Effect of Sodium Saccharin on Urinary Bladder Epithelial Regenerative Hyperplasia following Freeze Ulceration

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ABSTRACT

Sequential observations of the light and scanning electron microscopic appearances and labeling index of rat urinary bladder epithelium following freeze ulceration were performed for 8 weeks, and the effect of sodium saccharin on this process was assessed. In all rats treated with freeze ulceration of the bladder developed nodular and papillary hyperplasia around the ulcer by the fourth day. Under scanning electron microscopy, there were uniform and pliciform microvilli on the hyperplastic cell surfaces for the first 14 days after the ulcer. The labeling index of the bladder epithelium ([³H]thymidine injected 1 hr prior to sacrifice) was 10 to 20% after 1 day, and it rapidly diminished to 0.5 to 1.0% by the seventh day. When rats treated with freeze ulceration were fed control diet, the incidence of the light and scanning electron microscopic lesions rapidly diminished after the 14th day, and they were present at very low incidence after 56 days. The labeling index also decreased to the normal level (0.02 to 0.06%) by the 21st day. In contrast, rats fed sodium saccharin, either immediately after ulceration or beginning after 2 weeks of control diet following ulceration, developed nodular and papillary hyperplasia and luminal surface abnormalities detectable by scanning electron microscopy, and the incidences of these abnormalities remained high for the entire 8 weeks of this experiment. The labeling index in these groups also remained elevated. The rats fed control diet without ulceration had normal bladders. However, rats fed sodium saccharin developed mild simple hyperplasia and an increased labeling index.

Another experiment evaluated the effect of delaying the beginning of sodium saccharin administration until 8 weeks after ulceration. Surprisingly, the development of nodular and papillary lesions detected by light microscopy, surface abnormalities detected by scanning electron microscopy, and increased labeling index determined by autoradiography were similar to results after sodium saccharin administered immediately or beginning 2 weeks after ulceration. The results of these experiments suggest that sodium saccharin prolongs the regenerative hyperplastic changes following ulceration and maintains an increased proliferative rate in the epithelium. These changes appear to contribute to the eventual induction of bladder neoplasms in rats fed sodium saccharin following ulceration.

INTRODUCTION

Two-stage carcinogenesis based on the initiation-promotion model initially described in mouse skin (3, 32) has been demonstrated in experimental models of urinary bladder cancer (6, 9, 12, 15-18, 27-31). In experimental models, sodium saccharin has been shown to be a tumor promoter of bladder carcinogenesis (9, 12, 15-18, 28, 29) and has also been reported to be a weak bladder carcinogen (1, 8). Its promoting activity in vitro fibroblast systems has also been demonstrated (24). Moreover, sodium saccharin feeding has been shown to induce mild bladder epithelial hyperplasia in rats (13, 25), a general property of promoting agents (3, 32).

Recently, in our laboratory, long-term feeding of sodium saccharin was shown to induce an increased incidence of bladder tumors if fed to rats with a proliferating bladder mucosa induced by either the freeze ulceration technique or cyclophosphamide injection (11). Ulceration followed by 2 weeks of 0.2% N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide, a potent mutagen, and then by 102 weeks of 5.0% sodium saccharin induced bladder tumors at approximately the same incidence as did ulceration followed by 2 weeks of control diet followed by 102 weeks of sodium saccharin or ulceration followed immediately by sodium saccharin for 104 weeks (11). These results help to explain the carcinogenicity of sodium saccharin in 2-generation and pellet implantation experiments (2, 4-6, 8). In utero, the bladder epithelium is rapidly proliferating, and the surgical procedure involved in the insertion of the pellet into the bladder induces the same type of epithelial proliferation with approximately the same time course as that following freeze ulceration (14). However, the bladder mucosa following freeze ulceration (14), cyclophosphamide administration (11), or surgical incision and placement of a suture (14) returns to normal and remains normal if sodium saccharin or other promoters is not administered.

In this experiment, sequential observation of the bladder in rats fed sodium saccharin after freeze ulceration was performed to investigate the early effects of sodium saccharin on the regenerative hyperplasia and repair process, utilizing not only light microscopy for the detection of hyperplastic lesions but also the more sensitive techniques of autoradiography and scanning electron microscopy for the detection of increased rates of proliferation and foci of proliferation.

