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# ANTI-TUMOR EFFECT OF NANO-PARTICULATED BACILLUS CALMETTE-GUERIN (BCG) CELL WALL COMPLEX ON BLADDER CANCER CELL LINE

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**Abstract** Bacillus Calmette-Guerin (BCG) has been widely accepted as an effective treatment for CIS and superficial carcinoma of the bladder. However, it has considerable side effects and toxicity. We thought if effective bacterial cell wall elements as substitutes for live bacteria were identified, useful treatment with lower toxicity while maintaining strong anti-tumor effects might be possible. For these reasons we generated a nano-particulated BCG complex, which does not contain live bacteria. Here, we present its direct *in vitro* anti-tumor effect on bladder cancer cell lines. Tokyo 172 BCG sub-strain was disrupted by French press with monitoring the particle distribution by the particle analyzer. After removing the not-disrupted bacteria by centrifuge at 6,800×g, supernatant was centrifuged at 18,000×g. Then the supernatant (Sup) and the precipitate (CW) were lyophilyzed to obtain nano-particulated BCG complex. Bladder cancer cell lines, J82 and KK47, were co-cultured with BCG, Sup, CW or Sup+CW (mix) for 5 days, then viable cell numbers were counted. In J82 cells, when separately added to the culture medium, both CW and Sup reduced cell number to about 70% of control cells. While they were mixed together, they reduced cell number equally compared with BCG; 59.2% (mix) vs 60.2% (BCG) in J82 cells, 67.3% vs 68.8% in KK47 cells. These preliminary *in vitro* experiments demonstrated the identical direct anti-tumor effect of nano-particulated BCG to that of live BCG. *In vivo* tumor assays are warranted for clinical application of nano-particulated BCG.

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**Key words:** nano-particulated BCG; direct anti-tumor effect; bladder cancer cell line

Intravesical Bacillus Calmette-Guerin (BCG) therapy is known to be very effective in the treatment of and prophylaxis against carcinoma in situ (CIS) and superficial carcinoma of the bladder, however the exact molecular mechanism of action is not clear. We previously reported whether BCG effects against urinary bladder carcinoma cell line T24 correlated with apoptosis *in vitro*.[1)] BCG suppressed the growth of T24 directly by co-incubation for 96 hr. Cell cycle analysis showed increased apoptotic cell levels by BCG addition, but apoptotic DNA ladder was not seen and few apoptotic cells were detected by an in situ apoptosis detection kit. To confirm and to clarify this mechanism of growth suppression, we examined whether BCG decreases telomerase activity in bladder cancer cell lines.[2)] Cell viability in T24 cells significantly decreased to 54.2% when compared with non-BCG-treated cells ($p<0.05$), and J82 cells decreased to 53.3% ($p<0.05$). Thus, it was the fact that BCG had a direct anti tumor effect against bladder cancer cell lines. Telomerase activity in T24 cells treated with BCG for 5

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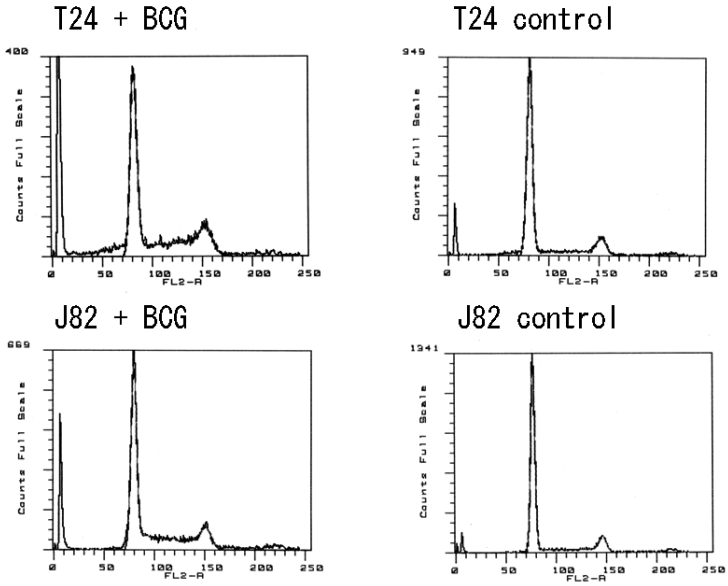


**Figure 1** FACS profile of BCG-treated bladder cancer cell lines. The percentage of apoptotic cells were 37.8% in T24 cells treated with BCG, 7.8% in T24 control cells, 13.8% in J82 cells treated with BCG, 3.9% in J82 control cells.

