる酸化的ストレスに対するNrf2の関与の可能性は低いと考えられた。また、p53蛋白の有無に関わらず、遺伝子障害性は認められなかった。8-OHdGレベルも上昇が認められず、本マウスがC57BL/c系統であり、*nrf2*欠損マウスはICR系統であることを考慮に入れると、PBOの酸化的ストレス産生には種差と系統差があることが示された。酸化的ストレスを誘発する非遺伝毒性肝発がん物質の中にもその生体内防御機構にNrf2蛋白の関与するものとしないものとがあることが判明し、Nrf2に転写調節を受ける遺伝子にはヒト遺伝子多型が数多く報告されていることを考慮すると、本研究結果は、この種の動物用医薬品のヒト危険度評価の精度向上に役立つものと考えられた。本研究結果より、酸化的ストレスを誘発する非遺伝毒性肝発がん物質の中にもその生体内防御機構にNrf2蛋白の関与するもの（DC）としないもの（PBO）とがあることが明らかとなった。また、DCは*gpt*遺伝子変異頻度を上昇させたのに対し、PBOはp53欠損下においてもその上昇を引き起こさなかった。本試験系がこの種の動物用医薬品のヒト危険度評価の精度向上に大きく貢献できる可能性が示された。

10. 牛脊柱からの背根神経節の除去に関する研究（九郎丸）

1. 牛の脊柱からの背根神経節の除去

背根神経節の脊柱からの除去率は、2007年3月から2010年2月の間で平均87%であった。3年間を通して、第11胸神経（平均70%）、第4（30%）、5（37%）、6（50%）腰神経、及び第1（66%）、2（69%）、3（45%）仙骨神経の背根神経節の除去率の低さが顕著であった。上記の7部位を除いた残りの25部位は85%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。

また、第4腰神経～第3仙骨神経の背根神経節が除去されにくい理由であるが、第1仙骨神経～第3仙骨神経の背根神経節は脊柱管外にあり、孔（腹側仙骨孔）の外の脂肪層内に位置する。したがって、断面にした脊柱管の内側から孔の外にある背根神経節を引き出そうとする場合、神経節を孔の外から内に通過させなければならない。この際に、神経節が剥がれ落ちる危険性が高いことが除去率を著しく低下させていると考えられる。同様のことが、第4腰神経～

第6腰神経の背根神経節の除去率の低さについても推測できる。なお、第4および5仙骨神経の背根神経節は孔の外に位置するにもかかわらず高い除去率を示したが、これはこれらの部位の神経節がかなり小さく、孔を比較的容易に通過できるためと考えられる。

2. 牛の品種別及び牡牝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。「交雑種去勢牡」、「交雑種牝」、「ホルスタイン去勢牡」、及び「和牛（黒毛、褐毛和種）去勢牡、ホルスタイン牝、etc.」のいずれの除去率も、85～90%であり、明瞭な差は認められなかった。

1. では2007年3月から2010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87%であった。除去率の極端に低い第11胸神経及び第4腰神経～第3仙骨神経の背根神経節の除去率が向上しない限り、完全除去達成は困難である。

第4腰神経～第3仙骨神経の背根神経節は孔の外に位置すると考えられることから、これらの部位の除去率を100%に近づけることは、物理的に極めて困難であると思われる。しかし、第1頚神経～第3腰神経の背根神経節（前半の24個）の除去率の3年間の平均は95%であり、前半の24/32、即ち脊柱全体の3/4の有効利用を目指して（後半の1/4の脊柱は従来通り廃棄）、前半24個の背根神経節の除去率を100%に近づけることは、今後十分可能だと思われる。

また、牛の品種別及び牡牝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。今後機会があれば、前半3/4の脊柱利用をめざした取り組みが現実的であると考える。

11. 各種動物における腎糸球体濾過量（GFR）測定の基礎検討（古濱）

血糖異常の原因として、GFLXの膵への高い移行性とK_{ATP}チャネル阻害により発現することが明かになった。

Iodixanolの単回静注・1回採血法で得られた健常5種動物のGFR値は既知文献値とよく一致していた。

D. 健康危険情報

特になし

E. 研究発表

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F. 知的財産権の出願・登録状況

1．特許取得
なし

2．実用新案登録
なし

3．その他
なし

別添４

研究成果の刊行に関する一覧表レイアウト

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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′-ACCGATCCCCAAGCTCATCAT-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′-GGCGATGCCCATTGAAGTGT-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

