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Neoplastic transformation and DNA-binding of 4,4′-methylenebis(2-chloroaniline) in SV40-immortalized human uroepithelial cell lines

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The tumorigenic transformation of certain occupationally significant chemicals, such as N-hydroxy-4,4′-methylenebis[2-chloroaniline] (N-OH-MOCA), N-hydroxy-*ortho*-toluidine (N-OH-OT), 2-phenyl-1,4-benzoquinone (PBQ) and N-hydroxy-4-aminobiphenyl (N-OH-ABP) were tested *in vitro* using the well established SV40-immortalized human uroepithelial cell line SV-HUC.PC. SV-HUC cells were exposed *in vitro* to varying concentrations of N-OH-MOCA, N-OH-OT, N-OH-ABP and PBQ that caused approximately 25% and 75% cytotoxicity. The carcinogen treated cells were propagated in culture for about six weeks and subsequently injected subcutaneously into athymic nude mice. Two of the fourteen different groups of SV-HUC.PC treated with different concentrations of N-OH-MOCA, and one of the three groups exposed to N-OH-ABP, formed carcinomas in athymic nude mice. ^{32}P-postlabeling analyses of DNA isolated from SV-HUC.PC after exposure to N-OH-MOCA revealed one major and one minor adduct. The major adduct has been identified as the N-(deoxyadenosin-3′,5′-bisphospho-8-yl)-4-amino-3-chlorobenzyl alcohol (pdAp-ACBA) and the minor adduct as N-(deoxyadenosin-3′,5′-bisphospho-8-yl)-4-amino-3-chlorotoluene (pdAp-ACT). Furthermore, SV-HUC.PC cytosols catalyzed the binding of N-OH-MOCA to DNA, in the presence of acetyl-CoA, to yield similar adducts. The same adducts were also formed by chemical interaction of N-OH-MOCA with calf thymus DNA, suggesting that the aryl nitrenium ion may be the ultimate reactive species responsible for DNA binding. The tumorigenic activity of N-OH-MOCA in this highly relevant *in vitro* transformation model, coupled with the findings that SV-HUC.PC cells formed DNA-adducts *in vitro* and contained enzyme systems that activated N-OH-MOCA to reactive electrophilic species that bound to DNA, strongly suggest that MOCA could be a human bladder carcinogen. These findings are consistent with the International Agency for Research on Cancer's classification of MOCA as a probable human carcinogen.

Introduction

Human urinary bladder cancer is the fourth most common form of cancer among men (1). It is one of the few malignancies in which occupational and environmental exposure to chemicals have been documented as major risk factors (2–6). The bladder cancer risk is increased ~50- to 60-fold in individuals working in dye industries, compared to the normal population (3). Similarly, production workers in rubber, coal, gas, textile, cable industries, leather processing and painting are at a greater risk for bladder cancer resulting from exposure to N-substituted arylamines (4,5). Bladder cancers arising from occupational exposure to chemicals have been estimated to account for ~30% of all cases (7). A few of the chemicals responsible for the carcinogenic activities have been identified and several others are suspected to be probable human carcinogens (8–13). The agents that are of particular significance for occupational safety include 4,4′- methylenebis[2-chloroaniline] (MOCA*), *ortho*-toluidine (OT) and *ortho*-phenylphenol (OPP) and its derivative 2-phenyl-1,4-benzoquinone (PBQ). Recent epidemiologic studies involving retrospective analyses among workers exposed to OT and aniline revealed a six-fold increase in the incidence of bladder cancer (8). Similarly, an excess of mortality from bladder cancer was reported in male workers exposed to MOCA, a chemical used as a curing agent for polyurethane elastomers (9,10). Additionally, MOCA and OPP have been shown to induce bladder tumors in dogs and rats, respectively (14–16). Although these agents cause tumors in experimental animals, there are severe limitations to the extrapolation of the data to humans. To assess the occupational hazards of these suspected human bladder carcinogens, we tested their biological effects using a well established *in vitro* transformation model comprised of human uroepithelial cells, the target cells for the carcinogenic action of these chemicals.

The carcinogenicity of these chemicals require bioactivation to reactive electrophilic species which then bind to DNA resulting in the mutational activation or inactivation of putative cancer genes, such as the oncogenes and tumor suppressor genes, respectively (17). With arylamines like OT and MOCA, the first step involves N-oxidation to generate N-hydroxyarylamines (18–20). N-oxidation is followed by conjugation of the N-hydroxy function with acetate, sulfate or glucuronate (21–24). Unlike the acetate and sulfate esters, the N-glucuronide conjugates are relatively stable and are excreted in urine as a transport form of these arylamines (24,25). Under acidic pH conditions, the N-hydroxyarylamines might be converted to aryl nitrenium ions, which interact with DNA to form covalent adducts (24). In the case of MOCA, N-hydroxy-MOCA (N-OH-MOCA) has been suggested to be the

*Abbreviations: MOCA, 4,4′-methylenebis[2-chloroaniline]; N-OH-MOCA, N-hydroxy-4,4′-methylenebis[2-chloroaniline]; OT, *ortho*-toluidine; N-OH-OT, N-hydroxy-*ortho*-toluidine; N-OH-ABP, N-hydroxy-4-aminobiphenyl; OPP, *ortho*-phenylphenol; PBQ, 2-phenyl-1,4-benzoquinone; SV-HUC, SV40-immortalized human uroepithelial cell line; dAp-ACBA, N-(deoxyadenosin-3′-phospho-8-yl)-4-amino-3-chlorobenzyl alcohol; dAp-ACT, N-(deoxyadenosin-3′-phospho-8-yl)-4-amino-3-chlorotoluene; pdA-ACBA, N-(deoxyadenosin-5′-phospho-8-yl)-4-amino-3-chlorobenzyl alcohol; pdA-ACT, N-(deoxyadenosin-5′-phospho-8-yl)-4-amino-3-chlorotoluene; pdAp-ACBA, N-(deoxyadenosin-3′,5′-bisphospho-8-yl)-4-amino-3-chlorobenzyl alcohol; pdAp-ACT, N-(deoxyadenosin-3′,5′-bisphospho-8-yl)-4-amino-3-chlorotoluene; TLC, thin layer chromatography; HPLC, high pressure liquid chromatography.

