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Mutagenic Activation of Urinary Bladder Carcinogens by CYP4B1 and the Presence of CYP4B1 in Bladder Mucosa

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ABSTRACT. We investigated the mutagenic activation of 2-naphthylamine (2-NA), 3,2′-dimethyl-4-aminobiphenyl (DMAB), and 3,3′-dichlorobenzidine (DCB), bladder carcinogens, by renal and bladder microsomes and by purified P450s using the *umu* gene expression system, which detects DNA damage. Mouse renal microsomes had high mutagenic activation toward DCB and low activity toward 2-NA. Purified mouse Cyp4b1 also had high mutagenic activity toward DCB. Anti-Cyp4b1 antibody efficiently inhibited DCB bioactivation by mouse renal microsomes with a high Cyp4b1 content. Lauric acid, a substrate of Cyp4b1, efficiently inhibited DCB bioactivation by renal and bladder microsomes of the mouse and by purified Cyp4b1. To assess the contribution of CYP4B1 to bladder carcinoma, further investigation was done with the *umu* test and an immunochemical study. Ten forms of purified rat P450s including rat CYP4B1 were used in the *umu* test for 2-NA, DMAB, and DCB. CYP4B1 had the highest activity toward DMAB and DCB. Other P450s had activities of less than 20% that of CYP4B1. CYP4B1 also activated 2-NA, but its activity was about 10% of that toward DMAB or DCB. Rat bladder epithelium was stained specifically with anti-Cyp4b1 antibody, indicating the presence of CYP4B1 in the rat bladder mucosa. Also, CYP4B1 mRNA was detected by northern blotting and reverse transcription–polymerase chain reaction (RT–PCR). These findings suggested that CYP4B1 could contribute to the initiation of carcinogenesis in rat and mouse bladder by activation of aromatic amines. BIOCHEM PHARMACOL **54**;6:677–683, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. cytochrome P450; CYP4B1; rat bladder; 3,3′-dichlorobenzidine; 3,2′-dimethyl-4-aminobiphenyl; *umu* gene expression

Primary aromatic amines such as benzidine and 2-NA[‖] have been identified as human bladder carcinogens that also produce bladder cancer in animals [1, 2]. Carcinogenic aromatic amines are thought to require activation to electrophilic species before exerting carcinogenic effects [3]. Benzidine has been studied extensively, and several different metabolic pathways involving N-oxidation leading to DNA binding have been described [4]. After benzidine is N-acetylated, it can then be N′-hydroxylated, and this appears to be the major activation pathway in humans [5–7]. On the contrary, biphenyl amine, which is also a bladder carcinogen, is inactivated by N-acetylation [8]. Benzidine is strongly activated by prostaglandin H synthetase, and it has been suggested that this enzyme is involved in the initiation of bladder cancer by benzidine [9]. DCB is a benzidine derivative and also a potent bladder carcinogen in rats and humans, although benzidine is a weak carcinogen for rat bladder [10]. DCB is not activated by prostaglandin H synthetase, and, unlike benzidine, DCB as well as acetylated DCB has high mutagenic activity [10]. The activation pathway of DCB may be different from that of benzidine. Iba [10] suggested that cytochrome P450 contributes to the mutagenic activation of DCB.

Several carcinogenic aromatic amines are metabolized to toxic and carcinogenic compounds by cytochrome P450s [11]. The liver has the highest P450 content, and most studies of aromatic amine metabolism have used the liver as the target [12]. Little information is available regarding the isoforms of cytochrome P450 in extrahepatic organs or tissues. Studies of aromatic amine activation in target tissues have demonstrated that bovine bladder mucosa microsomes contain P450 and mediate the N-hydroxylation of 4-aminobiphenyl [13]. Vanderslice *et al.* [14] have found that a P450 in rabbit bladder mucosa activates 2-aminofluorene to a mutagenic product. These findings suggest that P450s contribute to bladder carcinogenesis by chemical carcinogens. However, there is little evidence for the metabolism of biphenyl amine or benzidine derivatives by P450s.

