Acrolein Initiates Rat Urinary Bladder Carcinogenesis

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ABSTRACT

Acrolein, a reactive, \(\alpha,\beta\)-unsaturated aldehyde which is ubiquitous in the environment, forms DNA adducts, is mutagenic, and is teratogenic. However, studies have not indicated a carcinogenic effect in rodent bioassays. Since it is present in cigarette smoke and is the toxic metabolite of cyclophosphamide with respect to the urinary tract, we investigated the possibility that acrolein might have carcinogenic activity toward the rat urinary bladder. We also evaluated whether it possessed initiating and/or promoting activity.

To evaluate initiating activity, acrolein was administered at a dose of 2 mg/kg i.p. twice a week for 6 weeks followed by uracil as 3% of the diet for 20 weeks and then control diet for 6 weeks. \(N\-{4-[5-Nitro-2-furyl]-2-thiazolyl formamide (FANFT) as 0.2% of the diet followed by uracil. Acrolein followed by uracil produced an incidence of 18 of 30 rats (60%) with papilloma compared to 8 of 30 rats (27%) treated with solvent control followed by uracil. FANFT followed by uracil produced an incidence of 70% carcinosmas and 30% papillomas, clearly indicating that it is a much more potent initiating agent than acrolein. Acrolein for 6 weeks followed by control diet produced no tumors.

To evaluate promoting activity, groups of rats were fed FANFT for 6 weeks followed by acrolein. Acrolein administered during the initial 6 weeks and continued for the second phase of the experiment (to evaluate complete carcinogenic activity) resulted in severe toxicity. Administration of acrolein had to be terminated after 21 weeks of the experiment. The animals were maintained for 53 weeks of the experiment without further chemical treatment, and there was no evidence of papilloma or carcinoma development.

This study clearly indicates that acrolein has initiating activity for the urinary bladder when administered by i.p. injection to the male F344 rat, but toxicity precluded evaluation of its promoting or complete carcinogenic activity.

INTRODUCTION

Acrolein is structurally the simplest member of the class of \(\alpha,\beta\)-unsaturated aldehydes, which are ubiquitous in our environment (1–6). Acrolein is formed during combustion of organic matter, is an important industrial chemical (estimated annual production, 60 million pounds), is a nonsolated intermediate in the industrial synthesis of acrylic acid and various acrylates, and is present in relatively large quantities in cigarette smoke (10–140 \(\mu g/cigarette)). Acrolein is also considered to be the toxic metabolite of the chemotherapeutic and immunosuppressive drugs, cyclophosphamide and its analogues (7–9). It is a highly reactive chemical which is mutagenic in a variety of short-term assays (3, 10, 11), including Salmonella TA104, Chinese hamster V79 cells, and fibroblasts from xeroderma pigmentosum patients, but not in normal human fibroblasts.

Acrolein does not require metabolic activation but reacts directly with DNA to produce its mutagenic effects. It produces oxocyclic adducts with bases in DNA in vivo and in vitro. Some of these products have been identified, and there is evidence that DNA cross-links are formed in the presence of acrolein (3).

Previous studies evaluating the carcinogenicity of acrolein were inconclusive due to the small number of animals used, the short duration of the experiment, or inadequate histological evaluation of organs at autopsy (3, 12, 13). Other highly reactive \(\alpha,\beta\)-unsaturated aldehydes (Fig. 1) exhibit weak carcinogenic activity. Crotonaldehyde is a liver carcinogen in rats (14), and malondialdehyde (\(\beta\)-hydroxy acrolein) has initiating activity in female Swiss mice (15, 16). Structurally related alkyl halides, such as vinyl chloride, have been identified as carcinogens in animals and humans (17).

Acrolein’s reactivity makes it a highly toxic chemical. In cell culture, cytotoxicity is observed at concentrations as low as 0.1 \(\mu M (11)\). Toxicity in animals has varied, depending on the route of administration. The 50% lethal dose p.o. for acrolein has been reported to be 46 mg/kg (12), but we have observed that a single intragastric instillation of 25 mg/kg was lethal to rats within 48 h of administration (18). Death appeared to be due to severe gastric toxicity and hemorrhage. A single i.p. injection of 1 mg/kg acrolein produced marked peritonitis, and multiple injections at this dose were lethal (18).