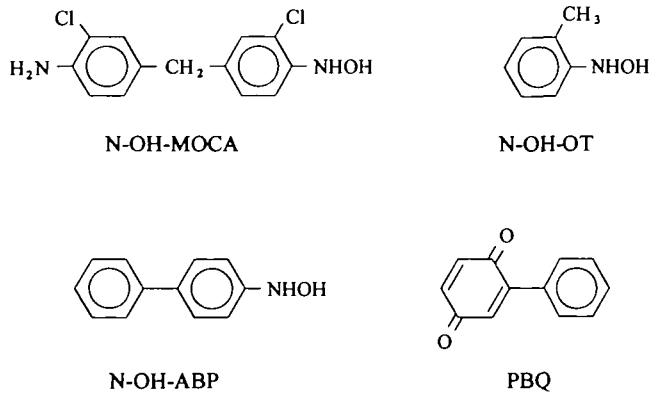

Fig. 1. Chemical structures of N-OH-MOCA, N-OH-OT, N-OH-ABP and PBQ.

penultimate carcinogenic species and on interaction with DNA it has been demonstrated to yield two major adducts (26,27). These adducts have been identified as N-(deoxyadenosin-8-yl)-4-amino-3-chlorobenzyl alcohol (dA-ACBA) and N-(deoxyadenosin-8-yl)-4-amino-3-chlorotoluene (dA-ACT) (27). The dA-ACBA adduct has also been detected in exfoliated urothelial cells of a worker exposed to MOCA (28). These adducts are also generated *in vivo* after administration of MOCA to rats and dogs (29–31).

In the present studies, we examined the neoplastic effects of certain occupationally relevant chemicals, N-OH-MOCA, PBQ (a putative reactive metabolite of OPP), and N-OH-OT (Figure 1) on SV40-immortalized cell lines of HUC. The results show that N-OH-MOCA causes neoplastic transformation of nontumorigenic SV-HUC to tumorigenicity. Furthermore, following exposure of SV-HUC.PC to N-OH-MOCA, two adducts (dA-ACBA and dA-ACT) have been identified by ^{32}P-postlabeling. These same adducts were also formed *in vitro* on incubation of N-OH-MOCA with SV-HUC.PC cytosol in the presence of acetyl CoA. The DNA binding studies on N-OH-MOCA, in conjunction with the neoplastic transformation of SV-HUC, strongly implicate MOCA might be a bladder carcinogen for humans. Hence the health hazards associated with its occupational exposures warrant careful evaluation.

Materials and methods

Chemicals and enzymes

N-OH-OT was prepared from 2-nitrotoluene by reduction with zinc dust in aqueous ammonium chloride (32). N-OH-OT was identified by desorption probe positive ion (M^+ at m/z = +123) mass spectrometry. N-OH-MOCA was synthesized by a modification of a published method (33,34) and its structural authenticity confirmed by mass spectrometry. These N-hydroxy derivatives were stored under argon at –70°C. HPLC analysis showed that N-OH-MOCA and N-OH-OT were greater than 95% pure. The following reagents and enzymes were obtained from the sources indicated: 2-nitrotoluene and PBQ (Aldrich Chemical Co., Milwaukee, WI); deoxyadenosine 3′-monophosphate (dAp), micrococcal nuclease, spleen phosphodiesterase type I, apyrase, calf thymus DNA and nuclease P1 (Sigma Chemical Co., St Louis, MO); T4 polynucleotide kinase (GIBCO/BRL, Gaithersburg, MD); [γ-^{32}P]ATP (6000 Ci/mmol) (DuPont, Boston, MA); Macherey–Nagel PEI-cellulose thin-layer chromatography (TLC) plates (Alltech, Deerfield, IL).

Cell culture methods

The two cell lines (SV-HUC.PC and SV-HUC.BC) used for the transformation studies are isogenic, both having been derived from SV-HUC, a clonal near diploid line of SV40-immortalized HUC (35–37). These two cell lines differ in their responsiveness to transformation by N-hydroxy metabolites of the human bladder carcinogen 4-aminobiphenyl; the SV-HUC.PC being responsive whereas the SV-HUC.BC is refractive (38). In the present studies, we used cryopreserved cells at passages 22 or 49 and 14 or 21, for SV-HUC.PC and SV-HUC BC, respectively. Both these original stocks were nontumorigenic when inoculated into athymic nude mice (38).

Cytotoxicity measurements

The cytotoxic effects of N-OH-MOCA, PBQ and N-OH-OT were first determined as described earlier (39), by quantitative measurement of inhibition of growth following exposure of SV-HUC to the chemicals for 24 h, relative to the control cultures treated under the same conditions containing only the solvent. The cytotoxicity tests were done in triplicates and experiments were done twice. Based on the cytotoxicity data, two or three effective doses representing approximately 10%, 25% and 75% cell survival were determined and these concentrations were used for the transformation studies.

