Enhanced antitumor effect of combination intravesical mitomycin C and bacillus Calmette-Guerin therapy in an orthotopic bladder cancer model

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Abstract. Intravesical immunotherapy with bacillus Calmette-Guerin (BCG) is currently the most successful adjuvant agent for the treatment and/or prophylaxis of non-muscle-invasive bladder cancer (NMIBC). However, NMIBCs recur in 60-70% of cases and 30% of these recurrent tumors present with a higher grade and more invasive properties. Patients that do not respond to intravesical BCG therapy are considered to be a challenge for urologists. Thus, novel conservative possibilities should be explored. To test the efficacy of a novel therapeutic approach, we examined the antitumor effect of combination therapy by intravesical administration of mitomycin C (MMC) plus BCG, infusing the two drugs simultaneously, in an orthotopic bladder cancer model. Intravesical BCG and MMC administration showed a dose-dependent survival (n=8 per group). The combination of MMC and BCG provided a significant survival advantage compared to the BCG-alone (p=0.035) and MMC-alone groups (p=0.040) (n=8 per group). The group with combined MMC/BCG exhibited a survival period similar to that achieved with an amount eight times higher that of BCG (n=10 per group). Ki-67 labeling index of cancer cells, showing tumor proliferation, was significantly lower in the combined group compared to the BCG-alone (p<0.05), MMC-alone (p<0.01) and control groups (p<0.01). No difference was detected between the combined group and the BCG-alone group with regard to CD3, T-cell infiltration and CD68 macrophage activity. The combined MMC/BCG treatment decreased the tumor appearance rate, improved the survival period and reduced the cellular proliferation rate in tumors compared to the BCG-alone treatment. The results suggest that the combined intravesical MMC/BCG treatment induced an enhanced antitumor effect against bladder tumors. The combined MMC/BCG treatment also showed a survival period similar to that achieved using a dose eight times higher of BCG-alone.

Introduction

Bladder cancer accounts for approximately 4% of cancer cases worldwide. Bladder cancer is the fourth most commonly diagnosed cancer in males (approximately 7%), and the sixth overall (approximately 5%) in the US (1).

The majority of patients with bladder tumors (70-80%) present with low-grade, non-muscle-invasive or superficial tumors confined to the mucosa. The tumors are typically managed conservatively by transurethral resection, followed by intravesical adjuvant treatment (2).

The current published clinical guidelines recommend that patients with an intermediate or high risk of recurrence and an intermediate risk of progression should be treated with bacillus Calmette-Guerin (BCG) or mitomycin C (MMC) in an adjuvant setting following transurethral resection of the bladder tumor (TURBT) (3). Intravesical immunotherapy with BCG is currently the most successful adjuvant agent for treating non-muscle-invasive bladder cancer (NMIBC) (4). However, 60-70% of NMIBCs recur despite intravesical BCG therapy and 30% of these recurrent tumors present with a higher grade and more invasive properties, since BCG does not have long-lasting tumor-specific immunity (5,6). In addition, BCG has a higher incidence of side effects, often increasing with the number of instillations, leading to the discontinuation of intravesical instillation treatment (4,7).

The data show that improvement in the current standard of intravesical BCG treatment regimens is required. Thus, novel strategies are crucial for the treatment of NMIBC with a superior antitumor effect, compared to that of conventional intravesical BCG therapy.

Numerous clinical trials have shown that combined treatments are more effective than the single use of any particular modality. In a study on a bladder cancer cell line,

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a combination of BCG, such as the Connaught strain, and MMC inhibited cell growth more significantly than either agent separately *in vitro* (8). In a previous study, bladder urothelial disruption induced by an intravesical cytostatic agent enhanced the fibronectin-mediated BCG attachment in murine experiments (9).

We hypothesized that combined intravesical BCG with MMC instillation has a positive effect on the adherence of BCG particles to the bladder wall and consequently stimulates the induction of the BCG-related immune response. Moreover, the combined MMC and reduced dose of BCG maintains an antitumor benefit compared to the standard dose of BCG, possibly with less toxic and fewer side effects than intravesical BCG instillation.