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‖ *Abbreviations:* 2-NA, 2-naphthylamine; CYP, cytochrome P450; 3-MeO-AAB, 3-methoxy-4-aminoazobenzene; DCB, 3,3′-dichlorobenzidine; DMAB, 3,2′-dimethyl-4-aminobiphenyl; DLPC, dilauroylphosphatidylcholine; and RT–PCR, reverse transcription–polymerase chain reaction.

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In this study, we investigated the mutagenic activation of the aromatic amine bladder carcinogens 2-NA, DMAB, and DCB by P450s using the *umu* test that detects DNA damage. 2-NA is a potent bladder carcinogen in humans but a weak one in rodents [2]. DMAB and DCB induce bladder carcinoma in the rat [10, 15–17]. In addition, we studied the presence and localization of CYP4B1 in rat bladder by means of immunostaining and northern blotting to evaluate the role of CYP4B1 in the induction of bladder cancer.

MATERIALS AND METHODS

Chemicals

Salmonella typhimurium NM2009 for the *umu* test was a gift from Dr. T. Shimada of the Osaka Prefectural Institute of Public Health. DCB was purchased from the Tokyo Chemical Industry Co. (Tokyo, Japan). 3-MeO-AAB was synthesized by a method described previously [18]. 2-NA, DMAB, $NADP^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and DLPC were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). The purities of 3-MeO-AAB, 2-NA, DMAB, and DCB were more than 95%. Other chemicals and reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Animals and Treatment

Male F344 rats (6 weeks old) were obtained from Charles River (Shiga, Japan) and housed in an air-conditioned room at 23 ± 1° under a relative humidity of 36 ± 6% and with a 12 hr light–12 hr dark cycle. Rat bladders were fixed in Carnoy's fixative, embedded in paraffin, sectioned at 4 μm, and stained immunohistochemically to investigate the localization of CYP4B1.

Purification of P450 and Preparation of Antibody

Male BALB/c mice (7 weeks old) were obtained from Japan Clea (Tokyo). Kidney and urinary bladder microsomes of rats and mice were prepared as described [19]. CYP1A1, 1A2, 2A2, 2B1, 2C11, 2D1, 2E1, 3A2, 4A2, and 4B1 were purified from hepatic, renal, and pulmonary microsomes of male rats and have been characterized previously [19–22]. Cyp4b1 was purified from renal microsomes of male mice. This P450 was characterized and its cDNA was cloned as described [23]. Originally, we purified three P450s from renal microsomes of male mice and designated them MK-1, MK-2, and MK-3 [23]. These forms could not be distinguished by peptide mapping, N-terminal amino acid sequence, catalytic activity, or mobility on SDS–PAGE [23]. Also, mouse Cyp4b1 expressed in yeast cells had properties similar to those of MK-1, MK-2, and MK-3 (data not shown). In this study, we used MK-2 as mouse Cyp4b1. Cytochrome b_5 and NADPH-P450 reductase were purified as described [24]. Antibodies against P450 were raised in a rabbit, and immunoglobulin G (IgG) was prepared as reported [24].

Immunochemical Study

Immunoblotting was done by a method reported previously [24]. Detection of protein on a nitrocellulose membrane blotted from acrylamide gel was done by means of chemiluminescence (ECL; Amersham, Buckinghamshire, England) following the manufacturer's instructions. Immunohistochemistry proceeded as follows. The avidin–biotin–peroxidase complex (ABC) method described by Hsu *et al.* [25] was used to demonstrate CYP4B1 immunohistostaining. After deparaffinization, tissue sections were immersed sequentially in normal serum (as a control) or anti-Cyp4b1 antibody (1:750, reaction time: 2 hr, room temperature), biotin-labeled goat anti-rabbit IgG (1:400), and ABC. The sites of peroxidase binding were detected with diaminobenzidine. The sections were then counterstained with hematoxylin for microscopic examination.