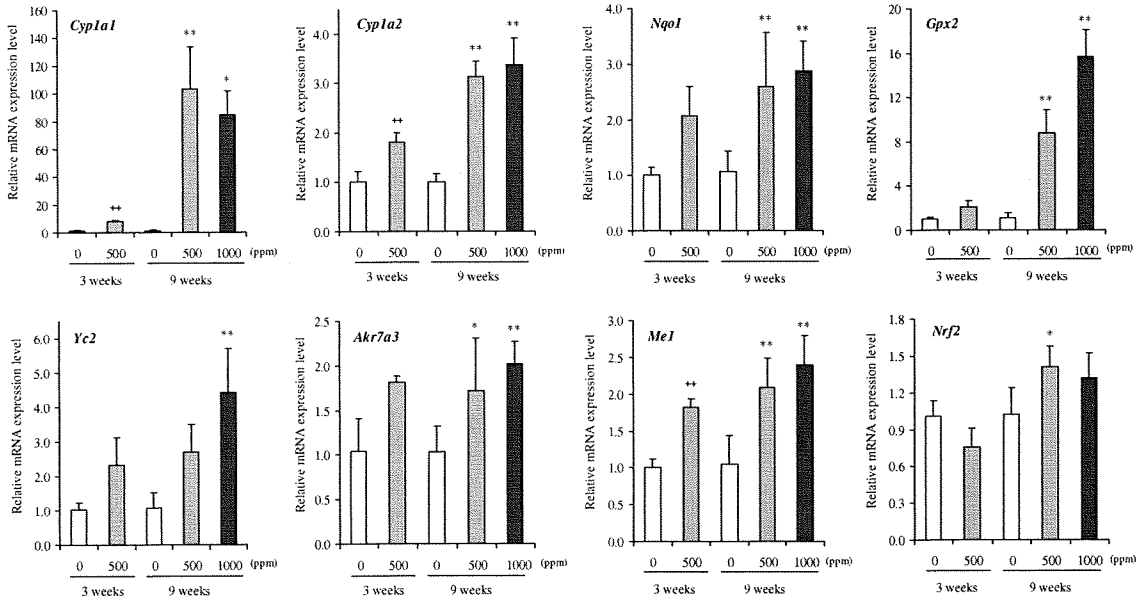

Fig. 1 The mRNA expression levels of *phase I* and *phase II* xenobiotic enzymes and *Nrf2*-related molecules in OX-treated rats quantified by real-time RT-PCR. Each column represents the mean + SD of three (3-week treatment) or five (9-week treatment) animals. ++, *, ** indicate significant differences 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*

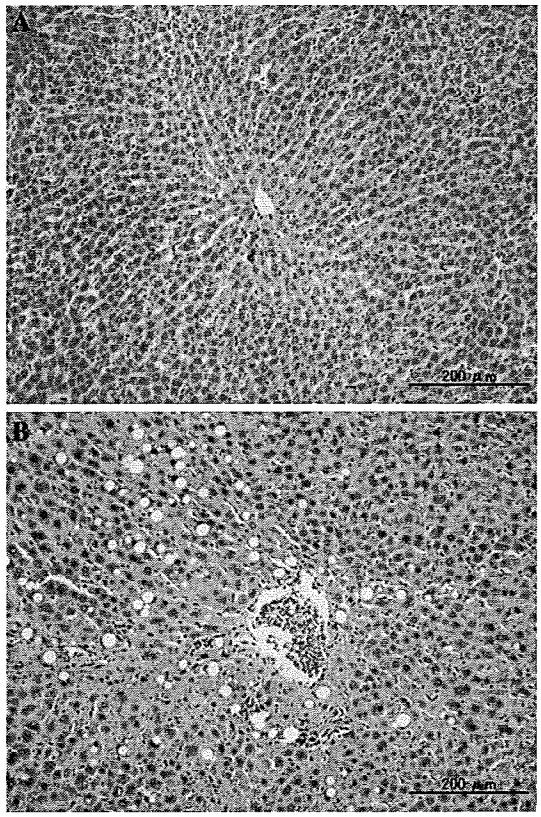

Fig. 2 Representative histopathological changes of H/E-stained liver sections of rats are given **a** basal diet, and **b** 1,000 ppm OX for 9 weeks. OX-induced slight hypertrophy and vacuolation of hepatocytes

