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Title

Mechanism responsible for the antitumor effect of BCG-CWS using the LEEL method in a mouse bladder cancer model

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Abstract

We previously reported on the development of a water soluble formulation of the cell wall skeleton of BCG (BCG-CWS), a major immune active center of BCG, by encapsulating it into a nanoparticle (CWS-NP). The CWS-NP allowed us to clarify the machinery associated with the BCG mediated anti-bladder tumor effect, especially the roles of bladder cancer cells and dendritic cells (DCs) in the initial step, which remains poorly understood. We show herein that the internalization of BCG-CWS by bladder cancer cells, but not DCs, is indispensable for the induction of an antitumor effect against bladder cancer. Tumor growth was significantly inhibited in mice that had been inoculated with mouse bladder cancer (MBT-2) cells containing internalized BCG-CWS. On the other hand, the internalization of BCG-CWS by DCs had only a minor effect on inducing an antitumor effect against MBT-2 tumors. This was clarified for the first time by using the CWS-NP. This finding provides insights into our understanding of the role of bladder cancer cells and DCs in BCG therapy against bladder cancer.

Keywords: nanoparticle; delivery system; bladder cancer; BCG-CWS; cancer immunotherapy

1. Introduction

Intravesical *Bacillus Calmette-Guérin* (BCG) therapy has been clearly shown to be effective against superficial bladder cancer and carcinoma in situ [1,2], and is one of the strongest and most successful types of immunotherapy. Although this form of treatment is quite effective, serious side-effects associated with the use of live mycobacteria can occur [3]. Thus, a non-infectious immunotherapeutic drug instead of live BCG would be highly desirable. The cell wall skeleton of BCG (BCG-CWS), the main immune active center of BCG [4], is a potent candidate. BCG-CWS is a huge assembly of peptidoglycans, arabinogalactans and mycolic acids [5]. It is known to be an adjuvant that is recognized by the toll-like receptors (TLR) 2 and 4 that are located on the cell surface, and induces anti-tumor immunity in an animal model and in humans [4,6-8]. However, it is quite difficult to formulate a suitable water-soluble drug without the BCG-CWS undergoing aggregation, because BCG-CWS is insoluble in both aqueous and non-aqueous solvent.

We recently succeeded in developing, for the first time, a water soluble formulation of BCG-CWS by encapsulating BCG-CWS into nanoparticles using the liposome evaporated via emulsified lipid (LEEL) method [9]. The nanoparticle encapsulating BCG-CWS (CWS-NP) was highly uniform and could be easily dispersed without any detectable aggregation. CWS-NP efficiently delivered BCG-CWS into cells and showed significant anti-bladder tumor effects in mouse bearing bladder tumors and in a rat with naturally developed bladder cancer. Our success is significant in two way. One is that CWS-NP is a potent candidate for new immunotherapeutic drug against bladder cancer. The second is that CWS-NP could be used better understand how BCG is involved in the anti-bladder tumor effect. Despite nearly four decades of clinical use, the mechanism of BCG therapy is still under investigation [10]. Because BCG is composed of many components, BCG infections can induce complicated responses. As a result, BCG components should be used for clarifying the machinery associated with BCG therapy. Although an immune response against BCG components in vitro using peripheral blood mononuclear cells has been demonstrated [11], a definitive understanding of the mechanism of BCG therapy is not available. The reason for this is that it is currently not possible to deliver the main immune active component of BCG to cells in an efficient manner. That is, there is no technology for delivering BCG components, other than the use of live BCG. Our earlier report, however, indicates that it is quite possible to efficiently deliver BCG components, similar to live BCG. Our approach to this problem promises to clarify the machinery associated with the BCG mediated anti-bladder tumor effect at the molecular level by using CWS-NP.

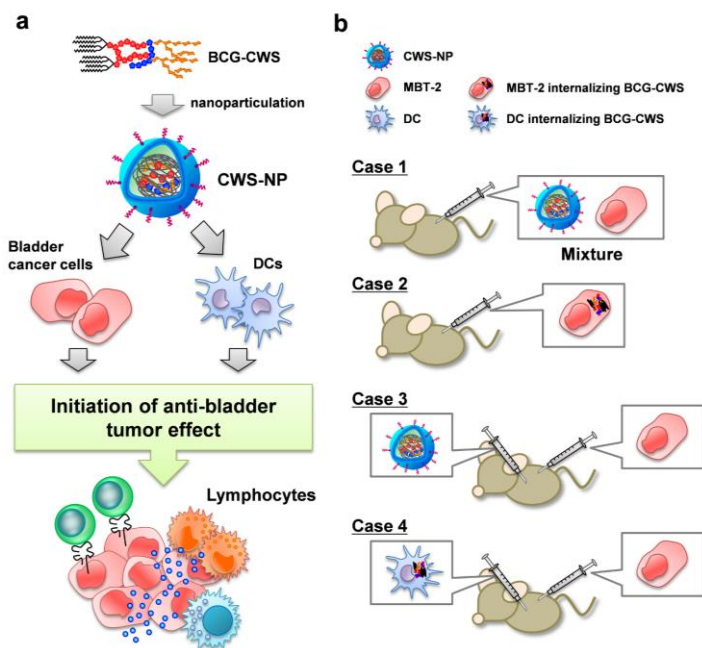
Immune cells, especially antigen presenting cells (APCs), urothelial cells and bladder cancer cells are all present in the bladders of bladder cancer patients. Although it is unclear which cells initiate the development of anti-bladder immunity after interaction with the instilled BCG, it is likely that bladder cancer cells are the initiators, based on the following body of evidence. The induction of an effective anti-bladder tumor effect appears to require live BCG, but not heat-killed BCG [12,13]. This