Preparation of RNA and Northern Blotting

Bladders were removed rapidly from rats and immediately frozen in liquid nitrogen. The total RNAs were isolated as described by Chomczynski and Sacchi [26]. The total RNA was analyzed by electrophoresis with 1% agarose gel and blotted onto a nylon membrane (Genescreen, NEN Research Products, Boston, MA, U.S.A.), which was hybridized with a CYP4B1 cDNA probe [23]. Nucleic acid hybridization was done at 65° in 0.9 M NaCl and 0.09 M sodium citrate (pH 7.0), containing heat-denatured salmon sperm DNA (50 mg/mL) and 0.5% SDS. Membranes were washed with 0.3 M NaCl and 0.03 M sodium citrate (pH 7.0) containing 0.5% SDS.

RT–PCR

cDNA was synthesized from total RNA (1 μg) by means of the reverse transcriptase reaction (total 20 μL) using an RNA PCR kit (Perkin–Elmer, Norwalk, CT, U.S.A.) according to the manufacturer's instructions. PCR reactions were done in a 100-μL final volume consisting of 1 × Taq DNA polymerase buffer (50 mM KCl, 10 mM Tris–HCl, pH 9.0, Triton X-100, 1.25 mM $MgCl_2$), a 0.2 mM concentration of each deoxyribonucleotide triphosphate, 10 pmol of each forward and reverse primer, 1 unit of Taq DNA polymerase (Perkin–Elmer), and 20 μL of the cDNA reaction mixture. The PCR was done for 30 cycles by sequence temperature: 1 min denaturation at 94°, 1 min annealing at 54°, and 2.5 min extension at 72°. The forward and reverse primers for rat CYP4B1 were 5′-CCAGTACCATAATGACTTCA-3′ and 5′-TAGAGG CGGAAGCACTCCTT-3′, respectively, and predicted to produce a 444-bp fragment (nucleotide positions 689–1132 of the reported rat CYP4B1 cDNA [27]). PCR products

TABLE 1. Mutagenic activation of procarcinogens by microsomes and Cyp4b1 from the mouse

	3-MeO-AAB	2-NA	DCB
Microsomes			
Kidney	241 ± 16	23 ± 3	269 ± 14
Bladder	—	—	16 ± 1
Purified Cyp4b1	1240	90	1620

Values of microsomes (means ± SD for 3 samples) are expressed as units/min/mg of protein. Values of purified P450 are expressed as units/min/nmol of P450. Renal microsomes (20 μg), bladder microsomes (50 μg), or purified Cyp4b1 (10 pmol) were reacted with procarcinogens (10 μM) under the conditions described in Materials and Methods. Key: 3-MeO-AAB, 3-methoxy-4-aminoazobenzene; 2-NA, 2-naphthylamine; DCB, 3,3'-dichlorobenzidine; and —, not assayed.

(10 μL) were analyzed directly by electrophoresis on a 1.0% agarose gel and stained with ethidium bromide.

Analytical and Assay Methods

The activation of *umu* gene expression by activated metabolites of procarcinogens in the microsomes and in a reconstituted monooxygenase system was measured as described [28]. In brief, the standard reaction mixture (final volume, 1.0 mL) contained 50 mM potassium phosphate buffer (pH 7.25); substrate (10 μM) such as DCB dissolved in dimethyl sulfoxide (10 μL); a mixture (NADPH-generating system) of $NADP^+$ (0.25 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (0.5 unit), and $MgCl_2$ (1.5 mM); either microsomes (20–50 μg) or a reconstituted system containing purified P450 (10–30 pmol), NADPH-P450 reductase (0.3 unit), cytochrome b_5 (10–30 pmol), and DLPC (5 μg); and a suspension of *S. typhimurium* (0.75 mL). The reaction at 37° for 120 min was stopped by rapidly cooling the mixture in an ice-water bath. A portion (0.2 mL) of the reaction mixture was assayed for β-galactosidase activity, and *umu* gene expression was measured as the specific β-galactosidase activity per minute per nanomole of P450 [28]. Individual CYP forms are designated by the systematic nomenclature of Nelson *et al.* [29].

