The effect of curcumin on bladder tumor in rat model

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Abstract. – OBJECTIVE: Bladder cancer is the most commonly malignant tumor in the urogenital tract, only next to prostate cancer with a higher incidence in China. Curcumin is the major component of curcuma longa and has multiple biological effects including anti-tumor. This study aimed to investigate the effect of curcumin on bladder cancer.

MATERIALS AND METHODS: SPF-grade Wistar rats were used for establishing bladder cancer model through injection of N-methyl-N-nitrosourea (MNU). Rats were then randomly divided into experimental, model and control group. 160 μmol/L curcumin were applied in the experimental group while model group received an equal volume of saline. General condition, morphology changes and cell cycle of bladder cancer cells were examined. Meanwhile, apoptotic proteins including Bcl-2, Bax and Survivin were also measured by Western blot.

RESULTS: Model rats displayed fever, hematuria, decreased food and water intake, dispersed fur, lower body mass and decreased activity. Under microscopy, the bladder wall was thickened with the cauliflower-like lesion, in which significant necrotic and hemorrhagic lesions were found. Experimental group rats improved general condition without decrease of body mass. The only minor lesion was found without significant necrosis or hemorrhage without invasion into the muscular layer. The number of G1 phase cells was increased while S phase cell number was decreased after drug intervention, suggesting suppression of G1/S transition (p < 0.05). In curcumin-treated rats, the expression of Bcl-2 and Survivin were significantly decreased while Bax protein expression was significantly elevated (p < 0.05).

CONCLUSIONS: Curcumin can inhibit the growth and invasion of rat bladder cancer cells, possibly through the arresting of G1/S transition and subsequently increased apoptosis.

Key Words: Curcumin, Bladder cancer, Apoptosis, Bcl-2, Bax, Survivin.

Introduction

The incidence of bladder cancer is rapidly increasing. The major approach for treating early stage bladder cancer is the surgical resection via urinary tract combined with intra-bladder chemotherapy or immune therapy, such as bCG vaccine infusion. It has a satisfactory response at the initial stage, but with a rapid recurrence and progression of tumors. Other adverse effects such as inflammation, fever and granuloma prostatitis also may occur in some patients1, 2. Mitomycin is a common drug for preventing the recurrence of bladder tumor, but cannot significantly increase survival rate or inhibit tumor progression3. A previous work has confirmed the involvement of curcumin in cell growth, proliferation, differentiation, invasion, migration and angiogenesis, as well as in the inhibition of tumor progression4. In a further study, curcumin has been found to have a wide spectrum of anti-tumor effects with less adverse reactions, suggesting it could be widely applied in tumor prevention and new drug development5. This paper selected Wistar rat as the experimental animals to establish bladder cancer model through injection of N-methyl-N-nitrosourea (MNU), followed by curcumin intervention. The general condition and microscopic morphology of rat bladder tissues were observed, along with measuring the apoptosis and related proteins’ expression, to evaluate the effect of curcumin on bladder cancer.

Materials and Methods

Animals

A total of 60 SPF grade healthy Wistar rats (30 males and 30 females, aged 8 weeks, body weight at 180 g ± 20 g) were provided by labora-
The effect of curcumin on bladder tumor in rat model

Morphology Observation

Bladder tissues of rats were fixed in 10% formalin, followed by routine dehydration, immersion and paraffin embedding. Tissues were then sectioned into 3–4 μm slices for H&E staining. Images were observed under an inverted microscope.

Cell Isolation and Culture

Bladder tissues were removed and homogenized. Tissues were rinsed in streptomycin/penicillin solution for 15 min, followed by Hanks solution for 20 min digestion. Supernatants and cell debris were discarded and the digestion was quenched in DMEM containing 10% fetal bovine serum (FBS). Cells were re-suspended in DMEM at 10^6 per mL in a humidified chamber at 37°C with 5% CO₂. Cells were passaged at 1:2 with medium change every other day. Cell morphology was examined under the microscope.

Cell Cycle Assay by Flow Cytometry

PI staining was used to detect cell cycle. Cells were collected and digested. After adjusting to 10^6/L, 0.49 mL buffer was added to the cells, followed by the addition of 5 μl PI dye. Staining was performed at room temperature in the dark for 10 min, followed by dual-parameter analysis by flow cytometry.

Western Blotting

Cells were collected for extracting proteins, which were loaded with 40 μg per lane on 8% SDS-PAGE gel. Proteins were transferred to the membrane, which was blocked at room temperature for 1 h. Rabbit anti-human Bel-2, Bax and Survivin monoclonal antibody (1:1 200 dilution) were added and incubated at 4°C overnight. The secondary antibody was then added for 1 h incubation, followed by color development using enhanced chemiluminescence reagents.

Statistical Analysis

SPSS17.0 software (SPSS Inc., Chicago, IL, USA) was used for data processing. Measurement data were presented as mean ± standard deviation (SD) and processed in one-way analysis of variance (ANOVA) with Newman-Keuls multiple comparison post-hoc analysis. The statistical significance was defined when p < 0.05.

