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Introduction of 65 kDa Antigen of *Mycobacterium tuberculosis* to Cancer Cells Enhances Anti-Tumor Effect of BCG Therapy

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***Abstract*: Bacillus Calmette Guerin (BCG) immunotherapy has anti-tumorigenic effects against bladder cancer. To improve the efficacy of BCG therapy, we introduced the gene encoding the 65 kDa heat shock protein (hsp) of *Mycobacterium tuberculosis* into a mouse malignant melanoma cell line (B16). An expression vector harboring the 65 kDa antigen gene was transfected into B16 using Lipofectamine, then expression of the antigen was confirmed by RT-PCR and Western blotting. Several cell lines expressing 65 kDa antigen were established (B16/65kDa). We also established a control cell line transfected with the vector alone (B16/con). All cell lines (B16, B16/con, B16/65kDa) were injected intraperitoneally into syngeneic mice with or without BCG prior immunization and the development of tumor ascites was examined. To analyze the mechanism of the anti-tumor effect, CD4 T cells or CD8 T cells were depleted *in vivo* by administering the corresponding monoclonal antibody. B16/65kDa expressed the 65 kDa hsp of *M. tuberculosis*. The tumor growth of B16/65kDa was slightly retarded in naive mice, but significantly inhibited by BCG. The anti-tumor effect was totally abrogated in mice deficient in CD4 T cells, suggesting that CD4 T cells are involved in this process. The 65 kDa hsp of *M. tuberculosis* was expressed after gene transduction in a malignant melanoma cell line and significantly enhanced the anti-tumor effect of BCG immunotherapy. CD4 T cells play an important role in this anti-tumor effect.**

***Key words*: BCG, Gene therapy, 65 kDa antigen**

Immunotherapy with BCG has anti-tumor effects against bladder cancer. The response rate to BCG instillation therapy by carcinoma *in situ* (CIS), which was thought to be an indication for radical cystectomy, is about 70% (25). BCG instillation therapy is also effective for patients with T1 grade 3 superficial bladder cancer, which frequently develops into invasive bladder cancer (11). However, the exact mechanism of BCG instillation therapy remains obscure. To improve and understand the process of BCG immunotherapy, the anti-tumor mechanism must be identified.

We investigated the usefulness of immune-gene therapy using cytokines such as interleukin-2 (IL2) (3, 5), IL12 (4, 17, 18, 27) and IL18 (8). These studies showed that tumor cells expressing cytokines significantly retarded tumor growth in syngeneic mice. Moreover, they also worked as tumor vaccines in preventing a response to a subsequent challenge with parental cells. Indeed introducing cytokine genes is one type of immune gene therapy. Another is to have the host more efficiently recognize tumor cells as being immunogenic. To improve the BCG therapy, we introduced the gene encoding the major antigen, 65 kDa heat shock protein (hsp), of *Mycobacterium tuberculosis* into a mouse melanoma cancer cell line. We then analyzed the anti-tumor effect of BCG against this cell line.

Materials and Methods

Cell line and animals. The mouse melanoma cell

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Abbreviations: BCG, Bacillus Calmette Guerin; CIS, carcinoma *in situ*; hsp, heat shock protein; CTL, cytotoxic T lymphocyte; MEM, minimum essential media; PCR, polymerase chain reaction.

line of C57BL/6 origin, B16, was maintained in minimum essential medium (MEM) containing 5% fetal calf serum. Female 6-week-old C57BL/6 mice were purchased from Clea Japan.

Gene manipulation. A gift from Dr. T.M. Shinnick (21), the gene encoding the 65 kDa protein of *Mycobacterium tuberculosis*, was inserted into a mammalian expression vector plasmid (pCEXV3) (16).

