The Feasibility of BCG and Sunitinib Combination Therapy for Transitional Cell Carcinoma

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Objective: Currently, intravesical bacille Calmette-Guérin (BCG) instillation is the standard treatment for patients with an intermediate to high risk of bladder cancer. Nevertheless, BCG-refractory generation is a common process during treatment. We hypothesized that sunitinib, a vascular endothelial growth factor (VEGF) receptor inhibitor, can synergistically enhance the immunotherapeutic effect of BCG on bladder tumor growth.

Materials and Methods: The 50% inhibitory concentration (IC50) of BCG and sunitinib in various transitional cell carcinoma (TCC) cell lines was determined by the MTT method. The therapeutic effects of BCG, sunitinib, and their combination on MBT-2 tumors were investigated in both orthotopic bladder cancer and subcutaneously inoculated tumor models in mice. Evaluated parameters included the tumor growth rate and tumor burden in the bladder, tumor volume in the subcutaneous site, survival rate, serum cytokine changes, and mean vascular density in tumor tissues.

Results: BCG and sunitinib showed in vitro cytotoxicity to tested TCC cell lines with slight variations in IC50. Sunitinib had an in vitro synergistic effect on BCG cytotoxicity in TCC cells. BCG and sunitinib had individual tumor suppression activities in mice MBT-2 tumors in both the orthotopic and subcutaneous models. Surprisingly, synchronous combination treatment using BCG and sunitinib did not demonstrate synergistic suppression efficacy in animal tumor models. Inhibition of tumor growth, increased survival rate, and decreased mean vascular density were observed in mice treated with BCG, sunitinib, and synchronous cotreatment. In contrast, no therapeutic efficacy was seen in mice treated with BCG and sunitinib metachronously.

Conclusions: BCG and the VEGF receptor inhibitor sunitinib had significant suppressive effects in mice bladder cancer models when administered individually. The underlying mechanisms by which they counteract cytotoxicity and the interaction of BCG and sunitinib during cotreatment require further investigation.

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KEY WORDS:
BCG; bladder cancer; sunitinib; synergism; vascular endothelial growth factor
1. Introduction

Transitional cell carcinoma (TCC) of the bladder is the fifth most common cancer in the US with an estimated 68,810 new cases and 14,100 deaths in 2008.\(^1\)\(^2\) Annually, approximately 336,000 people are diagnosed with bladder cancer worldwide. In Taiwan, 681 victims died of bladder cancer in 2006 according to the registration statistics of the Department of Health, Executive Yuan. TCC is histopathologically classified into three types: superficial (papillary tumors), confined to the bladder wall (pT1 and pTa tumors), and invasive (stages T2–T4).\(^3\) Approximately 30% of patients with papillary tumors will progress to invasive TCC, for which a radical cystectomy is standard therapy. Unfortunately, bladder cancer has the highest rate of recurrence, at 50–80%, of any cancer. Half of patients with muscle-invasive bladder cancer will develop metastatic disease.\(^4\) The treatment goal for superficial bladder cancer is to reduce tumor recurrence and prevent tumor progression, which would require additional aggressive therapies.

Bladder cancer is the most expensive of all cancers to treat because of the high recurrence rate. For bladder cancer, intravesical chemo/immunotherapy is widely used as adjuvant therapies after surgical transurethral resection of the superficial bladder, while systemic therapy is typically reserved for higher-stage, muscle-invading, or metastatic disease. The goal of intravesical therapy is to eradicate existing or residual tumors through direct cytoreduction or immunostimulation. A good response can usually be obtained with mitomycin C or bacille Calmette-Guérin (BCG) instillation. Nevertheless, disease relapse and progression are frequently observed in approximately 15% of clinical patients.\(^5\) This fact has prompted strong demands to develop more effective therapies for superficial bladder cancer after transurethral resection. Therefore, new targets need to be identified to treat a greater range of patients. Targeted therapy with novel agents directed at specific molecular pathways is a promising avenue to achieve such progress.