fact indicates that cells need to be invaded in order to achieve an effective anti-bladder tumor effect. Moreover, bladder cancer cells are exposed to the lumen of the bladder, because such cells are usually less differentiated, less polarized, exhibit a diminished expression of extracellular proteins and contain a minimal mucin layer [14]. That is, BCG can easily gain access to bladder cancer cells compared to urothelial cells. Interestingly, BCG-treated bladder cancer cells upregulate the expression of major histocompatibility complex (MHC) class II and the intracellular adhesion molecule 1 (ICAM-1), and produce cytokines, along with APCs [10]. Therefore, we hypothesized that the internalization of BCG-CWS by bladder cells is important for the initiation of anti-bladder tumor immunity.

In this study, we investigated the role of bladder cancer cells and DCs in terms of the internalization of BCG-CWS in the initiation of the anti-bladder tumor effect by using CWS-NP (Fig. 1a). We designed an experimental scheme to investigate the role of internalization of BCG-CWS by bladder cancer cells and DCs in anti-bladder tumor effects *in vivo* (Fig. 1b). Consequently, we were able to determine which specific cell-type is indispensable for the initiation of the anti-bladder tumor effect by using CWS-NP.

Figure 1: Concept and Experimental design

(a) Concept of this study. The role of bladder cancer cells and dendritic cells (DCs) in the initiation of anti-bladder tumor effect was investigated using nanoparticles encapsulating BCG-CWS (CWS-NP). (b) Case 1: Mice were subcutaneously administrated with a mixture of CWS-NP and mouse bladder cancer cells (MBT-2). Case 2: MBT-2 cells were exposed to CWS-NP and the uninternalized CWS-NP was then washed out. The MBT-2 cells internalizing CWS-NP were subcutaneously inoculated to mice. Case 3: CWS-NP and MBT-2 cells were subcutaneously administrated at a different location on the back of mice. Case 4: DCs were exposed to CWS-NP and the uninternalized CWS-NP was then washed out. MBT-2 cells and the DCs internalized CWS-NP were subcutaneously inoculated each at a different location on the back of mice.



2. Materials and Methods

2.1. Materials

Egg phosphatidylcholine (EPC) was purchased from NOF Corporation (Tokyo, Japan). Cholesterol (Chol), N- (7-nitro-2-1,3-benzoxadiazol-4-yl)- 1,2- dioleoyl- *sn*- glycerol- 3-phosphoethanolamine (NBD-DOPE) was purchased from AVANTI Polar Lipids Inc. Stearylated octaarginine (STR-R8) was synthesized by KURABO. BCG-CWS (SMP-105) was generously provided from MBR Co., Ltd. SMP-150' starting raw material is a killed BCG (Tokyo 172 strain) which strain can only be supplied by Japan BCG Laboratory.

2.2. Preparation of CWS-NP

CWS-NP was prepared by the LEEL method, as described previously [9]. Briefly, octaarginine-modified liposomes (R8-Lip), composed of EPC/Chol/STR-R8 (70:30:2 molar ratio), were prepared by the hydration method. R8-Lip contained 1 mol% of NBD-DOPE, when NBD-labeled CWS-NP was prepared for investigating a cellular uptake by MBT-2 cells and DCs. BCG-CWS was dissolved in pentane, and the resulting solution and R8-Lip were mixed. The mixture was sonicated with a probe type sonicator (BRANSON) to produce an O/W lipid emulsion. The pentane was removed with a rotary evaporator under reduced pressure to prepare CWS-NP. The CWS-NP was finally extruded through polycarbonate membrane filters with pore sizes varying from 0.4 to 0.2 μm using Mini-Extruder. Diameter, polydispersity index and zeta-potential was measured by a ZETASIZER Nano (ZEN3600,

Malvern Instruments). The diameter, polydispersity index and zeta-potential of the CWS-NP were 173 ± 8 nm, 0.241 ± 0.02 and 41 ± 1 mV.

2.3. Animal

C3H/HeN mice (female, 8-10 weeks) were obtained from Japan SLC, Inc (Sizuoka, Japan). Experiments using mice were approved by the Pharmaceutical Science Animal Committee of Hokkaido University.

2.4. Preparation of bone marrow derived dendritic cells (BMDCs) of mice

BMDCs were prepared as previously reported [15,16]. Briefly, bone marrow cells of C3H/HeN (female, 8-10 weeks) mice were cultured for 4 hours in RPMI1640 medium containing 10 mM HEPES, 50 μ M 2-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin and 10% fetal bovine serum (FBS). Non-adherent cells were collected and cultured in the same medium supplemented with 10 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D systems, Basel, Switzerland). On day 2 and 4, non-adherent cells were removed and adherent cells were cultured in fresh medium containing 10 ng/ml GM-CSF. On day 6, non-adherent and loosely adherent cells were used as immature BMDCs.

2.5. Internalization of CWS-NP into MBT-2 cells or BMDCs

MBT-2 cells (2×10^6) were incubated with CWS-NP (0.1 mg BCG-CWS) or nanoparticles without BCG-CWS (NP w/o CWS) in RPMI1640 medium without FBS for 1 hour. The cells were then washed twice and were further incubated in RPMI1640 medium for 1 hour. After harvesting, the cells were used as MBT-2 cells internalizing CWS-NP or NP w/o CWS.