To improve the efficacy of intravesical BCG therapy, a combination of MMC and BCG against bladder tumors was examined using an orthotopic animal model. Additionally, whether the combined treatment of MMC/BCG induces a beneficial antitumor effect was examined.

Materials and methods

Tumor cell line and reagents. The murine bladder tumor cell line, MBT-2, was cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc., Rockville, MD, USA) at 37°C in a humidified, 5% CO_2 atmosphere.

MMC (Kyowa Hakko Kirin Ltd., Tokyo, Japan) was used as the chemotherapeutic agent. The reagents were diluted using phosphate-buffered saline (PBS; Gibco, Invitrogen) to the working concentrations.

BCG (Connaught substrain, Immucyst, $3.77x10^8$ colony-forming units, dry weight 81 mg; Nihon Kayaku, Inc., Tokyo, Japan) was used.

Animals. Female C3H/HeN mice (8 weeks old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The animal procedures were reviewed and approved by the Animal Research Committee of Saitama Medical School.

Assessment of mitomycin C cytotoxicity to MBT-2 cells in vitro. To assess the cytotoxic effects of MMC on the growth of MBT-2 cells, exponentially-growing MBT-2 cells were seeded at a density of $2x10^5$ per 6-well plate and the cells were cultured in the absence or presence of MMC (1-100 μg/ml) for the designated time period (24-72 h; n=3 each). At the indicated time points, the cells were harvested and counted using a hemocytometer.

Intravesical implantation of murine bladder cancer cells. Subconfluent MBT-2 cells were trypsinized and >95% cell viability was confirmed using the trypan blue exclusion method. Syngeneic female C3H/HeN mice were anesthetized using an intraperitoneal injection of 50 mg/kg of sodium pentobarbital. Prior to each instillation, the bladder was emptied by gently pressing the bladder between the fingertips. A 24-gauge Teflon-coated catheter was introduced into the lumen of the bladder through the urethra. An amount of 1 million MBT-2 cells in a 100 μl suspension of PBS were then injected into the bladder, as previously reported (10). The contact between the MBT-2 cells and the bladder membrane,

for the implantation of the syngeneic orthotopic model, lasted for ~2 h, until the anesthesia wore off.

Toxicology of intravesical mitomycin C instillation in mice. The concentration of intravesical MMC instillation in clinical practice is ~1 mg/ml. The mice were arbitrarily placed into four groups. The animals (n=6) received 0 μg (PBS: control), 50 μg (0.5 mg/ml), 100 μg (1 mg/ml) or 200 μg (2 mg/ml) of MMC intravesically five times at 3-day intervals. On day 28, following the first instillation, body weights were compared to the initial body weights. Then, new mice (n=6) that received combined MMC and BCG intravesically were examined in a similar manner.

Intravesical therapy in an orthotopic bladder cancer model. To assess the antitumoral effect of intravesical administration treatment, the mice were arbitrarily placed into groups on day 5 following tumor implantation. The instillations of each experiment were repeated 5 times at 3-day intervals commencing on day 5 after tumor implantation under anesthesia. Each instillation lasted for ~2 h until recovery from anesthesia and the start of spontaneous voiding. On day 60, the surviving mice were sacrificed and necropsied. The animal experiments and the examination of the antitumor effects among the various treatment groups were performed in the manner described below.

Mitomycin C dose optimization (3 groups). Intravesical MMC was administered transurethrally at various doses: 0 (control), 25 and 50 μg in a final volume of 100 μl of PBS (n=8 mice per group), resulting in a concentration between 0 and 0.5 mg/ml. The intravesical instillation treatment was performed as described above.

