ABSTRACT

The promoting effect of sodium L-ascorbate on two-stage urinary bladder carcinogenesis in F344 rats initiated with N-butyl-N-(4-hydroxybutyl)nitrosamine at levels of 0.01 and 0.05% in drinking water was studied. Administration of 5.0% but not of 1.0% sodium L-ascorbate in the diet significantly increased the incidence and number of preneoplastic lesions, papillary or nodular hyperplasia, papilloma, and cancer of the urinary bladder. In groups given 5.0% sodium L-ascorbate, the urine was characterized by an apparent elevation of pH, a decrease of osmolality, and an increase of MgNH4PO4 crystalline. Addition of sodium L-ascorbate to the diet also resulted in increase in the content of ascorbic acid and its metabolite, dehydroascorbic acid, in the urine. These results show that an extremely high content of ascorbic acid and its metabolite, dehydroascorbic acid, appeared to have promoting activity in urinary bladder carcinogenesis under the present experimental conditions, while a high dose (1.0%) does not.

INTRODUCTION

There have been many reports on the relationship between vitamins and the development of cancers (21, 28), and ascorbic acid (vitamin C) has attracted particular attention with regard to the prevention of cancer (4). Ascorbic acid is known to block nitrosamine formation by reaction with nitrites and amines (19), and it has also been shown to inhibit bacterial mutagenesis by N-nitroso compounds (12). Therefore, it seems possible that its addition to foods may prevent cancer in humans.

The two-stage process of chemical carcinogenesis, initiation and promotion, is now known to occur in many organs, including the urinary bladder (2, 5, 9, 13, 20). Previously, we studied in a screening experiment the promoting activities of various chemicals in urinary bladder carcinogenesis in male rats (10). In these preliminary experiments, sodium L-ascorbate, the ionic form of ascorbic acid, appeared to have promoting activity in urinary bladder carcinogenesis.

This paper reports studies on the promoting effect of sodium L-ascorbate on urinary bladder carcinogenesis in rats.

MATERIALS AND METHODS

Animals. Male 8-week-old F344 rats (Charles River Japan, Inc., Kanagawa, Japan) were used in the present experiments. The rats were housed 5 per plastic cage (33.5 x 37.5 cm, 17.5 cm deep) with wood chips for bedding in an animal room with a 12-hr light-12-hr dark cycle at 22 ± 2°C (S.D.) and 60% relative humidity. Body weights, food consumption, and water intake were measured once a week. The amounts of food and water consumed for 2 consecutive days of a week were measured on a per cage basis.

Chemicals. BBN4 was obtained from Izumi Chemical Co., Yokohama, Japan. Sodium L-ascorbate, obtained from Wako Pure Chemical Ind., Ltd., Osaka, Japan, was reagent and food additive grade and was 99.8% pure (Lot DPP3987) in Experiment 1 and 99.2% pure (Lot DPG1180) in Experiments 2 and 3. It was added to Oriental M powdered basal diet (Oriental Yeast Co., Tokyo, Japan) at levels of 1.0 and 5.0%. Analysis of a food sample showed that the actual levels of sodium L-ascorbate added at levels of 1.0 and 5.0% were 0.94 and 4.91% initially and 0.92 and 4.81% after 2 weeks at room temperature (Japan Food Research Laboratories, Tokyo, Japan).

Experiment 1. Rats were randomly divided into 4 groups of 20 rats each. Groups 1 and 2 were given drinking water containing 0.01% BBN for 4 weeks, 7 days a week, and then powdered diet containing 5.0% (Group 1) or 1.0% (Group 2) sodium L-ascorbate for 32 weeks, 7 days a week. Group 3 was given only BBN for 4 weeks. Group 4 was given drinking water without BBN for 4 weeks and then powdered diet containing 5.0% sodium L-ascorbate. The total observation period was 36 weeks.

Experiment 2. Rats were randomly divided into 3 groups of 30 rats each. Groups 1 and 2 were given 0.05% BBN in their drinking water for 4 weeks and then given powdered diet containing sodium L-ascorbate at levels of 5.0 and 1.0%, respectively, for 32 weeks. Control Group 3 was given only 0.05% BBN for 4 weeks. The total observation period was 36 weeks.