**Table 1**. BCG suppressed cellular growth and telomerase activity against bladder cancer cell lines

| cell line | cell number | telomerase activity |
|---|---|---|
| 5637 | $p<0.05$ | $p<0.05$ |
| T24 | $p<0.05$ | $p<0.05$ |
| ACHN | NS | NS |
| Caki-II | $p<0.05$ | NS |
| PC-3 | NS | NS |
| HeLa | $p<0.05$ | NS |
| NHDF | NS | ND |

NS: not significant, ND: not determined.

days significantly decreased to 80.2% when compared with non-treated T24 cells ($p<0.05$) and similarly it significantly decreased to 71.1% in J82 cells ($p<0.05$). In T24 cells treated with BCG for 5 days, the percentage of apoptotic cells was 37.8%, which was a remarkable increase compared with 7.8% of non-treated T24 cells. Within the cell cycle, the cells of G1/S and S phases decreased, but those of G2/M phase showed no change. Similarly, the percentage of apoptotic cells in J82 cells was 13.8% as opposed to 3.9 % of control cells. (Figure 1) Thus, BCG induced apoptosis against bladder cancer cell lines. As shown in Table 1, BCG suppressed cellular growth and telomerase activity against bladder cancer cell lines. However, as telomerase activity did not change in human prostate cancer and renal cell carcinoma cells, BCG may have some specific mechanism of effect that is unique in bladder cancer cells.

The mechanisms of BCG effect have been thought to be due to both direct and indirect actions. We have also previously reported the indirect mechanisms through BCG activated lymphocytes (BAK cells) against T24 *in vitro*.[3] Certainly BCG is widely recognized as

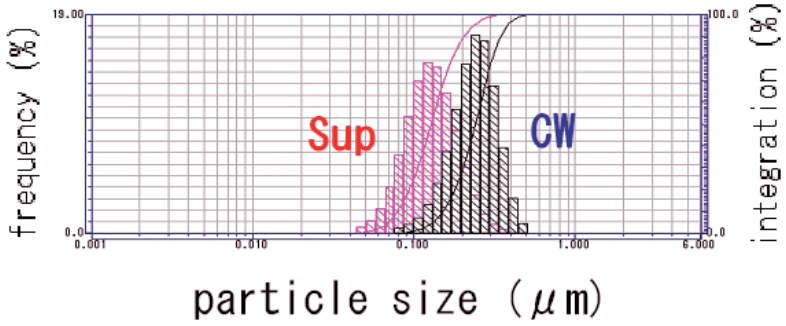


**Figure 2** Particle dirtibution of nano-particulated BCG. Sup consisted of micro-particles sized 42 to 390 nm (median 127.9 nm). CW consisted of micro-particles sized 75 to 500 nm (median 240.7 nm).

"immunotherapeutic" drug. What is the reason to focus on the "direct " anti tumor effect? Ikeda et al[4] reported that BCG induced the augmented expression of surface antigens, such as MHC Class II, CD1, CD80 and ICAM-1, of bladder tumor cells. Furthermore, BCG-treated MBT-2 cells could stimulate BCG-sensitized lymphocytes to produce IL-2 and IFN-gamma. Thus, BCG attachment or internalization to tumor cells is the trigger to induce immune response, in other words, it is an essential first step in the anti tumor effect of BCG. On the other hand, BCG therapy remains the problem of recurrence in about 30% of patients. Therefore, the direct anti tumor effect itself may be expected if patient's immune response is difficult to activate.