Bacillus Calmette-Guerin dose optimization (5 groups). The concentration of intravesical Connaught substrain BCG instillation in clinical practice is ~2 mg/ml. Our intravesical BCG was administered transurethrally at various doses: 0 (control), 100, 200, 400 and 800 μg in a final volume of 100 μl of PBS (n=8 mice per group), resulting in a concentration between 0 and 8 mg/ml. The intravesical instillation treatment was performed as described above.

Intravesical mitomycin C and/or bacillus Calmette-Guerin therapy (4 groups). For the intravesical administration, 100 μl of PBS, 50 μg of MMC in 100 μl of PBS, 100 μg of BCG in 100 μl of PBS, or a combination of 50 μg of MMC plus 100 μg of BCG in 100 μl of PBS were instilled transurethrally (n=8 mice per group). The intravesical instillation treatment was performed as described above.

Combined intravesical mitomycin C/bacillus Calmette-Guerin vs. various bacillus Calmette-Guerin doses (5 groups). For the intravesical administration, 100 μl of PBS, a combination of 50 μg of MMC plus 100 μg of BCG in 100 μl of PBS, 100 μg of BCG in 100 μl of PBS, 400 μg of BCG in 100 μl of PBS or 800 μg of BCG in 100 μl of PBS were instilled transurethrally (n=10 mice per group). The intravesical instillation treatment was performed as previously described.

Bladder tumor following single intravesical administration (4 groups). The mice were arbitrarily placed into four groups 17 days after the intravesical implantation of $1x10^6$ MBT-2 cells. A single intravesical administration of 100 μl of PBS, 50 μg of MMC in 100 μl of PBS, 100 μg of BCG in 100 μl of PBS or a combination of 50 μg of MMC plus

Table I. Toxicology of intravesical mitomycin C instillation in mice (n=6).

Dose (μg)	Death after instillation	Body weight at initiation (g)	Body weight at end point (g)	Body weight difference (g)	Survival (days)
Mitomycin C					
50	0	19.4±0.4	21.2±1.4	1.8±1.1	28
100	5 (83%)	19.0±0.8	14.3±1.6	-4.7±1.6	14.0±9.0
200	6 (100%)	20.9±0.4	15.6±0.7	-5.3±0.5	7.3±5.0
50 + BCG 100	0	20.7±0.9	21.4±2.3	0.7±1.4	28
Control (PBS)	0	19.8±1.3	22.7±1.8	2.9±1.1	28

Data presented as mean ± standard deviation.

Table II. Dose optimization of mitomycin C in an orthotopic bladder cancer model (n=8).

Dose (μg)	Death due to bladder tumor	Failure to produce tumor	Tumor-taking rate (%) (no. of tumor appearance/ no. of observation subjects)	Survival (days)
25	8	0	100% (8/8)	34.8±7.0
50	6	2	75% (6/8)	40.0±13.2
Control (PBS)	8	0	100% (8/8)	26.4±3.2

Data presented as mean ± standard deviation.

100 μg of BCG in 100 μl of PBS were instilled transurethrally (n=5 mice per group). The mice were sacrificed 1 day after the single intravesical administration and the whole bladder tissue was obtained. Following removal, the bladders were filled with 10% formalin and subsequently the samples were embedded in paraffin. The sections were routinely stained with hematoxylin and eosin.

Immunostaining (Ki-67, CD68 and CD3). Immuno-histochemical staining was performed in a standard manner according to the manufacturer's instructions (11). For Ki-67, a primary mouse monoclonal antibody at a dilution of 1:50 (M7249; Dako, Tokyo, Japan) was used overnight at 4°C. For CD68 (M0876; Dako) and CD3 (A0452; Dako), primary mouse polyclonal antibodies at a dilution of 1:50 (Dako) were also used overnight at 4°C.

To quantify the Ki-67 expression, the mean of at least four representative fields was evaluated for each treatment group. The number of cells was counted under a microscope and the results were recorded as the percentage of positively-stained cells.

To quantify the CD68 and CD3 expression, the number of positive cells were counted manually and the number of control bladder-positive cells were subtracted from the number of infiltrating cells in the tumor. The numbers were then normalized to those for 1 mm², expressed as a percentage to the total cell number per mm².