Transformation of SV-HUC

The transformation protocols were similar to those described earlier (38,40). The target cells (SV-HUC.PC and SV-HUC.BC) were exposed to two or three concentrations of each test chemical, as indicated. In addition, a positive control comprised of treatment with N-hydroxy-4-aminobiphenyl (N-OH-ABP), and a negative control involving treatment with the solvent alone were included in the experimental design. The cells were seeded at a density of about 1×10^6 viable cells per 100-mm Corning tissue culture dish using three independent dishes for each concentration. Following attachment of cells to the dishes, they were rinsed with a serum-free F12+ media. The cells were subsequently exposed to the test chemical for 24 h. As these chemicals are relatively unstable ($t_{1/2}$ in the range of few hours), a fresh stock (200-fold concentrated than the final dose) solution was prepared by dissolving them in dimethyl sulfoxide and added immediately to the media. Following incubation, the media was changed to F12+ with 1% FBS and the cells were allowed to grow to semi-confluence. At the first passage after carcinogen exposure, cells of each treatment set were split one to four and reseeded into new 100 mm dishes. For the next 5 weeks, the cells were propagated by reseeding into new 100 mm dishes at weekly intervals, keeping each set of dishes separate. By 6 weeks, about 50×10^6 cells were obtained, and used for inoculation into nude mice.

The inoculations were done subcutaneously and anterior dorsally into 4–6 week old female athymic nude mice (nu/nu; Harlan–Sprague–Dawley, Madison, WI) as described (38). For injection, the cells were dispersed gently at a density of about 25×10^6 cells/ml and 0.2 ml was injected dorsally into both the right and left side of the nude mouse. Cells from each set of dishes were individually injected into two different animals. The animals were housed under environmentally controlled rooms in laminar flow hoods and were routinely monitored for tumor development. The animals were sacrificed after ~9 months. The tumor growths were monitored weekly using a micrometer and were dissected when they reached ~1 cm in diameter. Representative sections were fixed in 10% formalin for histology and specimens were processed by the Surgical Pathology Clinic at University of Wisconsin Hospitals and Clinics. The slides were read by pathologist Dr Kennedy Gilchrist of the Department of Pathology, University of Wisconsin.

Binding of N-OH-MOCA to DNA

The DNA was isolated from 20×10^6 SV-HUC.PC cells using standard procedures (41,42) 24 h after treatment with N-OH-MOCA. Additionally, N-OH-MOCA was bound to calf thymus DNA by chemical interaction at pH 5.0 at 37°C for 20 h. The incubation mixture contained 5 mg/ml calf thymus DNA in 10 mM potassium citrate buffer, pH 5.0, containing 0.1 mM EDTA and 0.5 mM N-OH-MOCA. After the reaction, the DNA was extracted with diethyl ether, precipitated with ice-cold ethanol and redispersed in water. Also, N-OH-MOCA was enzymatically bound to calf thymus DNA using SV-HUC PC cytosol in the presence of acetyl-CoA. The incubate contained 2 mg of calf thymus DNA in 1 ml of 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM N-OH-MOCA, 1.0 mM acetyl-CoA and 1 mg of SV-HUC.PC cytosol. After incubation at 37°C for 30 min, the mixture was extracted three times with 2 ml of chloroform:isoamyl alcohol:phenol (24:1:25) and five times with 3 ml of diethyl ether. The DNA was precipitated with ethanol. A control experiment carried out in the absence of acetyl CoA in the incubation mixture showed ~1% of the amount of adduct compared to the samples incubated with acetyl-CoA.

^{32}P-Postlabeling analyses of DNA adducts of N-OH-MOCA

The adduct analysis was conducted on three different sets of DNA samples described below: SV-HUC.PC treated with N-OH-MOCA; N-OH-MOCA bound to calf thymus DNA by chemical reaction (pH 5.0); or by enzymatic reaction with SV-HUC.PC cytosol in the presence of acetyl-CoA. Routinely ~10 μg of DNA samples were hydrolyzed to 3′-nucleotides by digestion for 3 h at 37°C with 0.5 μg each of micrococcal nuclease and spleen phosphodiesterase per 1 μg of DNA. Following hydrolysis, the samples were incubated with or without nuclease P1 for 1 h (42). The adducts were ^{32}P-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP under conditions similar to those

described by Randerath and coworkers (43,44). ^{32}P-Labeled adducts were purified on PEI-cellulose TLC plates by overnight development onto a wick in 1.7 M sodium phosphate buffer, pH 6.0 (D1). The thin layer chromatogram was developed in the second direction (D2) at 90° to the first using the same buffer system, as described by Kaderlik and co-workers (28). The adducts were located on the TLC plates based on autoradiography using Fuji RX film. Further characterization of these adducts was made by high pressure liquid chromatography (HPLC) analyses after extraction three times with 3.7 M ammonium hydroxide in isopropyl alcohol. Structural identifications of these adducts were based on the chromatographic characteristics of these samples in comparison to the standards prepared by chemical synthesis.

Chemical synthesis of dAp-ACBA and dAp-ACT

Synthetic dAp-ACBA and dAp-ACT were prepared by reaction of 2'-deoxyadenosine-3'-monophosphate with N-OH-MOCA at pH 5.0 in 10 mM potassium citrate buffer for 20 h. The adducts were separated from the normal nucleotide by HPLC using conditions described below. The column fractions were evaporated to dryness under vacuum and redissolved in water. The eluates containing the adducts were labeled using T4 kinase and [γ-^{32}P]ATP to generate the bisphospho derivatives, *pdAp-ACBA and *pdAp-ACT.