MATERIALS AND METHODS

Five-week-old inbred male Fischer 344 rats (Charles River Breeding Laboratories, Wilmington, Mass.) were used and maintained at 24° and 50% humidity on a 12-hr light-dark cycle, and they had food and
water available ad libitum. Sodium saccharin (Sigma Chemical Co., St. Louis, Mo.) was mixed in a diet (Charles River rat chow) at a level of 5.0% by weight and pelleted. The pelleted form of the chow without sodium saccharin was fed during "control" periods. Freeze ulceration of the urinary bladder was performed with the rats under Nembutal anesthesia by the method of Shirai et al. (33, 34). A steel rod, 5 mm in diameter, frozen in dry ice-acetone, was applied twice in the same place to the serosal surface of the ventral portion of the urinary bladder for 2 sec each time, with 5 sec between each application.

For the first experiment, the rats were divided into 5 groups as illustrated in Chart 1. In Group 1, freeze ulceration was followed immediately by feeding of sodium saccharin. In contrast, in Group 2, freeze ulceration was followed by 2 weeks of control diet and then followed by sodium saccharin. Group 3 received freeze ulceration followed by control diet alone. Group 4 was fed sodium saccharin alone, and Group 5 was fed control diet alone without ulceration. In each group, 5 to 13 rats were sacrificed at each of various time periods up to 8 weeks after the beginning of the experiment as shown in Chart 1. The bladders (the number for each examination at each time period is shown in Tables 1, 2, and 3) were processed for light microscopy, scanning electron microscopy, or autoradiography.

For scanning electron microscopy, each bladder was inflated transurethrally through a needle with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) under constant pressure and processed as described previously (10, 19, 20). For autoradiography, each rat received a single i.p. injection of [methyl-3H]thymidine (New England Nuclear; 20 Ci/mmol) at a dose of 1 Ci/g body weight 1 hr before sacrifice. All rats were killed at the same time of day to avoid variations in the mitotic rate due to the circadian rhythm of the rat bladder (38). The bladder was inflated with Bouin’s fixative, cut into longitudinal strips, washed 3 to 5 times in 70% ethanol, and then processed as described previously (9, 11, 15). Several serial sections of all strips of each bladder were examined, and at least 5000 epithelial cells were counted per bladder. Slides processed for autoradiography were also used for light microscopic evaluation. The bladder of some rats were used for all examinations. In those cases, the rat received [methyl-3H]thymidine 1 hr before sacrifice, and the bladder was inflated with glutaraldehyde in situ and divided in half sagittally. One half was processed for scanning electron microscopy, and the other half was processed for autoradiography and light microscopic evaluation.

The light microscopic criteria for distinguishing simple hyperplasia from nodular and papillary hyperplasia have been described (10, 13, 14, 25, 37), as have the criteria for distinguishing the surface features observed by scanning electron microscopy (13, 14, 19, 20, 25). By each method, the bladders are tabulated only once in each table based on the most advanced lesion present. Autoradiographs were evaluated as described previously (13, 14, 25). The labeling index was expressed as the number of labeled urothelial cells per 100 urothelial cells evaluated. No attempt was made to determine the index in the ulcerated bladders based on distance from the ulcer, although the rate of labeled cells was higher closer to the ulcer (33). Thus, the data as expressed in the tables are an average which will underestimate the proliferative rate near the ulcer.