Angiogenesis plays an essential role in tumor growth, invasion, and metastasis. Vascular endothelial growth factor (VEGF) and its receptors are considered to be important regulators of angiogenesis. They are mediated through the VEGF-specific tyrosine-kinase receptors (TKRs), and blocking this pathway is a promising therapeutic strategy for inhibiting angiogenesis and tumor growth. Various clinical trials have validated the clinical importance of anti-VEGF and anti-VEGF receptor (VEGFR) therapy. Currently, the humanized monoclonal antibody, bevacizumab (which blocks VEGF-A), and the tyrosine kinase inhibitors (TKIs), sunitinib and sorafenib (which inhibit VEGFRs), are approved for patients with various malignancies, and several others are expected in coming years. Sunitinib and sorafenib were recently demonstrated to improve progression-free survival in patients with metastatic renal cell carcinoma.\(^6\)\(^–\)\(^9\) Therefore, we speculate that these agents should also be beneficial for bladder carcinoma.

The specific hypothesis of this study is that sunitinib can synergistically enhance the immunotherapeutic effect of BCG on bladder tumor growth both in vitro and in vivo.

2. Materials and Methods

2.1. TCC cell lines and agents

The human TCC cell lines, TCC8701, TCC8702, TCC9202, and TCC8301, were established in our laboratory from clinical specimens of patients with bladder cancer. T24 and the mouse TCC cell line, MBT2 (purchased from American Type Culture Collection [ATCC], Manassas, VA, USA), were used in this study. They were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA), 100 units/mL penicillin, and 100 mg/L streptomycin (both from Sigma, St. Louis, MO, USA) at 37°C, in an air atmosphere with 5% CO\(_2\). The Connaught strain of Mycobacterium bovis BCG (ImmuCyst, SinoFil Pasteur Limited, Toronto, Ontario, Canada) was used in this study. It is a freeze-dried preparation of an attenuated strain of M bovis containing viable bacteria of the Connaught strain of BCG, which is formulated to contain 81 mg (dry weight) of BCG and 5% w/v monosodium glutamate. This amount of BCG provides at least 180 million colony-forming units. It promotes a local inflammatory reaction with histiocytic and leucocytic infiltration of the urinary bladder. The local inflammatory effects are associated with an apparent elimination or reduction of superficial cancerous lesions of the urinary bladder. Sunitinib malate (Sutent\(^\circledR\), SU11248, NY, USA) is an orally bioavailable small-molecule inhibitor of multiple receptor tyrosine kinase domains, including VEGFR-1 and -2, platelet-derived receptor -\(\alpha\) and -\(\beta\), and the KIT receptor. It inhibits multiple signaling pathways, resulting in a dual action of antiproliferation and antiangiogenesis. Sunitinib used in this study was kindly provided by Pfizer (Taipei, Taiwan) with no mutual financial conflict or interest.

2.2. Mice and animal care

Pathogen-free 6–8-week-old female C3H/HeN mice (Animal Center, National Defense Medical Center, Taipei, Taiwan) were used for these experiments. Animal care and experimental procedures were in accordance with institutional guidelines.

