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Immunohistochemical Detection of Carcinogen-DNA Adducts in Normal Human Prostate Tissues Transplanted into the Subcutis of Athymic Nude Mice: Results with 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 3,2′-Dimethyl-4-aminobiphenyl (DMAB) and Relation to Cytochrome P450s and *N*-Acetyltransferase Activity

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Human prostate tissue transplanted into nude mice was examined immunohistochemically for DNA adducts formed after administration of 3,2′-dimethyl-4-aminobiphenyl (DMAB) or 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Positive staining for DMAB- or PhIP-DNA adducts was evident in 70–95% of both epithelial and stromal cells in human prostate xenografts. Reverse transcription-polymerase chain reaction (RT-PCR) analysis revealed a normal human prostate epithelial cell line (PrEC) to express both cytochrome P450 1A2 (CYP1A2) and *N*-acetyltransferase 2 (NAT2) mRNA, while a normal human prostate fibroblast cell line (NHPF) expressed NAT2, but not CYP1A2 mRNA. In addition, NAT2 and to a lesser extent CYP1A2 mRNAs were also found in four cases of normal human prostate tissues. The results suggest that initial activation of chemicals by liver CYP1A2 and subsequent metabolism by prostate NAT2 is a major pathway of DNA adduct formation in human prostate cells. Thus, the data suggest that human prostate has the potential to be targeted by environmental carcinogens.

Key words: PhIP — DMAB — DNA adducts — Human prostate — Immunohistochemistry

Most human cancers are believed to be caused by the exposure of individuals to environmental carcinogens, with diet playing a particularly important role.[1,2] 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a heterocyclic amine, isolated from cooked fish and meat,[3,4] and also detected in cigarette smoke condensate[5] and in beer and wine.[6] This compound has been found to be carcinogenic in the mammary glands of female rats, in the colon, prostate and thymus of male rats, and in lymphoid tissue in mice.[7–9] Organs targeted by PhIP-carcinogenicity in rodents are common sites of human malignancy in Western countries, where meat consumption is relatively high. Thus, it is highly likely that such carcinogens are important as environmental factors contributing to neoplasia in humans.

Cytochrome P450 (CYP) enzymes play essential roles in the activation and detoxification of a variety of environmental and dietary carcinogens[10,11] and the reactive intermediates generated have been implicated in cancer causation. PhIP bioactivation is highly dependent upon the CYP1A2-mediated *N*-hydroxylation of the parent amine to the corresponding *N*-hydroxy-PhIP.[12,13] *N*-Hydroxy-PhIP is subsequently esterified by acetyltransferases which generate highly electrophilic *O*-acetyl esters. These esters are capable of covalently binding to DNA.[14]

The immunohistochemical approach using DNA adduct-specific antibodies makes possible the precise localization of adduct formation at the cellular level, thus offering a tool for elucidation of the organotropism of carcinogens. Recently we have established antibodies against PhIP-DNA adducts which can be applied to paraffin-embedded sections.[15] To investigate the possible relationship of PhIP with human prostate carcinogenesis, we investigated whether PhIP-DNA adducts are formed in human prostatic cells *in vivo* using a normal human prostate xenograft model.[16] A preliminary finding on PhIP-DNA adduct formation in transplanted human prostate tissue has been reported.[17] An analysis of carcinogen-DNA adducts formed by the synthetic aromatic amine, 3,2′-dimethyl-4-aminobiphenyl (DMAB), which produces prostate tumors as well as colon, Zymbal gland and urinary bladder cancer in rodents was also presented. In addition, expression of associated metabolizing enzymes in human prostate was analyzed using immunohistochemistry and the reverse transcription-polymerase chain reaction (RT-PCR).