Drugs and Equipment

Curcumin (Lot. HB108YHSY, Daquan, China); MNU Sigma-Aldrich (St. Louis, MO, USA); DMEM culture medium, streptomycin/penicillin, fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA); Rabbit anti-human Bel-2, Bax and Survivin monoclonal antibody, goat anti-rat secondary antibody (Baode Biotech, Jiangmen, China). Incubator (Thermo Scientific, Waltham, MA, USA); CO₂ incubator, -80°C fridge and flow cytometry apparatus (SANYO, Moriguchi, Osaka, Japan); Inverted microscope (Nikon, Minato-ku, Tokyo, Japan); High-speed centrifuge (Beckman, Brea, CA, USA).

Bladder Cancer Model

A total of 60 rats were randomly divided into three groups (N = 20 each). Experimental and model groups were infused with different amounts of MNU at different time points, while the control group received no treatment. Details of schedules were as follows:

Experimental group: 1.5 mg MNU were infused via bladder at 0, 2, 4, 6 and 8 week, along with 160 μmol/L curcumin infusion at 1, 3, 5, 7, 9, and 11 week.

Model group: 1.5 mg MNU were infused via bladder at 0, 2, 4, 6 and 8 week, along with 0.2 mL NaCl solution (0.9%) containing 0.75% DMSO.

Control group: Equal volume of 0.9% NaCl solution at 0, 2, 4, 6, and 8 week, along with 0.2 mL NaCl solution (0.9%) containing 0.75% DMSO.

Sample Collection

Rats were sacrificed by cervical dislocation and bladder tissues were collected from each rat. One part of the extracted tissues was fixed in neutral buffered formaldehyde for analysis of the morphological change of bladder tissues by H&E staining. Other tissues were frozen for further use.
Results

General Condition of Rats
In all 60 rats, no significant changes have been observed in control group. Model rats, however, displayed fever, blood urea, and decreased food/water intake. In some rats, no food was taken and the fur was abolished, leading to significantly decreased body mass (more than 50%), as well as lower activity or even immobility. Experimental rats displayed fever and lower food intake, but without significant body mass loss.

Tissues Morphology
In control group, bladder wall showed a smooth morphology, without ulcer, hemorrhage, thinning or neoplastic lesion. In model rats, their bladder wall were significantly thickened, with the cauliflower-like neoplastic lesion, accompanied with significant necrosis and hemorrhage. Experimental rats had thickening of the bladder wall, which had minor tubular lesion without significant necrosis or hemorrhage. H&E staining confirmed the malignant transformation of bladder mucous. In model rats, with elongated induction by MNU, the differentiation grade of tumors was even lower, suggesting higher malignancy. Abnormal hypertrophy was found in mucous, with gradual formation of shallow cancer or even invasion of muscular layer. In the experimental group, malignancy can be observed in bladder mucous but without muscular invasion (Figure 1).

Cell Cycle Alternation
Bladder cells were isolated and cultured for analysis of cell cycle by flow cytometry. Results showed that compared with model or control group, experimental group displayed more cells at G1 phase but few S phase cells, suggesting the inhibition of G1/S transition (p < 0.05). Bladder cells in control group grew well with normal cell cycle (Table I).

Bcl-2, Bax and Survivin expression in bladder cells
Western blotting was used to detect the expression level of Bcl-2 and Bax in bladder cells. Results showed that in curcumin-treated bladder cells, the expression of Bcl-2 and Survivin were significantly decreased while Bax expression was

<table>
<thead>
<tr>
<th>Group</th>
<th>GI</th>
<th>S</th>
<th>G2/M</th>
<th>AP</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment</td>
<td>92.6 ± 1.2*</td>
<td>7.6 ± 0.8**</td>
<td>0.3 ± 0.7**</td>
<td>1.1 ± 0.5#</td>
<td>17.8 ± 1.8**</td>
</tr>
<tr>
<td>Model</td>
<td>83.2 ± 0.7*</td>
<td>11.8 ± 1.1*</td>
<td>5.4 ± 0.8*</td>
<td>15.3 ± 1.5*</td>
<td>8.2 ± 1.4*</td>
</tr>
<tr>
<td>Control</td>
<td>74.7 ± 0.5</td>
<td>18.7 ± 1.3</td>
<td>7.6 ± 1.1</td>
<td>0</td>
<td>24.5 ± 2.5</td>
</tr>
</tbody>
</table>

Note: *, p < 0.05 compared with control group; #, p < 0.05 compared with model group.
The effect of curcumin on bladder tumor in rat model

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... elevated \((p < 0.05)\). However, the model group had decreased the expression of Bcl-2 and Survivin, as well as elevated Bax expression compared with control group \((p < 0.05, \text{Table II, Figure 2})\).