Gene transfection. The expression vector harboring the 65 kDa gene (pCEXV3/65kDa) was co-transfected with pSV2neo (selection gene) into B16 cells using Lipofectamine. Briefly, 1.8×10^5 B16 cells were seeded into 60 mm dishes 16 hr before transfection. Purified pCEXV3/65kDa (9 μg) or pCEXV3 (9 μg; mock transfection) were transfected with 48 mg of Lipofectamine reagent and 3 ml of serum-free OPTI-MEM (Life Technologies, Inc., Gaithersburg, Md., U.S.A.). The plasmids were co-transfected with 1 μg of pSV2neo as a selection marker (24). Colonies were selected using 1 mg/ml Geneticin (Sigma, St. Louis, Mo., U.S.A.) 3 days after transfection, picked up by cloning cylinders and expanded into cell lines.

Cell proliferation assay. To compare the *in vitro* proliferation of B16 sublines, 5×10^3 cells of each cell line were seeded in each well of 12-well plates, and the number of cells in each cell line was counted daily by triplicate.

Polymerase chain reaction (PCR). Total RNA was isolated from transfected cell lines using acid-guanidinium thiocyanate-phenol-chloroform. Complementary DNA was synthesized from 2 μg of RNA by extension with oligo dT in 20 μl of reverse transcriptase reaction buffer (pH 8.3) containing 0.01 M DTT, 0.5 mM deoxynucleotide phosphate and 200 U of Superscript II (GIBCO BRL, Grand Island, N.Y., U.S.A.). The mixture was incubated at 42 C for 50 min, then at 70 C for 15 min.

The cDNA for 65 kDa protein of *Mycobacterium tuberculosis* was detected by PCR amplification in separate reactions, using oligonucleotide primers. PCR proceeded using a Perkin Elmer Cetus Gene Amp PCR system 9600 (Norwalk, Conn., U.S.A.) in 25 μl of reaction mixture for 30 cycles (1 min at 95 C, 1 min at 62 C and 2 min at 72 C) using rTth DNA polymerase (Perkin Elmer). Non-specific priming and oligomerization was minimized using hot-start PCR. Cycling was concluded with a final extension of 7 min at 72 C. A sample of each reaction (10 μl) was analyzed in a 2% agarose gel and visualized by ethidium-bromide fluorescence staining. The primers were:

5′-ACGATCACCAACGATGGTGTG-3′

5′-AGCTGACCAGCAGGATGTAGG-3′ (corresponding size: 529 bp)

Western blotting. Expression of the 65 kDa protein in transfected cells was confirmed by Western blotting. Cells were lysed in NP-40 lysis buffer (150 mM NaCl, 1% NP-40, 50 mM Tris pH 8.0, 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 15,000 rpm at 4 C, lysates were resolved by electrophoresis on 4–20% SDS-polyacrylamide gels, and then transferred to nitrocellulose membranes at a constant current of 35 mA for 4 hr. Non-specific binding on the membranes was blocked in PBS containing 10% powdered nonfat milk at 4 C overnight. The membranes were then incubated for 1 hr with an anti-65 kDa protein monoclonal antibody (7) in PBS containing 1.0% BSA, and washed twice for 5 min each with PBS containing 0.01% Tween 20. The membranes were then incubated for 1 hr with horseradish peroxidase-conjugated anti-mouse IgG antibody (DAKO, A/S, Denmark). After washing as described above, specific proteins were detected using diaminobenzidine (Wako Chemical, Tokyo).

Animal model of BCG therapy. To establish a BCG immunotherapy model, C57BL/6 mice were administered with 1 mg of live BCG intraperitoneally once each week for 2 weeks. The mice were challenged with tumor cells (5×10^6 cells) administered intraperitoneally one week after the last BCG administration. The presence of cancer ascites was examined 3 times each week and the percentage of ascites in free mice was calculated.