HPLC analyses of adducts

Samples were analyzed on a Hewlett-Packard 1090M high pressure liquid chromatograph equipped with a diode array detector and interfaced to a radioactivity detector (Radiomatic Instruments and Chemicals, Co. Inc., Tampa, FL). The instrument was fitted with a 30 cm C_{18} reverse-phase Varian MCH-10 column and eluted at a flow rate of 1 ml/min. Bisphosphate adducts were analyzed using the following solvent program: 20% methanol–80% 50 mM potassium phosphate, pH 5.3, 0–10 min; linear gradient to 40% methanol 10–15 min; 40% methanol 15–20 min; linear gradient to 70% methanol 20–35 min; 70% methanol 35–40 min; linear gradient to 20% methanol 40–45 min. Monophosphate adducts were purified by separation of normal nucleotide(s), by elution with 5% methanol–95% 100 mM ammonium formate, pH 3.5, for 25 min. Adducts were subsequently recovered by elution with 70% methanol–30% water; fractions were collected from the HPLC, dried *in vacuo*, and redissolved in distilled water.

Results

Cytotoxic effects of bladder carcinogens

Three chemicals, N-OH-MOCA, PBQ and N-OH-OT, were selected for the present studies based on their historical significance and anticipated occupational relevance to humans (12,13,46–48). The rationale for choosing the N-hydroxy derivatives of MOCA and OT instead of the parent compounds is based on the findings that these procarcinogens are metabolized in the liver to form the N-hydroxy derivatives, and the latter are transported via urine to the bladder to serve as the penultimate carcinogenic intermediates (24,25). Similarly, PBQ is believed to be the carcinogenic metabolite resulting from hepatic metabolism of OPP, which is widely used as a household fungicide and germicide (49). Hence, these chemicals were selected to test their carcinogenic effects directly on their target cell types, namely human uroepithelia. As a first step towards testing the neoplastic transformation of human uroepithelia, we determined the cytotoxicities of these agents so that suitable concentrations of chemicals could be determined for the *in vitro* experiments. Figure 2 shows the effect of varying concentrations of these chemicals on the relative survival of SV40-immortalized arylamine-responsive (SV-HUC.PC) human uroepithelial cells. The cytotoxicity curves were computed relative to the control cultures (treated only with the solvent vehicle). As shown in Figure 2, these compounds exhibited a dose-dependent toxicity and the LD_{50} for PBQ, N-OH-MOCA and N-OH-OT were, 2.8, 4.5 and 33.3 µM, respectively for the SV-HUC.PC. The LD_{50} values were ~2-fold lower for SV-HUV.BC. Based on these cytotoxicity response estimates, generally two specific concentrations that manifest approximately 25% and 75% cell kill were chosen for the transformation studies, unless stated otherwise.

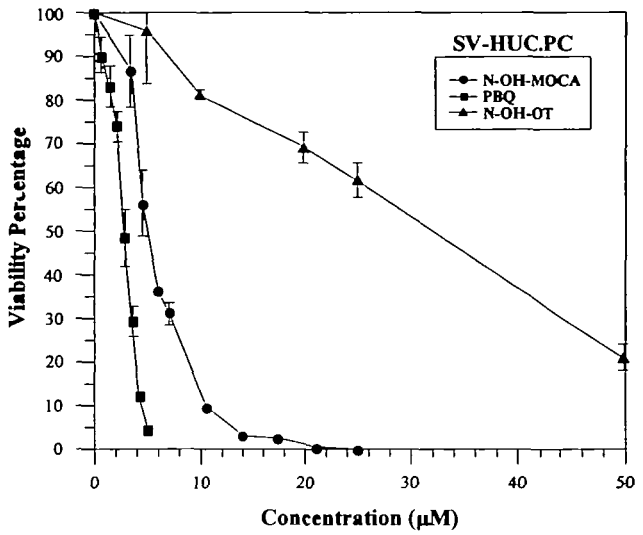

Fig. 2. Effect of varying concentrations of N-OH-MOCA, N-OH-OT and PBQ on the survival of SV-HUC.PC. Experiments were done in triplicate and the data points represent the mean with the error bar showing the ± SD.

Table I. Neoplastic transformation of SV-HUC

Chemical	Exp. set	Concentration (µM)	SV-HUC.PC Injection sites[a]	Tumors
DMSO (0.5%)	I	–	6	0
	II	–	6	0
PBQ	I	1	6	0
	I	2.5	6	0
	I	3.5	6	0
N-OH-MOCA	I	2.5	6	0
	I	5	4[b]	0
	I	10	6	2
	II	5	9	0
	II	10	9	0
N-OH-OT	II	15	6[b]	0
	II	45	9	0
N-OH-ABP	II	10	9	1

[a]For each chemical and for each concentration three culture dishes were independently treated and propagated as described in Materials and methods. The samples from each of these were inoculated in duplicate (Exp. set I) or triplicate (Exp. set II) into athymic nude mice. Each mouse had two injection sites

[b]The reason for the discrepancy in the number of injection sites in these groups is due to the loss of one of the starting culture dishes.

