Lack of Inhibitory Effects of an Anti-angiogenesis Drug, TNP-470, on Rat Urinary Bladder Papillomatosis Induced by Mechanical Stimulation

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Abstract: The purpose of this study was to clarify whether the anti-angiogenesis drug TNP-470 may exert inhibitory effects on rat urinary bladder papillomatosis. A total of 46, six-week-old, male F344/DuCrj rats were used. In groups 1 and 2, 20 male F344/DuCrj rats each underwent intravesical instillation of 5 beads (1.5 mm diameter) via the abdomen to cause mechanical irritation. Group 3 was included as a control group without beads. Rats in group 1 were then treated with subcutaneous (s.c.) injections of TNP-470 at the dose of 30 mg/kg body wt, 3 times a week for 4 weeks. The vehicle alone was injected s.c. for groups 2 and 3. Subgroups of animals (10 rats each in groups 1 and 2, and 3 in group 3) were killed at weeks 2 and 4 after the beginning of the experiment. Urinary bladder weights of rats treated with intravesical instillation of beads (groups 1 and 2) were significantly increased compared with normal control rats (group 3). There was no difference between urinary bladder weights and calculi weights of groups 1 and 2 at 4 weeks. Histologically, the urinary bladder epithelium of rats suffering mechanical irritation due to the beads (groups 1 and 2) was significantly hyperplastic, demonstrating papillomatosis. There were no significant differences in the degrees or extents of epithelial lesions between groups 1 and 2. Furthermore, elevation of ODC and SAT activities, biomarkers of cell proliferation, in the epithelium was similar in both groups. TNP-470 thus did not inhibit epithelial proliferation in the urinary bladder in this experiment. (J Toxicol Pathol 2002; 15: 197–201)

Key words: urinary bladder papillomatosis, anti-angiogenesis drug, TNP-470, ornithine decarboxylase, cell proliferation, angiogenesis

Introduction

Angiogenesis, the process of new blood vessel formation, is essential for the growth of solid tumors1. If growth of new capillaries is inhibited, tumor growth is impaired, restricting the tumor nodules to 0.4 mm in diameter in one experiment2. Inhibition of angiogenesis may therefore be useful as an effective means to cancer prevention.

Moreover, an angiogenic switch can be speculated during the early stage preceding the appearance of solid tumors, extensive vascularization with ongoing angiogenesis becoming apparent in end-stage tumors in transgenic mouse models3,4. Thus, angiogenesis might be an important factor for cancer promotion.

TNP-470, a semisynthetic analogue of fumagillin derived from Aspergillus fumigatus, possesses potent anti-angiogenesis activity in vitro5 and in vivo6–8, exerting its effects via inhibition of the proliferation of endothelial cells6,9. However, effects of TNP-470 in experimental chemical carcinogenesis models have been investigated in only a few cases10–14. The urinary bladder epithelium of rodents readily proliferates in response to mechanical irritation, for example in the presence of urinary calculi15–18 or foreign bodies19, after freeze-ulceration20 or even intravesical instillation of physiological saline21. Dietary supplementation with uracil at a concentration of 3% rapidly results in formation of calculi in the urinary bladder15–18. These cause mechanical stimulation and diffuse reactive hyperplasia of the epithelium of urinary bladder, called papillomatosis15. This features hyperplastic epithelium with increase in stroma including blood vessels17. Angiogenesis also appears to be
needed for formation of papillomatosis from the early stage (preparation for submission).

In a previous experiment conducted in our laboratory, papillomatosis induced by dietary supplementation with uracil was inhibited by administration of the anti-angiogenesis drug, TNP-470. However, calculi formation in the urinary bladder of the rats treated with TNP-470 was decreased as compared with that of vehicle treated animals. For that reason, the exact mechanisms of inhibition could not be clarified whether anti-angiogenesis effects of TNP-470 inhibited the growing of papillomtosis or the decreased calculi formation by TNP-470 treatment influenced the development of papillomatosis (submitted for publication).

In the present study, to overcome this problem with rat urinary bladder papillomatosis, we selected intravesical instillation of beads into the urinary bladder as a source of mechanical irritation which would not be expected to be directly influenced by any additional treatment.

Materials and Methods

Chemical and preparation
TNP-470, kindly provided by Takeda Chemical Industries, Ltd., Osaka, was suspended in a vehicle composed of 1% ethanol plus 5% arabic gum in saline.

Animals and their maintenance
A total of 46, five-week-old, male F344/DuCrj rats (Charles River Japan, Inc., Hino) were housed 5 per cage in an animal facility with a 12-hr light-12-hr dark cycle at a temperature of 22 ± 2°C and 44 ± 5% relative humidity and were given access to tap water and food (Oriental MF; Oriental Yeast Co., Tokyo). The animals were observed daily and body weights were measured weekly throughout the duration of the experiment.

Experimental procedure
In groups 1 and 2, 20 male F344/DuCrj rats each underwent the operation of intravesical instillation of 5 teflon beads (1.5 mm diameter) through opening the abdomen and cutting of the head of urinary bladder under ethanol anesthesia. After instillation of teflon beads, the abdomen and cutting of the head of urinary bladder were sutured. Animals in group 1 were then treated by subcutaneous (s.c.) injection with TNP-470 at a dose of 30 mg/kg body wt, 3 times a week for 4 weeks. The vehicle alone was injected s.c. for groups 2 and 3. Subgroups (10 rats each in groups 1 and 2, and 3 rats in group 3) were killed at weeks 2 and 4 after the beginning of the experiment. For pathological analysis, urinary bladders were inflated by intraluminal injection of 10% phosphate-buffered formalin solution. After fixation, the urinary bladders were divided sagittally and weighed. Calculi including beads in the urinary bladder were also weighed. For histological analysis the urinary bladders were cut into eight strips, routinely processed for embedding in paraffin, sectioned and stained with hematoxylin and eosin. To assess ornithine decarboxylase (ODC) and spermidine/spermine N'-acetyltransferase (SAT) activities, biochemical markers of epithelial proliferation in the rat bladder22, epithelial samples were obtained by scraping and frozen in liquid nitrogen, with storage at –80°C in a deep freezer until analysis.