What is the reason to focus on the cellular components of BCG? As BCG is the live bacteria, it has the potential for undesirable side effects and toxicity. Many attempts have been made to increase the clinical efficacy while decreasing side effects. Morales et al.[5] reported that mycobacterial cell wall extract (MCWE) demonstrated clear clinical activity against bladder cancer. Hayashi et al.[6] reported that immunotherapy with BCG-CWS (BCG-cell wall skelton) was effective for the prolongation of survival in cancer patients. Zlotta et al[7]. reported that numerous BCG subcomponents provided positive stimuli Th1 cell differentiation and enhanced non-MHC-restricted cytotoxicity against bladder tumor cells. Also we have been tried to find subcomponents that have the equally anti tumor effect to live BCG. BCG was disrupted by sonication or autoclave, then centrifuged and separated to supernatant and precipitate. These fractions were co-cultured with bladder tumor cell line for 5 days, and then counted viable cell numbers by trypan blue staining. Supernatant fraction reduced viable cell number significantly compared with non-treated control, however, these effect was not sufficiently compared with effect of live BCG. BCG was disrupted by French press, then centrifuged at 6,760×g. Supernatant was centrifuged at 18,000×g and 60,000×g, and then various obtained precipitate and supernatant were also co-cultured with bladder cancer cell line. As a result, supernatant fraction at 60,000 ×g was most effective, however, also its effect was not sufficiently compared with effect of live BCG.[7]

Tokyo 172 BCG sub-strain was disrupted by French press with monitoring the particle distribution by the particle analyzer. After removing the not-disrupted bacteria by centrifuge at 6,800×g, supernatant was centrifuged at 18,000×g. Then the supernatant (Sup) and the precipitate (CW) were lyophilyzed to obtain nano-particulated BCG complex. CW consisted of micro-particles sized 75 to 500 nm (median 240.7 nm). Sup consisted of micro-particles sized 42 to 390 nm (median 127.9 nm). (Figure 2) Bladder

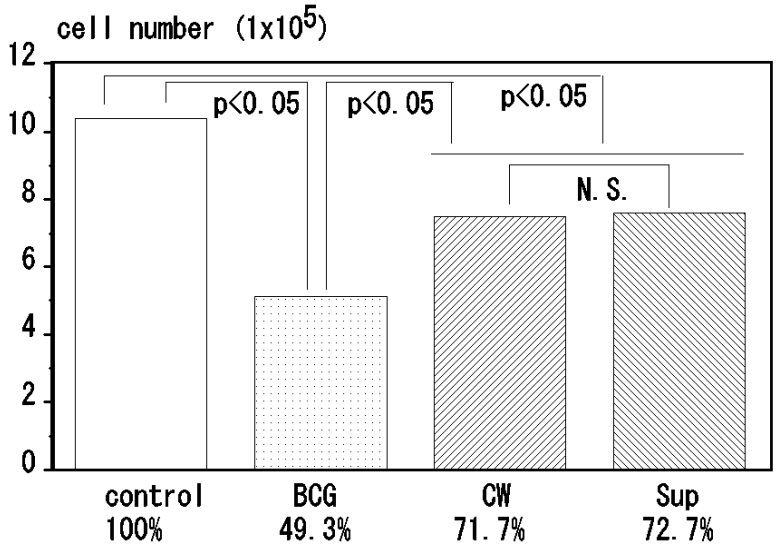


**Figure 3** CW or Sup was insufficiently when separately added in J82 cells. In J82 cells, when separately added to the culture medium, both CW and Sup reduced cell number to about 70% of control cells.