Immunodeficient mice. T cells were depleted *in vivo* by the intraperitoneal administration of rat MAb GK1.5 (anti-CD4; IgG_{2b}) and MAb 2.43 (anti-CD8; IgG_{2b}) (both hybridomas were acquired from the ATCC). These MAbs were used as ascites fluids (titer, >1:10,000 according to mouse thymocyte staining by flow cytometry). The MAb preparations (0.2 ml) were injected i.p. on day −3 and every 7 days thereafter. Throughout our experiments, respective T cell sub-populations were depleted by >97% according to indirect immunofluorescence staining and cytofluorometric analysis (EPICS Elite, Coulter, Fla., U.S.A.) of splenocytes using the MAbs GK1.5 (CD4) or 2.43 (CD8), respectively (Fig. 1).

Results

Expression of 65 kDa Antigen in Transfected Cells

The presence of 65 kDa antigen mRNA was screened in transfected clones by RT-PCR. Parental and mock-transfected B16 (B16/con) did not express 65 kDa antigen mRNA. Clones B16/65kDa-1 and B16/65kDa-2 expressing 65 kDa antigen mRNA were selected for use in further experiments (Fig. 2). Expression of 65 kDa antigen protein was confirmed by Western blotting in B16/65kDa-1, B16/65kDa-2 (Fig. 3). We then compared the proliferation rates *in vitro* among the B16