For urine examination in Experiments 1 and 2, samples of urine were obtained from 5 rats in each group in both experiments 2 days before sacrifice. Rats were individually housed in metabolic cages without food or water for 4 hr in the early morning. The pH, osmolality, and other parameters of the samples were measured, and 2 ml of the remainder of the samples were concentrated and examined microscopically for urinary sediment.

The liver and kidneys were removed, weighed, and fixed in 10% phosphate-buffered formalin solution. The urinary bladder was inflated by intraluminal injection of 10% phosphate-buffered formalin solution and was then cut into 8 strips. The urinary bladder was examined histologically. For quantitative analysis, urinary bladder lesions were counted by light microscopy, the total length of the basement membrane was measured with a color video image processor (VIP-21CH; Olympus-Ikegami Tsushin Co., Tokyo, Japan), and numbers of lesions were expressed per 10 cm of basement membrane (20). The liver and kidney were also examined histologically.

Experiment 3. Rats were randomly divided into 3 groups of 10 rats each. Sodium L-ascorbate was administered ad libitum to rats at levels of 0, 1.0, and 5.0% in Oriental M powdered basal diet. Rats were individually placed in metabolic cages with glass collection tubes surrounded by ice without food or water for 4 hr in the early morning (5 a.m. to 9 a.m.) before sacrifice at Day 7. The urine was stored in the freezer. At the time of sacrifice, blood samples were collected from the abdominal aorta for blood analysis. One day later, sodium L-ascorbate contents in the blood and urine were measured by a calorimetric method by Japan Food Research Laboratories.

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Promoting Effects of Sodium L-Ascorbate on Two-Stage Urinary Bladder Carcinogenesis in Rats 1

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1 The abbreviations used are: BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; PN hyperplasia, papillary or nodular hyperplasia.
RESULTS

Experiment 1. Rats given sodium L-ascorbate had no toxic symptoms such as diarrhea. Data on the body weight, urinary bladder weight, BBN intake, water consumption, and sodium L-ascorbate intake are given in Table 1. The increase in average body weight in Groups 1, 2, and 3 given BBN was not different from that of controls during BBN administration. The average body weight in rats of Group 4 treated with sodium L-ascorbate without BBN was slightly less in Week 28 and was clearly less than those of the other groups when rats were weighed before sacrifice at Week 36 after overnight starvation. The urinary bladder weight was significantly higher in Group 1 than in Control Group 3. There were no differences in the average water consumption or BBN intake of groups given BBN for 4 weeks, but groups given 5.0% sodium L-ascorbate, with or without BBN, drank more water than did controls from Week 5 to the end of the experiment.

In urinalysis in Week 36, an apparent elevation of pH was observed in groups given 5.0% sodium L-ascorbate. Treatment with 1.0% sodium L-ascorbate also resulted in slight elevation of pH in the urine. Groups of rats treated with 5.0% sodium L-ascorbate showed a decrease of osmolality and increase of crystals in the urinary sediment. This was not the case with 1.0% sodium L-ascorbate. Crystals were of MgNH₄PO₄ based on their morphological appearance by light microscopy (22). Other parameters were within normal ranges.

The histopathological lesions of the urinary bladder observed in rats are summarized in Table 2. As described previously (11), the lesions found in the urinary bladder epithelium were classified into 4 types: simple hyperplasia, PN hyperplasia, papilloma, and cancer. Simple hyperplasia consisted of diffuse or focal thickening of the epithelium with 4 to 8 layers of transitional epithelial cells. In PN hyperplasia, the epithelium was 6 to 8 cells thick, and in most cases the changes were strictly localized. Cellular atypism and mitotic figures were not seen in the areas of proliferating cells. Hyperplasia showed either exophytic growth, with a delicate fibrovascular core and protrusion into the lumen of the urinary bladder, or endophytic growth. These lesions are a form of preneoplastic hyperplasia (11, 17, 18). Papilloma was defined as a benign epithelial tumor in which the transitional epithelial cells were arranged in branched finger-like processes with a delicate fibrovascular core or showed an endophytic growth pattern. Cellular irregularity was slight and few mitotic figures were present in the epithelial proliferation of papillomas. Cancer was of the transitional cell type showing varying degrees of atypia. Mitotic figures were frequently seen. The incidences of simple hyperplasia and PN hyperplasia were significantly higher in Group 1, given BBN followed by 5.0% sodium L-