Tumorigenic transformation of SV-HUC

Following exposure of SV-HUC.PC and SV-HUC.BC stocks to varying concentrations of N-OH-MOCA, PBQ and N-OH-OT, the cells were propagated *in vitro* for 6 weeks and then injected into athymic nude mice to test for tumorigenicity. Tumorigenic transformation was observed with two independent sets of SV-HUC.PC treated with N-OH-MOCA (Table I). The positive control group, SV-HUC.PC treated with N-hydroxy-4-aminobiphenyl (N-OH-ABP), also induced tumors in nude mice. However, none of the other test chemicals or the negative control samples manifested activities in these cell lines. Furthermore, N-OH-ABP and N-OH-MOCA were ineffective in the SV-HUC.BC stock (data not shown). The latency period of detection of tumors for N-OH-MOCA and N-OH-ABP were 17 and 24 weeks, respectively, after injection into nude mice. The histopathological characteristics of the N-

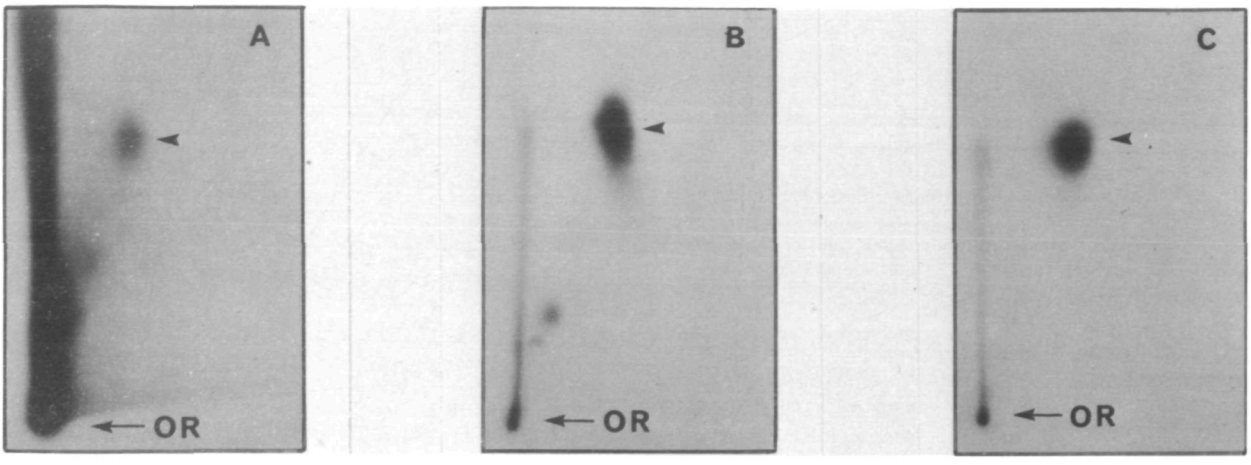

Fig. 3. Autoradiogram profiles of the TLCs of the ^{32}P-postlabeled adducts obtained from **(A)** SV-HUC.PC treated with N-OH-MOCA; **(B)** N-OH-MOCA bound to calf thymus DNA in the presence of SV-HUC.PC cytosol and acetyl-CoA; and **(C)** N-OH-MOCA chemically bound to calf thymus DNA at pH 5.0. D1 development was from bottom to top and D2 was from left to right; ◄, indicates the position of the adduct(s); OR, origin.

OH-MOCA induced tumors revealed that they were poorly differentiated carcinomas. In contrast, N-OH-ABP induced tumors showed features characteristic of focal squamous cell carcinomas (data not shown).

Analyses of MOCA-DNA adducts

Since N-OH-MOCA exhibited tumorigenic transformation activity and has been shown to form DNA-adducts *in vitro* and *in vivo* (27), we examined their formation in SV-HUC.PC using ^{32}P-postlabeling after nuclease P1 enrichment. The autoradiogram of the two dimensional TLC of SV-HUC.PC exposed to N-OH-MOCA in culture for 24 h, shows one major adduct (Figure 3A). The chromatographic mobility of the adduct formed with SV-HUC.PC in culture matched with the hydrolysates of the DNA sample obtained after incubation of DNA with SV-HUC.PC cytosol and N-OH-MOCA in the presence of acetyl-CoA (Figure 3B). During the acetyl-CoA mediated activation of the N-OH-MOCA, the reactive acetoxy-amino derivative is conceivably formed which breaks down to generate the aryl nitrenium ion. The latter has been shown to interact with deoxyadenosine (dA) to form dA-ACBA and dA-ACT derivatives. Since the same aryl nitrenium intermediate is also generated by chemical reaction of N-OH-MOCA under acidic conditions, we compared the DNA adducts generated by chemical reaction with those obtained using intact SV-HUC.PC or its subcellular fraction (Figure 3A and 3B). Figure 3C shows that an adduct with similar chromatographic characteristics is also detected in the DNA sample interacted with N-OH-MOCA under acidic conditions, suggesting that these might be structurally the same.

To confirm the above possibility, the adducts were eluted from the TLC plates and subjected to HPLC. Figure 4 shows the profile of the product obtained from intact cells (Figure 4A), along with those obtained by enzymatic reaction using SV-HUC.PC cytosol (Figure 4B) and chemical reaction (Figure 4C). All these samples revealed a major peak at ~8 min, suggesting that they might be the same. Earlier, one major adduct (dA-ACBA) and a minor adduct (dA-ACT) were reported in the DNA treated *in vitro* with N-OH-MOCA (27). The latter was shown to be sensitive to nuclease P1 whereas the former was resistant to hydrolysis by nuclease P1, and thus, in the nuclease P1 enriched samples, mainly one main adduct was detected as expected. To examine whether the minor adduct is also formed in this system, we analyzed the