Measurement of ODC and SAT activities
ODC and SAT activities were measured by the methods of Otani et al.23 and Masui et al.24, respectively. Frozen pieces of rat bladder epithelium (each 5 urinary bladders) were suspended in 0.5 ml of 50 mM Tris (pH 7.5) containing 0.25 M sucrose, disrupted in a homogenizer for a few minutes, then centrifuged at 100,000 g for 30 min. The resultant supernatant was assayed for ODC and SAT activities by measurement of the amount of radioactive putrescine produced from (5–14C)ornithine and the amount of acetyl moiety transferred from (1–14C)acetyl coenzyme A to spermidine, respectively.

Statistical analysis
Statistical analysis was accomplished with Stat-view software for Macintosh computers, and significant differences were determined by the use of Dunnett’s t-test.

Results
Table 1 summarizes data for body and urinary bladder weights and calculi weights including beads at 2 and 4 weeks. The general condition was good in each group. However, the body weights of group 1(TNP-470 treated

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<tr>
<th>Group</th>
<th>Treatment</th>
<th>2 Weeks</th>
<th>4 Weeks</th>
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<tbody>
<tr>
<td>1</td>
<td>Beads + TNP-470</td>
<td>136 ± 16&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.28 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>Beads + Vehicle</td>
<td>185 ± 13</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>Sham + Vehicle</td>
<td>207 ± 6</td>
<td>0.09 ± 0.01</td>
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<sup>a</sup> Mean ± SD.  
<sup>b</sup> Significantly different from group 2 at p<0.01 (Dunnet’s t-test).
group) were significantly decreased compared with those of group 2. The toxicity of TNP-470 was apparent from the decrease of food intake in group 1 (data not shown). The Urinary bladder weights of rats undergoing intravesical instillation of beads (groups 1 and 2) were significantly increased compared with normal control values (group 3). There were no significant differences between urinary bladder weights and calculi weights of groups 1 and 2 at 4 weeks, although the urinary bladder weights of rats treated with TNP-470 at week 2 were slightly higher than those of vehicle treated animals. A similar tendency was noted for calculi volume.

Histologically, the urinary bladder epithelium of rats subjected to intravesical instillation of beads (groups 1 and 2) was significantly hyperplastic and revealed diffuse papillary growing with narrow stroma, demonstrating papillomatosis (Fig. 1). However, there were no significant differences in the epithelial lesions between groups 1 and 2 (Fig. 1). There was no significant difference in blood vessels between groups 1 and 2. Table 2 shows ODC and SAT activities of the urinary bladder epithelium. Again, there were no significant differences in values between groups 1 and 2.

Fig. 1. Histopathology of the urinary bladder lesions in the rats with instillation of beads. A shows the TNP-470 treated rat. B shows vehicle-treated rat. Both A and B show diffuse papillary growth of urinary bladder epithelium with narrow stroma (papillomatosis). No significant difference is observed between these lesions. H&E. × 60.
Table 2. ODC and SAT Activities of Urinary Bladder Epithelium

<table>
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<tr>
<th>Group</th>
<th>Treatment</th>
<th>2 Weeks</th>
<th>4 Weeks</th>
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<tr>
<td></td>
<td></td>
<td>ODC (pmol/hr/mg)</td>
<td>SAT (pmol/10 min/mg)</td>
</tr>
<tr>
<td>1</td>
<td>Beads + TNP-470</td>
<td>1390 ± 721a</td>
<td>165 ± 134</td>
</tr>
<tr>
<td>2</td>
<td>Beads + Vehicle</td>
<td>2679 ± 1441</td>
<td>295 ± 16</td>
</tr>
</tbody>
</table>

a: Mean ± SD.

Discussion

In our previous experiment, urinary bladder papillomatosis caused by dietary supplementation with uracil in rats was inhibited by TNP-470 (prepared for submission). However, an important complicating factor was the reduction in calculi in the urinary bladders of rats receiving the TNP-470. In the present experiment, we therefore used intravesical instillation of beads to stimulate the urinary bladder epithelium. Clearly, TNP-470 did not inhibit epithelial proliferation of the urinary bladder in this case.

The urinary bladder epithelial lesions induced by beads were almost the same as those caused by dietary supplementation with uracil in rats. They showed diffuse papillary epithelial growth with narrow stroma (papillomatosis). There were no significant difference in the blood vessels in each lesion (we compared the beads induced lesion with uracil induced lesions in the previous experiment).

One of the mechanisms by which TNP-470 exerts antiangiogenesis effects is to inhibit the expression of cyclin D1 in endothelial cells. In tumor development, an angiogenic switch appears to be induced at an early stage. Also, with the urinary papillomatosis caused by mechanical stimulation, angiogenesis occurs early in the process. ODC activity is significantly increased as compared to the control level and PCNA-positive cells become more numerous. These proliferating cells need an abundant blood supply to obtain energy. In the present experiment neither histopathological evidence of proliferation nor ODC activity of the urinary epithelium was influenced by the TNP-470 treatment. However, since the ODC level was about 30 times the control level at 2 weeks after bead instillation (ODC level of normal urinary bladder epithelium was not examined in this experiment, so the normal level is that obtained by personal communication) there is a possibility that the mechanical stimulation was too strong to allow any significant effects of the anti-angiogenesis drug. Further studies are necessary to clarify this point.

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