Statistical methods. The values are presented as the mean ± standard deviation (SD). Variations among the groups were assessed using a one-way ANOVA. Statistical signifi-cance was determined as $p < 0.05$. The survival curves were generated using the Kaplan-Meier method and were compared using the log-rank test.

Results

Cytotoxic effect of mitomycin C against MBT-2 cells. MMC exerted moderate cytotoxic effects against MBT-2 cells *in vitro*. After 1 day of exposure to MMC at concentrations of 0 (control), 1, 10 or 100 μg/ml, the number of MBT-2 cells ($\times 10^5$) were 2.60±0.10, 1.63±0.40, 1.47±0.25 and 0.23±0.06, respectively. After 2 days of exposure, the number of cells were 4.83±0.61, 0.73±0.15, 0.53±0.25 and 0.10±0.0, respectively. After 3 days of exposure, the number of cells were 15.50±2.34, 0.63±0.29, 0.27±0.06 and 0.0±0.0, respectively. Compared to the untreated cells, MMC caused both a dose- and a time-dependent inhibition of growth during the 3-day culture period.

Intravesical instillation in an orthotopic bladder cancer model
    Toxicology of intravesical mitomycin C instillation in mice. Of the 6 mice in each group treated with a dose of 0 (control), 50, 100 or 200 μg of MMC, 6, 6, 1 and 0 mice survived, respec-tively, (Table I). Of the 6 mice that received combined MMC (50 μg) and BCG (100 μg), all 6 survived (Table I).

The pathological findings for the murine bladders of the mice that succumbed due to adverse events associated with intravesical MMC instillation showed acute inflammation, submucosal edema and detachment of the urothelium membrane on the bladder. The bladders in the 200 μg group exhibited these findings more significantly compared to the

Table III. Dose optimization of bacillus Calmette-Guerin in an orthotopic bladder cancer model (n=8).

Dose (μg)	Death due to bladder tumor	Failure to produce tumor	Tumor-taking rate (%) (no. of tumor appearance/ no. of observation subjects)	Survival (days)
100	6	2	75.0% (6/8)	37.9±17.4
200	6	2	75.0% (6/8)	41.8±14.3
400	5	3	62.5% (5/8)	42.3±16.7
800	4	4	50.0% (4/8)	44.3±16.9
Control (PBS)	7	1	87.5% (7/8)	31.3±13.4

Data presented as mean ± standard deviation.

Table IV. Bacillus Calmette-Guerin and/or mitomycin C therapy in an orthotopic bladder cancer model (n=8).

Treatment (μg)	Death due to bladder tumor	Failure to produce tumor	Tumor-taking rate (%) (no. of tumor appearance/ no. of observation subjects)	Survival (days)
BCG (100)	6	2	75.0% (6/8)	33.4±16.8
MMC (50)	6	2	75.0% (6/8)	39.5±13.5
BCG (100) + MMC (50)	5	3	62.5% (5/8)	51.5±8.1
Control (PBS)	7	1	87.5% (7/8)	29.6±13.0

Data presented as mean ± standard deviation. BCG, bacillus Calmette-Guerin; MMC, mitomycin C; PBS, phosphate-buffered saline.

100 μg group. In addition, the survival period in the 200 μg group (7.3±5.0 days) was significantly shorter than that of the 100 μg group (14.0±9.0 days).

Based on the preceeding toxicology experiment, 25 or 50 μg of MMC were used for subsequent MMC dose optimization studies on the orthotopic bladder cancer model.

Dose optimization of mitomycin C (5 groups). Of the 8 mice in each group treated with a dose of 0 (control), 25 or 50 μg of MMC, 0, 0 and 2 mice, respectively, failed to develop tumors and survived (Table II). The mean survival period in the 50 μg treatment group was 40.0±13.2 days, while the mean survival periods in the 25 and 0 μg (control) groups were 34.8±7.0 and 26.4±3.2 days, respectively. The survival advantage for intravesical MMC in the 50 μg administration group was statistically significant, compared to the control group (p=0.002).