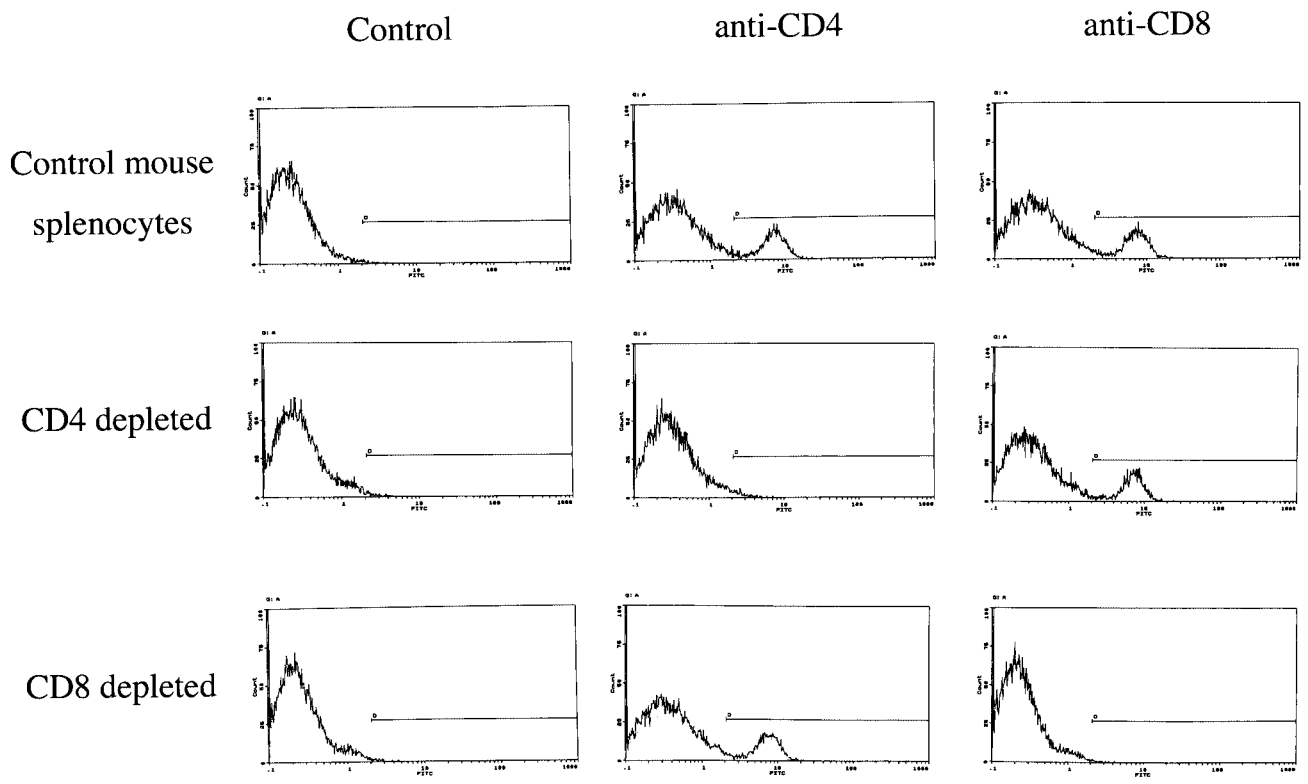

Fig. 1. Fluorometric analysis (EPICS Elite) of control, CD4-depleted and CD8-depleted mouse. Splenocytes were stained with rat MAbs GK1.5 (CD4) or 2.43 (CD8) followed by FITC conjugated anti-rat IgG absorbed with mouse IgG.

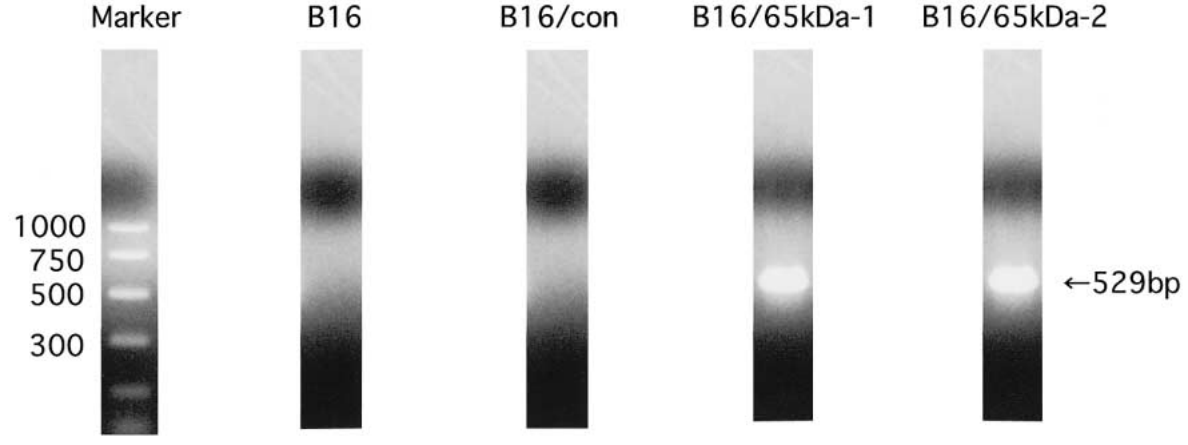

Fig. 2. RT-PCR of parental and B16 cell lines transfected with 65 kDa gene expressing 65 kDa mRNA. The corresponding band was observed in transfected cell line (B16/65kDa-1, B16/65kDa-2), but not in parental and mock-transfected lines (B16, B16/con).

sublines. There was no significant difference in cell proliferation *in vitro* among these cell lines (data not shown).

Tumorigenicity of 65 kDa Antigen Expressing B16 in Syngeneic Mice

To examine the effect of 65 kDa antigen on tumor growth *in vivo*, 5×10^6 cells of B16, B16/con, B16/65kDa-1 and B16/65kDa-2 were injected intraperitoneally to syngeneic C57/Bl mice. B16 and B16/con developed a similar degree of ascites. On the other hand, B16/65kDa-1 and B16/65kDa-2 slightly retarded ascites formation (Fig. 4) and in the following experiments, the results were the same. Thus, representative data of B16/65kDa-1, described as B16/65kDa are shown.

