Production of Urothelial Tumors in the Heterotopic Bladder of Rats by Instillation of N-Glucuronosyl or N-Acetyl Derivatives of N-Hydroxy-2-aminofluorene

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ABSTRACT

Male F344 rats which had been implanted with a heterotopic bladder were randomly divided into four groups and their heterotopic bladders were instilled once a week for 30 weeks with 0.5 ml phosphate-buffed saline-dimethyl sulfoxide solution (4:1), or this solution containing 2 \( \mu \)mol N-methyl-N-nitrosourea, 1 \( \mu \)mol N-hydroxy-2-acetylaminofluorene (N-OH-AAF), or 1 \( \mu \)mol N-hydroxy-N-glucuronosyl-2-aminofluorene (N-OH-N-GI-AF). These bladders were then instilled once a week for an additional 23 weeks with phosphate buffered saline solution without the addition of dimethyl sulfoxide. The animals were killed at the end of 53 weeks. Transitional cell carcinomas were observed in five of 37, 36 of 37, 15 of 35, and 36 of 38 rats of the control, N-methyl-N-nitrosourea, N-OH-AAF, and N-OH-N-GI-AF groups, respectively. No histological alteration was observed in their natural bladders and no tumor was observed in the liver. As judged by kinetic measurements of the radioactive compounds, N-OH-AAF was removed much faster than N-OH-N-GI-AF from the fluid of heterotopic bladder. The pH of the fluid in the bladder was between 7.1 and 7.4. The present study demonstrates the carcinogenicity of N-OH-N-GI-AF and N-OH-AAF for rat bladder.

INTRODUCTION

The arylamines 4-aminobiphenyl, 2-aminonaphthalene, and benzidine have been associated with high incidences of human bladder cancer. They induce tumors of the bladder as well as other organs in experimental animals (1, 2). The induction of urinary bladder tumors by arylamines has been shown to be due to the metabolites excreted in the urine (3, 4). Both humans and experimental animals exposed to carcinogenic arylamines or N-arylacemides excrete N-hydroxy metabolites in the urine (5-14). Depending on the hepatic N-acylation and N-deacetyl-cation capacities, these metabolites appear mainly as the glucuronic acid conjugates of hydroxylamines or N-arylated-hydroxamic acids. The \( \beta \)-glucuronide of N-OH-AAF is not carcinogenic when injected s.c. to the rat (15) and it does not induce UDS in cultured urothelial cells unless in the presence of \( \beta \)-glucuronidase (16). Depending on the species, the deglucuronidated hydroxamic acids can be activated by urothelial cells through N-deacetylation (16, 17) or \( \beta \)-O-acetyltransferase of \( \beta \)-glucuronidated hydroxamic acids can be activated by urothelial cells through N-deacetylation (16, 17) or \( \beta \)-O-acetyltransferase and produce UDS in cultured urothelial cells (16-18) secondary to modification of DNA. Because of the low level of \( \beta \)-glucuronidase in the urine, \( \beta \)-glucuronides are subject to little met.

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4 The abbreviations used are: N-OH-AAF, N-hydroxy-2-acetylaminofluorene; N-OH-N-GI-AF, N-hydroxy-N-glucuronosyl-2-amino- fluorene; N-OH-AAF, N-methyl-N-nitrosourea; N-OH-N-GI-AF, the \( \beta \)-glucuronide of N-OH-AAF; MNU, N-methyl-N-nitrosourea; PBS, phosphate-buffered saline (10 mM sodium phosphate-0.8% NaCl, pH 7.0); UDS, unscheduled DNA synthesis; DMSO, dimethyl sulfoxide.