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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GENOTOXICITY AND CARCINOGENICITY

Involvement of oxidative stress in hepatocellular tumor-promoting activity of oxfendazole in rats

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Abstract The tumor-promoting effects of oxfendazole (OX), a benzimidazole anthelmintic, were investigated using a medium-term rat hepatocarcinogenesis model. Six-week-old male F344 rats received an intraperitoneal injection of *N*-diethylnitrosamine (DEN) and were given a powdered diet containing 0 or 500 ppm OX for 6 weeks from 2 weeks after DEN treatment. All animals were subjected to two-thirds partial hepatectomy 1 week after OX treatment. The numbers and areas of glutathione *S*-transferase placental form (GST-P)-positive foci were significantly increased in the livers of rats treated with OX, with concomitantly increased cell proliferation, compared with those in the livers of the DEN alone group. Quantitative real-time RT-PCR analysis revealed that OX induced not only mRNA expression of phase I enzymes *Cyp1a1*, *Cyp1a2*, but also Nrf2-regulated phase II enzymes such as *Gpx2*, *Nqo1*, *Yc2*, *Akr7a3* and *Gstm1*, presumably due to an adaptive response against OX-induced oxidative stress. Reactive oxygen species production increased in microsomes isolated from the livers of OX-treated rats. Furthermore, OX enhanced oxidative DNA damage (as assessed by 8-hydroxydeoxyguanosine; 8-OHdG) and lipid peroxidation (as assessed by thiobarbituric acid-reactive substances; TBARS). These results suggest that administration of OX at a high dose and for a long term enhances oxidative stress responses, which may contribute to its tumor-promoting potential in rats.

Keywords Oxfendazole · Reactive oxygen species · Oxidative stress · Tumor-promotion · Rat

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Introduction

Fenbendazole [methyl 5-(phenylthio)-2-benzoimidazolecarbamate] (FEN) and oxfendazole [methyl 5-(phenylsulfinyl)-2-benzimidazolecarbamate] (OX) are benzimidazole anthelmintics that have 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 these chemicals depend on their inhibition of glucose uptake and tubulin polymerization in parasitic worms (Delatour and Parish 1986). 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 (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 carcin-

ogenic potential of OX could be attributed to its administration at lower dose levels (WHO 1991). In addition, OX exerted a tumor-promoting activity when administered to *N*-diethylnitrosamine (DEN)-treated rat livers at relatively high and repeated doses (Mitsumori et al. 1997). OX increased the number of hepatocellular foci positive for the placental form of glutathione *S*-transferase (GST-P), as a novel preneoplastic marker for chemical carcinogens (Kitahara et al. 1984), and decreased the levels of connexin 32, a protein responsible for gap junctional intercellular communication, which are considered to be a biological hallmark of tissues treated with tumor-promoting chemicals (Klaunig and Ruch 1990). Moreover, the same results were obtained in a two-stage carcinogenesis study of FEN in the livers of rats (Shoda et al. 1999). Considering that OX and FEN have no mutagenic activity in short-term genotoxicity assays (WHO 1991), these results suggest that OX exerts its tumor-promoting activity in the rat liver in a non-genotoxic or indirect genotoxic manner; however, its molecular mechanism has not yet been fully elucidated.

