Role of CYP2E1 in Diethylnitrosamine-Induced Hepatocarcinogenesis In vivo

Jin Seok Kang, Hideki Wanibuchi, Keiichirou Morimura, Frank J. Gonzalez, and Shoji Fukushima

Abstract

CYP2E1 metabolizes many low–molecular weight toxins and carcinogens. Some in vitro experiments suggest that CYP2E1 may be involved in the metabolic activation of diethylnitrosamine. However, there has been no direct evidence demonstrating a role for CYP2E1 in diethylnitrosamine-mediated carcinogenesis in vivo. To clarify this, we carried out a diethylnitrosamine-induced hepatocarcinogenesis experiment using Cyp2e1-null mice. Male 14-day-old wild-type and Cyp2e1-null mice were treated with diethylnitrosamine (10 mg/kg of body weight) and killed at weeks 24 and 36 after diethylnitrosamine treatment for investigation of tumors and at 6, 24, and 48 h for examination of apoptosis and gene expression. Liver weights of Cyp2e1-null mice were significantly different at weeks 24 and 36 compared with wild-type mice (P < 0.01). Liver tumor incidences of Cyp2e1-null mice were significantly decreased at weeks 24 and 36 compared with wild-type mice (P < 0.01). Cyp2e1-null mice showed significant decrease in the multiplicities of hepatocellular adenoma at weeks 24 and 36 (P < 0.05 and P < 0.01, respectively), and of hepatocellular carcinoma at week 36 (P < 0.01) compared with wild-type mice. Apoptotic index and caspase-3 and/or Bax mRNA expression of Cyp2e1-null mice were significantly different at 6, 24, and 48 h after diethylnitrosamine treatment compared with wild-type mice (P < 0.05). We conclude that Cyp2e1-null mice show lower tumor incidence and multiplicity compared with wild-type mice in diethylnitrosamine-induced hepatocarcinogenesis. It is suggested that CYP2E1 completely participates in diethylnitrosamine-induced hepatocarcinogenesis, and high frequency of tumors in wild-type mice could be associated with the increased apoptosis.

Introduction

Biotransformation of chemical carcinogens is catalyzed by the cytochrome P450 (CYP) system. CYPs are a superfamily of membrane-bound heme containing enzymes that catalyze the oxidation of a wide variety of endogenous and exogenous compounds, including drugs, carcinogens, and other xenobiotic chemicals (1). Many CYPs also activate carcinogens to electrophilic derivatives that bind to cellular macromolecules, such as DNA resulting in initiation of chemical carcinogenesis (2, 3). CYPs from liver microsomes activate N-alkylnitrosamines, and in particular, CYP2E1 has a primary role in the activation of N-alkylnitrosamines (4, 5). Using knockout mice model, it was shown that CYP2E1 metabolizes many low–molecular weight toxicants and carcinogens, such as nitrosamines, benzene, carbon tetrachloride chloroform, and vinyl chloride (6).

Diethylnitrosamine has been found in a variety of products, including tobacco smoke (7), meat, and whiskey (8). It has had extensive use as a carcinogen in experimental animal model systems (9), especially in several mouse strains treated at 14 to 15 days of age (10–13). It is hydroxylated by CYPs in the liver through an alkylation mechanism to become bioactive (14) and is principally oxidized to reactive products by CYP2E1 in rat liver microsomes as shown by the Salmonella typhimurium test system (15). From these, it is suggested that CYP2E1 may be involved in the metabolic activation of diethylnitrosamine. However, other CYPs may activate diethylnitrosamine under some conditions (5, 16), and there has been no direct evidence about the role of CYP2E1 in diethylnitrosamine-mediated carcinogenesis in vivo. To clarify this, we investigated diethylnitrosamine-induced hepatocarcinogenesis experiment using Cyp2e1-null mice.