A second experiment was then performed as illustrated in Chart 2. Group 1 was given sodium saccharin beginning 8 weeks after ulceration. Group 2 was given sodium saccharin beginning 2 weeks after ulceration, the ulceration procedure being performed 6 weeks after the

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**Table 1**

Sequential incidences of bladder lesions

<table>
<thead>
<tr>
<th>Group 1, UI→Sac</th>
<th>Group 2, UI→2 wk Cont→Sac</th>
<th>Group 3, UI→Cont</th>
<th>Group 4, Sac</th>
<th>Group 5, Cont</th>
</tr>
</thead>
<tbody>
<tr>
<td>Period (days)</td>
<td>Effective no.</td>
<td>Simple hyperplasia</td>
<td>Effective no.</td>
<td>Simple hyperplasia</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
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<td>4</td>
<td>4</td>
<td>1</td>
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<td>5</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>2</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>17</td>
<td>5</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>21</td>
<td>13</td>
<td>11</td>
<td>13</td>
<td>7</td>
</tr>
<tr>
<td>35</td>
<td>13</td>
<td>6</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>56</td>
<td>13</td>
<td>3</td>
<td>10</td>
<td>14</td>
</tr>
</tbody>
</table>

*UI, ulcer; Sac, sodium saccharin; Cont, control diet.
beginning of the experiment. Group 3 was fed sodium saccharin for the last 8 weeks of the experiment, and Group 4 was fed control diet for the entire 16 weeks of the experiment. The number of rats examined in each group by light microscopy, scanning electron microscopy, and autoradiography is shown in Table 4. The procedures used were as described above.

RESULTS

Experiment 1. For the first 14 days after ulceration, there was no difference in the morphological findings of the urinary bladders whether sodium saccharin was administered or not. The appearances were similar to those described previously (14, 33, 34), except that the ulcer and the hyperplasia lasted for a slightly longer time. The incidence of histological hyperplastic bladder lesions observed in each group at different periods is summarized in Table 1. The hyperplastic lesions were divided into 2 categories, simple hyperplasia and nodular and papillary hyperplasia as described previously (9, 11, 15).

One day after freezing, moderate simple hyperplasia occurred at the edge of the ulcer. That was followed by marked nodular and papillary hyperplasia appearing around the ulcer by the fourth day with eventual reepithelialization and repair of the ulcer by the 21st day after ulceration. When freeze ulceration was followed by control diet (Group 3), the incidence of nodular and papillary hyperplasia rapidly diminished after the 14th day, and it was found in only one of 13 and one of 11 rats at the 35th and the 56th day, respectively. However, simple hyperplasia was more persistent.

In contrast, in the groups in which ulceration was followed by sodium saccharin, either immediately (Group 1) or after 2 weeks of control diet (Group 2), the incidence of nodular and papillary hyperplasia remained high throughout the 8 weeks of this experiment. Simple hyperplasia was observed in almost all rats in Groups 1 and 2 at all periods of this experiment.

Continuous feeding of sodium saccharin without ulceration (Group 4) induced mild simple hyperplasia in one of 5 rats at the 14th day, and after that it was observed continuously at an incidence of 40 to 75%. One or 2 rats in each period after the 21st day also showed nodular and papillary hyperplasia in this experiment. In rats fed control diet only (Group 5), no hyperplastic lesions of the urinary bladder were found except for one rat with mild simple hyperplasia at the 56th day.

The luminal surface of the urinary bladder from each rat was observed by scanning electron microscopy. Normal superficial cells are polygonal and are covered with peaked microridges (14, 19). However, almost immediately after freeze ulceration, smaller, round cells covered with ropy, rounded microridges and short uniform microvilli were observed around the ulcer. Small, round cells with pleomorphic microvilli also appeared at the fourth to seventh day in all rats treated with freeze ulceration. The incidence of lesions detected by scanning electron microscopy observed in each group at different periods is summarized in Table 2. Each rat is tabulated on the basis of the most advanced lesion found on the luminal surface of the urinary bladder. When freeze ulceration was followed by control diet (Group 3), the incidence of uniform microvilli or pleomorphic microvilli rapidly decreased after the 14th day, and they were found in only one of 9 rats at the eighth week. In contrast, in the group treated with freeze ulceration followed immediately by sodium saccharin (Group 1), the incidence of
these lesions remained high. In the group treated with freeze ulceration followed by 2 weeks of control diet and then sodium saccharin (Group 2), these lesions were also persistent. Sodium saccharin administration without freezing (Group 4) also induced these changes at an incidence of 10 to 20% after the 21st day.