2.3. In vitro combined treatment of bladder cancer cells with BCG and sunitinib

Cells were exposed to BCG, sunitinib, or their combination for 72 hours. Cellular chemosensitivity was assayed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl
tetrathiazolium (MTT; Sigma) assay to determine cell viability in vitro. In brief, MBT-2 cells (5,000 cells/well) in 100 μL of culture medium were seeded into 96-well microplates and incubated at 37°C for 24 hours before drug exposure. Plated cell numbers were calculated to keep control cells growing in the exponential phase throughout the incubation period. For concurrent treatment, cells were treated with agents (each in 100 μL of culture medium) and incubated for 72 hours. At that point, 50 μL of MTT (2 mg/mL in RPMI medium) was added to each well and allowed to react for 2.5 hours. The blue formazan crystals formed were pelleted to the well bottoms by centrifugation (3,000 rpm/5 minutes), separated from the supernatant, and dissolved in 150 μL of DMSO. The optical density was determined by absorbance spectrometry at 492 nm using a microplate reader (MRX-2; Dynex Technologies, Chantilly, VA, USA). Three separate experiments with triplicate runs for each run were performed to obtain the mean cell viability. Drug concentrations in flasks by trypsinization and adjusted to the required concentration of 5 × 10^6 cells/mL. Mice were anesthetized in groups of five with a single dose of an intraperitoneal injection of ketamine HCl/xylcaine (100 mg/15 mg body weight per mouse) before the study. The bladder was catheterized via the urethra with a 24-gauge plastic intravenous cannula under sterile conditions. The bladder was then traumatized by instilling 0.1 mL of a 0.1 N HCl solution for 15 seconds, it was neutralized with 0.1 mL 0.1 N KOH and then flushed with sterile saline. The tumor cell suspension (5 × 10^6 cells in 0.1 mL 50% normal mouse serum) was instilled via the cannula. The urethra was compressed with a serrefine clamp for 30 minutes to prevent premature bladder evacuation. Under these conditions, macroscopic tumors usually developed in mice which resulted in death within 6 weeks without treatment.

2.6. Immunotherapy of orthotopic bladder tumors using BCG, sunitinib, or their combination

For intravesical therapy, mice were anesthetized, catheterized, and administered BCG (0.18 mg/mL), sunitinib (0.25 mg/mL), or their combination in 0.1 mL PBS and retained for 1 hour. Treatment began 3 days after MBT-2 tumor implantation, and it was given twice weekly for a total of six doses. In addition, negative control mice were only injected with 0.1 mL PBS. The activity, body weight, and survival of mice were monitored daily. After a mouse died, it was frozen and kept for further histopathological evaluation until the end of the study. Cumulative survival rates of treated MBT-2-bearing mice were periodically determined during 42 days of observation. A total of 10 to 12 mice were used per experiment group, and each study was repeated twice.

2.7. Treatment of subcutaneously inoculated bladder tumors in mice

The therapeutic effects of BCG, sunitinib, and their combination were also evaluated in a subcutaneous tumor model. The subcutaneous tumor model was performed by subcutaneous needle inoculation of 10^7 MBT-2 tumor cells in 0.5 mL of a PBS solution. Tumor nodules were visible at approximately 10 days, and treatment began twice weekly with an intraperitoneal injection of BCG (0.18 mg/mL), sunitinib (0.25 mg/mL), or their combination in 0.1 mL PBS for six injections in total. The activity, body weight, and survival of mice were monitored daily. The volume of tumor growth was monitored weekly. The tumor volume was calculated using the following formula: volume = 0.52 × L × W × H, where L is the length or largest diameter, W is the width or smallest diameter, and H is the height (all in mm). After 42 days, mice were sacrificed, tumors were sent for histopathological examination, and sera were collected for cytokine detection.
2.8. Histological examination

Bladder sections (4 μm) were assessed by hematoxylin and eosin staining for intravesical tumors. Briefly, mice were sacrificed, and the bladders were perfused and fixed with 10% neutral buffered formalin. Standardized cross-sections were obtained from the bladder tissue border, stained in hematoxylin and eosin, and evaluated on a Presage image analysis system (Advanced Imaging Concepts, Princeton, NJ, USA). The tumor incidence, tumor loci, and histological characteristics of bladder sections were examined.

2.9. Quantification of MVD

Mean vascular density (MVD) in the tumor was counted after immunostaining with an anti-CD34 monoclonal antibody. Five microscopic fields at 400× magnification from selected specimens were examined for the presence of microvessels, and MVD was determined as the average number of microvessels per 400× field.

2.10. Serum cytokine determination

Blood samples of mice were centrifuged. Serum was recovered at the study completion and examined for the concentration of various individual cytokines. Interleukin (IL)-1, IL-2, VEGF, regulated on activation normal T cell expressed and secreted (RANTES), and monocyte chemoattractant protein (MCP)-1 levels were measured with a quantitative enzyme-linked immunosorbent assay kit (R and D Bioscience, San Diego, CA, USA) according to the manufacturer’s directions. Each experiment was performed in duplicate.