Materials and Methods

Animals and treatment. Cyp2e1-null mice were generated as described previously (17) and bred onto a C57BL/6N background. Wild-type C57BL/6N mice were obtained from Charles River Japan, Inc., and housed in a room maintained on a 12-h light/dark cycle at constant temperature and humidity. Mice were allowed free access to pellet chow diets (CE-2, Oriental Yeast Co.) during the experiment. Male Cyp2e1-null mice were bred with C57BL/6N females, yielding F1 heterozygotes. F2 homozygotes were produced by crossing the F1 generation. F3 wild-type or Cyp2e1-null mice were produced by mating F2 homozygotes. In each step, genotyped were carried out according to the protocols of Genotyping Protocol for Cyp2e1<sup>tm1Gonz</sup> provided by Jackson Laboratory and confirmed by Western blotting using CYP2E1 antibody and CYP2E1 standard kindly provided by Dr. Yoshihiko Funae (Department of Chemical Biology, Osaka City University Medical School, Japan).

Expression of Cyp2e1 checked by PCR genotyping showed lower PCR products (125 bp) represent wild-type mice. A 280-bp was diagnostic for the Cyp2e1<sup>tm1Gonz</sup> mouse. Western blotting of CYP2E1 confirmed that wild-type mice showed a clear band, whereas Cyp2e1-null mice had no band. Representative patterns are illustrated in Fig. 1 for genotyping and Fig. 1B for Western blotting.

In long-term study, F3 male wild-type and Cyp2e1-null mice were treated with diethylnitrosamine (10 mg/kg of body weight, dissolved in 0.9% saline by a single i.p. injection) at 14 days of age and were sacrificed at weeks 24 and 36 after diethylnitrosamine treatment for examination of liver tumors. Thirty-eight wild-type mice were sacrificed at both time points, and twenty-four and twenty-six Cyp2e1-null mice were sacrificed at weeks 24 and 36, respectively. All liver tissues with or without nodules were fixed.

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in 1% phosphate-buffered formalin for 24 h and were routinely processed and embedded in paraffin, and 4-μm sections were stained with H&E for histopathologic examination. The total numbers of tumors in all liver sections per each animal were counted after histopathologic classification.

In short-term study, male wild-type and Cyp2e1-null mice were treated with the same dose of diethylnitosamine or 0.9% saline at the same time and were killed at 6, 24, and 48 h for examination of apoptosis, caspase-3, and Bax mRNA expression. Five wild-type and five Cyp2e1-null mice were treated with saline or diethylnitosamine and killed at every time point. At necropsy, half of diethylnitosamine-treated or saline-treated livers were processed for histopathologic examination, and the remaining samples from all the animals were snap-frozen in liquid nitrogen for RNA extraction and subsequent analysis. All procedures were approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School.

Immunohistochemical examination and quantification of apoptosis. The avidin-biotin complex method was used to show apoptosis in sections (4 μm) of liver tissue thawed with xylene and hydrated through a graded ethanol series. Sections were treated sequentially with proteinase K and 3% hydrogen peroxide and were treated with equilibration buffer, TdT enzyme, and anti-digoxigenin peroxidase conjugate according to the manufacturer’s instruction (ApopTag peroxidase in situ apoptosis detection kit, Chemicon). Immune complexes were visualized with 3,3′-diaminobenzidine tetrahydrochloride as a chromogen. As a negative control, PBS was used instead of TdT enzyme treatment. The sections were counterstained with Mayer’s hematoxylin to facilitate examination under light microscopy.

Sections were analyzed blinded for counts of apoptosis-positive cells. Quantification of apoptosis-positive hepatocytes was performed by scoring over 1,000 cells from 10 random different fields from each animal at 400× magnification. The results were expressed as apoptotic index relative to the total hepatocytes.

RNA isolation and real-time reverse transcription–PCR analysis. About 30 mg of frozen tissues was homogenized, total RNA was isolated from frozen liver using ISOGEN (Nippon Gene Co. Ltd.), and isopropanol was precipitated, dissolved in DEPC-treated distilled water, and stored at −80°C until use. RNA concentrations were determined with a spectrophotometer (Ultraspex: 3000, UV/Visible Spectrophotometer; Pharmacia Biotech). The quality of the isolated RNA was assessed by measuring the absorbance at 260 nm, analyzing the A260/A280 ratio (1.7–2) and evaluating the integrity of the 28S and 18S RNA band on 1% agarose gels.