Sequential changes in the labeling index (labeled cells/100 cells) in each group are summarized in Table 3. All rats treated with ulceration of the bladder showed a very high labeling index immediately after freezing: 20 to 25% around the ulcer; and 7 to 15% in other parts of the bladder after the first day. It rapidly diminished by the seventh day (0.3 to 0.6%), and the labeling index became similar in hyperplastic and nonhyperplastic areas. In Group 3, it decreased to the normal level (0.02 to 0.06%) by the 21st day. In contrast, in groups fed sodium saccharin following freeze ulceration (Groups 1 and 2), it remained at an elevated level (0.3 to 0.7%). The group fed sodium saccharin alone without ulceration (Group 4) showed a continuous, slightly elevated labeling index from the first week through the end of this experiment, but it was at a level less than the groups fed sodium saccharin following freeze ulceration. The group fed control diet alone (Group 5) had the usual low level (0.02 to 0.08%) of labeling continuously throughout this experiment.

**Experiment 2.** The incidence of light microscopic and scanning electron microscopic lesions and the labeling index determined by autoradiography in each group are shown in Table 4. The incidence of nodular and papillary hyperplasia detected by light microscopy in the group given sodium saccharin beginning 8 weeks after ulceration (Group 1) was 21%, in contrast to 43% in the group given sodium saccharin beginning 2 weeks after ulceration (Group 2). However, the incidence of pleomorphic microvilli detected by scanning electron microscopy and increased labeling index determined by autoradiography were similar between both groups. Sodium saccharin feeding without ulceration (Group 3) did not induce nodular and papillary hyperplasia in this experiment, and the incidence of pleomorphic microvilli and labeling index in this group also was less than in Groups 1 and 2. In the group fed control diet alone following ulceration (Group 4), nodular and papillary hyperplasia was found in only one of 14 rats. In this group, uniform microvilli and pleomorphic microvilli were not found, and the labeling index was at normal levels.

**DISCUSSION**

In the present study, sodium saccharin was shown to prolong the regenerative hyperplastic changes following freeze ulceration and to maintain an increased proliferative rate in the bladder epithelium. These changes appear to contribute to the eventual induction of bladder neoplasms by sodium saccharin in rats in which the bladder epithelium is rapidly proliferating (11).

Single-generation feeding studies with sodium saccharin in rats, mice, hamsters, and monkeys have failed to demonstrate a carcinogenic effect or have resulted in very low incidences of bladder tumors (1, 8). However, the administration of sodium saccharin over 2 generations, including administration during pregnancy and lactation, resulted in high incidences of bladder tumors in male rats when fed at levels of 5 or 7.5% of the diet (2, 8). The carcinogenic effect of sodium saccharin has also been demonstrated using the pellet implantation technique in

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**Table 3**

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 17</th>
<th>Day 21</th>
<th>Day 35</th>
<th>Day 56</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ul—Sac</td>
<td>10.05 ± 3.32</td>
<td>4.80 ± 4.06</td>
<td>0.62 ± 0.27</td>
<td>0.45 ± 0.57</td>
<td>0.91 ± 0.56</td>
<td>0.74 ± 0.29</td>
<td>0.31 ± 0.19</td>
<td>0.31 ± 0.19</td>
</tr>
<tr>
<td>Ul—2 wk Cont—Sac</td>
<td>0.26 ± 0.31</td>
<td>0.63 ± 0.49</td>
<td>0.59 ± 0.41</td>
<td>0.27 ± 0.03</td>
<td>0.7 ± 0.04</td>
<td>0.7 ± 0.03</td>
<td>0.7 ± 0.03</td>
<td>0.7 ± 0.03</td>
</tr>
<tr>
<td>Ul—Cont</td>
<td>11.13 ± 2.73</td>
<td>4.69 ± 2.53</td>
<td>0.29 ± 0.24</td>
<td>0.10 ± 0.06</td>
<td>0.04 ± 0.02</td>
<td>0.07 ± 0.04</td>
<td>0.07 ± 0.03</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Sac</td>
<td>0.08 ± 0.03</td>
<td>0.28</td>
<td>0.19 ± 0.17</td>
<td>0.51 ± 0.39</td>
<td>0.33 ± 0.22</td>
<td>0.15 ± 0.06</td>
<td>0.15 ± 0.06</td>
<td>0.15 ± 0.06</td>
</tr>
<tr>
<td>Cont</td>
<td>0.04 ± 0.03</td>
<td>0.10 ± 0.11</td>
<td>0.08 ± 0.06</td>
<td>0.06 ± 0.03</td>
<td>0.07 ± 0.03</td>
<td>0.07 ± 0.03</td>
<td>0.07 ± 0.03</td>
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</tr>
</tbody>
</table>