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MATERIALS AND METHODS

Animals Male KSN (*nu*/*nu*) athymic nude mice, purchased from Japan SLC, Inc. (Shizuoka) at 12–14 weeks old, were used in the present studies. The animals were maintained and treated in accordance with institutional guidelines in compliance with national and international law. They were housed in plastic cages with hard wood chips for bedding, and fed standard diet (Oriental MF, Oriental Yeast Co., Ltd., Tokyo) and water *ad libitum*. Mice were kept in an environmentally controlled room maintained at a temperature of 22±2°C, and a relative humidity of 55±10%, with a 12 h light/dark cycle.

Tissue processing and transplantation Prostate tissues were obtained from 4 patients (45, 46, 54 and 71 years of age) who underwent total cystoprostatectomy for the treatment of bladder cancers. Prostate tissue preparation and transplantation into the nude mice were conducted as described elsewhere.[16] Briefly, prostate glands were separated from the bladder and placed in culture medium (Dulbecco's MEM with 12.5 m*M* HEPES, Gibco BRL, Rockville, MD), and macroscopically divided into peripheral and transition zones. Small portions were fixed in formalin for histological evaluation as 0-week samples, and the remaining tissues were cut into approximately 5-mm cubes. Trimmed tissues from the peripheral zone were implanted into the subcutis of left and right lateral regions of nude mice. Five mice were used for each human prostate sample.

Carcinogen treatment of nude mice PhIP hydrochloride and DMAB were synthesized by the NARD Institute (Osaka), with purities of >99.9% and >98.0%, respec-

Table I. Immunohistochemical Demonstration of PhIP-DNA Adducts in Human Prostate Transplanted into Nude Mouse Subcutis

Tissues			PhIP (200 mg/kg)	Corn oil
Transplant human prostate	1 (45 years of age)	Epithelial cell	+ (80–90)	–
		Stromal cells	+ (80–90)	–
	2 (46 years of age)	Epithelial cell	+ (75–85)	–
		Stromal cells	+ (75–85)	–
	3 (54 years of age)	Epithelial cell	+ (85–95)	–
		Stromal cells	+ (85–95)	–
	4 (71 years of age)	Epithelial cell	+ (80–90)	–
		Stromal cells	+ (80–90)	–
Nude mouse prostate	Ventral		++ (85–95)	–
	Dorso-lateral		++ (85–95)	–
	Anterior		++ (85–95)	–
	Seminal vesicle		++ (85–95)	–
Nude mouse liver			++ (85–95)	–

–, negative; +, weakly positive; ++, strongly positive.
The percentage of positive cells is in parentheses.

Table II. Immunohistochemical Demonstration of DMAB-DNA Adducts in Human Prostate Transplanted into Nude Mouse Subcutis

Tissues			DMAB (200 mg/kg)	Corn oil
Transplant human prostate	1 (45 years of age)	Epithelial cell	+ (80–90)	–
		Stromal cells	+ (70–80)	–
	2 (46 years of age)	Epithelial cell	+ (80–90)	–
		Stromal cells	+ (70–80)	–
Nude mouse prostate	Ventral		++ (80–90)	–
	Dorso-lateral		+ (70–80)	–
	Anterior		–	–
	Seminal vesicle		+ (80–90)	–
Nude mouse liver			+ (80–90)	–

–, negative; +, weakly positive; ++, strongly positive.
The percentage of positive cells is in parentheses.

tively. Mice were given a single intragastric injection of 200 mg/kg of PhIP or DMAB dissolved in corn oil for 5–14 days after receiving implants. Control mice received corn oil alone. All mice were killed 24 h after administration of a carcinogen, and transplanted human prostate glands and the liver and prostate of each mouse were removed. Half of each was fixed in acetone and the remaining tissues were immediately frozen in liquid nitrogen and stored at −80°C until processed.