BCG Immunotherapy Model (Fig. 4)

Injecting the peritoneal cavity of syngeneic mice with 5×10^6 B16 cells caused tumor ascites to form within 2 weeks. However, two prior intraperitoneal injections

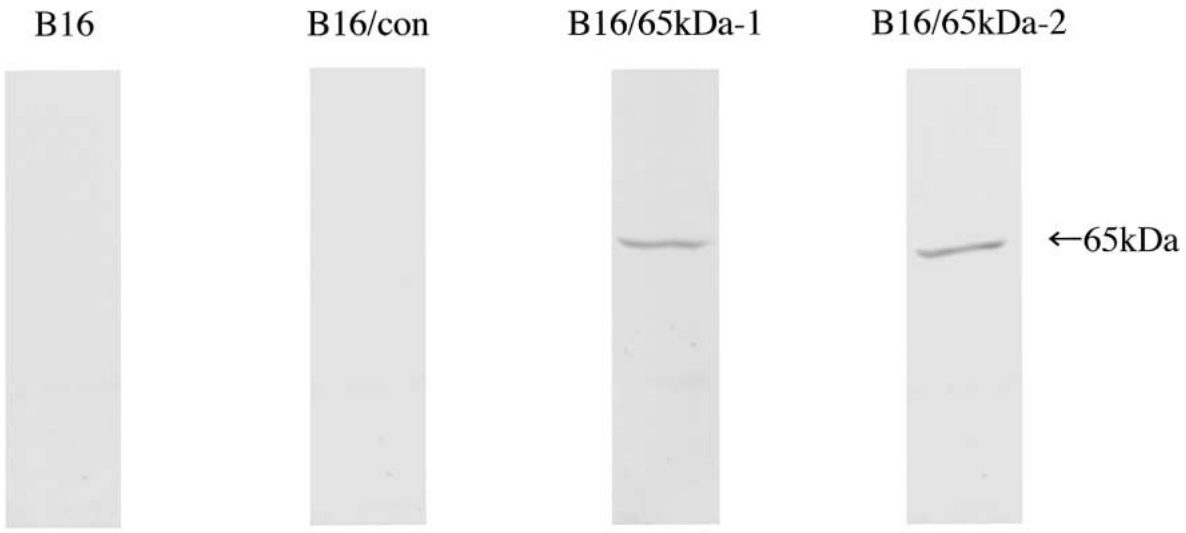

Fig. 3. Western blots of parental and B16 cell lines transfected with the gene expressing 65 kDa protein. The corresponding band was observed in transfected cell lines (B16/65kDa-1, B16/65kDa-2), but not in parental and mock-transfected lines (B16, B16/con).