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**Table 4**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Simple hyperplasia</th>
<th>Nodular and papillary hyperplasia</th>
<th>No. of rats</th>
<th>Ropy microvilli</th>
<th>Uniform microvilli</th>
<th>Pleomorphic microvilli</th>
<th>No. of rats</th>
<th>LIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ul—8 wk Cont—Sac</td>
<td>14</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>0.32 ± 0.12c</td>
<td></td>
</tr>
<tr>
<td>Ul—2 wk Cont—Sac</td>
<td>14</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>0.33 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Sac</td>
<td>14</td>
<td>5</td>
<td>2</td>
<td>0</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>0.19 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Cont</td>
<td>14</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>4</td>
<td>0.06 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

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LI, labeling index (number of labeled cells per 100 cells); Ul, ulcer; Sac, sodium saccharin; Cont, Control diet. Mean ± S.D.
mice (4–6). In our laboratory, long-term feeding of sodium saccharin also induced bladder tumors if administered to rats with a rapidly proliferating bladder epithelium induced by the freeze ulceration technique or by a single i.p. injection of cyclophosphamide (100 mg/kg) (11). Ulceration followed by 5% sodium saccharin in the diet for 2 years induced a 20 to 30% incidence of bladder tumors, whether given immediately after ulceration or beginning 2 weeks after ulceration. Sodium saccharin or freeze ulceration alone did not induce bladder tumors. The induction of bladder tumors in these 3 types of experiments appear to have a common mechanism. Sodium saccharin exposure in utero, by pellet implantation, or by feeding to rats after ulceration of the bladder all involve exposure to rats in which the bladder epithelium is rapidly proliferating.

We have previously shown that sodium saccharin without prior initiation induced mild hyperplasia in the urinary bladder epithelium (13) and that this effect followed a dose response (25). In the present experiment, continuous feeding of sodium saccharin also induced mild hyperplastic lesions, and these changes were increased by pretreatment with freeze ulceration. However, in contrast to rats fed sodium saccharin without prior ulceration, the combined treatment resulted in the formation of numerous nodular and papillary hyperplastic lesions, which are considered preneoplastic in experimental bladder carcinogenesis.

Sodium saccharin has also been shown to act as a promoting agent in 2-stage urinary bladder carcinogenesis in rats following initiation with methyl nitrosourea (16–18), N-(4-(5-nitro-2-furyl)-2-thiazolyl)formamide (9, 12, 15), or N-butyl-N(4-hydroxybutyl)nitrosamine (27–31) and has also shown promoting activity in an in vitro assay system using fibroblasts (24). All of the initiators used in these experiments are potent mutagens. Sodium saccharin is not mutagenic in a variety of bacterial and other assays (8, 22, 35), it is not metabolized (7, 21, 36), and it does not interact with DNA (23). In the 2-generation, pellet implantation, and ulceration experiments with sodium saccharin, no mutagenic exposure is evident. The induction of bladder tumors in these experiments not involving the use of a mutagenic initiating agent may be due to the action of sodium saccharin in maintaining an increased proliferative rate in the bladder epithelium and maintaining the presence of nodular and papillary hyperplastic lesions in the bladder. Somewhat surprisingly, however, the effects of sodium saccharin were similar whether administered immediately after ulceration or 2 or 8 weeks after ulceration. By 8 weeks after ulceration, the bladder mucosa has returned essentially to normal by light and scanning electron microscopy and by autoradiography. These results suggest that an irreversible event occurred during ulceration and/or during repair which was responsive to the effects of subsequent sodium saccharin.

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REFERENCES


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