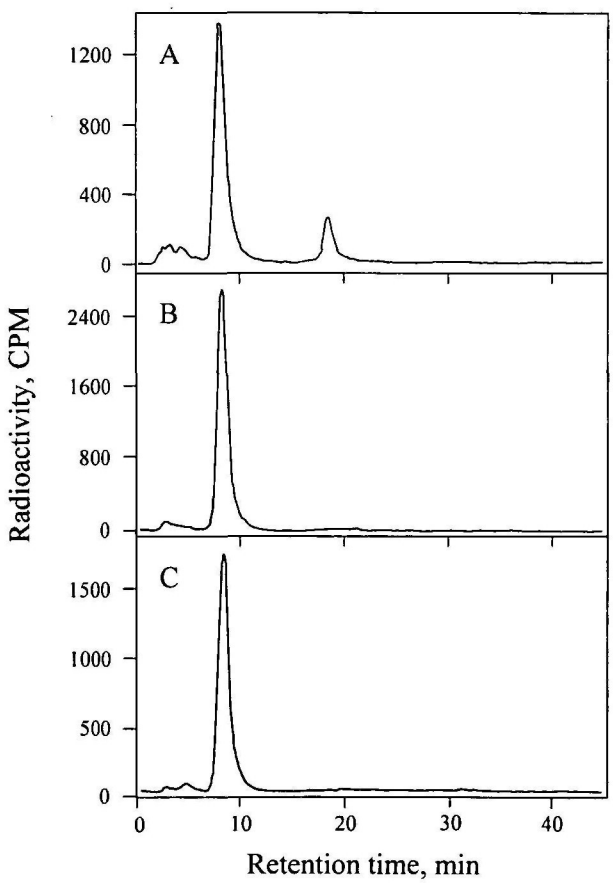

Fig. 4. HPLC radioactivity profiles of the ^{32}P-postlabeled adducts extracted from the two-dimensional TLCs of **(A)** SV-HUC.PC treated with N-OH-MOCA; **(B)** N-OH-MOCA bound to calf thymus DNA in the presence of SV-HUC.PC cytosol and acetyl-CoA; and **(C)** N-OH-MOCA chemically bound to calf thymus DNA at pH 5.0. The HPLC conditions are described in Materials and methods.

DNA hydrolysates without nuclease P1 digestion. The two-dimensional TLC analyses poorly resolved the two adducts (data not shown). However, by HPLC they were well resolved. Figure 5 shows the HPLC profile of the bisphospho adducts extracted from two-dimensional TLC samples obtained by enzymatic reaction (Figure 5A) and chemical reaction (Figure

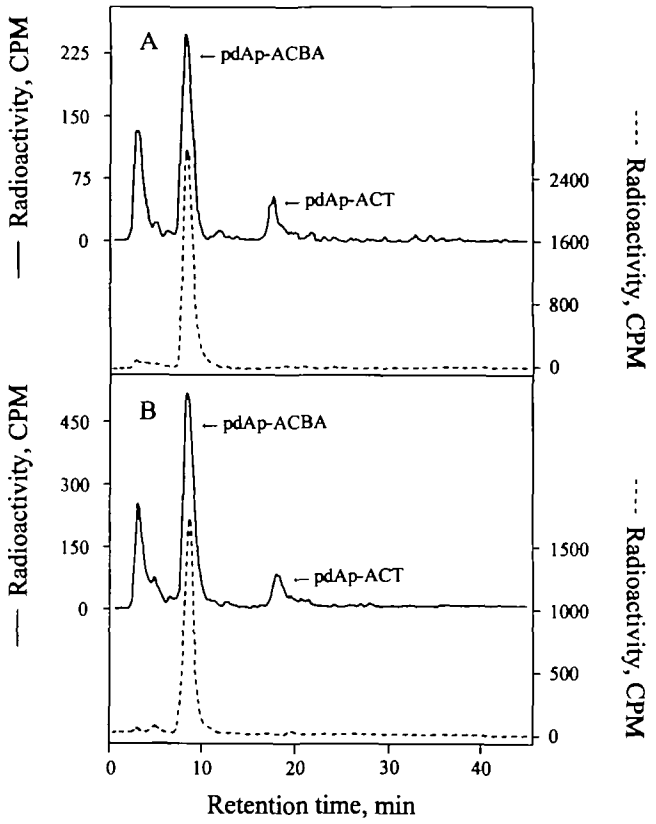

Fig. 5. HPLC radioactivity profiles of the ^{32}P-postlabeled adducts from the two-dimensional TLCs of (A) N-OH-MOCA enzymatically bound to calf thymus DNA by SC-HUC.PC cytosol and acetyl-CoA and (B) N-OH-MOCA chemically bound to calf thymus DNA at pH 5.0. In both panels, the solid line represents the profile obtained by postlabeling the DNA without nuclease P1 treatment and the dashed line represents the profile obtained following nuclease P1 enrichment of the DNA samples In the labeling experiments where the nuclease P1 enrichment step was not included, about 100 ng of DNA was used for hydrolysis to minimize nonspecific incorporation of label into normal nucleotides. HPLC conditions are the same as for Figure 4.

5B) with and without nuclease P1 digestion prior to ^{32}P-labeling. As shown in Figure 5, three radioactive peaks were observed at 3, 8 and 18 min prior to nuclease P1 digestion, whereas only one major peak at 8 min was detected after nuclease P1 treatment.