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Body wt (g)</th>
<th>Urinary bladder wt</th>
<th>Sodium l-ascorbate intake (Weeks 5 to 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BBN (%)</td>
<td>Sodium l-ascorbate (%)</td>
<td>Initial</td>
<td>Final</td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>------------------------</td>
<td>---------</td>
<td>-------</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>1</td>
<td>0.01</td>
<td>5.0</td>
<td>107</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.01</td>
<td>1.0</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.01</td>
<td>5.0</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.01</td>
<td>5.0</td>
<td>109</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>1</td>
<td>0.05</td>
<td>5.0</td>
<td>127</td>
</tr>
<tr>
<td></td>
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<td>0.05</td>
<td>1.0</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.05</td>
<td>1.0</td>
<td>133</td>
</tr>
</tbody>
</table>

a Mean ± S.D.
b Significantly different from Group 3 at p < 0.05.
c Significantly different from Group 3 at p < 0.01.
d Significantly different from Group 3 at p < 0.001.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Papillary or nodular hyperplasia</th>
<th>Papilloma</th>
<th>Cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BBN (%)</td>
<td>Simple hyperplasia incidence</td>
<td>Incidence No./10 cm of BM a</td>
<td>Incidence No./10 cm of BM b</td>
</tr>
<tr>
<td>-------</td>
<td>-----------</td>
<td>-------------------------------</td>
<td>----------</td>
<td>--------</td>
</tr>
<tr>
<td></td>
<td>Sodium l-ascorbate (%)</td>
<td>Effective no. of rats</td>
<td>Incidence</td>
<td>25 (100)b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>25 (100)b</td>
<td>7.0 ± 0.3 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29</td>
<td>20 (89.6)</td>
<td>11 (37.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30</td>
<td>17 (56.7)</td>
<td>8 (28.7)</td>
</tr>
</tbody>
</table>

a BM, basement membrane.
b Significantly different from Group 3 at p < 0.001.
c Numbers in parentheses, percentage.
d Significantly different from Group 3 at p < 0.01.
e Mean ± S.D.
sodium L-ascorbate, than in Control Group 3. Moreover, quantitative analysis of the lesions showed that the number of lesions of PN hyperplasia per 10 cm of basement membrane was significantly increased in Group 1. Treatment with 1.0% sodium L-ascorbate tended to increase the number of PN hyperplasia. However, there were no differences in the incidences or numbers of papillomas in Groups 1, 2, and 3. The mucosa of rats treated with 5.0% sodium L-ascorbate (Group 4) was characterized by normal histology. Lymphocytic infiltration in the submucosa of the urinary bladder was observed in 1 of 20 rats in Group 4. No inflammation, calculous deposit, or foreign body reaction was observed histologically. Other histopathological changes were not seen.

**Experiment 2.** Rats given sodium L-ascorbate at low and high doses showed slight reduction in body weight (Table 1). The intake of BBN in different groups was almost the same. The average food consumption of groups given sodium L-ascorbate was also similar to that of the control, but water consumption of rats given 5.0% sodium L-ascorbate was more than that of controls from Week 5 to Week 36 (Table 1).

Urinalysis showed a shift to alkaline pH, a decrease of osmolality, and an increase of MgNH4PO4 crystals in rats given 5.0% sodium L-ascorbate. Urine of rats in Group 1 also contained occult blood and an increased number of RBC in the urinary sediment. Other urinary parameters examined were within normal ranges.

Macroscopically, rats in Group 1, given BBN followed by 5.0% sodium L-ascorbate, had more tumors of the urinary bladder than did rats in Group 3 given BBN alone. The absolute and relative weights of the urinary bladder were significantly increased in Group 1 (Table 1).

