Korean J Biol Sci 8: 117-123, 2004

Administration of Agonistic Anti-4-1BB Monoclonal Antibody Inhibits Melanoma Metastasis Via IFN-γ Production

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Key Words:
4-1BB
IFN-γ

The purpose of this study was to analyze inhibitory effects of anti-4-1BB monoclonal antibody on melanoma metastasis. The 4-1BB (CD137) T cell molecule is a member of the TNF receptor family and its activation by either 4-1BB ligand or antibody induces T cell activation and growth. In the present study, administration of anti-4-1BB mAb induced inhibition of melanoma metastasis. Agonistic anti-4-1BB mAb induced not only $CD8^+4\text{-}1BB^+$ T cells but also $CD8^+IFN\text{-}\gamma^+$ T cell population. In the presence of anti-CD3 antibody, lymphocytes produced high levels of IFN-γ and low levels of IL-4 in anti-4-1BB mAb treated group. Exposure of melanoma cells to IFN-γ induced expression of MHC-I molecules. Thus, the increase in number of $CD8^+$ T cells and enhanced MHC-I expression on B16F10 cells by augmented IFN-γ production in response to anti-4-1BB mAb may result in suppression of tumor growth and metastasis.

Tumors are usually recognized by uncontrolled proliferation and spread of clones of transformed cells. Cancers are fully developed (malignant) tumors with a specific capacity to invade and destroy the underlying mesenchyme. In addition, it is believed that malignant tumors evade or overcome the host immune systems. Immunotherapy is treatment by immunological means. In active immunotherapy the tumor bearer's own immune system should induce tumor-specific T cell-mediated immunity that can recognize tumor antigen and block the growth and metastasis of malignant tumor cells (Boon et al., 1992). However, such aim of immunotherapy has been difficult to achieve because tumors are poorly immunogenic and suppress immune response of the host (Boon et al., 1992; Li et al., 1996). To overcome this problem, it is essential to activate immune cell function at the tumor site (Hamaoka et al., 1987).

T lymphocytes require two signals for optimum activation: TCR ligation and costimulation. Costimulation provides an independent stimulus that influences the outcome of T cell-APC interaction. Failure to receive this signal has been shown to render T cells anergic (Mueller et al., 1989). The 4-1BB, which is an inducible T cell Ag present on $CD4^+$, $CD8^+$, and NK1.1 cells, provides CD28-independent costimulation of T cell activation (Melero et al., 1998; Vinay et al., 1998). Murine 4-1BB is a 30-kDa glycoprotein that belongs to the TNF receptor superfamily (Kwon et al., 1989).

There is substantial evidence that 4-1BB may play a role in the development of antitumor immunity. Monoclonal Abs (mAbs) have been developed that bind to 4-1BB and provide a costimulatory signal to activated T cells. It has been demonstrated that *in vivo* administration of anti-4-1BB mAbs results in the regression of established s.c. and pulmonary P815 mastocytoma and AG104A sarcoma (Melero et al., 1997).

In an attempt to investigate the role of 4-1BB stimulation in the tumor metastasis, we assessed the effects of anti-4-1BB mAb on productions of IFN-γ by murine leukocytes in B16 melanoma mice. Malignant melanoma is the prototype of extremely aggressive tumors against which many immunotherapeutic approaches have been tried. Our data obtained from *in vivo* experiments using agonistic anti-4-1BB mAb have revealed 4-1BB stimulation by mAb and suggested that this might be an efficacious way to block metastasis in tumor-bearing hosts. Taken together, these data suggest that the effect of anti-4-1BB mAb is dependent on enhanced IFN-γ secretion.

Materials and Methods

Animals and antibodies

Male C57BL/6 mice (Jackson) 7 wk of age were maintained in specific-pathogen-free facility. 3H3, an agonistic anti-4-1BB mAb was is a generous gift of Dr. Mittler (Klussman et al., 1997) and was purified from

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ascitic fluids of Balb/c nude mice by affinity chromatography on protein G Agarose column (Gibco). Clone 3H3 were used to treat tumor and clone 3E1 was used to stain 4-1BB. Purified rat IgG, Fc receptor-blocking mAb 2.4G2, PE-CD8, FITC-H-2K^b were purchased from BD PharMingen.