BMDCs (1×10^6) were incubated with CWS-NP (0.1 mg BCG-CWS) or NP w/o CWS in RPMI1640 medium without FBS for 30 min. The cells were then washed twice and were further incubated in RPMI1640 medium for 1 hour. After harvesting, the cells were used as BMDCs internalizing CWS-NP or NP w/o CWS.

NBD-labeled CWS-NP was used, when we evaluated the cellular uptake of CWS-NP by MBT-2 cells and BMDCs. MBT-2 cells (2×10^6) or BMDCs (1×10^6) were incubated with NBD-labeled CWS-NP (0.1 mg BCG-CWS) in RPMI1640 medium without FBS for 1 hour or 30 min, respectively. The cells were then washed twice and were further incubated in RPMI1640 medium for 1 hour. After harvesting, the fluorescent intensity in the cells was measured by flow cytometry (Gallios, Beckman Coulter, Brea, CA) and was then analyzed using the Kaluza software package (Beckman Coulter, Brea, CA).

2.6. Cell growth of MBT-2 cells

MBT-2 cells (5×10^6) internalizing CWS-NP or NP w/o CWS were seeded in 35 mm dish and were cultured. After 3 days, the cells were re-seeded in 100 mm dish and were further cultured for 2 days. After harvesting the cells, the cells were stained with trypan blue and the number of live cells was counted.

2.7. Antitumor effect

C3H/HeN mice (female, 8-10 weeks) were subcutaneously inoculated with MBT-2 cells (1×10^6), MBT-2 cells (1×10^6) mixed with CWS-NP (0.1 mg BCG-CWS) or NP w/o CWS, or MBT-2 cells (1×10^6) internalizing CWS-NP or NP w/o CWS. Tumor growth was then monitored.

MBT-2 cells (1×10^6) were subcutaneously inoculated to C3H/HeN mice (female, 8-10 weeks). In addition, CWS-NP (0.1 mg BCG-CWS) or NP w/o CWS was subcutaneously injected in a different place of back of the same mouse. The tumor growth was then monitored.

MBT-2 cells (1×10^6), and BMDCs (100 or 5000 cells) internalizing CWS-NP were subcutaneously inoculated each in a different place of back of C3H/HeN mice (female, 8-10 weeks). The tumor growth was then monitored.

Tumor volume was calculated by the following formula: $(\text{major axis} \times \text{minor axis}^2) \times 0.52$.

2.8. Histological analysis of MBT-2 syngraft tumor tissue

C3H/HeN mice (female, 8-10 weeks) were subcutaneously inoculated with MBT-2 cells (1×10^6) mixed with CWS-NP (0.1 mg BCG-CWS) or NP w/o CWS, or MBT-2 cells (1×10^6) internalizing CWS-NP. Mice were then subcutaneously inoculated with MBT-2 cells (1×10^6), followed by the subcutaneous injection of CWS-NP (0.1 mg BCG-CWS) in a different location on the back of the same mouse. Tumor tissues were collected from the mice 10 days after inoculation. The tumor tissues were fixed with 4% paraformaldehyde. After embedding in paraffin, 3 μm tissue sections were prepared. The tissue sections were then stained with hematoxylin-eosin (HE) or giemsa and observed by microscopy. Total tumor area and necrotic tumor area in the HE-stained tissue sections were measured with NIH Image (ver. 1.44p, Wayne Rasband, National Institutes of Health, USA). The numbers of Macrophages, lymphocytes, granulocytes and mast cells in the 5 views of high-power field (HPF, objective 40 $\times$) were counted and the numbers of infiltrated cells in 1 view of HPF were calculated by averaging the cell numbers in the 5 views.

2.9. Statistical analysis

Comparisons between multiple treatments were made by one-way analysis of variance, followed by Tukey-Kramer test. The unpaired *t*-test was used to compare the results for the two treatments. In the case of the comparison of tumor volumes, the two-way repeated analysis of variance

was used, followed by Tukey-Kramer test. A P value of <0.05 was considered to be a significant difference.

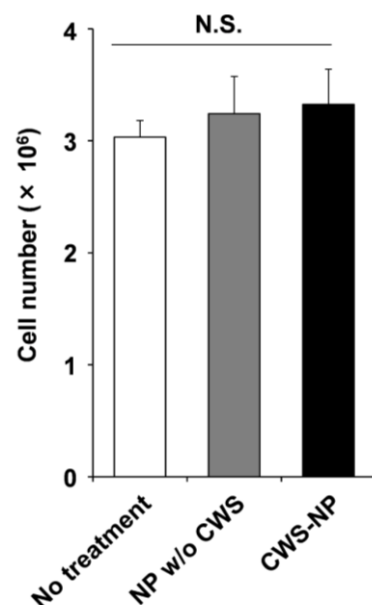
3. Results

3.1. Direct cytotoxicity of BCG-CWS against MBT-2 cells

It has been reported that the exposure of BCG to bladder cancer cells resulted in a decreased cell growth and the induction of cell death [17-19]. Hence, to investigate the direct cytotoxicity of CWS-NP and NP w/o CWS against MBT-2 cells, we compared the cell growth of MBT-2 cells treated with CWS-NP (0.1 mg BCG-CWS) and NP w/o CWS. MBT-2 cells internalizing CWS-NP and NP w/o CWS were cultured for 5 days and the number of cells counted. The number of MBT-2 cells internalizing CWS-NP was comparable to that of MBT-2 cells internalizing NP w/o CWS and non-treated MBT-2 cells (Fig. 2). This result indicates that the internalization of BCG-CWS had no direct cytotoxicity against MBT-2 cells.