For cDNA synthesis, 3 μg of total RNA were heated to 70°C for 10 min and then placed immediately on ice for 10 min. To each sample, 4 μL of 5× first strand buffer, 2 μL of 0.1 mol/L DTT, 4 μL of 2 mmol/L each deoxynucleotide triphosphate mix, 1 μL of oligo(dT) primer, and 1 μL of Superscript II reverse transcriptase (Invitrogen) were added. Reverse transcription was then carried out at 42°C for 50 min and followed by heating to 70°C for 15 min, and cDNA samples were stored at −20°C until assayed. cDNAs were amplified using specific probes and primers for mouse caspase-3 and Bax using Taqman Gene Expression Assays according to the manufacturer’s instruction (Applied Biosystems). The PCR program cycles were set as follows: initial denaturing at 95°C for 20 s, followed by 40 cycles (95°C for 3 s, 60°C for 30 s).

β-Actin mRNA was used as an internal standard, and caspase-3 and Bax mRNA expressions were determined by real-time reverse transcription–PCR (RT-PCR) and normalized against β-actin mRNA levels. All PCR products were amplified in a linear cycle. Data are the mean ± SD from all samples per group of three independent experiments.

Statistical analysis. Statistical analyses were performed with Student’s t test using the JMP program (SAS Institute). For all comparisons, probability values of <5% (P < 0.05) were considered to be statistically significant.

Results

Body and liver weight in long-term study. During experiment, there were no deaths. There were no differences of body weight between wild-type and Cyp2e1-null mice during experimental periods (data not shown). However, liver weight of Cyp2e1-null mice were significantly different at weeks 24 and 36 compared with wild-type mice (P < 0.01; Table 1).

Liver tumors in long-term study. Macroscopically, several nodules in wild-type mice were shown; however, there were no masses viewed in Cyp2e1-null ones at week 24. Many nodules in wild-type mice were shown, whereas there were a few mass detected in Cyp2e1-null mice at week 36. Representative macroscopic pictures of the livers from wild-type and Cyp2e1-null mice are shown in Fig. 2A and B, respectively.

Histopathologic examination revealed that most tumors were hepatocellular adenomas (HCA) at week 24 in wild-type mice with no tumors observed in Cyp2e1-null mice (Table 2). At week 36, there was increase of hepatocellular carcinoma (HCC) in wild-type mice, whereas few HCC were found in Cyp2e1-null mice. Representative HCC from the livers of wild-type mice at 36 weeks after diethylnitosamine treatment is shown in Fig. 2C.

Liver tumor incidences of Cyp2e1-null mice were significantly decreased at weeks 24 and 36 compared with wild-type mice (P < 0.01). Cyp2e1-null mice showed a significant decrease in multiplicities of HCA at weeks 24 and 36 (P < 0.05 and P < 0.01, respectively).

<table>
<thead>
<tr>
<th>Wk after diethylnitosamine</th>
<th>CYP2E1 genotype</th>
<th>No. mice</th>
<th>Final body weight (g) mean ± SD</th>
<th>Relative liver weight (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>+/-</td>
<td>38</td>
<td>32.7 ± 4.5</td>
<td>50.0 ± 0.4</td>
</tr>
<tr>
<td>36</td>
<td>+/-</td>
<td>24</td>
<td>32.1 ± 2.8</td>
<td>4.4 ± 0.4*</td>
</tr>
<tr>
<td>36</td>
<td>-/-</td>
<td>38</td>
<td>34.6 ± 5.1</td>
<td>6.2 ± 2.2</td>
</tr>
<tr>
<td>36</td>
<td>-/-</td>
<td>26</td>
<td>35.4 ± 5.2</td>
<td>4.3 ± 0.6*</td>
</tr>
</tbody>
</table>

*Significantly different from wild-type mice at P < 0.01.
respectively) and HCC at week 36 compared with wild-type mice ($P < 0.01$; Table 2).