2.11. Statistical analysis

Tumor growth inhibition was analyzed with the generalized estimating equation test from the calculated mean ± standard deviation of tumor weights. The Mann-Whitney U-test was used for statistical comparison of cytokine changes, with differences considered significant at p < 0.05. For cumulative survival rates, Kaplan-Meier estimates were calculated for multiple-comparison methods, with differences considered statistically significant at p < 0.05.

3. Results

3.1. In vitro cytotoxic effects of BCG, sunitinib, and their combination

The toxic effects of BCG and sunitinib individually were significant after culture for 72 hours (Table 1). A lower concentration of BCG (0.18 mg/mL) had higher cytotoxicity than a higher concentration of BCG (0.36 mg/mL) (data not shown). Among various cell lines, IC50 values of sunitinib were 0.70 in TCC8701, 0.92 in TCC8702, and 0.97–1.85 μM in the TCC9202 and TCC8301 cell lines. The IC50 value for BCG was 0.054 μg/mL in T24 TCC cells (Table 1). The combination of BCG and sunitinib also had a cytotoxic effect on TCC cells. Median effect analysis for the in vitro combined treatment revealed that sunitinib had a strong synergistic effect on BCG cytotoxicity toward T24 and MBT-2 TCC cells with CIs of 0.28 and 0.22, respectively (Figure 1). The combined effects were analyzed at low cytotoxic concentrations of BCG (0.05 mg/mL) with sunitinib (0.1, 1, and 10 μM).

3.2. Tumor incidence in the orthotopic bladder cancer model

The tumor incidence were 86%, 61%, 35%, 67%, and 80% in the control, BCG, sunitinib, synchronous cotreatment, and metachronous cotreatment groups, respectively. There were markedly decreased tumor growth incidences in mice treated with sunitinib, BCG, and synchronous cotreatment compared with those in controls and mice subjected to metachronous cotreatment (p < 0.01, Figure 2).

3.3. Tumor burden comparison

In the orthotopic bladder cancer model, bladder wet weights at week 6 were 1,800 ± 955, 970 ± 537, 680 ± 407, 820 ± 686, and 1,580 ± 1,247 mg in the control, BCG, sunitinib, and synchronous and metachronous cotreatment groups, respectively (Figure 3A). Tumor sizes were much smaller in mice treated with BCG, sunitinib, and synchronous cotreatment compared with those in controls and mice subjected to metachronous cotreatment (p < 0.01).

Table 1  IC50 values of BCG (bacille Calmette-Guérin) and sunitinib in various transitional cell carcinoma cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>BCG (mg/mL)</th>
<th>Sunitinib (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCC8301</td>
<td>0.028</td>
<td>1.85</td>
</tr>
<tr>
<td>TCC8701</td>
<td>0.032</td>
<td>0.70</td>
</tr>
<tr>
<td>TCC8702</td>
<td>0.023</td>
<td>0.92</td>
</tr>
<tr>
<td>TCC9202</td>
<td>0.025</td>
<td>0.97</td>
</tr>
<tr>
<td>T24</td>
<td>0.054</td>
<td>1.17</td>
</tr>
<tr>
<td>MBT-2</td>
<td>0.680</td>
<td>16.33</td>
</tr>
</tbody>
</table>
3.4. Survival rate

Survival rates of mice with orthotopic bladder tumors at week 6 were 30%, 63%, 78%, 55%, and 33% in the control, BCG, sunitinib, and synchronous and metachronous cotreatment groups, respectively (Figure 4A). Mice treated with sunitinib and BCG had much higher survival rates than those in the control group and mice treated with metachronous cotreatment ($p<0.001$).

In the subcutaneous tumor model, survival rates at week 6 were 33%, 88%, 75%, 67%, and 20% in the control, BCG, sunitinib, and synchronous and metachronous cotreatment groups, respectively (Figure 4B). Mice treated with BCG, sunitinib, and synchronous cotreatment had much higher survival rates than those in the control group and mice treated with metachronous cotreatment ($p<0.001$).
3.5. Changes in mouse body weight during therapy

There was no significant difference in body weights before and after the experiment in any study group (data not shown). The average body weight was decreased in all mice compared with that in control mice, which may have been due to stress or the treatment process.