Immunohistochemistry Immunohistochemical analyses

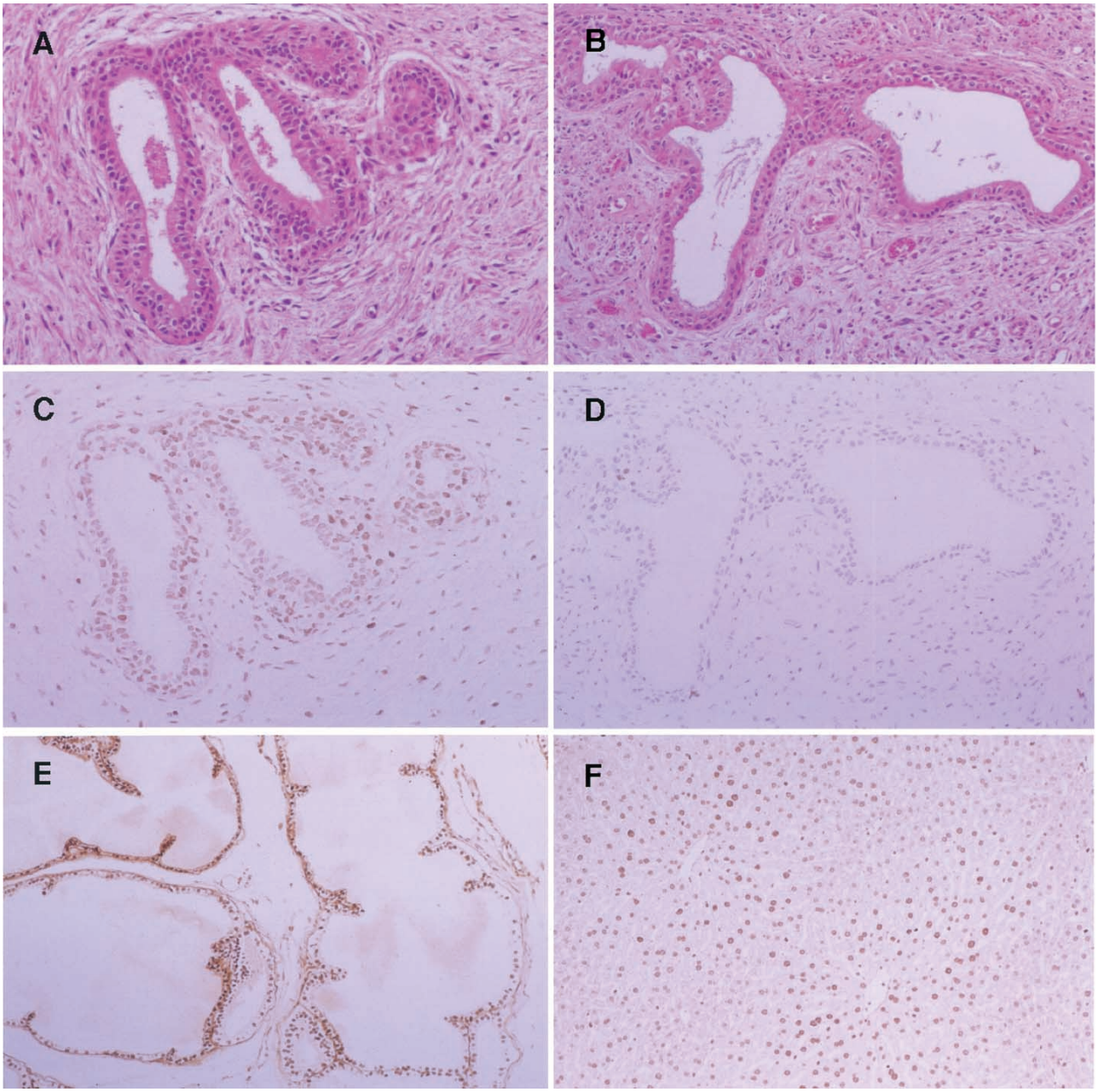

Fig. 1. Immunohistochemical demonstration of PhIP-DNA adducts in normal human prostate tissues transplanted subcutaneously into nude mice. Nude mice were killed 24 h after intragastric administration of PhIP at a dose of 200 mg/kg (A, C, E, F) or corn oil (B, D). A, B: HE staining of the serial sections to C, D. C, D: transplanted human prostate tissues. E: ventral prostate of a nude mouse. F: liver of a nude mouse.