**Histopathologic and immunohistochemical examination and quantification of apoptosis in short-term study.** In short-term study, H&E staining revealed apoptotic figures featured more frequently in liver of wild-type mice than that of $Cyp2e1$-null mice (Fig. 3A and B), and TUNEL staining confirmed these differences between livers of wild-type and $Cyp2e1$-null mice (Fig. 3C and D). The apoptotic index of $Cyp2e1$-null mice were significantly different at 6 ($P < 0.05$) and 24 h ($P < 0.01$) compared with those of wild-type mice, but there were no differences at 48 h after diethylnitrosamine treatment (Fig. 4).

**Quantitative RT-PCR analysis in short-term study.** Quantitative RT-PCR analysis showed that caspase-3 mRNA expressions in $Cyp2e1$-null mice treated with diethylnitrosamine was significantly different at 6 ($P < 0.01$) and 24 h ($P < 0.05$) compared with that of wild-type mice (Fig. 5A), and Bax mRNA expression in $Cyp2e1$-null mice was significantly different at 6, 24, and 48 h after diethylnitrosamine treatment compared with that of wild-type mice ($P < 0.01$; Fig. 5B).

**Discussion**

It was reported that diethylnitrosamine was hydroxylated by P450 isozymes in the liver to a bioactive metabolite (14). CYP2E1 converts diethylnitrosamine to reactive products in vitro (15). However, the role of CYP2E1 in the carcinogenicity of diethylnitrosamine in vivo is not known. To determine whether CYP2E1 is required for diethylnitrosamine carcinogenicity, wild-type and $Cyp2e1$-null mice were compared in a liver carcinogenesis bioassay. Both liver tumor incidence and multiplicity were significantly decreased in $Cyp2e1$-null mice compared with wild-type mice at week 24 and/or week 36. Liver weights of wild-type mice were significantly different compared with that of $Cyp2e1$-null mice at weeks 24 and 36, which was likely due to more tumors in wild-type mice. Thus, diethylnitrosamine carcinogenicity in $Cyp2e1$-null mice was very less sensitive compared with wild-type mice.

There were no tumors at week 24 in $Cyp2e1$-null mice and about one-half the incidence of tumors at week 36 in the null mice.

<table>
<thead>
<tr>
<th>Wk after diethylnitrosamine</th>
<th>CYP2E1 genotype</th>
<th>Tumor incidence</th>
<th>HCA multiplicity</th>
<th>HCC multiplicity, mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>+/-</td>
<td>18/38 (47.4%)</td>
<td>1.5 ± 2.9</td>
<td>0.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>0/24 (0%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>+/-</td>
<td>38/38 (100%)</td>
<td>10.4 ± 5.5</td>
<td>3.6 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>-/-</td>
<td>17/26 (65.4%)</td>
<td>2.3 ± 3.2*</td>
<td>0.0 ± 0.2*</td>
</tr>
</tbody>
</table>

*Significantly different from wild-type mice at $P < 0.01$.
† Significantly different from wild-type mice at $P < 0.05$. 

![Figure 2.](image) Macroscopic and microscopic findings in liver of wild-type and $Cyp2e1$-null mice at week 36. Note that many tumors were in the liver from a wild-type mouse (A), but few and small size tumors in the liver from a $Cyp2e1$-null mouse (B). C, representative figure for hepatocellular carcinoma from the liver of a wild-type mouse at 36 wk after diethylnitrosamine treatment. Bar, 100 μm.
indicating that CYP2E1 is the main diethylnitrosamine-activating P450 responsible for diethylnitrosamine-induced hepatocarcinogenesis. Cyp2e1-null mice are also resistant to other toxins and carcinogens that are activated by CYP2E1 (3). At week 36, production of HCC, as well as HCA, was inhibited, suggesting that there may be an inhibition of initiation and promotion in diethylnitrosamine-induced hepatocarcinogenesis.

It seems that the low incidence of tumors at week 36 in Cyp2e1-null mice may be due to a low level of the metabolite that causes the liver tumors. However, the loss of the enzyme does not prevent induction of tumors and probable production of a carcinogenic metabolite, suggesting that there may be another CYP involved in diethylnitrosamine metabolic activation. Although CYP2E1 was thought to be mainly responsible for diethylnitrosamine biotransformation, it was reported that other CYPs may metabolize diethylnitrosamine under some conditions (5, 16). In this connection, it was reported that diethylnitrosamine was oxidized to genotoxic products by CYP2A6, as well as CYP2E1, and the relatively high contribution of CYP2A6 to the activation of diethylnitrosamine was seen in mice and human (18, 19).