3.6. Cytokine levels after intravesical instillation

Markedly increased serum IL-1 and IL-6 levels were observed in mice subjected to BCG and sunitinib cotreatment, either synchronously or metachronously (Table 2). In contrast, all measured cytokines, including IL-1, IL-2, VEGF, RANTES, and MCP-1, were markedly suppressed in mice treated with BCG or sunitinib alone. Interestingly, VEGF levels were also decreased in mice treated with BCG compared with those in the control group (316.2 ng/mL vs. 592.0 ng/mL, p < 0.001) and sunitinib-treated mice (422.5 ng/mL vs. 592.0 ng/mL, p < 0.01).

3.7. Changes in MVD in tumor tissues

Tumors in the BCG and sunitinib groups had markedly decreased MVDs compared with those in the control and cotreatment groups, in both the orthotopic bladder and subcutaneous tumor models (p < 0.01) (Table 3, Figure 5).

4. Discussion

Intravesical immuno- and chemotherapy, surgery, and systemic chemotherapy are all critical elements in managing patients with bladder cancer. Despite advances in these modalities, newer treatment paradigms are sought to improve patient outcomes. Targeted therapy with novel agents directed at specific molecular pathways is a theoretically promising avenue to achieve such progress.

Experimental and clinical data, including approval of the multi-targeted drugs sunitinib and sorafenib, indicate that exciting results, including tumor regression, can be expected from the combined targeting of different
BCG and sunitinib therapy for bladder cancer

Table 2  Serum cytokine changes in mice 6 weeks after BCG (bacille Calmette-Guérin), sunitinib, or their combined treatment in a subcutaneous tumor model*

<table>
<thead>
<tr>
<th>Study group</th>
<th>Cytokines (μg/mL)</th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-1</td>
<td>IL-6</td>
<td>VEGF</td>
<td>RANTES</td>
<td>MCP-1</td>
</tr>
<tr>
<td>Control (n = 8)</td>
<td>22.0 ± 1.1</td>
<td>570.6 ± 10.8</td>
<td>592.0 ± 14.6</td>
<td>139.3 ± 27.8</td>
<td>94.0 ± 9.9</td>
</tr>
<tr>
<td>BCG (n = 20)</td>
<td>0</td>
<td>34.7 ± 3.2</td>
<td>316.2 ± 20.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sunitinib (n = 17)</td>
<td>0</td>
<td>51.1 ± 3.7</td>
<td>422.5 ± 44.7</td>
<td>7.7 ± 4.5</td>
<td>0</td>
</tr>
<tr>
<td>Synchronous cotreatment (n = 12)</td>
<td>447.6 ± 71.7</td>
<td>617.5 ± 19.1</td>
<td>398.7 ± 31.6</td>
<td>10.5 ± 8.4</td>
<td>48.4 ± 0.4</td>
</tr>
<tr>
<td>Metachronous cotreatment (n = 5)</td>
<td>373.7 ± 84.9</td>
<td>1129.9 ± 27.2</td>
<td>391.1 ± 53.4</td>
<td>91.6 ± 37.4</td>
<td>50.4 ± 5.4</td>
</tr>
</tbody>
</table>

*Data presented as mean ± standard deviation. IL = interleukin; VEGF = vascular endothelial growth factor; RANTES = regulated on activation normal T cell expressed and secreted; MCP = monocyte chemoattractant protein.