were performed as reported previously[15] with minor modifications. Deparaffinized sections were sequentially treated with 0.5% H_2O_2 for 30 min, 0.05% Tween 20 for 3 min twice, 250 μg/ml RNase for 1 h at 37°C, 2.5 *N* HCl for 20 min, 0.1% trypsin for 15 min at 37°C, and then 5% skim milk for 1 h. The sections were incubated with diluted anti-PhIP-DNA adduct antibody (1:10000), anti-DMAB-DNA adduct antibody[18] (1:1000) or anti-CYP1A2 antibody (1:400, Chemicon International Inc., Temecula, CA) for 2 h at room temperature and sequentially exposed to biotin-labeled goat anti-rabbit IgG and ABC (Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA). The sites of peroxidase binding were visualized with diaminobenzidine. The numbers of cells with carcinogen-DNA adducts were evaluated as percentages of the cell population.

Cell culture Human prostate epithelial (PrEC) and stromal (PrSC) cells, purchased from Clonetics Co. (Walkersville, MD, San Diego, CA), were maintained in "PrEGM" and "FGM" media (Clonetics Co., San Diego, CA), respectively. Cells were harvested at 70–80% confluence by trypsinization and centrifugation, and cell pellets were frozen in liquid nitrogen and stored at −80°C until processed.

RT-PCR Complementary DNA (cDNA) was synthesized with Moloney murine leukemia virus (MMLV)-reverse transcriptase primed with oligo(dT) primers from 1 μg of mRNA from cell lines or total RNA from transplanted human prostate tissues as the template. The same volumes of cDNA solutions were amplified by PCR to measure the amounts of cytochrome P450 1A2 (CYP1A2), *N*-acetyltransferase-2 (NAT2) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNAs. Primers were designed to span exon-intron boundaries to avoid amplification of genomic DNA, and were synthesized by Nihon Gene Research Laboratories Inc. (Sendai): 1) 5′-GAAGATTGTCAACCTTGTCA-3′ and 2) 5′-ATGTAGAAGCCATTCAGCGT-3′, for human CYP1A2 cDNA,[19] 3) 5′-TGCATTTTCTGCTTGACA-3′ and 4) 5′-TGATGTGGTTATAAATGA-3′, for human NAT2 cDNA,[20] and 5) 5′-AACGGATTTGGTCGTATTGG-3′ and 6) 5′-CATACTTCTCATGGTTCACA-3′, for human GAPDH cDNA,[21] PCR amplification was performed with 45 cycles at 94°C for 60 s, 56°C (for CYP1A2), 48°C (for NAT2) and 62°C (for GAPDH) for 90 s and 72°C for 90 s, followed by a 5 min extension at 72°C.

RESULTS

Immunohistochemical demonstration of PhIP- or DMAB-DNA adducts and CYP1A2 in human prostate and mouse tissues The characteristics of transplanted human prostate tissues were reported earlier.[16] Data on immunohistochemical localization of PhIP- or DMAB-DNA adducts in transplanted human prostate and mouse tissues are summarized in Tables I and II. Both epithelial and stromal cells in human prostate xenografts obtained from each individual demonstrated positive nuclear staining for anti-PhIP-DNA (Fig. 1C) or anti-DMAB-DNA adducts (Fig. 2A). No signals were detected in the sections of vehicle-treated human prostate tissue xenografts (Figs. 1D, 2B). With PhIP-DNA adducts the positivities were 75–95% for both epithelial cells and fibroblasts. Strong positive signals were also found in epithelial cells of all lobes of the mouse prostate gland, seminal vesicles and hepatocytes in liver (Fig. 1, E and F). In addition, positive signals for DMAB-DNA adducts were found in 70–90% of both epithelial and stromal cells in 2 cases of human prostate xenografts, and also observed in epithelial cells of