6 abolic activation in the bladder and are, therefore, considered as detoxified metabolites (16, 17). On the other hand, the N-glucuronides of hydroxylamines can be hydrolyzed to free hydroxylamines in neutral or mild acidic media, and the hydrolyzed products can react with macromolecules nenzymatically (12, 19, 20) or through further conjugation (21, 22). This is supported by the observation that both the N-glucuronides and free hydroxylamines induce UDS in cultured urothelial cells of various species (16, 17). Therefore, the N-glucuronides of hydroxylamines are considered to be responsible for the induction of bladder tumors (8, 12, 16, 17, 19, 20). However, direct evidence to support this hypothesis has not been available. Although intravesical instillation of N-hydroxy-2-amino-naphthalene to the dog produces urinary bladder tumors (23), it is still not certain whether the tumors are in fact induced by this hydroxylamine. This is due to the observations that the instilled carcinogens can be rapidly absorbed through bladder wall, metabolized in the liver and reexcreted in the urine (23-26). The problem of secondary exposure can be avoided by using a heterotopic bladder model in the rat developed in Oyasu’s laboratory (27). Previously, bladder tumors have been induced in the implanted bladder by periodical instillation with MNU (28-30). This model is also capable of detecting modifiers of bladder carcinogenesis (31, 32). The present study utilized this model to examine the direct carcinogenicities of N-OH-N-GI-AF and N-OH-AAF for rat bladder.

MATERIALS AND METHODS

Chemicals. MNU was purchased from Pfaltz and Bauer, Flushing, NY. N-OH-AAF and N-OH-N-GI-AF were synthesized according to the reported methods (16, 33). N-OH-[ring-\( ^3 \)H]Naf was synthesized from labeled [ring-\( ^3 \)H]nitrofluorene and was used for the synthesis of labeled N-OH-AAF and the N-glucuronide of N-OH-AAF. The purity of these compounds, as determined by radiochromatogram on thin-layer chromatography systems (33), was greater than 95%.

Preparation of Reservoir-Bladder Unit. Male Fischer rats, approximately 200 g, were obtained from Charles River Breeding Co., Wilmington, MA. Transplantation of the bladder from a donor to a recipient was performed according to Oyasu’s procedure (26-30). In short, the rats were anesthetized with i.p. injection of sodium pentobarbital. The bladder was removed from the donor rat and ligated to a connecting tube of a reservoir, and then inflated with 0.5 ml of PBS solution containing 0.15 mg gentamycin sulfate. After removal of the gluteal muscle of the recipient rat, the prepared reservoir-bladder was implanted into the cavity thus produced and the skin was closed over the apparatus. A week later the fluid in the reservoir-bladder unit was withdrawn and replaced with 0.5 ml of fresh PBS-gentamycin solution with a needle and a syringe, and this procedure was repeated once a week for 4 weeks before the rats were used for the following experiment.

Animal Experiment. Since this was the first experiment to test the carcinogenicities of N-OH-AAF and N-OH-N-GI-AF in heterotopic bladders, a maximum concentration of N-OH-AAF in PBS-DMSO was used to maximize the carcinogenic potential. An equal molar concentration of N-OH-N-GI-AF was also used in order to compare carcinogenicities of these two compounds. The test compounds were dissolved in DMSO and then added to four volumes of PBS-gentamycin solution to make solutions containing 4 \( \mu \)mol/ml MNU, or 2 \( \mu \)mol/ml N-OH-AAF or N-OH-N-GI-AF. The solutions were used...
immediately for injections, and those that were not used within 10 min after preparation were discarded. The rats that carried a heterotopic bladder were randomly divided into four groups and injected once a week for 30 weeks with 0.5 ml of the injection solution, or this solution containing MNU, N-OH-N-Gl-AF or N-OH-AAF. The heterotopic bladder was then injected with 0.5 ml PBS-gentamycin once a week for an additional 23 weeks. The animals were killed at the end of 53 weeks and complete necropsies were performed. Heterotopic bladders were isolated from the surrounding tissues and inflated with 10% phosphate buffered formalin solution, fixed, and cut into six to eight strips. They were embedded, cut and stained by hematoxylin and eosin for histological evaluation. Natural bladders were also similarly processed and evaluated.