Cigarette smoking and cyclophosphamide therapy have been implicated as causative agents of bladder cancer (19–22). Cigarette smoke contains a number of carcinogenic chemicals, including polycyclic aromatic hydrocarbons and aromatic amines as well as acrolein (3). It is not clear which of these agents contributes to the causation of human bladder cancer. Acrolein is the metabolite of cyclophosphamide that apparently produces urinary tract cytotoxicity (7–9). Cyclophosphamide administered to rodents or humans produces hemorrhagic cystitis with a regenerative hyperplastic response. Bladder hyperplasia is also observed in individuals who smoke cigarettes (23), and i.p. injection of acrolein produces mild hyperplasia of the bladder in the rat (18). Since acrolein is a factor common to cigarette smoking and cyclophosphamide therapy, we evaluated the potential urinary bladder carcinogenicity of acrolein, including its initiating and promoting activities.

MATERIALS AND METHODS

FANFT* was obtained from Dr. George T. Bryan (University of Wisconsin, Madison) and purified by crystallization from hot dimethylformamide (25). The recrystallized FANFT was identified by nuclear magnetic resonance with purity greater than 97% (24). Acrolein was obtained from Aldrich Chemical Co. (Milwaukee, WI) and was found to be 97% pure, containing approximately 3% water and 200 ppm hydroquinone. Uracil was purchased from Sigma Chemical Company (St. Louis, MO). Single batches of acrolein and uracil were used as received.

FANFT and uracil were mixed in certified Prolab 3200 diet. FANFT was mixed as 0.2% of the diet by weight and pelleted at the University of Nebraska-Mead Farm facility within 10 weeks of being fed. Uracil

* Abbreviation used: FANFT, \(N\-{4-[5-Nitro-2-furyl]-2-thiazolyl formamide."

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3 Present address: Department of Urology, Nagoya City University Medical School, Nagoya, Japan.
were weighed immediately prior to i.p. injections to determine the dose until the end of the experiment. In addition, rats in appropriate groups the experiment. The rats were weighed at the beginning of the experi- during weeks 2, 4, 6, 8, and 10 and then every 4 weeks until the end of the second phase (weeks 20 and 21 of the experiment). Acrolein was not adminis-

groups 5-8, the second phase was intended to be 100 weeks, but the
tim; at the end of weeks 1, 2, 4, 6, and 10; and then every 4 weeks

The concentration of the acrolein solution was adjusted during

were weighed at the beginning of the experiment, according to the body weights of the

animals, so that the volume of the injections was 1.0-2.0 ml/rat/
injection. The concentration of the acrolein solution was adjusted during

the course of the experiment, according to the body weights of the

rats in groups 5-8 were killed at the end of 53 weeks of the experiment.

Food and water consumption was determined over 7-day intervals
during weeks 2, 4, 6, 8, and 10 and then every 4 weeks until the end of the experiment. The rats were weighed at the beginning of the experi-
ment; at the end of weeks 1, 2, 4, 6, and 10; and then every 4 weeks until the end of the experiment. In addition, rats in appropriate groups were weighed immediately prior to i.p. injections to determine the dose of acrolein to be administered.

When moribund or at terminal sacrifice, a complete autopsy was performed. Sacrifice was by an overdose of Nembutal. All tissues were observed grossly, but only the stomach, lungs, esophagus, liver, kidneys, and bladder were routinely processed for histopathological examination. The tissues were fixed in 10% formalin, including in situ inflation of the stomach, lungs, esophagus, and bladder with the fixative. Any other tissues with grossly visible lesions was also placed in fixative for further examination. Sections of these tissues were processed for routine embedding in paraffin, sectioned, stained with hematoxylin and eosin, and examined histopathologically. Only one section of each tissue was taken except for the bladder, which was bisected midsagittally, and each half was further cut into 3–4 strips. Three sets of serial sections of the bladder were examined, and the lesions were classified as previously described (28–32) into the categories of normal, simple hyperplasia, papillary and/or nodular hyperplasia, papilloma, or carcinoma.

Statistical evaluations (Statistical Analysis System; SAS Institute, Cary, NC) of data collected on body weights, food consumption, and water consumption were performed using Duncan’s test for comparison of means (33). Bladder histological diagnoses were statistically evaluated using Fisher’s exact test (one tail) (34).

RESULTS

The rats in the different groups consumed similar amounts of diet and water during the experiment. Total consumption of FANFT was approximately 1.3 g/rat for the 6-week period the was administered. During the first 6 weeks of the experiment, body weight gain was slightly less in rats fed FANFT in group 2, but not in the rats fed FANFT in groups 6 or 7, compared to the control group (group 8).

Acrolein, in contrast, caused significant retardation of growth in all rats to which it was administered (groups 1, 3, and 5). At 6 weeks, this amounted to a 7–10% lower body weight than in the control rats. Acrolein administration during the second phase of the experiment was particularly toxic. Administration of the chemical had to be interrupted and the dose reduced several times during the course of the experiment, eventually resulting in early termination, as described in “Materials and Methods.” Total acrolein administered to groups 1 and 3 was 3.3 mg/rat, and for groups 5 and 6 it was 11.9 and 8.3 mg/rat, respectively.