Histopathological lesions of the urinary bladder are shown in Table 2. The incidence of simple hyperplasia was significantly higher in Group 1 given 5.0% sodium L-ascorbate than in the control group. The incidence and the number of PN hyperplasia per 10 cm basement membrane were also significantly increased in Group 1. The incidence and the number of papilloma and cancer per 10 cm basement membrane were also significantly increased by 5.0% sodium L-ascorbate. However, 1.0% sodium L-ascorbate did not significantly increase the occurrence of any bladder lesions in rats pretreated with BBN.

**Experiment 3.** The sodium L-ascorbate contents in the blood and urine of the various groups are presented in Table 3. Values of total ascorbic acid and dehydroascorbic acid in the blood of rats treated with sodium L-ascorbate at levels of 1.0 or 5.0% were significantly higher than those of the control. In the urine, total ascorbic acid increased significantly in rats treated with sodium L-ascorbate. These values clearly demonstrated a dose dependency. Values of dehydroascorbic acid in the urine were also significantly elevated when compared to the control.

**DISCUSSION**

It is clear that PN hyperplasia is a preneoplastic lesion of rat urinary bladder (11, 17, 18). Our study was done to evaluate the promoting activity of sodium L-ascorbate in urinary bladder carcinogenesis in a relatively short-term experiment. Effects were determined on the induction of preneoplastic lesions rather than advanced stages of urinary bladder carcinogenesis in Experiment 1 after initiation with a low dose of BBN. In this experiment, administration of 5.0% but not of 1.0% sodium L-ascorbate increased the occurrence of PN hyperplasia in the urinary bladder of rats pretreated with BBN. This is consistent with our preliminary findings (10). In Experiment 2, we examined the promoting activity of sodium L-ascorbate on urinary bladder carcinogenesis after initiation with a higher dose of BBN. In this experiment, addition of sodium L-ascorbate at a level of 5.0% to the diet resulted in a marked increase not only in PN hyperplasia but also in papilloma and cancer. Thus, the promoting effect of an exceedingly high dose of sodium L-ascorbate was more clearly demonstrated after stronger initiation.

Moreover, the inductions of papilloma and cancer in the group treated with 5.0% sodium L-ascorbate were significantly higher in Experiment 2 than in Experiment 1. Thus, although both doses of BBN caused definite initiation of urinary bladder carcinogenesis, the promoting activity of sodium L-ascorbate was clearer after the stronger initiation in Experiment 2. These results are consistent with results on the promoting effect of saccharin in 2-stage carcinogenesis in rat urinary bladder initiated by 2 different doses of BBN (16).

Ascorbic acid has been shown to have protective effects against nitrosation, the reaction of nitrite with secondary amine in vitro and in vivo (19, 27). It also inhibited mutagenesis induced by N-methyl-N‘-nitro-N-nitrosoguanidine and dimethylnitrosamine in the Ames test (12). Furthermore, it was found to inhibit the conversion of N-hydroxy-N-acyetyl-2-aminofluorene to 2 more potent carcinogens in vitro (6). Ascorbate also prevented induction of urinary bladder carcinogenesis by implantation of a tryptophan metabolite, 3-hydroxyanthranilic acid, into mouse bladder (23) and inhibited the initiation of skin tumors by application of 7,12-dimethylbenz(a)anthracene (24). Thus, ascorbic acid is recognized to be important for cancer prevention. Consistent with this, ascorbate treatment significantly prolonged the life of patients with cancer (3). However, unexpectedly, in the present study, an extremely large dose of sodium L-ascorbate promoted bladder carcinogenesis in rats. Moreover, ascorbate was recently found to promote induction of sarcoma by 3-methylcholanthrene in guinea pigs (1). It is known that ascorbic acid is synthesized in rat liver and excreted by the kidney, and it is present in large amounts with urea in the urine. In the present study, addition of sodium L-ascorbate to the diet resulted in an increase in the

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**Table 2.** The incidence of simple hyperplasia was significantly higher in Group 1 given 5.0% sodium L-ascorbate than in the control group. The incidence and the number of PN hyperplasia per 10 cm basement membrane were also significantly increased in Group 1. The incidence and the number of papilloma and cancer per 10 cm basement membrane were also significantly increased by 5.0% sodium L-ascorbate. However, 1.0% sodium L-ascorbate did not significantly increase the occurrence of any bladder lesions in rats pretreated with BBN.