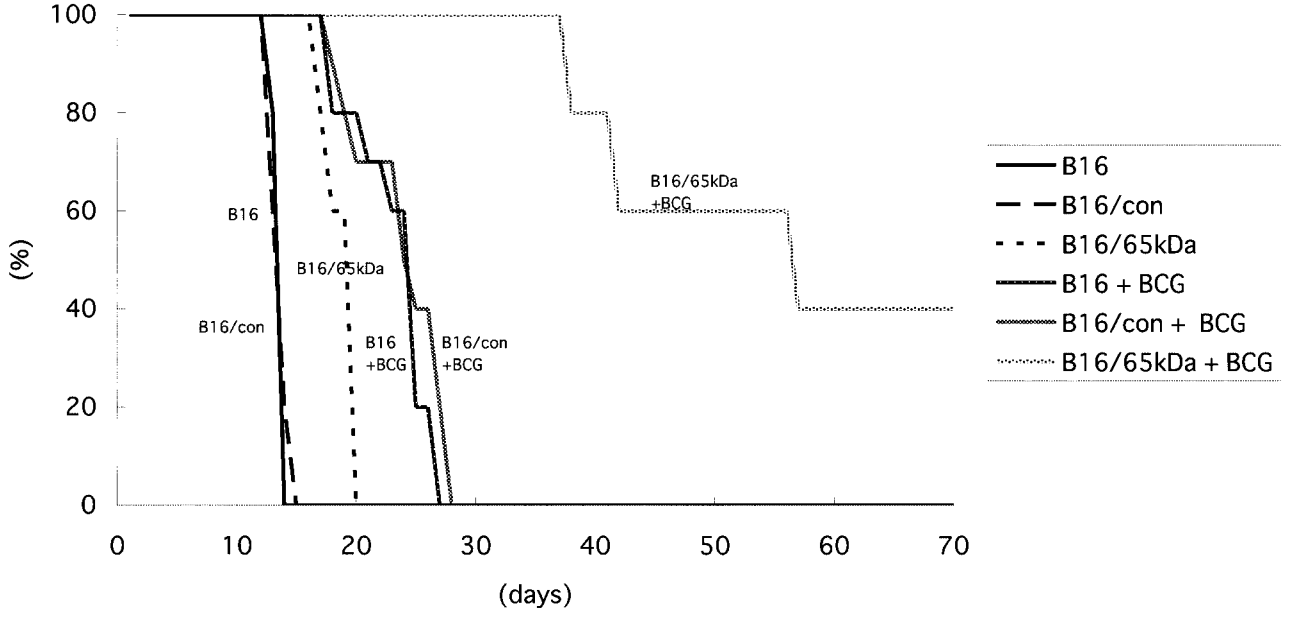

Fig. 4. Growth of tumor ascites in mice intraperitoneally injected with 5×10^6 B16, B16/con and B16/65kDa cells.
Formation of tumor ascites was examined 3 times each week and the percentage of ascites free mice was calculated. Y axis: percentage of ascites free mice. X axis: days after injection of tumor cells. Mice pre-immunized with 1 mg of live BCG twice are described as B16+BCG, B16/con+BCG and B16/65kDa + BCG.

of 1 mg of BCG significantly inhibited ascites formation. B16/con elicited a similar anti-tumor effect. To confirm that BCG specifically caused the anti-tumor effect, we injected the same amount of *E. coli* using the same schedule. However, tumor growth *in vivo* was not affected (data not shown). None of the mice injected with either BCG or *E. coli* alone developed ascites.

We examined how the expression of 65 kDa antigen in tumor cells affects BCG immunotherapy. The anti-tumor effect of BCG immunotherapy was remarkably enhanced by 65 kDa antigen expression. Although BCG immunotherapy inhibited tumor formation by parental B16 cells, all of the mice eventually developed ascites. On the contrary, 40% of mice injected with B16/65kDa completely rejected the tumor.

Mechanism of BCG Immunotherapy

To elucidate the mechanism of BCG immunotherapy, immuno-deficient mice were established using a monoclonal antibody against the corresponding CD molecule