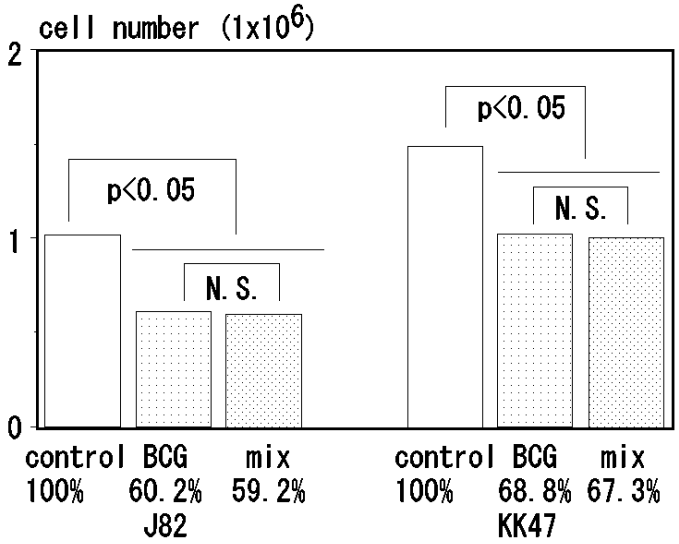


**Figure 4** Nano-particulated BCG was equally effective to BCG when they were mixed. When CW and Sup were mixed together, they reduced cell number equally compared with BCG; 59.2% (mix) vs 60.2% (BCG) in J82 cells, 67.3% vs 68.8% in KK47 cells.

cancer cell lines, J82 and KK47, were co-cultured with BCG, Sup, CW or Sup+CW (mix) for 5 days, then viable cell numbers were counted. In J82 cells, when separately added to the culture medium, both CW and Sup reduced cell number to about 70% of control cells. (Figure 3) While they were mixed together, they reduced cell number equally compared with BCG; 59.2% (mix) vs 60.2% (BCG) in J82 cells, 67.3% vs 68.8% in KK47 cells. (Figure 4)

What is difference between live BCG and nano-particulated BCG? Nano-particulated BCG is not alive and smaller than live BCG. Therefore, nano-particulated BCG may reduce

side effect because there is no live mycobacterial infection. Nano-particulated BCG may be delivered to inside of tumor, therefore it may be expected as new drug against invasive bladder cancer, live BCG-refractory bladder cancer, or other tissues' cancer. These preliminary *in vitro* experiments demonstrated the identical direct anti-tumor effect of nano-particulated BCG to that of live BCG. *In vivo* tumor assays are warranted for clinical application of nano-particulated BCG.

## Reference

1) Sasaki A, Kudoh S, Mori K, Takahashi N, Suzuki T. Are BCG effects against urinary bladder carcinoma cell line T24 correlated with apoptosis in vitro? Urol Int 1997;59:142-8.

2) Saitoh H, Mori K, Kudoh S, Itoh. H, Takahashi N, Suzuki T. BCG effects on telomerase activity in bladder cancer cell lines. Int J Clin Oncol 2002;7:165-70.

3) Takahashi J, Kudoh S, Liu XX, Mori K, Suzuki T. BCG (bacille Calmette-Guerin)-activated lymphocytes (BAK cells) induce apoptosis in urinary bladder carcinoma cell line T24 in vitro. Int J Clin Oncol 2000;5:253-61.

4) Ikeda N, Toida I, Iwasaki A, Kawai K, Akaza H. Surface antigen expression on bladder tumor cells induced by bacillus Calmette-Guerin (BCG): A role of BCG internalization into tumor cells. Int J Urol 2002;9:29-35.

5) Morales A, Chin JL, Ramsey EW. Mycobacterial cell wall extract for treatment of carcinoma in situ of the bladder. J Urol 2001;166:1633-38.

6) Hayashi A, Doi O, Azuma I, Toyoshima K. Immuno-friendly use of BCG-cell wall skelton remarkably improves the survival rate of various cancer patients. Proc Japan Acad Ser B 1998;74:50-5.

7) Zlotta AR, Van Vooren J-P, Denis O, et al. What are the immunologically active components of bacilli Calmette-Guerin in therapy of superficial bladder cancer? Int J Cancer 2000;87:844-52.

8) Mori K, Rahman NATM, Ishimura H, et al. Anti-tumor effects of cell wall component fractionated from BCG. Annals Soc BCG BRM Immunother 2003;27:33-41. (Articles in Japanese)