As nuclease P1 treatment was earlier shown to dephosphorylate the minor adduct (dA-ACT) and not the dA-ACBA analog, this suggested that the early eluting component which is insensitive to nuclease P1 might be the dA-ACBA. To verify this possibility, the N-OH-MOCA chemically modified DNA was digested to 3'-mononucleotides and the adducts were purified by HPLC. The purified adducts were ^{32}P-labeled, the bisphospho derivatives were recovered from the two-dimensional TLC and subjected to nuclease P1 digestion. Figure 6 shows the HPLC profile of the samples prior to and after digestion with nuclease P1. Following nuclease P1 digestion, the later eluting 18 min component (*pdAp-ACT) was hydrolyzed to generate the 5'-monophosphate analog (retention time 22 min, Figure 6B), whereas the component eluting at 8 min was resistant to hydrolysis. Further confirmation was obtained by chemical synthesis of dAp-ACT with dAp and N-OH-MOCA. Chemical interaction of dAp with N-OH-MOCA yielded primarily the dAp-ACT derivative and was purified from the reaction mixture by HPLC. The 3'-

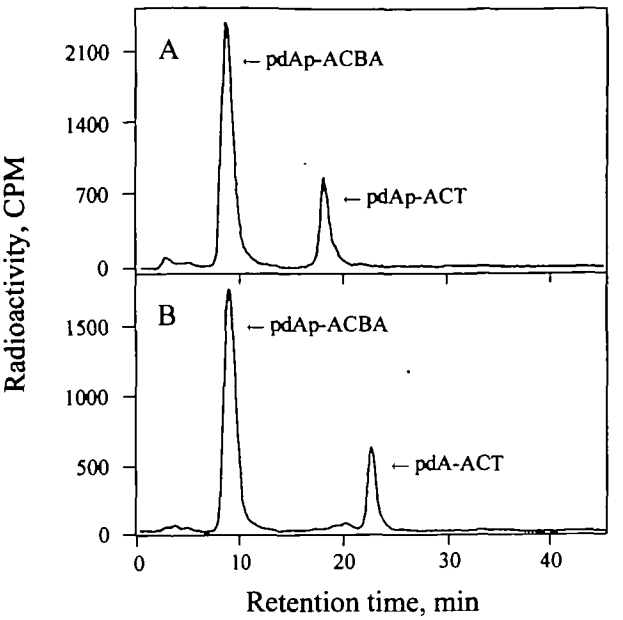

Fig. 6. HPLC radioactivity profile of the bisphosphate adducts obtained from N-OH-MOCA chemically bound calf thymus DNA (postlabeled after HPLC purification of the 3'-monophosphate adducts) without (A) or with (B) subsequent digestion of the labeled adducts with nuclease P1. HPLC conditions are described in Materials and methods.

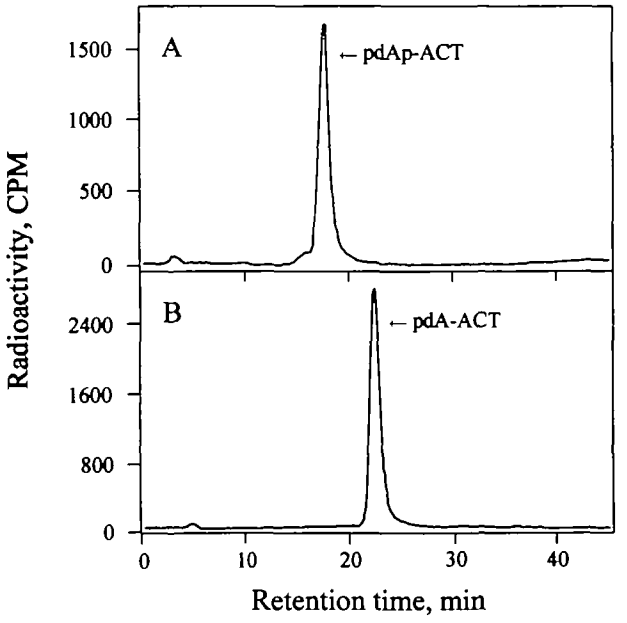

Fig. 7. HPLC radioactivity profile of the labeled bisphosphate adduct (obtained by ^{32}P-labeling the purified product obtained by reaction of N-OH-MOCA with deoxyadenosine-3'-monophosphate) without (A) or with (B) digestion with nuclease P1. HPLC conditions are the same as for Figure 4.

monophosphate analog was ^{32}P-postlabeled to generate the bisphospho derivative (*pdAp-ACT). Figure 7 shows the HPLC profile of the bisphospho derivative before (Figure 7A) and after (Figure 7B) digestion with nuclease P1. Similar to the product derived from chemical reaction of N-OH-MOCA with DNA, the synthetic product from dAp was also sensitive to nuclease P1 digestion. Based on the nuclease P1 sensitivity of these adducts, the 8 min and 18 min components have been identified as pdAp-ACBA and pdAp-ACT, respectively.