Table 3  Mean vascular density in tumor tissues of orthotopic bladder and subcutaneous MBT-2 tumors in C3H mice

<table>
<thead>
<tr>
<th>Model</th>
<th>Mean vascular density (×400)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Orthotopic bladder</td>
<td></td>
</tr>
<tr>
<td>tumors (n)</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>(7)</td>
<td>(18)</td>
</tr>
<tr>
<td>Subcutaneous bladder</td>
<td></td>
</tr>
<tr>
<td>tumors (n)</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>(8)</td>
<td>(20)</td>
</tr>
</tbody>
</table>

*Data presented as mean ± standard deviation; (n) represents animal number; †p < 0.01. BCG = bacille Calmette-Guérin.

pathways in the tumor angiogenesis scenario, especially with certain types of cancer. Sunitinib malate and sorafenib are novel US Food and Drug Administration (FDA)-approved antiangiogenic agents which were recently demonstrated to improve progression-free survival in patients with metastatic renal cell carcinoma.14–16 However, the two small-molecule TKIs currently FDA-approved for metastatic renal cell carcinoma (sunitinib and sorafenib) are not TKR-specific. Despite the many trials reporting clinical efficacies of this new class of therapeutics, the exact molecular mechanism accounting for their clinical effects is still largely unknown. While the clinical efficacy of molecular-targeted therapy in patients with metastatic renal cell carcinoma (mRCC) was found to be impressive in some patients, approximately 60% of patients with mRCC do not respond to these TKIs. It is now clear that there are inherent limitations and disadvantages when using these therapeutics as monotherapy agents. It has been hypothesized that monotherapy with any single TKI can potentially be limited by tumor cell adaptation and compensation, with overexpression of non-targeted oncogenic growth factor or TKRs that confer resistance to tumor cells. This hypothesis is supported by the observation in those patients who show a response, the duration of clinical response is typically approximately 10–12 months,17 during which clonal expansion of resistant tumor cells may occur.

The main mechanisms of these new drugs are defined as preventing tumor growth by inhibiting angiogenesis and inducing apoptosis and necrosis by acting on different types of VEGFs, such as several agents that were demonstrated to be beneficial against bladder carcinoma. Silay et al suggested that these two new agents may also theoretically increase the progression-free survival of patients with advanced bladder carcinoma because of their antiangiogenic and tumor cell apoptotic effects.18 Bradley et al conducted an anti-VEGFR monotherapy phase II therapy trial using sunitinib in advanced bladder cancer patients after chemotherapy.19 Dreicer et al. reported that using sorafenib as a single agent has minimal activity in
patients with advanced urothelial cancer in the second-line setting.\textsuperscript{20} Gallagher et al. reported that sunitinib treatment did not reach the predetermined therapeutic threshold in metastatic urothelial cancer patients, but antitumor responses were observed.\textsuperscript{21}

To date, the precise mechanism of action of intravesical BCG remains unknown. Mycobacteria are thought to bind to the bladder wall via an interaction between the bacterial antigen 85 complex and fibronectin.\textsuperscript{22,23} A likely scenario is that exposure to BCG acts as a danger signal, which activates local dendritic cells.\textsuperscript{24} Activated dendritic cells may then migrate to local lymph nodes where peptides of BCG and TCC origin are presented to T lymphocytes. The differentiation of native CD4-positive T cells into the T-helper type 1 subset is indispensable for successful treatment of superficial bladder cancer with BCG.\textsuperscript{25} The activated T lymphocytes then migrate to the urothelium and lyse TCC cells, either directly through CD8-positive population or indirectly by activating natural killer cells. These facts emphasize the importance of secondary immune reactions in obtaining a cytotoxic effect of BCG against TCC tumor cells in addition to cytokine mediation.

To the best of our knowledge, this is the first study to examine the effects of the combined inhibition by BCG and sunitinib in bladder cancer cells. The specific hypothesis of this study is that sunitinib in conjunction with BCG decreases the recurrence incidence of superficial bladder cancer better than standard intravesical chemotherapy or immunotherapy with BCG alone. Theoretically, this combined regimen should also be beneficial for advanced bladder cancer.