Figure 2: Direct cytotoxicity of CWS-NP against MBT-2 cells

Mouse bladder cancer cells (MBT-2) were incubated with nanoparticle encapsulating BCG-CWS (CWS-NP) (0.1 mg BCG-CWS) or nanoparticle without BCG-CWS (NP w/o CWS) for 1 hour. After washing the cells, the MBT-2 cells (5×10^6) were cultured for 5 days. After harvesting the cells, the number of live cells was counted. Values are the means $\pm$ SEM ($n=3$). N.S.: not significant difference.



3.2. Effect of internalization of BCG-CWS by MBT-2 cells on antitumor effect

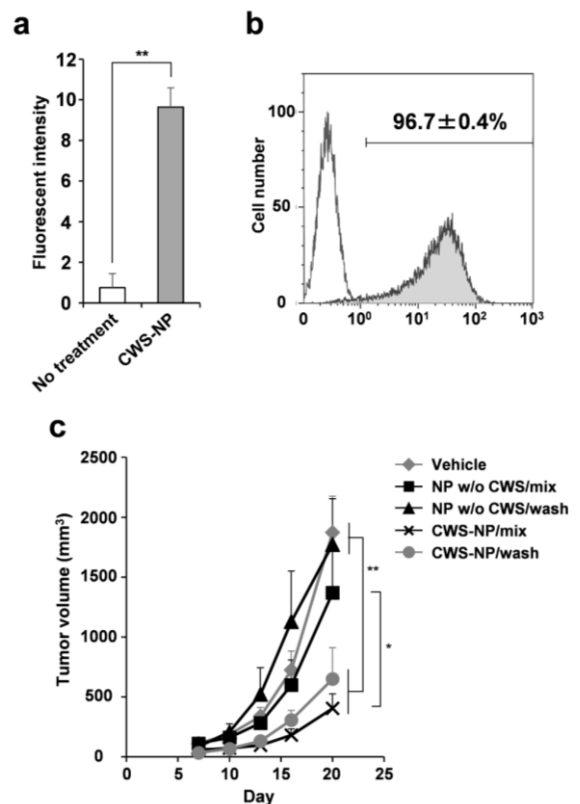
In a previous report, tumor growth was significantly inhibited in a group of mice that were inoculated with MBT-2 cells mixed with CWS-NP, compared to that of mice inoculated with MBT-2 cells only [9]. In this experimental design (Case 1 in Fig. 1b), CWS-NP appeared to interact with both MBT-2 and subcutaneous immune cells, particularly APCs, indicating that the initiation of the anti-bladder tumor effect involved bladder cancer cells and immune cells. Thus, it is difficult to conclude which cell type was mainly involved in the antitumor effect against MBT-2 tumors. In this study, we prepared MBT-2 cells internalizing CWS-NP or NP w/o CWS by washing out the un-internalized CWS-NP or NP w/o CWS (Case 2 in Fig. 1b). We first examined the internalization of CWS-NP by MBT-2 cells before injecting the mice with the washed cells. NBD-labeled CWS-NP was incubated with MBT-2 cells and the fluorescent intensity in the MBT-2 cells was then measured. The fluorescent intensity of MBT-2 cells was drastically increased after treatment with the CWS-NP (Fig. 3a).

The CWS-NP was taken up by 96.7% of the MBT-2 cells (Fig. 3b). Thus, these results clearly show that MBT-2 cells internalize sufficient CWS-NP before inoculation into mice.

Mice were subcutaneously inoculated with MBT-2 cells only (Vehicle), a mixture of MBT-2 cells and CWS-NP (CWS-NP/mix) or NP w/o CWS (NP w/o CWS/mix), MBT-2 cells internalizing CWS-NP (CWS-NP/wash) or NP w/o CWS (NP w/o CWS/wash). The CWS-NP/wash group showed a significant inhibition of MBT-2 tumor growth (Fig. 3). This inhibition of tumor growth was similar to that of the CWS-NP/mix group which was a positive control. This result clearly indicates that the internalization of BCG-CWS by MBT-2 cells has a substantial influence on the development of the antitumor effect.

Figure 3: Effect of internalization of BCG-CWS into MBT-2 cells in anti-bladder tumor effect

(a) Amount of nanoparticle encapsulating BCG-CWS (CWS-NP) taken up by mouse bladder cancer cells (MBT-2). NBD-labeled CWS-NP was pulsed to MBT-2 cells and the fluorescent intensity was measured by flow cytometry. The values are the mean $\pm$ SEM of at least three different experiments (** $P < 0.01$). (b) Histogram analysis of cellular uptake of CWS-NP by flow cytometer. White: no treatment, gray: CWS-NP treatment. The values are the mean $\pm$ SEM of at least three different experiments. (c) Mice were subcutaneously inoculated MBT-2 cells (1×10^6), MBT-2 cells (1×10^6) mixed with CWS-NP (0.1 mg BCG-CWS) or nanoparticle without BCG-CWS (NP w/o CWS), or MBT-2 cells (1×10^6) internalizing CWS-NP or NP w/o CWS. The tumor growth was then monitored. Values are the means $\pm$ SEM ($n=5-8$, * $P < 0.05$, ** $P < 0.01$).