RESULTS

Mutagenic Activation of Procarcinogens by Mouse Renal Microsomes and Purified Mouse Cyp4b1

We have found that mouse Cyp4b1 activates 3-MeO-AAB, 2-aminoanthracene, and 2-aminofluorene [23]. In the present study, we further investigated the mutagenic activation of the aromatic amines 3-MeO-AAB, 2-NA, and DCB by mouse Cyp4b1 using the *umu* test (Table 1). 2-NA induces bladder tumor in humans but not in rodents [2]. Mouse Cyp4b1 and renal microsomes had low activity toward 2-NA. However, Cyp4b1 had high mutagenic activation toward not only 3-MeO-AAB but also DCB, which induces bladder tumors in rats [15, 16]. Mouse renal microsomes contain Cyp4b1 as the major form, and it also has high activity toward DCB. Mouse bladder microsomes activated DCB, although to a lesser extent than mouse renal microsomes.

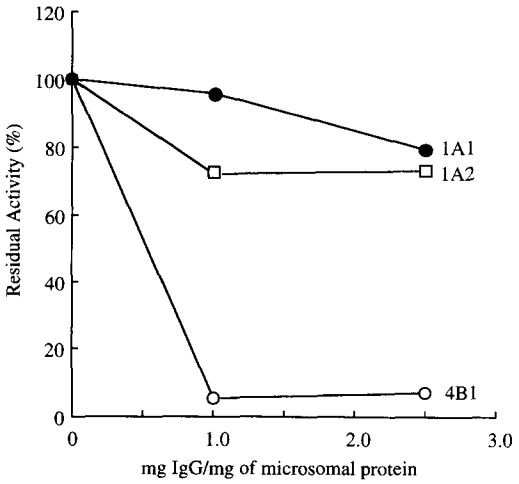

FIG. 1. Effects of antibodies on *umu* gene expression of DCB by mouse renal microsomes. Various amounts of immunoglobulin G (IgG) were incubated with renal microsomes and then the assay proceeded as described in the footnote to Table 1. 1A1, 1A2, and 4B1 indicate antibodies against rat CYP1A1, rat CYP1A2, and mouse Cyp4b1, respectively.

Effects of Cyp4b1 Antibody on umu Gene Expression of DCB by Mouse Renal Microsomes

Mouse renal microsomes and purified Cyp4b1 had high activity toward DCB. We investigated the effects of Cyp4b1 antibody on the bioactivation of DCB by renal microsomes (Fig. 1). Anti-Cyp4b1 antibody completely inhibited the mutagenic activation of DCB by mouse renal microsomes. CYP1A1 and 1A2 antibodies were also used for comparison. CYP1A1 and 1A2 activate many procarcinogens, including aromatic or heterocyclic amines [28]. The effects of these antibodies on DCB bioactivation were slight. These results indicated that Cyp4b1 plays a major role in the bioactivation of DCB in mouse renal microsomes. We also investigated the effects of CYP1A1, 1A2, and 4B1 antibodies on the bioactivation of DCB in bladder microsomes. Unfortunately, inhibition of *umu* activity by antibodies was undetectable because the activity of bladder microsomes was too low and the increased amount of microsomal protein might interfere with the inhibition by the antibody. However, the Cyp4b1 substrate, lauric acid [23], efficiently inhibited the *umu* activation of DCB by both mouse renal and bladder microsomes, suggesting that Cyp4b1 activates DCB in mouse bladder microsomes (Table 2).

Immunoblotting of Mouse Kidney and Bladder

Renal and bladder microsomes of the male mouse were subjected to immunoblotting with Cyp4b1 antibody (Fig. 2). Both microsomes gave a single staining band, indicating the presence of Cyp4b1 in the mouse bladder. The level of

TABLE 2. Effects of lauric acid on *umu* gene expression of DCB

Sodium laurate (μM)	Liver microsomes	Kidney microsomes	Bladder microsomes	Purified Cyp4b1
0	100 (146)	100 (309)	100 (15)	100 (1439)
1	93	103	91	100
10	90	96	74	88
100	80	42	24	34

Hepatic microsomes (20 μg), renal microsomes (20 μg), bladder microsomes (50 μg), and purified P450 (10 pmol) were used in the assay. Activities without sodium laurate were set at 100%, and values are expressed as a percentage of the residual activity. Values in parentheses indicate absolute activities and are expressed as units/min/mg of protein for microsomes and units/min/nmol of P450 for purified P450.