Discussion

All the three tested compounds (N-OH-MOCA, N-OH-OT and PBQ) showed a dose-dependent cytotoxic response in the target uroepithelial cell lines (Figure 1). There were significant differences in the potencies among these three chemicals, PBQ being highly cytotoxic followed by N-OH-MOCA and N-OH-OT. This contrasts with the effects observed on tumorigenic transformation in these cell lines. The N-OH-MOCA showed tumorigenic activity in the responsive cell line SV-HUC.PC (Table I), whereas it was inactive in SV-HUC.BC (data not shown). Among a total of fourteen independent groups that were prepared after treatment of SV-HUC.PC with varying concentrations of N-OH-MOCA, two of the groups induced tumors in athymic nude mice (Table I). These two groups comprised cells that were treated with the highest concentration (10 μM) of N-OH-MOCA, and these tumors were obtained on two independent animals. However, only one of the two sets of animals treated with 10 μM concentrations of N-OH-MOCA exhibited activity, implying the limitations of the transformation system. A number of factors (concentration, duration of exposure, stability of the chemical, rates of formation and detoxication of the reactive intermediates, rate of repair of DNA damage, passage of stock cultures at inoculation and carcinogen treatment, immune response characteristics of the animals, etc) could affect the frequency of *in vitro* transformation and tumorigenicity of these chemicals. Hence, demonstration of dose-dependent tumorigenicity, and a statistical evaluation of the effect of various factors on tumor response, will warrant the design of large scale studies using these animals. The observed tumorigenic effects of SV-HUC.PC exposed to N-OH-MOCA in these pilot studies in conjunction with the findings on the carcinogenic effects of MOCA in several experimental animal systems (14,15,50), lends further support to the view that MOCA might be a human bladder carcinogen. This view is further substantiated by DNA adduct formation in SV-HUC.PC, a step considered to be essential for the initiation of neoplasia. ^{32}P-Postlabeling analyses of DNA obtained from SV-HUC.PC after treatment with N-OH-MOCA showed one major and one minor adduct (Figure 4). The major adduct has been identified as pdAp-ACBA. The formation of this adduct in SV-HUC.PC is in conformity with the findings reported earlier by Segerback and Kadlubar (27). Subsequent reports have shown that these same adducts are generated in target tissues of susceptible species, namely the urinary bladder of dogs (31). Recently, the major MOCA-DNA adduct, pdAp-ACBA, was also found in exfoliated bladder cells of a worker with an accidental acute exposure to MOCA in a plastic manufacturing industry (28). Furthermore, the same adducts were formed by chemical interaction of N-OH-MOCA with calf thymus DNA or dAp (Figures 3–7), suggesting that the aryl nitrenium intermediate generated from N-OH-MOCA might be responsible for DNA binding. Incidentally, the reaction of N-OH-MOCA with DNA or dAp yields different proportions of products (Figures 6 and 7). The DNA sample obtained after incubation of N-OH-MOCA with acetyl-CoA and SV-HUC.PC cytosol also exhibited an adduct profile similar to that obtained from chemical reaction (Figures 3–5), implying that these target cells contain the enzyme systems essential for conversion of N-OH-MOCA to aryl nitrenium intermediate. Mechanistically, it is analogous to the metabolic activation of N-hydroxy aryl- or heterocyclic-amines, and involves acetyl-CoA-dependent activation of N-hydroxylamines by cytosolic acetyl transferases to form the N-acetoxyamino derivative, which breaks down to yield the nitrenium analog (19,23,51). The occurrence of such acetyl transferases in human uroepithelia and their role in the bioactivation of N-hydroxy metabolites of the human bladder carcinogen 4-aminobiphenyl have been well documented (52,53). Thus the observed DNA-adduct profile in SV-HUC.PC with N-OH-MOCA is consistent with the occurrence of acetyl CoA-dependent acetyl transferases in human uroepithelia and their role in the activation of the carcinogenic N-hydroxyarylamines.

^{32}P-postlabeling analysis of nuclease P1 enriched digest of the DNA sample isolated from SV-HUC.BC exposed to N-OH-MOCA revealed the presence of pdAp-ACBA, although quantitatively the adduct levels were about three-fold lower compared to SV-HUC.PC (data not shown). These results imply that the lack of tumorigenic response to N-OH-MOCA by SV-HUC.BC might not be due to differences in the activation but might be associated with differences in the repair of these adducts by these cell lines.

The characterization of the MOCA-DNA adducts reported here is partly based on sensitivities to digestion with nuclease P1. Earlier studies have shown that dAp-ACBA is not sensitive to the 3′-phosphatase activity of nuclease P1, whereas the 3′-phosphate of dAp-ACT is hydrolyzed by nuclease P1 (31). Hence, nuclease P1 digestion is usually adopted for enrichment and under these conditions primarily dAp-ACBA is detected as reported here. In the absence of nuclease P1 enrichment, three adducts with retention times of 3, 8 and 18 minutes are observed (Figure 5). Two of these adducts with retention times 3 and 18 min are sensitive to nuclease P1. The identity of the early eluting adduct remains to be established.

The effect of nuclease P1 on the hydrolysis of adducted nucleotides observed at the level of the DNA occurs also at the level of the bisphospho derivatives. This is illustrated in Figures 6 and 7 wherein the ^{32}P-labeled bisphosphate analogs *pdAp-ACBA and *pdAp-ACT were isolated, subsequently digested with nuclease P1 and the products analyzed for the formation of the labeled 5′-phospho derivatives. The results show that in contrast to *pdAp-ACT which is hydrolyzed to the 5′-monophosphate, *pdAp-ACBA was not hydrolyzed by nuclease P1 (Figures 6 and 7). These data lend additional support to the earlier reports on the nuclease P1 sensitivities of these adducts (27,31).

The human uroepithelial cell culture model that we have used in the present study is a highly relevant model for the study of bladder carcinogenesis by arylamines. The tumorigenic transformation by N-OH-MOCA, demonstration of its bioactivation by cytosolic enzymes, possibly by acetyltransferases similar to those described earlier with normal HUC (49–51) in the target cells, and the documentation of the formation of MOCA-DNA adducts in the target cells suggests that MOCA is probably a potent human carcinogen, as has been rightly classified by the IARC.

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