3.3. Effect of internalization of BCG-CWS by immune cells on antitumor effect

To investigate the influence of immune cells on the antitumor effect, we subcutaneously inoculated mice with MBT-2 cells and subcutaneously injected CWS-NP (CWS-NP/s.c.) or NP w/o CWS (NP w/o CWS/s.c.) in a different location on the back of the same mouse (Case 3 in Fig. 1b). CWS-NP appear to be internalized by only subcutaneous immune cells, including DC, because CWS-NP was subcutaneously injected at some distance from the point where the MBT-2 cells were inoculated. As

a result, the subcutaneous injection of CWS-NP resulted in no inhibition of MBT-2 tumor growth (Fig. 4). The antitumor effect was drastically decreased compared to the mice that received CWS-NP/mix or CWS-NP/wash.

In addition, we prepared BMDC and subcutaneously inoculated mice with the MBT-2, followed by the subcutaneous injection of BMDCs incorporating CWS-NP in a different location on the back of the same mouse (Case 4 in Fig. 1b). CWS-NP was internalized only by BMDCs. We also examined the internalization of CWS-NP by BMDCs before injecting the mice with washed cells. NBD-labeled CWS-NP was incubated with BMDCs and the fluorescent intensity in the BMDCs were then measured. The fluorescent intensity of BMDCs was drastically increased after the CWS-NP treatment (Fig. 5a). The CWS-NP was taken up by 97.5% of the BMDCs (Fig. 5b). Thus, these results clearly show that the BMDCs internalize sufficient CWS-NP before inoculation into mice.

The administration of BMDCs showed a significant antitumor effect (Fig. 5c). However, no difference regarding for the antitumor effect between the administration of BMDCs internalizing CWS-NP and the administration of non-treated BMDCs was not observed (Fig. 5c). Thus, these data suggest that the antitumor effect mediated by CWS-NP is not due to the direct interaction of BCG-CWS with subcutaneous immune cells or DCs.

Figure 4: Effect of subcutaneously-administered CWS-NP on the anti-bladder tumor effect

Mice were subcutaneously inoculated with mouse bladder cancer cells (MBT-2) (1×10^6). In addition, nanoparticle encapsulating BCG-CWS (CWS-NP) (0.1 mg BCG-CWS) or nanoparticle without BCG-CWS (NP w/o CWS) was subcutaneously injected in a different location on the place of back of the same mouse. Values are the means $\pm$ SEM (n=6-8, **P<0.01).

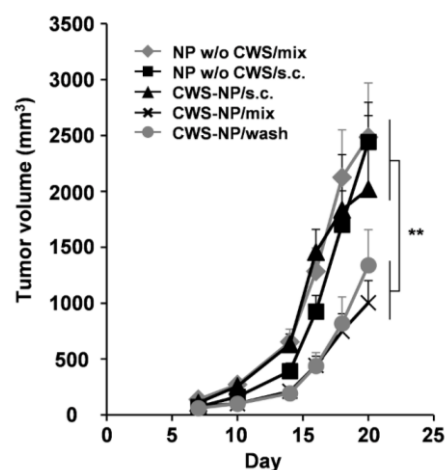
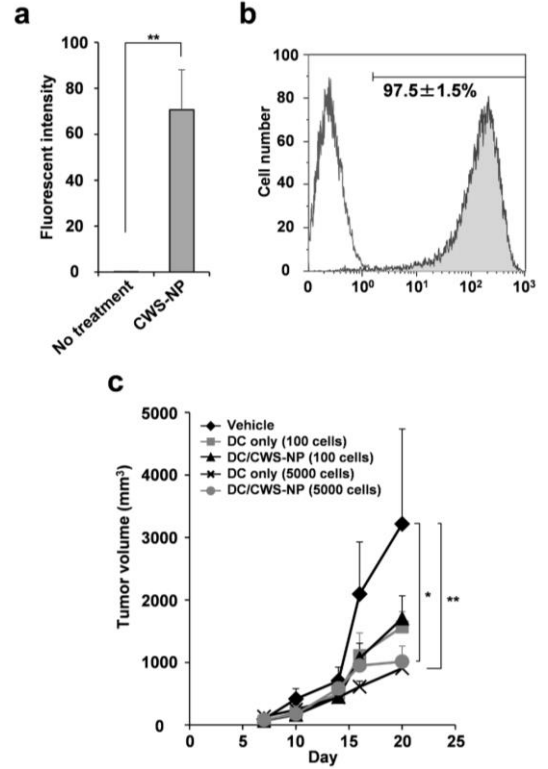


Figure 5: Effect of internalization of BCG-CWS into immune cells on anti-bladder tumor effect

(a) Amount of nanoparticle encapsulating BCG-CWS (CWS-NP) taken up by mouse bone marrow derived dendritic cells (BMDCs). NBD-labeled CWS-NP was pulsed to BMDCs and the fluorescent intensity was measured by flow cytometry. The values are the mean $\pm$ SEM of at least three different experiments (** $P < 0.01$). (b) Histogram analysis of cellular uptake of CWS-NP by flow cytometer. The values are the mean $\pm$ SEM of at least three different experiments. (c) BMDCs (1×10^6) were incubated with CWS-NP (0.1 mg BCG-CWS) for 1 hour. After washing the BMDCs, mouse bladder cancer cells (MBT-2) (1×10^6), and the BMDCs (100 or 5000 cells) were subcutaneously inoculated each in a different location on the back of the mice. Values are the means $\pm$ SEM ($n = 5-6$, * $P < 0.05$, ** $P < 0.01$).