Oxidative stress has been recognised as an important factor involved in the pathogenesis of degenerative and inflammatory diseases, aging, and cancer (Wiseman and Halliwell 1996; Trush and Kensler 1991). Indeed, reactive oxygen species (ROS) are believed to play a pivotal role in the etiology of liver cancer, and ROS overproduction and subsequent oxidative DNA damage have been implicated in enhancing the development of hepatocellular tumors caused by agents such as the drug fenofibrate (Nishimura et al. 2007), the insecticide piperonyl butoxide (Muguruma et al. 2007) and p,p′-DDT (Harada et al. 2003). These compounds have been shown to increase gene expression levels of the transcription factor Nrf2-regulated genes such as glutathione peroxidise 2 (*Gpx2*), a NAD(P)H dehydrogenase (*Nqo1*), Glutathione *S*-transferase Yc2(*Yc2*), aflatoxin B1-aldehyde reductase (*Akr7a3*), and Glutathione *S*-transferase mu1(*Gstm1*). It is well known that Nrf2 binds to the antioxidant response element (ARE), a cis-acting sequence found in the 5′-flanking regions of these genes encoding a group of detoxification and antioxidant enzymes (McMahon et al. 2001), which are involved in protection against oxidative stress (Thimmulappa et al. 2002; Kwak et al. 2003). Glutathione peroxidase is the antioxidant enzyme that scavenges hydrogen peroxide and organic hydroperoxides, and thus protects cellular components against oxidative stress (Brigelius-Flohe 1999). Furthermore, Gpx2 may be involved in mammary carcinogenesis and cell proliferation in both rats and humans (Naiki-Ito et al. 2007). Nqo1 catalyses the obligatory two-electron reduction and detoxification of endogenous and environmental quinones (Riley and Workman 1992; Talalay et al. 1995). Yc2 catalyses the conjugation of glutathione to a variety of endogenous and xenobiotic electrophils (Hayes and Pulford 1995) and represents an important cellular defence by acting as scavengers of ROS (Hayes and McLellan 1999). Akr7a3 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). *Gstm1* may play a protective role in defences against 4-hydroxynonenal- and hydrogen peroxide-induced oxidative stress in vitro (Raza et al. 2002) and both constitutive and oxidative stress-induced mRNA expression depends on the presence of Nrf2 in the liver (Chanas et al. 2002).

Recently, it has been suggested that repeated doses of the benzimidazole-derived anthelmintics albendazole and mebendazole induce lipid peroxidation and an imbalance of glutathione homeostasis in rat livers (Locatelli et al. 2004). These results imply the possible involvement of oxidative stress in OX- and FEN-induced rat hepatocarcinogenesis.

In the present study, the mechanism underlying the tumor-promoting ability of OX in the livers of rats was investigated with particular focus on gene expression and biochemical events affecting the cellular redox status. We have demonstrated that OX exhibits tumor-promoting activity that enhances oxidative stress and preneoplastic foci in a DEN-initiated hepatocarcinogenesis model in partially hepatectomized rats.

Materials and methods

Chemicals

Oxfendazole [methyl 5-(phenylsulfinyl)-2-benzimidazolecarbamate, OX; CAS No. 53716-50-0] and *N*-diethylnitrosamine [DEN; CAS No. 55-18-5] were purchased from Hayashi Pure Chemical Industries (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan) with purities of 99.7% and >99%, respectively.

Animals and experimental design

Animals received humane care in accordance with the Guide for Animal Experimentation by the Tokyo University of Agriculture and Technology. A total of 24 male F344/N rats aged 5 weeks were purchased from Japan SLC, Inc. (Shizuoka, Japan), maintained in an air-conditioned room with a 12-h light/dark cycle (room temperature, 24 ± 3°C; relative humidity, 55 ± 10%), and given free access to a powdered diet (Oriental MF; Oriental Yeast, Tokyo, Japan) and tap water. After a 1-week acclimatization period, a medium-term liver carcinogenesis bioassay (Ito et al. 2003) was performed by the following procedure. All animals received an intraperitoneal injection of DEN at a dose of 200 mg/kg body weight, and were fed a diet con-

taining 0% (basal diet) or 0.05% OX for 6 weeks starting 2 weeks after DEN initiation. All animals were subjected to two-thirds partial hepatectomy 1 week after OX treatment. Nine animals died after partial hepatectomy, while 15 animals survived (9 in the DEN alone group and 6 in the DEN + 0.05% OX group). Body weight and food consumption were measured once a week. At the end of the experiment, the rats were euthanized by exsanguination under ether anaesthesia, and the livers were excised and weighed; the sliced liver samples were fixed in 10% phosphate-buffered formalin for histopathology and immunohistochemistry. The remaining pieces of the livers were frozen in dry ice and stored at −80°C until further analysis.