Dose optimization of bacillus Calmette-Guerin (5 groups). Of the 8 mice in each group treated with a dose of 0 (control), 100, 200, 400 or 800 μg of BCG, 1, 2, 2, 3 and 4 mice, respectively, failed to develop tumors and survived (Table III). Compared to the PBS treatment (control), intravesical BCG administration resulted in a longer survival period, presumably demonstrating a beneficial antitumor effect in a dose-dependent manner.

Intravesical mitomycin C and/or bacillus Calmette-Guerin therapy (4 groups). Of the 8 mice in each group treated with PBS (control), MMC-alone, BCG-alone or combined MMC/ BCG, 1, 2, 2 and 3 mice, respectively, failed to develop tumors and survived (Table IV). The tumor-taking rates were 87.5, 75, 75 and 62.5% in the control group, the BCG-alone, the

MMC-alone and the combined MMC/BCG group, respectively. A significant survival advantage was observed for the combined MMC/BCG group (51.5±8.1 days), compared to the BCG-alone group (33.4±16.8 days, p=0.035, Fig. 1) and the MMC-alone group (39.5±13.5 days, p=0.040, Fig. 1).

Intravesical combined mitomycin C/bacillus Calmette-Guerin vs. various BCG doses (total of 5 groups). Of the 10 mice in each group treated with PBS (control), 100, 400 or 800 μg of BCG or combined MMC (50 μg) plus BCG (100 μg), 1, 3, 4, 6 and 4 mice, respectively, failed to develop tumors and survived (Table V). A significant survival advantage for the combined MMC/BCG group (47.7±11.9 days) was observed, compared to the 100 μg BCG group (34.5±17.9, p=0.04). The survival period of the combined MMC/BCG group was similar to that of the group treated with a dose eight times higher than that of BCG alone (46.7±12.3 days).

Immunostaining of cancers, macrophages and T-lymphocytes. Three antibodies reactive to Ki-67, CD68 and CD3 antigens were used to evaluate the response to the combined intravesical MMC/BCG treatment against inoculated bladder cancer cells. The Ki-67 antibody labeled the nuclei of proliferating cells. Antibodies to CD68 and CD3 labeled the cell membrane and cytoplasm of infiltrated macrophages and T-lymphocytes. Fig. 2 shows the statistical analysis regarding the difference among the treatment groups.

Combined treatment of mice by MMC/BCG resulted in a significant decrease of the Ki-67 labeling index of cancer cells, compared to the control (PBS: 55.7±1.2% vs. MMC/ BCG: 32.7±3.0%, p<0.01), BCG-alone (43.8±3.1%, p<0.05)

Table V. Combined bacillus Calmette-Guerin/mitomycin C vs. various bacillus Calmette-Guerin doses (n=10).

Treatment (μg)	Death due to bladder tumor	Failure to produce tumor	Tumor-taking rate (%) (no. of tumor appearance/ no. of observation subjects)	Survival (days)
BCG (100)	7	3	70% (7/10)	34.5±17.9
BCG (400)	6	4	60% (6/10)	42.4±16.8
BCG (800)	4	6	40% (4/10)	46.7±12.3
BCG (100) + MMC (50)	6	4	60% (6/10)	47.7±11.9
Control (PBS)	9	1	90% (9/10)	27.3±11.9