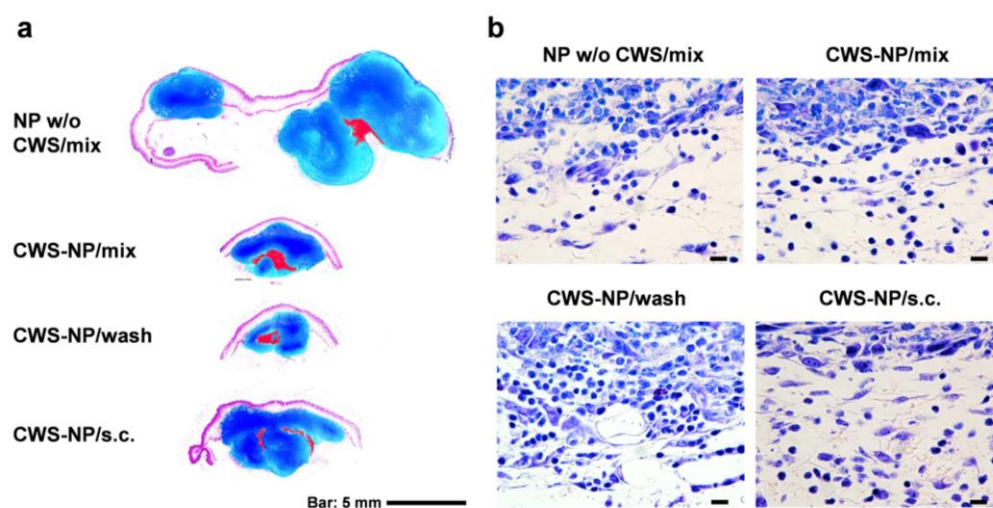
3.4. Histological analysis of MBT-2 syngraft tumor tissue

Finally, we performed a histological analysis of MBT-2 syngraft tumor tissues at 10 days after the inoculation of MBT-2 cells. Necrosis was efficiently induced in tumor tissue by inoculation with a mixture of MBT-2 cells and CWS-NP (CWS-NP/mix), the MBT-2 cells internalizing CWS-NP (CWS-NP/wash) or MBT-2 cells and CWS-NP separately (CWS-NP/s.c.) compared to tumor tissue inoculated with a mixture of MBT-2 cells and NP w/o CWS (NP w/o CWS/mix) (Fig. 6a and Table). Moreover, the infiltration of white blood cells (WBC) into tumor tissue was observed in the case of CWS-NP/mix, CWS-NP/wash and CWS-NP/s.c. at a comparable level (Fig. 6b and Table). We also counted the number of WBC that had infiltrated into tumor tissues (Table). In the case of CWS-NP/mix, CWS-NP/wash and CWS-NP/s.c., numerous WBC were found to have infiltrated into the tumor tissue. The infiltrated WBCs included macrophages, lymphocytes, neutrophils, eosinophils and mast cells (Table). These observations suggest that CWS-NP induces the infiltration of immune cells into tumor tissue.

Figure 6: Histological analysis of MBT-2 tumor tissue

Mice were subcutaneously inoculated with mouse bladder cancer cells (MBT-2) (1×10^6) mixed

with nanoparticle encapsulating BCG-CWS (CWS-NP) (0.1 mg BCG-CWS) or nanoparticle without BCG-CWS (NP w/o CWS), or MBT-2 cells (1×10^6) internalizing CWS-NP. And, MBT-2 cells (1×10^6) were subcutaneously inoculated to mice, followed by subcutaneous injection of CWS-NP (0.1 mg BCG-CWS) in a different place of back of the same mouse. Tumor tissues were collected from the mice 10 days after inoculation and tissue sections were then prepared. (a) Total tumor area and necrotic tumor area in the HE-stained tissue sections were measured with NIH Image. (b) Microscopic analysis of HE-stained tissue sections. Bars show 10 mm.



4. Discussion

Some reports of *in vitro* experiments showed that the exposure of BCG to bladder cancer cells decreased cell growth and induced cell death [17-19]. However, this type of cytotoxicity was induced only with very high ratios of BCG to bladder cancer cells. These ratios are unlikely to be found in a clinical setting. In addition, no evidence currently exists that the direct cytotoxicity of BCG occurs *in vivo*. The cytotoxicity of BCG against bladder cancer continues to be a controversial issue. The growth of MBT-2 cells was not inhibited by treatment with CWS-NP under our experimental conditions (Fig. 2), although CWS-NP was efficiently taken up by MBT-2 cells within 1 hour [9]. This result indicates that the internalization of CWS-NP by MBT-2 cells had no effect on cell growth. The experimental conditions for CWS-NP exposure to MBT-2 cells were common both *in vitro* and *in vivo*. Hence, we conclude that the direct cytotoxicity by CWS-NP is not associated with the anti-bladder tumor effect mediated by CWS-NP.

Figure 3c clearly shows that the internalization of BCG-CWS by bladder tumor cells substantially contributes to the induction of the anti-bladder tumor effect. BCG-CWS does not appear to

be involved in the process of attachment to bladder cancer cells, because the BCG-CWS in CWS-NP is covered with lipid layers and is located in an inner phase of the lipid nanoparticle. CWS-NP is taken up by bladder cancer cells, which is mediated by octaarginine, which is present on the surface of CWS-NP [9]. Thus, the processes that occur after cellular uptake appear to be involved in the anti-bladder tumor effect. BCG-CWS may be degraded in the lysosomal compartments, because the CWS-NP localized in acidic compartments after being taken up by MBT-2 cells [9]. It has been reported that murine bladder carcinoma cells present BCG to specific CD4⁺ T cells that are derived from bladder-draining lymph nodes following intravesical BCG administration [20]. Glucose monomycolate, a glycolipid derived from Mycobacteria, is present on the CD1 molecule and the presentation induces antigen specific T cell responses [21-23]. It is likely that the anti-bladder tumor effect was induced by presenting the component of degraded BCG-CWS to T cells. However, antigens that are present on T cells in the context of BCG or BCG-CWS for bladder cancer have not been identified. Further studies will be needed in this area.