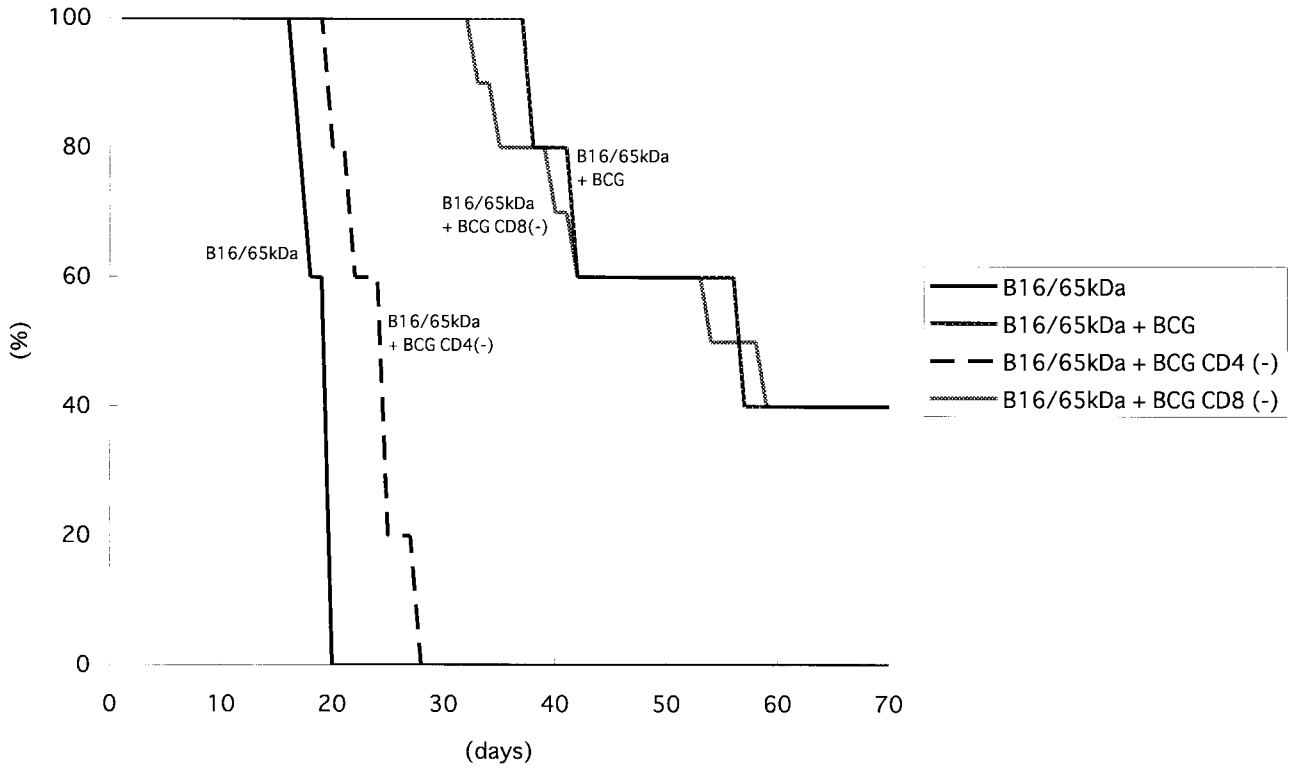

Fig. 5. Growth of tumor ascites in mice immunized with BCG (B16/65kDa+BCG) and then intraperitoneally injected with 5×10^6 B16/65kDa cells. Formation of tumor ascites was examined 3 times each week and the percentage of ascites free mice was calculated. Y axis: percentage of ascites free mice. X axis: days after injection of tumor cells. To elucidate the anti-tumor mechanism, the same experiments were performed in mice depleted of CD4 (B16/65kDa+BCG CD4 (−)), or CD8 (B16/65kDa+BCG CD8 (−)). Control curves show formation of tumor ascites in non-immunized mice intraperitoneally injected with 5×10^6 B16 cells.

(Fig. 1). The anti-tumor effect of BCG immunotherapy against B16/65kDa was abolished in mice depleted of CD4, but not of CD8 (Fig. 5). These results suggest that CD4 T cells, but not CD8 T cells, play an important role in the killing mechanism associated with BCG immunotherapy.

Discussion

BCG is an important clinical tool because of its profound immunostimulatory properties, as it can prevent tuberculosis and eradicate high-risk superficial bladder cancer. The presence of carcinoma *in situ* (CIS) in the urinary bladder had required total cystectomy, since CIS frequently progresses to invasive bladder cancer. BCG instillation therapy is believed to be the most successful immunotherapy against cancer because 70% of CIS patients can be cured without cystectomy (25). BCG instillation therapy also works well against T1b grade 3 bladder cancer, which is thought to have high malignant potential (11). However, some patients are refractory to BCG therapy and still require total cystectomy (9). To improve the efficiency of BCG against bladder cancer, the exact mechanism of its anti-tumor effect must be clarified. Here, we focused on the 65 kDa heat shock protein of mycobacteria, which can elicit powerful humoral and cellular immune responses. We reported that BCG instillation therapy raises a serum antibody against the 65 kDa protein (6), indicating that this protein plays an important role in BCG immunotherapy.