Data presented as mean ± standard deviation. BCG, bacillus Calmette-Guerin; PBS, phosphate-buffered saline.
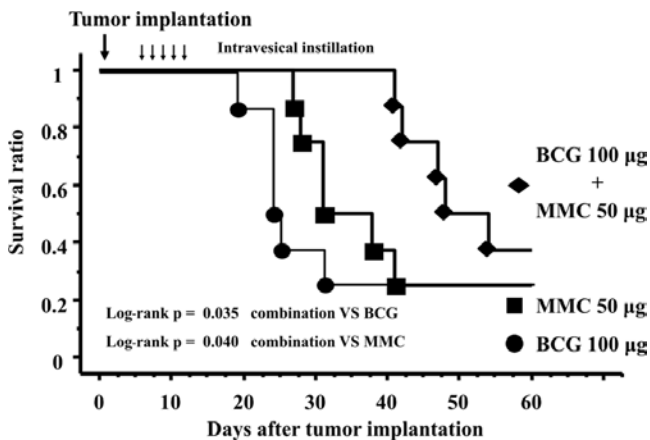

Figure 1. Kaplan-Meier analysis of the survival rates of mice according to five times intravesical instillation treatment, following MBT-2 cell implantation in an orthotopic bladder cancer model. A significant survival advantage in the combined BCG/MMC (◆) group was observed, compared to that in the BCG-alone (●) group (p=0.035) or the MMC-alone (■) group (p=0.040).

Figure 2. The mice were randomly allocated to four groups 17 days after the intravesical implantation of 1x10^6 MBT-2 cells. A single intravesical administration of 100 μl of PBS, 50 μg of MMC in 100 μl of PBS, 100 μg of BCG in 100 μl of PBS or a combination of 50 μg of MMC and 100 μg of BCG in 100 μl of PBS were instilled into the bladder. The bladders were removed and fixed with 10% formalin 1 day after the single intravesical instillation, and the bladder tumor specimens were then immunostained with the antibodies, Ki-67, CD68 and CD3. Statistical analysis among the various treatment groups (mean ± SD, n=4, ANOVA, Bonferroni's multiple correction) were examined. Cells labeled with Ki-67, CD68 and CD3 antibodies were expressed as the percentage to the total cell numbers per mm^2.

and MMC-alone groups (51.2±2.8%, p<0.01; Fig. 2, Ki-67). BCG-alone significantly reduced, while MMC-alone increased CD68$^+$ cells as compared to the control (p<0.0001 and p<0.01, respectively). This trend was the same when two drugs were infused into the test subjects when compared to MMC-alone treatment (p<0.0001; Fig. 2, CD68). Notably, more CD3$^+$ cells infiltrated into the tumors when BCG was injected, irrespective of the presence (p<0.05) or absence of (p<0.05) MMC (Fig. 2, CD3).

Discussion

BCG is currently regarded as the most successful immunotherapy for solid tumors. The intravesical instillation of BCG is the most effective means of prophylaxis for the recurrence and progression of non-muscle-invasive bladder cancer (4). Although the mechanism of the antitumor effects of BCG remains to be elucidated, it is generally accepted that intravesical BCG instillation causes the non-specific stimulation of the local immune system, inducing the local infiltration of the bladder wall with activated T cells (6). Repeat intravesical BCG insti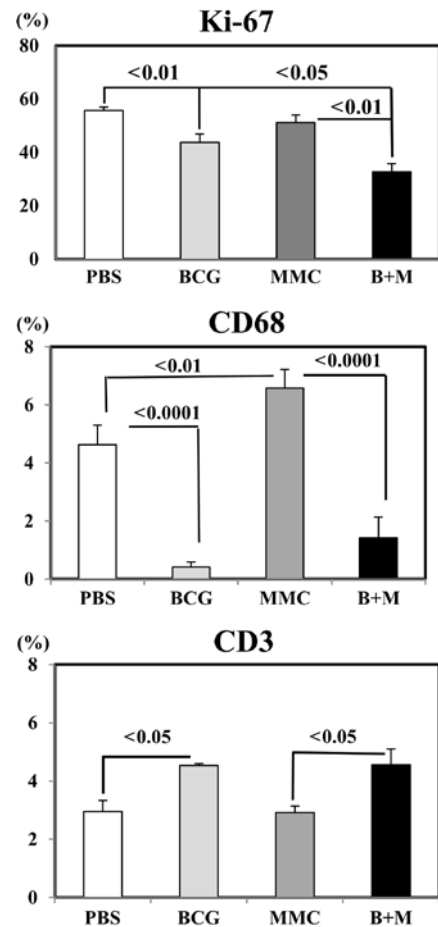llations induce a cascade of immunological reactions, including local cytokine and chemokine production which are probably released by T-lymphocytes and other cells infiltrating the bladder wall following intravesical BCG treatment (12).