CWS-NP/s.c. (Case 3 in Fig. 1b) showed no anti-bladder tumor effect (Fig. 4), suggesting that BCG-CWS must associate with MBT-2 cells in order to induce the anti-bladder tumor effect. This result is consistent with the requirement of BCG-mediated antitumor effects [24]. Moreover, the administration of DCs internalizing BCG-CWS failed to enhance the anti-bladder tumor effect, although DC administration enhanced the anti-bladder tumor effect (Fig. 5c). This result clearly indicates that the internalization of BCG-CWS by DCs is not required for the induction of the anti-bladder tumor effect. On the other hand, it is known that BCG-CWS activates DC, resulting in an enhancement in the effectiveness of cancer immunotherapy [25]. The question arises as to why the BMDCs exposed with CWS-NP failed to enhance an anti-bladder tumor effect. When delivered by CWS-NP, BCG-CWS did not appear to be involved in the binding step to DCs along with the case of MBT-2 cells. BCG-CWS appear to elude recognition by TLRs on the cell surface. As a result, it is likely that the DC activation by BCG-CWS did not occur. Consequently, this suggests that BCG-CWS internalized by DCs has no effect on the initiation of an anti-bladder tumor effect.

The present possible mechanism responsible for the anti-bladder tumor effect mediated by live BCG is briefly the follows [10]. For the details, see the cited review. BCG attaches to urothelial cells or bladder cancer cells and is then internalized by them. DCs may also be taken up and process live BCG. Following internalization, bladder cancer cells upregulate the expression of MHC class II and ICAM-1, and produce cytokines, along with DCs. The recruitment of immune cells, such as granulocytes, T cells, natural killer (NK) cells and macrophages, occurs at the tumor site, resulting in cytotoxicity to bladder cancer cells. In the latter part, the recruitment of granulocyte, T cells, NK cells, is likely [26-29]. However, the mechanism responsible for how BCG initiates the anti-bladder tumor immune responses is not yet clear. The findings reported herein indicate that the internalization of BCG-CWS by bladder

cancer cells, but not DCs, is clearly involved in the development of the anti-bladder tumor effect. The mechanism of BCG therapy can be discussed in reference to the results of this study, because the CWS-NP-mediated anti-bladder tumor effect appears to be similar to that induced by BCG in some respects. Lymphocytes and Granulocytes were involved in the CWS-NP mediated anti-bladder tumor effect, along with BCG therapy (Table). It is likely that the number of neutrophils were increased in the groups of CWS-NP/mix and CWS-NP/wash, although these differences did not reach the level of statistical significance. However, further investigations regarding the involvement of neutrophil is clearly called for. In addition, BCG and CWS-NP were taken up by bladder cancer cells via a similar pathway. It was recently reported that BCG is taken up by bladder cancer cells via macropinocytosis [30]. In the case of CWS-NP, macropinocytosis is involved in its uptake by bladder cancer cells [9]. Therefore, our findings provide support for how BCG initiates anti-bladder tumor immune responses. That is, the invasion of live BCG into bladder cancer cells, but not DCs, initiates development of an anti-bladder tumor effect in BCG therapy.

The findings reported herein provide a rational mechanism for the initiation of the anti-bladder tumor effect mediated by BCG-CWS. The effect involves immune responses caused by the internalization of BCG-CWS by bladder cancer cells, but not DCs. This finding provides new insights into the mechanism responsible for the initiation of the anti-bladder tumor immunity mediated by BCG. That is, the internalization of BCG-CWS contained in BCG by bladder cancer cells contributes to the initiation of anti-bladder tumor immunity. We indirectly deduced this from in vivo evidence for the first time, although our experimental conditions were different from the actual environment of the urinary bladder. However, further study using an orthotopic model of bladder tumor is clearly needed. Moreover, we clearly demonstrated that the above described delivery system, CWS-NP, is a powerful tool for investigating the machinery associated with the anti-bladder tumor effect mediated by BCG or BCG components.

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Table: Percentage of necrosis area and analysis of cellular infiltration in MBT-2 syngraft tumor tissue

Parameters		NP w/o CWS/mix	CWS-NP/mix	CWS-NP/wash	CWS-NP/s.c.
Necrosis area (%)		6.8±5.3	36.4±22.7	17.2±4.3	25.8±17.5
WBC count/HPF	Macrophage	4.9±1.7	51.1±1.5	55.1±6.0	43.8±5.1
	Lymphocyte	9.0±3.1	43.7±6.2	52.0±6.8	48.2±11.9
	Neutrophil	1.7±0.3	11.1±4.6	10.1±4.8	4.5±2.0
	Eosinophil	1.0±0.6	2.7±2.0	5.2±2.4	13.9±10.7
	Basophil	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Mast cell	1.8±0.6	3.7±0.8	3.4±1.2	3.2±0.7
	Total WBC count/HPF	18.4±2.4	112.3±8.8	128.8±18.3	113.6±22.7
Differential ratio of WBC (%)	Macrophage	29.2±10.7	46.2±4.6	44.6±4.1	40.4±6.1
	Lymphocyte	47.4±11.0	38.5±2.7	41.8±3.0	42.3±5.6
	Neutrophil	8.9±1.2	9.4±3.2	7.2±2.8	4.3±2.1
	Eosinophil	5.2±3.2	2.4±1.7	3.8±1.2	9.7±6.4
	Basophil	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
	Mast cell	9.3±1.9	3.5±1.0	2.6±0.6	3.3±1.1

The numbers of Macrophage, lymphocyte, granulocytes and mast cell in the 5 views of high-power field (HPF, objective 40×) were counted and the numbers of infiltrated cells in 1 view of HPF were calculated by averaging the cell numbers in from 5 views. Values are the means ± SEM (n=3).