Uracil administration in the second phase of the experiment also produced severe inhibition of growth, as previously described (27, 30). This was actually more severe than in the long-term acrolein-treated rats, but the animals showed few pronounced signs of overt toxicity than the long-term acrolein-treated rats. The growth of the uracil-treated rats was corrected to some degree during the last 6 weeks of the experiment, during which they were fed the control diet. The total consumption of uracil was 53, 59, and 53 g/rat for the 20 weeks of administration in groups 1, 2, and 4, respectively.

Direct exposure of the peritoneum to acrolein resulted in chemical peritonitis. Granulation tissue and scar formation were evident on the peritoneal surface, and there were numerous adhesions between the abdominal wall and the intestines. There was no evidence, however, of gastrointestinal obstruction. Inflammation was seen in a few rats in groups 5 and 6 in the skin and subcutaneous tissues at the site of injection.

In rats treated with uracil, calculi were not present by the end of the experiment, since the animals had been on the control diet for 6 weeks; the calculi which formed during uracil administration eventually dissolved when the animals were returned to the control diet (31, 32). Nevertheless, the animals did have calculi during the time of uracil administration, and this was reflected even at the end of the experiment by the presence of
ACROLEIN INITIATION

Table 1 Treatments and body weights during the experiment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial phase</th>
<th>Second phase</th>
<th>Body weight (g) during week*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Group 1</td>
<td>Acrolein</td>
<td>Uracil</td>
<td>74 ± 1</td>
</tr>
<tr>
<td>Group 2</td>
<td>FANFT</td>
<td>Acrolein</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>Group 3</td>
<td>Control</td>
<td>Acrolein</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>Group 4</td>
<td>Solvent</td>
<td>Acrolein</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>Group 5</td>
<td>Acrolein</td>
<td>Acrolein</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>Group 6</td>
<td>FANFT</td>
<td>Acrolein</td>
<td>74 ± 1</td>
</tr>
<tr>
<td>Group 7</td>
<td>Acrolein</td>
<td>Solvent</td>
<td>73 ± 1</td>
</tr>
<tr>
<td>Group 8</td>
<td>Control</td>
<td>Control</td>
<td>73 ± 1</td>
</tr>
</tbody>
</table>

* Body weights are listed as the mean ± SEM.

Table 2 Urinary bladder lesions in rats administered acrolein, FANFT, uracil and/or control

<table>
<thead>
<tr>
<th>Chemicals administered</th>
<th>Urinary bladder lesions</th>
<th>Normal</th>
<th>Simple hyperplasia</th>
<th>Papillary/Nodular hyperplasia</th>
<th>Papilloma</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>First phase (6 wks)</td>
<td>Second phase (26 or 47 wks)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 1</td>
<td>Acrolein</td>
<td>0</td>
<td>1</td>
<td>10^f</td>
<td>18^h</td>
<td>1</td>
</tr>
<tr>
<td>Group 2</td>
<td>FANFT</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Group 3</td>
<td>Acrolein</td>
<td>26</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 4</td>
<td>Solvent</td>
<td>0</td>
<td>0</td>
<td>21</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Group 5</td>
<td>Acrolein</td>
<td>14</td>
<td>14^e</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 6</td>
<td>FANFT</td>
<td>8</td>
<td>22^f</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 7</td>
<td>FANFT</td>
<td>15</td>
<td>14^e</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Group 8</td>
<td>Control</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* The second phase of groups 1–4 was 26 weeks, and for groups 5–8 it was 47 weeks.

DISCUSSION

Acrolein is a highly reactive \(\alpha,\beta\)-unsaturated aldehyde which chemically reacts with proteins and nucleic acids (36–38). It is mutagenic (3, 11) and teratogenic (3) and is acutely toxic to hydrouret and hydronephrosis in nearly all of the rats that received uracil during the experiment (only two rats in group 4 did not have hydronephrosis). These changes were observed only in rats treated with uracil and have been described previously (31, 32).

The histopathology of the urinary bladders of the rats in different groups are summarized in Table 2. The positive control group (group 2), treated with FANFT followed by uracil, had a high incidence of carcinomas (70%), and the remaining animals had multiple papillomas. Acrolein also had initiating activity, since there was a significant increase in the incidence of papillomas (60% in group 1) compared to the rats treated with solvent followed by uracil (group 4, 27%; \(P < 0.05\)). In each of groups 1 and 4 one rat had a carcinoma, and none of the rats in either of these groups had normal bladders. With the increased numbers of rats with papillomas in the group treated with acrolein followed by uracil (group 1), there was an apparent decreased incidence of papillary and nodular hyperplasia compared to group 4, since the animals are tabulated only by the most severe lesion present in each bladder. Thus any rat that had a carcinoma also had papillomas and areas of hyperplasia, and rats with papillomas also had areas of papillary and nodular hyperplasia. If the total incidence of papillary and nodular hyperplasia was tallied, rather than only listing the animal in the category of the most severe lesion, the incidence was 97% of the rats in group 1 and 100% of the rats in group 4.