In patients that do not respond to intravesical BCG therapy, cystectomy remains the treatment of choice, when patients are compliant. Patients in whom BCG fails are a challenge to urologists and careful individualization of therapy in experienced hands appears warranted. Therefore, new conservative possibilities should be explored.

The effector mechanisms of intravesical MMC (a cystostatic drug) and BCG (immunotherapy) differ. Local administration of low doses of various anticancer drugs (cystostatic drugs) at the site of antigenic stimulation may potentiate the generation of T-effector cells, as detected by the delayed-type hypersensitivity measurements in murine models (13). Evidence from murine experiments indicated that certain chemotherapy anticancer agents (cystostatic drugs), such as doxorubicin or gemcitabine, promote the activation of immune effectors (14,15).

The rationale for combining anticancer drugs is based on the need to increase efficacy and reduce the emergence of resistant malignant cells. MMC plays a dual role in that it promotes antitumor action and has a tissue-scarifying effect that enables BCG to attach more efficiently to the urothelium (9,16). Therefore, we hypothesize that an enhanced antitumor effect would be demonstrated by the combination of MMC and BCG. Although sequential intravesical administration of MMC and BCG has been used in patients with bladder cancer, our proposed combined intravesical administration of MMC/BCG regimen, infusing the two drugs simultaneously, has yet to be examined (17-20).

We hypothesized that the combination of MMC/BCG is beneficial as an alternate intravesical treatment following TURBT. Thus, we examined whether this combination strategy would augment intravesical BCG therapy in an orthotopic animal model. The orthotopic bladder cancer model closely mimics the clinical situation and this model has led to a substantially greater understanding of the mechanisms involved, a pre-requisite for a meaningful conclusion (21). The immunologic aspects of intravesical BCG-related experiments should be evaluated with a syngeneic orthotopic bladder cancer model, such as our MBT-2 cells/C3H mouse orthotopic model. However, there are significant shortcomings since the outcomes of a murine orthotopic bladder cancer model may present differently compared to human clinical practice.

The combined intravesical instillation of BCG and anti-fibrinolytic agents was reported to be a safe and effective method of improving BCG immunotherapy as noted in a pilot study of 257 patients with TURBT (22). From a clinical point of view, the intravesical instillation of MMC/BCG is an attractive strategy since it is simple to perform, cost-effective and easy to prepare without requiring additional time for catheterization.

In the present study, a significant survival advantage was observed in the combined instillation of the MMC/BCG group, compared to that in the BCG-alone and MMC-alone groups.

Clinical research in the field currently focuses on the means of reducing BCG-induced side effects. A number of authors have proposed lowering the dose of BCG by one third to reduce BCG-related adverse events while maintaining an antitumor effect against bladder tumors (23).

The number and severity of the side effects caused by 6 weeks of BCG were not significantly different between the sequential combination group, to which 4 weeks of MMC was administered prior to BCG administration, and the BCG monotherapy group, indicating that BCG-related toxicity does not increase when BCG instillations are started immediately after a period of intravesical MMC instillation (17).

In the present study, a repeated intravesical BCG instillation protocol exhibited a dose-dependent antitumor effect in an orthotopic bladder cancer model. A significant survival advantage was observed in the combined MMC/BCG group, compared to that in the BCG-alone and MMC-alone groups. A similar survival period was also noted between the combined MMC/BCG group and the BCG-alone group with a dose eight times higher than that of BCG.