Cyp4b1 in bladder microsomes was calculated to be 3.5 pmol/mg of the microsomal protein. The level was 2.5% that of mouse renal microsomes.

umu *Gene Expression of DCB by Purified Rat P450s*

We found that mouse Cyp4b1 had high activity toward DCB, a benzidine derivative. Little is understood about the bioactivation of benzidine and biphenyl amine derivatives by the multiple forms of P450. It is established that DMAB and DCB induce bladder carcinoma in rats [15, 17], and we have purified several forms of P450 from the rat liver [20]. Thus, we performed the *umu* test for DCB, DMAB, and 2-NA bioactivation using ten forms of purified rat P450s, including rat CYP4B1 (Fig. 3). Rat CYP4B1 had extremely high activity toward DCB and DMAB. Isoform-specificity toward these chemicals was high. Its activity is similar to that of mouse Cyp4b1. CYP1A1 and 1A2 also had activity toward DCB and DMAB, but their activity was less than 20% that of CYP4B1. 2-NA also was activated by CYP4B1, although its activity toward 2-NA was only 10% of that toward DMAB and DCB.

Immunostaining of Rat Bladder

We investigated localization of CYP4B1 in the rat bladder by immunohistochemistry. CYP4B1 was immunohistochemically demonstrated in the bladder epithelium, especially in the surface epithelium of the normal rat bladder (Fig. 4A). Procarcinogens in urine can be activated on the surface of the bladder epithelium. Non-immune serum did not produce any staining in rat bladder (Fig. 4B).

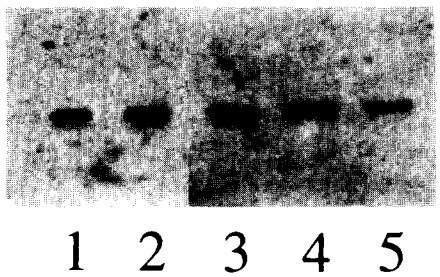

FIG. 2. Immunoblotting of mouse kidney and bladder microsomes. The renal microsomes (0.5 μg), bladder microsomes (20 μg), and purified Cyp4b1 (0.04 pmol) were resolved by SDS–PAGE. Lanes 1 and 2 are individual renal microsomes. Lanes 3 and 4 are individual bladder microsomes. Lane 5 is purified mouse Cyp4b1.

Northern Blotting and RT–PCR of Rat Bladder RNA

Total RNA was isolated from the rat bladder and subjected to agarose gel electrophoresis (Fig. 5A). RNA was blotted onto a membrane and hybridized with a CYP4B1 cDNA probe (Fig. 5B). A single band appeared, indicating the presence of CYP4B1 mRNA in the rat bladder. Furthermore, RNA was converted to cDNA and amplified by PCR.