Monitoring of microbial contamination of the heterotopic bladder was performed periodically. Fluid removed from the heterotopic bladder 7 days following injection was added to Difco nutrient broth, incubated at 37°C overnight to detect microbial contamination. The fluid was also tested for pH using pH paper (EM Laboratories, Inc., Elmford, NY, pH range: 4.0–7.0 and 6.5–10.0) (34).

Absorption of Carcinogens by Heterotopic Bladder. One mol of N-OH-[ring-3H]AAF or N-OH-N-Gl-[ring-3H]AAF (0.5 μCi) in 0.5 ml PBS-DMSO was injected into the heterotopic bladder. At various time intervals after the injection, the heterotopic fluid was removed with a needle and a syringe, and the bladder was washed 10 times with an injection of 0.5 ml PBS. The solutions were combined and the radioactivity was measured by a scintillation counting method.

RESULTS

Tumor Induction. Only one heterotopic bladder was found to have had a microbial infection during the experimental period. This rat was discarded and no attempt was made to identify the microorganisms. The natural bladders of rats in all groups were histologically normal. Histological alterations of the heterotopic bladders included inflammatory polyps, hyperplasias, and tumors. The classification of tumors is according to Oyasu et al. (29). Transitional cell carcinomas in heterotopic bladders were observed in all groups of rats. However, the incidences were greater in the treated groups than in the control group, and the differences were statistically highly significant (Table 1). Furthermore, while low grade and in situ tumors were observed in the control group, more malignant tumors were observed in the carcinogen-treated groups. Histologically, the tumors in the N-OH-AAF and N-OH-N-Gl-AF groups were indistinguishable from those of the MNU group (positive control). N-OH-N-Gl-AF is more carcinogetic than N-OH-AAF as judged by tumor incidence and the grade and stage of the lesions (Table 1).

Absorption of Carcinogens. The absorption of N-OH-AAF and N-OH-N-Gl-AF by the heterotopic bladders was determined by the disappearance of labeled compound from the vesical fluid. Approximately 97% of the injected radioisotope can be recovered immediately after the injection of 1 μmol of the labeled compounds. N-OH-AAF was rapidly absorbed through the bladder wall. It took approximately 18 min for half of this compound to be absorbed. In contrast, the absorption of N-OH-N-Gl-AF was much slower (Fig. 1).

pH of the Bladder Fluid. The pH of the vesical fluid was 7.25 ± 0.14 (N = 25). The pH range was between 7.1 and 7.4.

DISCUSSION

Repeated intravesical instillations of saline produce urinary stones or severe papillomatosis in natural bladders (35), but not in heterotopic bladders (27). Furthermore, bladder tumors are not produced following repeated instillation of saline in the heterotopic bladders (29). A 14% incidence of bladder tumors in the heterotopic bladders observed in the control group of the present study may be due to either gentamicin or DMSO, and a prolonged period of weekly instillations. Intravesical instillation of MNU into the natural bladders of rats has produced bladder tumors (36) and this phenomenon can be reproduced in heterotopic bladders (28, 29, 31, 32), even though heterotopic bladders may not be exposed to urinary factors that promote bladder carcinogenesis (32). The pH of the vesical content of the heterotopic bladders is close to that of the urine of rats fed commercial rat chow (34, 37). Based on the observations mentioned above, the heterotopic bladder appears to be a valid model for detecting urinary metabolites that may initiate bladder carcinogenesis.

In view of the observation that rats which have received intravesical injections into the heterotopic bladders of N-butyln-(3-carboxypropyl)nitrosamine, a proximate carcinogen of N-butyln-(4-hydroxybutyl)nitrosamine, develop tumors in the natural bladders but not in the heterotopic bladder (29), proximate urinary bladder carcinogens administered by this manner are very likely to produce tumors in natural bladders. Since the natural bladders of the rats receiving instillations of MNU, N-OH-AAF or N-OH-N-Gl-AF in heterotopic bladders do not develop histological alterations such as hyperplasias or papillomas that are associated with the development of urothelial tumors, this suggests that these chemicals, after being absorbed through the heterotopic bladders, are substantially detoxified before being excreted into urine. This is in agreement with the observation that N-hydroxy-2-aminonaphthalene is extensively metabolized following absorption from the natural bladder of the rat (25).