Thus, it can be speculated that a combined, locally-applied MMC/BCG regimen enables the BCG dose to be reduced, resulting in fewer adverse events as a result of BCG instillation, while maintaining an antitumor effect against bladder cancer. However, we must consider that a substantial safety concern exists for this combined instillation regimen, since the combination of BCG-induced bladder inflammation and MMC-induced bladder irritation may cause a breach in the normal bladder barrier function, enabling systemic exposure to high levels of MMC or systemic infection with BCG.

The antitumor mechanism of intravesical BCG immunotherapy is complex in that the cell death pathway induced by intravesical BCG administration in bladder tumors is associated with apoptosis, necrosis and a decrease in cell proliferation (24). On the other hand, the antitumor effect of BCG in the human bladder tumor cell line T24 was associated with a decrease in proliferation, and apoptosis was not considered to be a significant mechanism of antitumor effects (25). The Ki-67 immunostaining method is widely used as a cell proliferation marker in various types of tumors (26). Asakura *et al* reported that the Ki-67 labeling index was an independent predictor of the recurrence of non-muscle-invasive bladder tumor and of progression (27). Santos *et al* reported that a high Ki-67 labeling index and multifocality were significantly related to recurrence and progression-free survival, and were independent prognostic factors for non-muscle-invasive bladder tumor (28). In this study, the Ki-67 labeling index of the bladder tumors following intravesical instillation in the combined MMC/BCG group was found to be significantly lower than that in either the BCG-alone or MMC-alone groups. We consider Ki-67 immunostaining using TURBT specimens to aid analysis and to be a prognostic marker for patients following TURBT. However, the use of Ki-67 immunostaining as a marker for evaluating the response to intravesical therapy may be limited. Notably, no parameter for predicting the response to intravesical BCG treatment has been introduced clinically (4).

In an animal model using guinea pigs, the sequential combination of intravesical MMC and BCG was studied for immunological effects (16). A slight increase in the MHC class II expression on the bladder urothelium was detected when MMC and BCG treatment was combined. However, these authors concluded that MMC did not enhance the immune response of BCG, but that the increased antitumor activity was due more to the separate activity of the two drugs, i.e., a cytostatic effect of MMC on tumor cells and a local immune response in the bladder evoked by BCG.

Numerous aspects in our study, including a combined local administration, the use of an orthotopic murine bladder cancer model and varying concentrations of the two drugs, account for discrepancies between our results and previously reported animal studies examining the combination of MMC and BCG (16).

As anticipated, the cytotoxic effect of BCG on the murine bladder tumors was enhanced when injected together with MMC in an orthotopic bladder cancer model. We invetigated an underlying mechanism of this enhanced effect by evaluating macrophages (CD68 immunostaining) and T cells (CD3 immunostaining), since these agents play a significant role in cellular immunity. The number of macrophages decreased, while the T cells increased within tumors when BCG was given to animals. This is an unexpected result, as we assumed that BCG generates granuloma which involves macrophages. MMC, on the other hand, increased macrophages within tumors, in order to eliminate apoptotic cells.

Only macrophages within tumors, and not in the surrounding tissue were examined, due to the experimental methodological limitations of this study. Immunohistochemical analysis using samples following a single intravesical administration were regarded as unsuitable for the investigation of the mechanism among distinct treatment groups. It is presumed that an optimal immnostaining method for examining the mechanisms following multiple intravesical instillation using bladder specimens in orthotopic murine bladder cancer model has yet to be determined. Thus, it appears that our findings utilizing bladder tumor specimens may provide less valuable information regarding the response of multiple intravesical instillations protocol.

Although no difference was found between the combined group and the BCG-alone group with regard to CD3, T-cell infiltration and CD68 macrophage activity, our results suggest that T cells are responsible for attacking cancer cells, while macrophages remove dead cells. Therefore, it can be assumed that other cell populations, such as B-cells, natural killer cells or the cytokine or chemokine levels, may differ among different treatment groups, possibly contributing to the enhanced antitumor effect of the combined administration regimen.

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