<table>
<thead>
<tr>
<th>Dose in diet (%)</th>
<th>Total ascorbic acid (mg/100 ml)</th>
<th>Dehydroascorbic acid (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.74 ± 0.07*</td>
<td>0.43 ± 0.05*</td>
</tr>
<tr>
<td>1.0</td>
<td>1.06 ± 0.18*</td>
<td>0.61 ± 0.08*</td>
</tr>
<tr>
<td>5.0</td>
<td>1.61 ± 0.80*</td>
<td>0.80 ± 0.23*</td>
</tr>
</tbody>
</table>

*Significantly different from the control at p < 0.001.

---

**Table 3.** Ascorbate content in the blood and urine of rats treated with sodium L-ascorbate during 1 week.

<table>
<thead>
<tr>
<th>Dose in diet (%)</th>
<th>Total ascorbic acid (mg/100 ml)</th>
<th>Dehydroascorbic acid (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total ascorbic acid (mg/100 ml)</td>
<td>Dehydroascorbic acid (mg/100 ml)</td>
</tr>
<tr>
<td>0</td>
<td>2.23 ± 0.12</td>
<td>1.83 ± 0.21</td>
</tr>
<tr>
<td>1.0</td>
<td>29.30 ± 5.82</td>
<td>7.92 ± 0.66</td>
</tr>
<tr>
<td>5.0</td>
<td>86.82 ± 34.23</td>
<td>12.82 ± 2.53</td>
</tr>
</tbody>
</table>

*Significantly different from the control at p < 0.001.
content of ascorbic acid and its metabolites in the urine, and this excess of ascorbic acid in the urine may have contributed to induction of preneoplastic lesions, papilloma, and cancer in the urinary bladder. Investigations are required on why extremely high doses of sodium L-ascorbate promote urinary bladder carcinogenesis in rats.

In the present study, apparent elevation of pH, low osmolality, and increase of MgNH₄PO₄ crystal contents were established in urine after sodium L-ascorbate (5.0%) treatment. Administration of 5.0% sodium saccharin p.o., which promotes urinary bladder carcinogenesis, induces MgNH₄PO₄ crystalluria (8, 10). However, crystallurias have been induced by chemicals which do not possess promoting activity (10). Therefore, any correlation between crystalluria and promotion of urinary bladder carcinogenesis requires further study. It also has been reported that sodium o-phenylphenate and o-phenylphenol are carcinogenic for the rat urinary bladder (7, 14). Moreover, the carcinogenicity of sodium o-phenylphenate seems to be more potent than that of o-phenylphenol. This evidence supports the view that the alkalinity of urine relates to carcinogenic potency in the urinary bladder. In the present study, 1.0% sodium L-ascorbate did not induce crystalluria and only slightly increased the pH of the urine, while not showing promoting activity for the urinary bladder. Alterations in the composition of the urine could be responsible for tumor development. Thus, promoting activity of sodium L-ascorbate may be related not to its primary effect but rather to secondary events caused by the observed changes in composition of the urine. We need further investigation to resolve this point. It is of obvious importance to establish whether ascorbic acid may have promoting activity or not.

Sodium L-ascorbate is an antioxidant used as a food additive. Wattenberg (25, 26) demonstrated that antioxidants have inhibitory effects on chemical carcinogenesis. The induction of cancers in various organs by carcinogens was inhibited by concomitant administration of antioxidants in the diet. However, recently, we found that these antioxidants, such as butylated hydroxyanisole and butylated hydroxytoluene, possess promoting activity in 2-stage urinary bladder carcinogenesis in rats (15). The mechanism of promotion by sodium L-ascorbate was suggested to depend on its antioxidant properties. Therefore, many other antioxidants may possess promoting activities. Moreover, sodium L-ascorbate might act as a promoting agent merely because it is an anion, similar to saccharin, and not because it is an antioxidant.

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Promoting Effects of Sodium L-Ascorbate on Two-Stage Urinary Bladder Carcinogenesis in Rats

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