Discussion

Bladder tumor is the most common cancer in urinary surgery. Multiple approaches have been developed in clinics. However, the recurrence rate is still high even surgical approaches have been employed. Drug treatment had unfavorable efficacy with higher adverse effects\(^6,7\). Curcumin is the major component of curcuma longa and has multiple biological effects including anti-oxidation, anti-inflammation and anti-tumor. Recently, the anti-tumor potency of curcumin has been developed and become a new research focus\(^8\). Curcumin mainly inhibits tumor cell growth, proliferation, invasion, metastasis, leading to blockage of the angiogenesis of tumors, exerting satisfactory anti-tumor potency\(^9,10\).

In this study, rat bladder tumor model was established in both model and experimental groups. A previous study has proven the efficacy of MNU in inducing bladder cancer model. Experimental rats further received curcumin intervention. Our study found no significant changes in control rats with normal growth. Model rats, however, had fever, blood urea, decreased food/water intake or even refusing to feed, accompanied with dispersed fur and lower body mass, as well as low motility or even immobility. However, experimental rats displayed no significant body mass loss even with fever and lower food intake. We further examined tissue morphology of rat bladder under a light-field microscope. In control group, bladder wall showed a smooth morphology, without ulcer, hemorrhage, thickening or neoplastic lesion, whereas, in model rats, their bladder wall was significantly thickened, with the cauliflower-like neoplastic lesion, accompanied with significant necrosis and hemorrhage.

Abnormal hypertrophy was observed in mucous, with gradual formation of shallow cancer or even invasion of muscular layer. With the elongated induction of MNU, the differentiation grade of tumors was even lower, suggesting higher malignancy. Experimental rats had to thicken of the bladder wall, which had minor tubular lesion but without significant necrosis or hemorrhage. Malignancy can be observed in bladder mucous without muscular invasion. A previous study found that the intervention by high dosage of curcumin on various malignant tumors including breast cancer and skin cancer can decrease tumor lesion numbers, shrink tumor size and decrease the tumor formation rate\(^11\). A high concentration of curcumin within short time window can exert cytotoxicity for bladder cancer cells. Moreover, the intra-bladder infusion approach of drug delivery had a better efficacy for inhibiting tumor cell seeding\(^12\), which are consistent with our results.

During the process of tumor cell growth, cell cycle showed abnormality and over-proliferation, as well as inhibition of cell apoptosis\(^13\). A standard cell cycle followed G1-S-G2-M sequence and the dysfunction at arresting point G1/S or G2/M, leads to dysregulation of cell proliferation and growth, causing tumor formation\(^14,15\). In this work, we separated bladder cells, whose cell cycle was analyzed by flow cytometry. Results showed more cells at G1 phase but few S phase cells, suggesting the inhibition of G1/S transition in the experimental group compared with control or model group. These findings suggested that curcumin regulates cell cycle through prevention

Table II. Bcl-x, Bax and Survivin expression.

<table>
<thead>
<tr>
<th>Group</th>
<th>Bcl-2</th>
<th>Bax</th>
<th>Survivin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental group</td>
<td>0.14 ± 0.11*</td>
<td>0.76 ± 0.17*</td>
<td>0.18 ± 0.07*</td>
</tr>
<tr>
<td>Model group</td>
<td>0.53 ± 0.13*</td>
<td>0.33 ± 0.09*</td>
<td>0.53 ± 0.21*</td>
</tr>
<tr>
<td>Control group</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.07</td>
<td>0.08 ± 0.07</td>
</tr>
</tbody>
</table>

Note: *, \(p < 0.05\) compared with control group; **, \(p < 0.05\) compared with model group.
of the G1/S transition of bladder cancer cells, leading to inhibition of tumor growth and proliferation.

As one programmed suicide of cells, apoptosis is highly conserved and tightly regulated during cell evolution [16]. A previous report has shown that the occurrence of the malignant tumor is mainly due to the proliferation of tumor cells and imbalance of apoptosis. In this process, the pro-apoptotic gene was inhibited while anti-apoptotic genes were activated, leading to the long-term survival of tumor cells and inhibition of apoptosis [17]. Bcl-2 is mainly distributed in mitochondrial membrane and nuclear membrane. The elevated expression of Bcl-2 can inhibit apoptosis but does not affect mitosis [18]. As one pro-apoptotic factor, Bax could induce cell apoptosis via down-regulating Bcl-2 [19]. In this study, the expression of Bcl-2 and Survivin were significantly decreased while Bax expression was elevated in curcumin-treated bladder cells, suggesting that curcumin could decrease the expressions of Bcl-2 and Survivin while increase Bax expression. A basic study has found that curcumin could inhibit cell proliferation in human liver cancer cells, which had significantly increased apoptosis, elevated expression of Bax and Caspase-3 while decreased expression of Bcl-2 and Survivin [20], which are consistent with our study.

Conclusions

Curcumin could inhibit the growth and invasion of rat bladder cancer cells. Also, it can increase the number of G1 phase cells and decrease S phase cell number, thus impeding the G1/S transition. Moreover, curcumin also decreases the expression of anti-apoptotic proteins Bcl-2 and Survivin, while increases pro-apoptotic protein Bax expression. Our study suggests that curcumin may be used as a novel drug candidate in the treatment of bladder cancer.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

References

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