Due to their excretion in urine after exposure to arylamines (8, 12, 20) and reactivity with nucleic acids (19), the N-glucuronides of arylhydroxylamines have been suggested as the metabolites responsible for bladder carcinogenesis (12, 19, 20). However, due to problems associated with the lack of a bioassay
model and availability of compounds, the carcinogenicity of this class of compounds in urinary bladders has not been tested until now. The present study has demonstrated the bladder carcinogenicity of N-OH-AAF and N-OH-N-GI-AF. The histological features of the tumors produced by these two compounds and MNU in the heterotopic bladder are indistinguishable from that produced in natural bladders of rats treated with N-butyl-N-(4-hydroxybutyl)nitrosamine or N(4-(5-nitro-2-furyl))-2-thiazoylformamide.

Similar to the absorption of the O-glucuronide of N-OH-AAF in rabbit bladders (24) and of the N-glucuronide of N-hydroxy-2-amino naphthalene in rat bladders (25), N-OH-N-GI-AF was absorbed slowly in the heterotopic bladder. The absorption rate of the N-glucuronide of N-hydroxy-2-amino naphthalene in rat bladders is increased under the conditions which favor the hydrolysis of this N-glucuronide (25), suggesting that these glucuronic acid conjugates may be absorbed following hydrolysis. Because of the instability of N-OH-N-GI-AF observed during preparation of this compound, it is speculated that a significant fraction of the compound which is not absorbed within 4 hr might have been decomposed to inactive compounds. Even under this condition, this compound produced a greater incidence of urothelial tumors than N-OH-AAF did. A rapid absorption through and clearance from urothelial cells may result in insufficient activation of the N-OH-AAF and in only a low incidence of tumor. Although its O-glucuronide has been shown to be inactive in inducing UDS (16, 17) and is likely to be a detoxified metabolite, this compound is expected to be absorbed slowly in heterotopic bladders. Its activity in inducing bladder tumors, therefore, ought to be investigated. Nevertheless, the induction of bladder tumors in heterotopic bladders is consistent with the induction of UDS in cultured urothelial cells by N-OH-AAF and N-OH-N-GI-AF (16–18). These results demonstrate the usefulness of the UDS method for detections of metabolites of bladder carcinogens.

The present study has demonstrated the carcinogenicities of N-OH-AAF and N-OH-N-GI-AF in rat urothelial cells. However, several questions with regard to the N-glucuronides remain. It is envisioned that the pH of N-glucuronide solution will affect the hydrolysis of this compound, and consequently affect the rate of absorption. Furthermore, while N-OH-AAF generated from the glucuronide is capable of reaction with nucleic acids directly or indirectly through O-acetylation in urothelial cells, some hydroxylamines, such as N-hydroxy-2,3',

dimethyl-4-aminobiphenyl, can react with DNA only after further activation (21,22). It is not yet known whether these factors can significantly modify the carcinogenicity of this class of metabolites. Experiments designed to answer these questions are to be conducted.

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Fig. 1. Disappearance of labeled N-OH-AAF and N-OH-N-G1-AF from the content of heterotopic bladder. Half ml of ring labeled compounds, 1 μmol (0.5 μCi), was injected into the heterotopic bladder. At various time periods, the bladder fluid was removed for the determination of radioactivity (see "Materials and Methods" for detail). Points, values from each rat; —, N-OH-AAF; —, N-OH-N-G1-AF.

BLADDER TUMOR INDUCTION BY 2-AAF METABOLITES
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