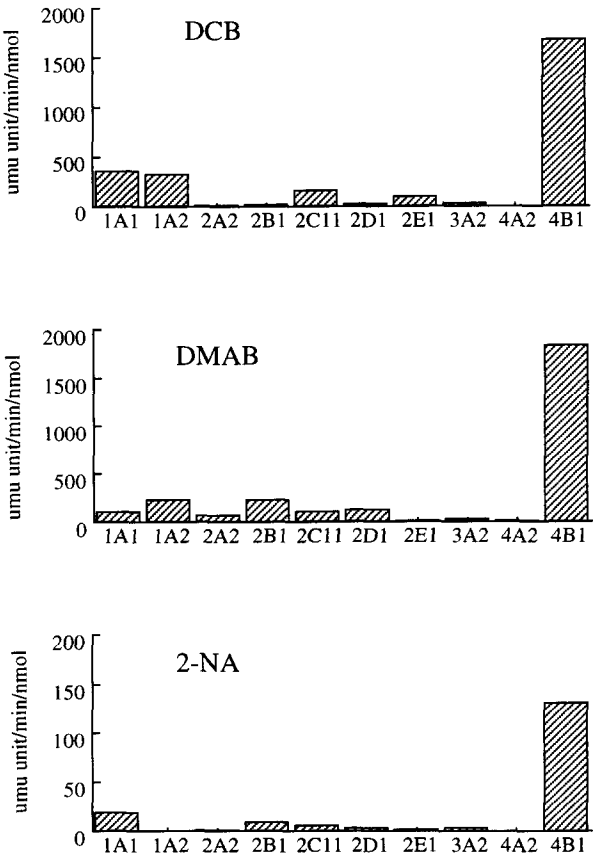

FIG. 3. *umu* Gene expression of DCB, DMAB, and 2-NA by purified rat P450s. Purified rat P450s (10 pmol) were reacted with procarcinogens (10 μM) under the conditions described in Materials and Methods.

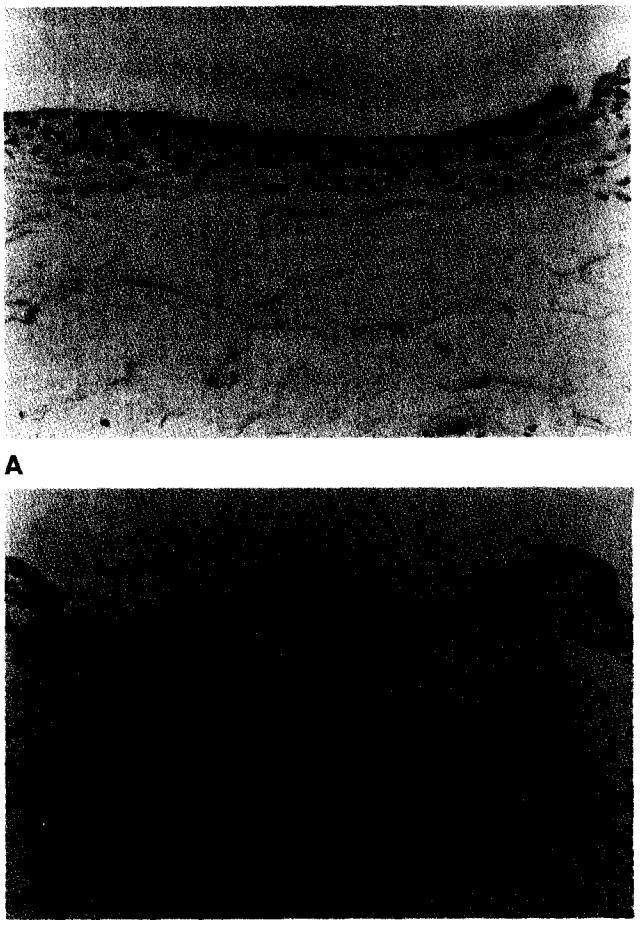

FIG. 4. Localization of CYP4B1 in rat bladder. The rat bladder was immunohistochemically stained with anti-Cyp4b1 antibody (A) and with non-immune serum (B). CYP4B1 was visualized with the avidin–biotin–peroxidase complex (ABC) and diaminobenzidine.

The predicted band (444 bp) migrated in an agarose gel (Fig. 5C). These results are further evidence for the presence of CYP4B1 in the rat bladder.

DISCUSSION

This study provides direct evidence that P450 can activate DMAB, a biphenyl derivative, and DCB, a benzidine derivative, to mutagenic substances. Vanderslice *et al.* [14] found that the bladder mucosa of the rabbit contained CYP4B1, which plays a major role in the metabolism of 2-aminofluorene, a carcinogenic aromatic amine, and that P450 is an important enzyme in carcinogenesis of the bladder as well as the liver, which has the highest P450 content. CYP4B1 also metabolizes 2-aminoanthracene and aflatoxin B_1 [30]. *CYP4B1* genes of rabbit and rat were first established by Gasser and Philpot [27]. Poupko *et al.* [13] found that the N-hydroxylation activity of 4-aminobiphenyl (a carcinogenic amine in the rat bladder) is inhibited by a chemical inhibitor of P450. Although the bladder has a