A wide range of mycobacterial species express the 65 kDa protein (2, 28). This antigen contains epitopes that are species-specific and those that are common to various species of mycobacteria. Identity has been demonstrated between *Mycobacterium tuberculosis* and BCG 65 kDa protein (21). The 65 kDa antigen has several features. The immune response to the BCG 65 kDa protein is involved in adjuvant arthritis in rats (26). Moreover, T cells from the synovial fluid of patients with rheumatoid arthritis can recognize the 65 kDa protein (1). Thus, this protein plays an important role in autoimmune disease. The 65 kDa protein is one of the heat shock proteins (hsp) induced by various types of stress to protect cells from environmental damage (22). Many hsp are also essential for cellular function under normal conditions and they can also function as molecular chaperones to fold and transport proteins.

We aimed to introduce the mycobacterial 65 kDa

antigen to tumor cells to increase their immunogenicity. The tumor growth of B16 (B16/65kDa) cells transfected with the 65 kDa gene was slightly retarded compared with parental B16 cells in naive mice. Moreover, introduction of the 65 kDa gene remarkably enhanced the anti-tumor effect of BCG immunotherapy. We believe that this strategy represents a breakthrough for BCG immune therapy for bladder cancer. Lukacs et al. originally reported the possibility of immune gene therapy using mycobacterial 65 kDa hsp (14). They introduced the 65 kDa hsp of *Mycobacterium leprae* to murine macrophage tumor cell lines (J774). The transfected cell line (J774-hsp65) was totally abrogated when injected into syngeneic Balb/c mice. This anti-tumor effect was also evident in athymic nude mice, indicating that the anti-tumor property of J774-hsp65 does not require T cells. Mice injected with J774-hsp65 resisted subsequent challenge with parental J774. On the contrary, both CD4 and CD8 T cells were essential for this acquired immunity. Lukacs also described *in vivo* gene therapy using liposome-mediated transfection with the same antigen (15). Kuromatsu et al. introduced the α antigen of *Mycobacterium kansasii* into a mouse bladder cancer cell line (13). The transfected cell lines were rejected in mice primed and unprimed with BCG. That study proved the induction of cytotoxic T lymphocyte (CTL) epitope-specific CD8 CTLs. The α antigen is a different protein from the 65 kDa hsp. However, both are highly immunogenic mycobacterial antigens that can elicit a powerful anti-tumor effect when expressed by tumor cells.

Our findings were somewhat different from those of Lukacs. The anti-tumor effect in our model was not as powerful as theirs, since the immunogenic properties of B16 and J774 are quite different. B16 is a tumor cell line with low immunogenicity. J774 is a macrophage tumor cell line that can present antigen, which might be associated with its powerful anti-tumor effect. Of interest is the effector cells in each model. J774 does not require T cells to reject tumors whereas the main effectors in BCG therapy against B16/65kDa were CD4 T cells. Shinomiya et al. also showed Th1 type CD4+ T cell clone, which was capable of recognizing purified protein derivative from *Mycobacterium tuberculosis*, had anti-metastatic activity against melanoma. This clone could secrete IFNγ, TNF and interleukin-2 which stimulated NK cell activity (23). Considering that CD4 T cells are powerfully stimulated by mycobacterial infection, humoral mediators such as interferon and TNF may play an important role in the killing mechanism of BCG immunotherapy.

In this paper, we successfully showed that the introduction of 65 kDa hsp gene enhanced BCG immunotherapy. The next step is to establish the effective method to introduce the gene into bladder cancer for clinical settings. Several papers reported the gene introduction by instillation therapy with adenovirus or liposome (10, 12, 20). Pagliaro et al. reported a phase I study of p53 gene therapy for patients with locally advanced bladder cancer and showed evidence or tumor response (19). In the future, we will try to establish instillation therapy combined with BCG and 65 kDa gene to improve the current BCG therapy.

In conclusion, expression of the 65 kDa mycobacterial hsp specifically enhanced the anti-tumor effect of BCG therapy against a malignant melanoma cell line with low immunogenicity. In addition, CD4 T cells but not CD8 T cells are involved in this mechanism.

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