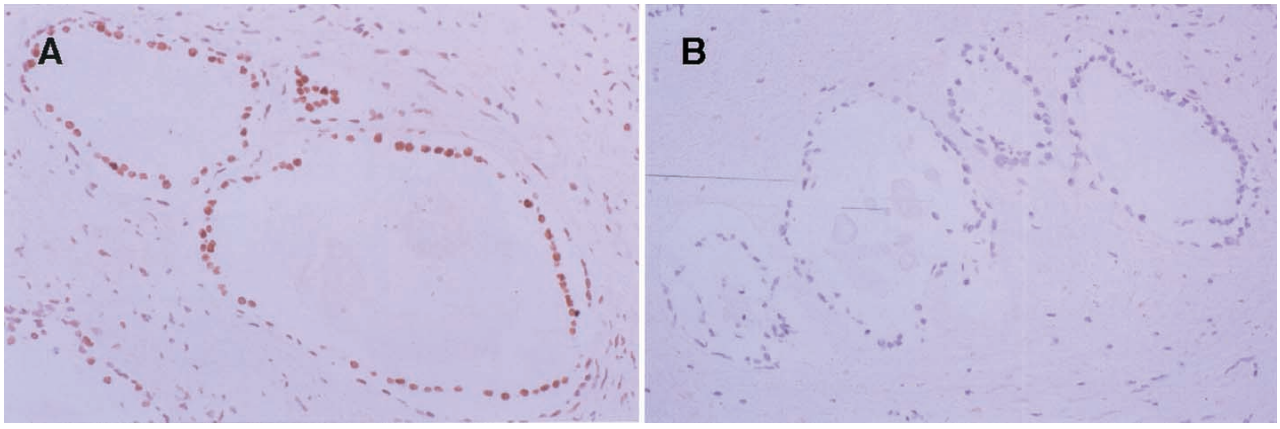

Fig. 2. Immunohistochemical demonstration of DMAB-DNA adducts in normal human prostate tissues transplanted subcutaneously into nude mice. Nude mice were killed 24 h after intragastric administration of DMAB at a dose of 200 mg/kg (A) or corn oil (B).

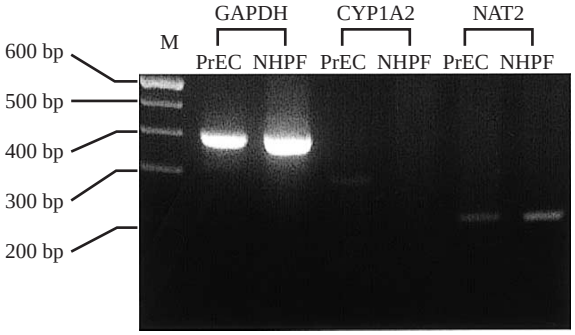

Fig. 3. RT-PCR analysis of CYP1A2 and NAT2 mRNA expression in normal human prostate cell lines (PrEC and NHPF). Sizes of the specific RT-PCR products are 225 bp for NAT2, 288 bp for CYP1A2 and 396 bp for GAPDH. Human GAPDH primers were used in a parallel PCR reaction to confirm RNA integrity.

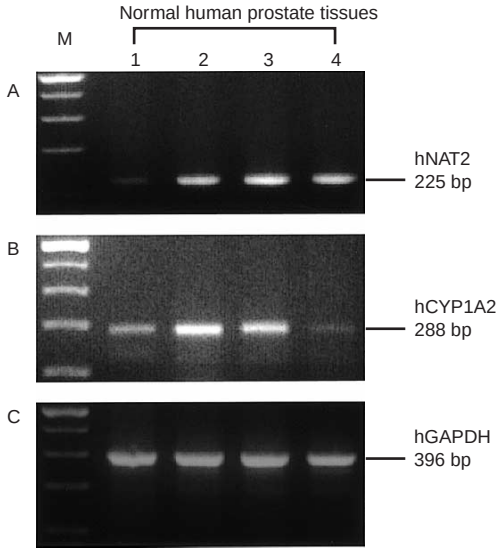

Fig. 4. Expression of mRNA for hNAT2 (A), hCYP1A2 (B) and human GAPDH (C) in normal human prostate tissues as revealed by RT-PCR. Representative samples are shown. Lanes 1–4, normal human prostate tissues from four different individuals.