In short-term study, a carcinogenic initiating dose of diethylnitrosamine increased the apoptotic index, caspase-3 mRNA, and Bax mRNAs in livers of wild-type mice, but not in Cyp2e1-null mice. High dose treatment with diethylnitrosamine induced DNA damage and mutations (14) and induction of HCA and HCC (13, 20–22). The higher apoptotic index in wild-type mice than Cyp2e1-null mice indicated that more DNA damage occurred in wild-type mice. These events may be early steps responsible for the initiation and promotion of hepatocarcinogenesis as indicated by Figure 3.
the eventual appearance of benign and/or malignant tumors. On the other hand, there were many proliferating cell nuclear antigen-positive labeled cells in both wild-type and Cyp2e1-null mice, and there were no differences in cell proliferation index between the two types of mice (data not shown).

There has been a report that diethylnitrosamine treatment increased lipid peroxidation and increased apoptotic response in livers of mice (23). Oxidative stress is the result of an increase in reactive oxygen species (ROS), such as hydrogen peroxide (H2O2), hydroxyl radicals (OH), superoxide anion radical (O2•−) and so on, and high intracellular levels of ROS can lead to damaged mitochondria, DNA modification, and lipid peroxidation, resulting in a number of disease states, including cancer in human (24).

Recent studies suggest that expression of CYP2E1 is involved in yielding oxidative stress from both hepatic cells (25–27) and nonparenchymal cells, such as Kupffer cell (28, 29). Moreover, liver injury in response to inflammation or diethylnitrosamine exposure elicits an inflammatory response in nonparenchymal cells, which secrete several cytokines and growth factors that promote compensatory proliferation of quiescent hepatocytes carrying diethylnitrosamine-induced mutations. This process allows for the transmission of genetic alterations to daughter cells, thereby favoring liver neoplastic progression, and the increased proliferation is followed by dysplasia, HCA, and HCC formation (30). Further studies will be required to investigate the DNA damage induced by diethylnitrosamine of hepatic cells or via indirect from nonparenchymal cells through CYP2E1.

Interestingly, even in the absence of substrate, expression of CYP2E1 can generate ROS (31). However, the mechanisms responsible for its strong effect are still a matter of debate. Thus, the decrease of the intensity of the oxidative stress in Cyp2e1-null mice could markedly reduce the DNA damage provoked by diethylnitrosamine in hepatocytes and, therefore, the frequency of cancer initiation and/or promotion.

In our study, quantitative RT-PCR analysis showed an increase of Bax mRNA after diethylnitrosamine treatment in wild-type mice only. Bcl-2 protein duels with its counteracting twin, a partner known as Bax. When Bax is in excess, cells execute a death command; however, when Bcl-2 dominates, the program is inhibited and cells survive (32). Ectopic expression of Bax induces apoptosis with apoptosis-associated morphologic alterations, caspase activation, and subsequent substrate proteolysis (33, 34). Increased cell turnover results in the selection of monoclonal hepatocyte populations that subsequently undergo genomic alterations that lead to the development of HCC (35). However, p53 showed no difference between the liver of wild-type and Cyp2e1-null mice treated with diethylnitrosamine (data not shown). Our study suggests that the apoptosis response may be induced by a p53-independent pathway. It was shown that p53 integrated numerous signals that control cell life (36), and some of the proapoptotic family members, such as Bax and Noxa or PUMA, were transcriptional targets of p53 (37).

In conclusion, because Cyp2e1-null mice showed lower tumor incidence and multiplicity compared with wild-type mice in diethylnitrosamine-induced hepatocarcinogenesis, it is suggested that CYP2E1 could strongly participate in diethylnitrosamine-induced hepatocarcinogenesis and the high frequency of tumors in wild-type mice could be associated with the increase of apoptosis.

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