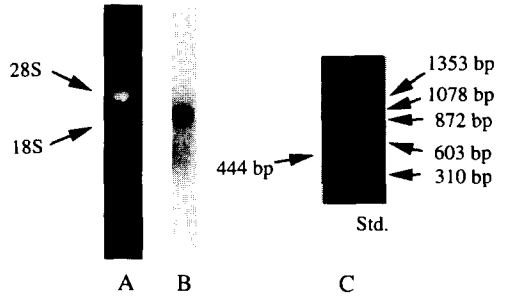

FIG. 5. Northern blots and RT–PCR analysis of CYP4B1 mRNA. Total cellular RNA was isolated from whole rat bladder. Total RNA (10 μg/well) was separated on 1% agarose gel (A) and subjected to northern blot analysis using a CYP4B1 cDNA probe (B). RT–PCR was done using total RNA with the CYP4B1 primers described in Materials and Methods. Amplified fragment oligonucleotide was resolved by electrophoresis with 1.0% agarose gel stained with ethidium bromide (C).

low content of P450, it may be sufficient to initiate bladder cancer upon long exposure to carcinogens in the urine. Human CYP4B1 cDNA was isolated by Nhamburo *et al.* [31]. They and Czerwinski *et al.* [31, 32] reported that the cDNA-expressed human CYP4B1 had no activity toward 2-aminofluorene and lauric acid, which are typical substrates for CYP4B1, and concluded that substrate specificity of human CYP4B1 is different from that in other species. However, human renal microsomes had lauric acid ω-hydroxylation activity, like mouse renal microsomes, and was inhibited efficiently by anti-CYP4B1 antibody, which did not cross-react with CYP4A forms (data not shown). CYP4B1 forms of rabbit, rat, mouse, and hamster have activity toward 2-aminofluorene [33]. Nhamburo *et al.* [31] reported the possibility of genetic polymorphism in the human *CYP4B1* gene. There may be human *CYP4B1* variants that have different catalytic specificity.

Aromatic amines are first metabolized in the liver by N-acetylation, N-oxidation, and other modification. N-Oxidation is the activation pathway of aromatic amines, and activated amines bind to protein or DNA [7, 34]. 4-Aminobiphenyl-hemoglobin adducts and DNA adducts in urothelial cells are detected in humans, and adduct levels are correlated with bladder cancer risk [35]. Part of the amines activated in the liver may reach the bladder through the bloodstream [34], and amines are also activated directly on the bladder surface [36–38]. N-Acetylation is important in the activation of aromatic amines. N-Acetylation of 4-aminobiphenyl seems to cause inactivation of its mutagenicity because slow acetylators have a high risk of bladder cancer [6]. On the contrary, benzidine may be activated after N-acetylation because almost all DNA adducts of benzidine in humans are acetylated derivatives and the acetylation activity of benzidine in humans seems to have no effect on the levels of urothelial DNA adducts [6]. DCB, as well as N-acetylated DCB, has high mutagenic activity, and DCB is also important in the initiation of bladder cancer [10]. The activation pathway of DCB may be different from that of benzidine. Benzidine is strongly

activated by prostaglandin H synthetase but not by hepatic P450s [9, 10]. However, DCB is not activated by prostaglandin H synthetase [10].

P450s, especially CYP4B1, can bioactivate DMAB and DCB, bladder carcinogens. CYP4B1 was revealed in rat and mouse bladder microsomes by means of immunoblotting and immunohistochemistry. Tissue-staining indicated that CYP4B1 was present in epithelial cells of the bladder. This is the first direct evidence of CYP4B1 in the bladder mucosa. These findings, together with the results of a metabolic study, indicated that DMAB and DCB are activated by CYP4B1 in the mucosa of the rat and mouse bladder. These results suggested that CYP4B1 could contribute to the initiation of carcinogenesis in rat and mouse bladder by activation of aromatic amines.

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