Histopathology and immunohistochemistry

The fixed liver slices were dehydrated in graded ethanol, embedded in paraffin, sectioned, and stained with haematoxylin and eosin (H.E.) for histopathological examinations. Immunohistochemical staining of GST-P and proliferating cell nuclear antigen (PCNA) was performed by the following procedure. The deparaffinized liver sections were treated with 0.3% H_2O_2 in methanol for 30 min to block endogenous peroxidase and then incubated overnight at 4°C with rabbit anti-GST-P antibody (1:2,000 dilution; Medical and Biological Laboratories Co., Ltd., Aichi, Japan) or mouse anti-PCNA antibody (1:500 dilution; Dako, Glostrup, Denmark). For PCNA staining, the sections were heated by microwave in 10 mmol/l sodium citrate buffer (pH 6.0) before quenching the endogenous peroxidase activity. The avidin-biotin-peroxidase complex method (Vectastain Elite ABC system; Vector Laboratories, Burlingame, CA) was then employed with 3,3′-diaminobenzidine as a chromogen, followed by light counterstaining with haematoxylin.

The numbers and areas of GST-P-positive foci (>0.2-mm diameter) and the total areas of the liver sections were quantified using Scion Image (Scion Corp., Frederick, MD, USA). PCNA-positive nuclei were examined in a total of 20 fields (approximately 300–400 hepatocytes in each field) per animal, and the cells with positive nuclei (dark brown) were counted to determine the PCNA labelling index.

qRT-PCR analysis

The expression levels of the genes listed in Table 1 were quantified using quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis. The gene products are involved in the detoxification of xenobiotics and defences against oxidative stress responses (Dewa et al. 2007). Briefly, total RNA from five rats per treatment group was extracted using TRIzol reagent (Invitrogen Corp.), according to the manufacturer's instructions. The total RNA was reverse transcribed using ThermoScript reverse transcriptase (SuperScript III First-Strand Synthesis System; Invitrogen). All PCR reactions were performed using SYBR Green I chemistry (Applied Biosystems, CA, USA) and were carried out under the following conditions using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): incubation at 50°C for 2 min followed by 95°C for 10 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. The forward and reverse primers listed in Table 1 were designed using the Primer Express 2.0 software following Applied Biosystems' instructions for optimal primer design. The relative differences in gene expression were calculated using cycle time (Ct) values that were first normalised to those of the hypoxanthine-guanine phosphoribosyltransferase (Hprt) gene, the endogenous control in the same sample, and then relative to a control Ct value by the 2-ddCt method (Livak and Schmittgen 2001). The data represent the average fold changes with standard deviation.

Table 1 Phase I and phase II enzyme/antioxidant genes examined in this study and their primer list

Accession no.	Gene description	Gene symbol	Forward	Reverse
X00469	Cytochrome P450, family 1, subfamily a, polypeptide 1	*Cypla1*	gccttcacatcagccacaga	ttgtgactctaaccacccagaatc
NM_012541	Cytochrome P450, family 1, subfamily a, polypeptide 2	*Cypla2*	aagcgccggttgcattg	tgcaggaggatggctaagaag
NM_183403	Glutathione peroxidase 2	*Gpx2*	accgalcccaagctcatca	tctcaaagttccaggacacatctg
NM_017000	NAD(P)H dehydrogenase, quinone 1	*Nqo1*	tccgccccaacttctg	tctgcgtgggccaataca
X78847	Glutathione S-transferase Yc2 subunit	*Yc2*	aagctgagcagggctgatgt	acaatgcctgggtccatctc
NM_013215	Aldo-keto reductase family 7, member A3 (aflatoxin aldehyde reductase)	*Akr7a3*	ccgctttcttgggaatccat	ggcgatgccattgaagtgt
NM_020540	Glutathione S-transferase, mu 1	*Gstm1*	gaacgttcgcggacttactca	acgtatctcttctcctcatagtttgaatct
NM_012600	Malic enzyme 1	*Me1*	cgaccagcaaagctgagtgtt	ctgccgctggcaaagatc
NM_012583	Hypoxanthine guanine phosphoribosyl transferase[a]	*Hprt*	gccgaccggttctgtcat	tcataacctggttcatcatcactaatc

[a] Control gene for normalization of relative gene expression