Rats treated with acrolein for 6 weeks followed by the control diet (group 3) generally had normal bladders by the end of the experiment, with only 4 of the 30 rats showing mild simple hyperplasia. More severe lesions were not observed in this group. Rats given long-term acrolein administration did not develop papillomas or carcinomas. However, many of the rats treated with long-term acrolein, with or without prior administration of FANFT (groups 5 and 6, respectively), had high frequencies of simple hyperplasia despite not having had acrolein administered during the final 32 weeks of the experiment. Thus, the hyperplasia induced by acrolein appears to be irreversible. Although not statistically significant, two rats treated with long-term acrolein without prior FANFT administration (group 5) had papillary and nodular hyperplasia. FANFT followed by solvent control also produced a high incidence of simple hyperplasia, and one rat in this group developed a carcinoma. Interestingly, rats fed FANFT and then acrolein (group 6) had a higher incidence of simple hyperplasia than rats given FANFT for 6 weeks followed by solvent control (group 7). Rats not administered any of the chemical treatments had normal bladders (group 8). These data clearly demonstrate initiating activity for acrolein in the male rat urinary bladder but provide only suggestive evidence for a promoting effect.

A single subcutaneous fibroadenoma in a rat in group 6 and a testicular mesothelioma in one rat in group 7 were the only other tumors detected in this experiment. Both commonly occur as spontaneous tumors in male F344 rats (35).
cells in vitro (11) and to animals in vivo, with toxicity dependent on the route of administration (3, 12, 18). Despite these findings, there has been no direct evidence for a carcinogenic effect (3, 12, 13). However, there is evidence that acrolein might be the reactive metabolite responsible for the carcinogenic effects of cyclophosphamide in rats and humans (7–9, 20–22). Acrolein has been identified as the causative metabolite responsible for the hemorrhagic cystitis produced by the administration of cyclophosphamide or its analogues. Also, oral ingestion of acrolein (25 mg/kg/day) produced benign adrenal cortical tumors in female F344 rats (39).

The reactivity and toxicity of acrolein pose serious difficulties in evaluating its carcinogenic potential, since it cannot be administered at high doses or for long periods of time (3). Nevertheless, a single i.p. injection of acrolein resulted in simple hyperplasia of the bladder epithelium in male rats within a few days (18). The present experiment provides evidence that acrolein administered i.p. for as short a period as 6 weeks has initiating activity for the rat urinary bladder.

Increased cell proliferation has been postulated to contribute to the carcinogenic risk from chemicals (40); we therefore hypothesized that acrolein would also have bladder tumor-promoting activity and complete carcinogenic activity in addition to initiating activity. Intravesical instillation of acrolein produces severe hyperplasia of the urothelium (41), and i.p. acrolein produces mild hyperplasia (18). However, the toxicity observed in the present experiment required premature termination of the experiment, precluding interpretation of the long-term promoting or carcinogenic potential of acrolein. Papillary and nodular hyperplasia, a preneoplastic lesion (28, 29), in 2 of the 30 rats treated with long-term acrolein (even though treatment was discontinued 32 weeks prior to sacrifice) implies that the aldehyde might have a weak carcinogenic effect. Also, the rats treated with FANFT followed by acrolein had a somewhat higher incidence of simple hyperplasia compared to rats treated with FANFT followed by solvent control. The hyperplastic lesions obviously had some degree of irreversibility. These findings do suggest that acrolein might have weak promoting and complete carcinogenic activity. Additional experiments are required to evaluate the complete carcinogenic activity of acrolein.

Injections of cyclophosphamide i.p. at levels of 10 mg/kg twice a week produced prominent nodular and papillary hyperplasia in rats, but administration had to be interrupted and the dose reduced because of toxicity similar to that observed in the present experiment with acrolein.2 Define carcinogenic activity or promoting activity at these doses could not be ascertained. However, administration p.o. at lower doses for 2 years did demonstrate carcinogenic activity in the rat (22), and this has been observed in humans (20, 21). As indicated above, previous studies point to acrolein as the carcinogenic metabolite of cyclophosphamide, and the present results provide evidence that it can account, at least partially, for the initiating activity of cyclophosphamide carcinogenesis.

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