In this study, the \textit{in vitro} effect of combined treatment with BCG and sunitinib was not compatible with \textit{in vivo} animal study results, including tumor growth inhibition and survival time prolongation. A synergistic effect of sunitinib on BCG cytotoxicity has not been observed in animal models of localized bladder cancer. This indicates that certain antitumor effects of BCG or sunitinib alone are specifically mediated and conducted in animal hosts, and these do not exist in an \textit{in vitro} culture environment. Hipp et al. reported that sorafenib (Nexavar), but not sunitinib, affects the function of dendritic cells and induction of primary immune responses.\textsuperscript{26} The number of regulatory T cells is reduced in peripheral blood mononuclear cells in mice treated with sunitinib. Those results indicate that sorafenib, but not sunitinib, is theoretically more suitable for combination with immunotherapeutic approaches to treat cancer patients. In contrast, the major cytotoxic effect of BCG on TCC cells is likely to be caused by a secondary immune response by activation of various killer cells.

The reason that we observed synergistic cytotoxicity of BCG and sunitinib in \textit{in vitro}-cultured TCC cells in this study was because of the combined direct cytotoxic effect of both reagents. Three pathways have been proposed for the direct killing effect of BCG on tumor cells as follows: (1) reactive oxygen species production with subsequent mitochondrial damage; (2) apoptosis activation including caspase activation, apoptosis-inducing factor, and DNA fragmentation; and (3) necrosis-associated chemokine expression, such as nuclear factor-κB, box 1, AP1 (activator protein 1), IL-6 (Interleukin-6), and c/EBP (CCAAT-enhancer-binding proteins).\textsuperscript{27,28} In our study, the decreased synergistic effect of BCG and sunitinib in orthotopic bladder and subcutaneous TCC models, with either synchronous or metachronous cotreatment, may have been due to redistribution or metabolism of sunitinib within animals or an inadequate dosage resulting in a lower efficacy, although BCG treatment can induce a more-powerful systemic immune response in the same time frame. The underlying causes need to be addressed through further research. The underlying mechanism of why metachronous sunitinib treatment can antagonize BCG’s effect on TCC tumor growth also needs further evaluation. This may be caused by cross linkages of sunitinib bystanders after intravesical instillation with BCG subantigens resulting in a decreased immune response locally and systemically in mice.

Our study findings suggest the possibility and feasibility of immuno-targeted combination therapy for bladder cancer in the future. Marked increases in IL-1 and IL-2 cytokines were observed in the serum of mice with BCG and sunitinib cotreatment. In contrast, general inhibition of measured cytokines, including IL-1, IL-2, VEGF, MCP-1, and RANTES, were observed with individual BCG and sunitinib treatment. These various manifestations may be related to the reagent dose or the limited number of cytokines that we measured. The therapeutic efficacy may originate from other major cytokines in BCG treatment or mediators in sunitinib treatment.

RANTES/CCL5 is a chemokine (CC motif) ligand that binds to several chemokine receptors and Duffy antigen receptor for chemokines.\textsuperscript{29} It is expressed by macrophages, endothelial cells, keratinocytes, and T cells, and plays a key role in inflammation, cell recruitment, and T cell activation. It is reasonable to expect that such broad expression relies on post-translational proteolytic processing as a means of modulating immunological activity and receptor binding. Both MCP-1 and RANTES have also been shown to induce the formation of trp RNA-binding attenuation protein-positive, multinuclear cells from macrophage colony-stimulating factor-treated monocytes.

Currently, a number of different strategies to inhibit VEGF signal transduction are in development, including development of humanized neutralizing anti-VEGF monoclonal antibodies, receptor antagonists, soluble receptors, antagonistic VEGF mutants, and inhibitors of VEGF receptor function. Experimental and clinical data, including the approval of the multitargeted drugs sunitinib and sorafenib, indicate that exciting results, including tumor regression, can be expected from the combined targeting of different pathways in the tumor angiogenesis scenario. One strategy is to employ sequential or combination
targeted therapy. Another approach is to use other classes of targeted therapies either in combination or in sequential therapy after the development of treatment resistance. However, most of these possibilities came from mRCC with few examples in bladder cancer, especially for topical intravesical therapy of superficial bladder cancer, which has relatively low angiogenic activity.

In conclusion, the feasibility of BCG combined with a VEGF inhibitor for treating localized bladder cancer did not show solid supporting evidence of synergistically decreasing the tumor incidence or growth rate from the preliminary results of our study.

Acknowledgments

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