Figure legend

Figure 1: Concept and Experimental design

(a) Concept of this study. The role of bladder cancer cells and dendritic cells (DCs) in the initiation of anti-bladder tumor effect was investigated using nanoparticles encapsulating BCG-CWS (CWS-NP). (b) Case 1: Mice were subcutaneously administrated with a mixture of CWS-NP and mouse bladder cancer cells (MBT-2). Case 2: MBT-2 cells were exposed to CWS-NP and the uninternalized CWS-NP was then washed out. The MBT-2 cells internalizing CWS-NP were subcutaneously inoculated to mice. Case 3: CWS-NP and MBT-2 cells were subcutaneously administrated at a different location on the back of mice. Case 4: DCs were exposed to CWS-NP and the uninternalized CWS-NP was then washed out. MBT-2 cells and the DCs internalized CWS-NP were subcutaneously inoculated each at a

different location on the back of mice.

Figure 2: Direct cytotoxicity of CWS-NP against MBT-2 cells

Mouse bladder cancer cells (MBT-2) were incubated with nanoparticle encapsulating BCG-CWS (CWS-NP) (0.1 mg BCG-CWS) or nanoparticle without BCG-CWS (NP w/o CWS) for 1 hour. After washing the cells, the MBT-2 cells (5×10^6) were cultured for 5 days. After harvesting the cells, the number of live cells was counted. Values are the means $\pm$ SEM (n=3). N.S.: not significant difference.

Figure 3: Effect of internalization of BCG-CWS into MBT-2 cells in anti-bladder tumor effect

(a) Amount of nanoparticle encapsulating BCG-CWS (CWS-NP) taken up by mouse bladder cancer cells (MBT-2). NBD-labeled CWS-NP was pulsed to MBT-2 cells and the fluorescent intensity was measured by flow cytometry. The values are the mean $\pm$ SEM of at least three different experiments (**P<0.01). (b) Histogram analysis of cellular uptake of CWS-NP by flow cytometer. White: no treatment, gray: CWS-NP treatment. The values are the mean $\pm$ SEM of at least three different experiments. (c) Mice were subcutaneously inoculated MBT-2 cells (1×10^6), MBT-2 cells (1×10^6) mixed with CWS-NP (0.1 mg BCG-CWS) or nanoparticle without BCG-CWS (NP w/o CWS), or MBT-2 cells (1×10^6) internalizing CWS-NP or NP w/o CWS. The tumor growth was then monitored. Values are the means $\pm$ SEM (n=5-8, *P<0.05, **P<0.01).

Figure 4: Effect of subcutaneously-administered CWS-NP on the anti-bladder tumor effect

Mice were subcutaneously inoculated with mouse bladder cancer cells (MBT-2) (1×10^6). In addition, nanoparticle encapsulating BCG-CWS (CWS-NP) (0.1 mg BCG-CWS) or nanoparticle without BCG-CWS (NP w/o CWS) was subcutaneously injected in a different location on the place of back of the same mouse. Values are the means $\pm$ SEM (n=6-8, **P<0.01).

Figure 5: Effect of internalization of BCG-CWS into immune cells on anti-bladder tumor effect

(a) Amount of nanoparticle encapsulating BCG-CWS (CWS-NP) taken up by mouse bone marrow derived dendritic cells (BMDCs). NBD-labeled CWS-NP was pulsed to BMDCs and the fluorescent intensity was measured by flow cytometry. The values are the mean $\pm$ SEM of at least three different experiments (**P<0.01). (b) Histogram analysis of cellular uptake of CWS-NP by flow cytometer. The values are the mean $\pm$ SEM of at least three different experiments. (c) BMDCs (1×10^6) were incubated with CWS-NP (0.1 mg BCG-CWS) for 1 hour. After washing the BMDCs, mouse bladder cancer cells (MBT-2) (1×10^6), and the BMDCs (100 or 5000 cells) were subcutaneously inoculated each in a different location on the back of the mice. Values are the means $\pm$ SEM (n=5-6, *P<0.05, **P<0.01).

Figure 6: Histological analysis of MBT-2 tumor tissue

Mice were subcutaneously inoculated with mouse bladder cancer cells (MBT-2) (1×10^6) mixed with nanoparticle encapsulating BCG-CWS (CWS-NP) (0.1 mg BCG-CWS) or nanoparticle without BCG-CWS (NP w/o CWS), or MBT-2 cells (1×10^6) internalizing CWS-NP. And, MBT-2 cells (1×10^6) were subcutaneously inoculated to mice, followed by subcutaneous injection of CWS-NP (0.1 mg BCG-CWS) in a different place of back of the same mouse. Tumor tissues were collected from the mice 10 days after inoculation and tissue sections were then prepared. (a) Total tumor area and necrotic tumor area in the HE-stained tissue sections were measured with NIH Image. (b) Microscopic analysis of HE-stained tissue sections. Bars show 10 mm.