the ventral and dorso-lateral prostates, seminal vescles and hepatocytes but not of the anterior prostate of mice (Table II).

Staining for CYP1A2 was weakly positive in epithelial cells but not in stromal cells in transplanted human prostate tissues. Strongly positive staining was found in the livers of nude mice.

Expression of CYP1A2 and NAT2 mRNA PrEC was found to express both CYP1A2 and NAT2 mRNA on RT-PCR analysis. Normal human prostate fibroblast cell line (NHPF) expressed NAT2, but not CYP1A2 mRNA (Fig. 3).

NAT2 and CYP1A2 mRNAs were expressed in four samples of transplanted human prostate tissues, all obtained from different individuals (Fig. 4).

DISCUSSION

Humans may be exposed to PhIP on a daily basis, because this is the most abundant heterocyclic amine in cooked meat and fish and it is found in the urine.[22, 23] The present study is the first to demonstrate clearly that PhIP- and DMAB-DNA adducts may be formed in human prostatic cells, both epithelial and stromal cells being positive in the *in vivo* model established in our laboratory. This very significant finding indicates that human prostate tissue has the capability to metabolically activate PhIP, and implies that PhIP may play an important role in human prostate cancer development.

PhIP bioactivation principally involves the enzymes CYP1A2 and NAT2 in man.[24] PhIP is initially metabolized to two major products, 2-hydroxyamino-PhIP (N^2-OH-PhIP) which is mutagenic, and 4′-hydroxyamino-PhIP (4′-OH-PhIP) which is non-mutagenic, by CYP1A2 in human liver.[12, 25, 26] N^2-OH-PhIP is further *O*-acetylated by NAT2 to the ultimate form, *N*-acetoxy-PhIP, which reacts with deoxyguanosine to form *N*-(2′-deoxyguanosin-8-yl)-PhIP.[27–29] The present RT-PCR analyses using human prostate culture cells showed expression of both NAT2 and CYP1A2 mRNAs to be considerably lower than that of GAPDH mRNA. The weak immunohistochemical positivity for CYP1A2 in human prostate xenografts presumably is a simple reflection of mRNA expression levels. The results suggest that initial activation in liver CYP1A2 and subsequent metabolism by prostate NAT2 is the major pathway of PhIP-DNA adduct formation in human prostate cells, although prostate epithelial cells *per se* are capable of directly metabolizing free PhIP as a minor pathway.

Immunohistochemical analysis of PhIP-DNA adducts has shown the distribution of DNA adducts in prostate glands to be essentially the same in rats and mice. However, mouse hepatocytes were found to be strongly positive in contrast to their rat counterparts.[15] These findings are consistent with the fact that PhIP induces liver tumors in mice,[30] but not in rats. Experiments on rat prostate carcinogenesis using PhIP or DMAB in our laboratory have demonstrated that these carcinogens induce only epithelial and not non-epithelial tumors in prostate glands, and a correlation with the cellular distribution of carcinogen-DNA adducts is thus lacking. It is generally accepted that DNA adduct formation is necessary, but in itself not sufficient for carcinogenesis. In this study, a very high dose of PhIP was applied to allow detection of PhIP-DNA adducts in human prostate tissues. Further investigations are now

needed to clarify what is the determinant factor for carcinogenic organotropicity and what is the level of PhIP involvement in human prostate carcinogenesis as an environmental risk factor, at doses to which people are exposed on a daily basis.

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