Tumor cell lines and animal experiments

B16F10 melanoma cells were a kind gift of Dr. I. J. Fidler (Texas M & D Anderson Medical Center) and cultured in RPMI 1640 medium supplemented with 10% FBS. For animal experiments, cells were passed two to five times with medium after their regrowth from frozen stocks. Cells in a log growth phase were detached from tissue culture flasks using a mixture of 0.25% trypsin and 0.03% EDTA. The cells were then washed and suspended in PBS just before inoculation to mice. 2×10^5 B16F10 cells in 0.2 ml suspension were injected into the tail vein of mice. Anti-4-1BB mAb was injected i.p. on 1, 3 and 5 days post tumor injection. Rat IgG was injected as a control. The number of black metastatic colonies in lungs was counted under a dissecting microscope (Yokoyama et al., 1986).

FACS analysis

For FACS analysis, lymphocytes were blocked with Fc receptor-blocking mAb 2.4G2 for 20 min at 4°C and stained with PE-conjugated CD8 or 4-1BB mAb. Expression of 4-1BB on lymphocytes was measured by staining with FITC-conjugated anti-4-1BB (clone 3E1) mAb. After washing, cells were analyzed by FACSCalibur™ (Becton Dickinson).

Cytokine ELISA

To measure cytokine production, 2×10^6 splenocytes from B16F10 mice were cultured in 200 µl culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in 96-well plates (Costar) precoated with 1 µg/ml hamster anti-mouse CD3 mAb (145-2C11; BD PharMingen) in PBS overnight at 4°C. Culture supernatants were collected after 48 h and assayed by ELISA kit (R&D) for IL-4 and IFN-γ.

Intracellular cytokine analysis

Splenocytes (2×10^6/well) were isolated from B16F10 bearing mice on 14 day and cultured 48 h with PMA (200 ng/ml) and ionomycin (500 ng/ml). Before the termination of cultures, cells were treated for 5 h with Golgi-stop (BD PharMingen) to inhibit cytokine release. Following surface staining for CD8 cells were fixed in Cytofix/Cytoperm solution (BD PharMingen) and then stained with FITC-conjugated anti-mouse IFN-γ diluted in 1X perm/wash solution (BD PharMingen). Samples were analyzed on a FACSCalibur (BD Biosciences) and analyzed by CellQuest software (BD Biosciences).

ELISA with sera of immunized mice

Sera from mice injected with rat IgG or 4-1BB mAb were taken on day 14. ELISA was performed to detect the melanoma antibody level as described elsewhere. Briefly, ELISA plates in a 96-well format were coated with B16F10 melanoma cells as target. Different serum dilutions were incubated on freshly coated ELISA plates. A secondary anti-mouse horseradish-coupled antibody was used for substrate reaction. The absorbance measured at the wavelength of 450 nm. Each measurement was carried out in triplicate.

IFN-γ treatment of B16F10 cells

B16F10 cells were cultured for 48 h in RPMI 1640 medium supplemented with 10% FBS containing 200 U/ml mouse rIFN-γ, then harvested and washed, and used for cytometry or cytotoxicity assays.

Assay of CTL activity

CTL activity was measured using a standard 4-h ^{51}Cr release assay with B16F10 targets at the indicated effector/target ratios. Splenocytes isolated from B16F10 mice were used as effector cells and B16F10 cells preincubated with 200 U/ml of IFN-γ for 24 h, were used as target cells.

Results

Agonistic anti-4-1BB mAb (3H3) inhibits pulmonary metastasis

To evaluate whether triggering 4-1BB by an agonistic mAb might further protect the host from tumor, various doses of anti-4-1BB mAb were tested. Three injections of 100 µg per mouse reduced the number of colonies in the lung remarkably compared to control antibody (Fig. 1A,B). This result suggests that the anti-4-1BB mAb treatment has inhibitony effects on pulmonary metastasis.

Anti-4-1BB mAb treatment inhibits antibody production

To determine the effect of anti-4-1BB mAb on the generation of humoral immunity to B16F10 cells, melanoma antibody production was measured. We injected B16F10 melanoma cells into C57BL/6 mice on day 0. Rat IgG or 4-1BB mAb were injected on day 1, 3 and 5. Fig. 2A shows the daily change of melanoma antibody titer in sera taken from the anti-4-1BB mAb or control IgG treated tumor-bearing mice. The sera were

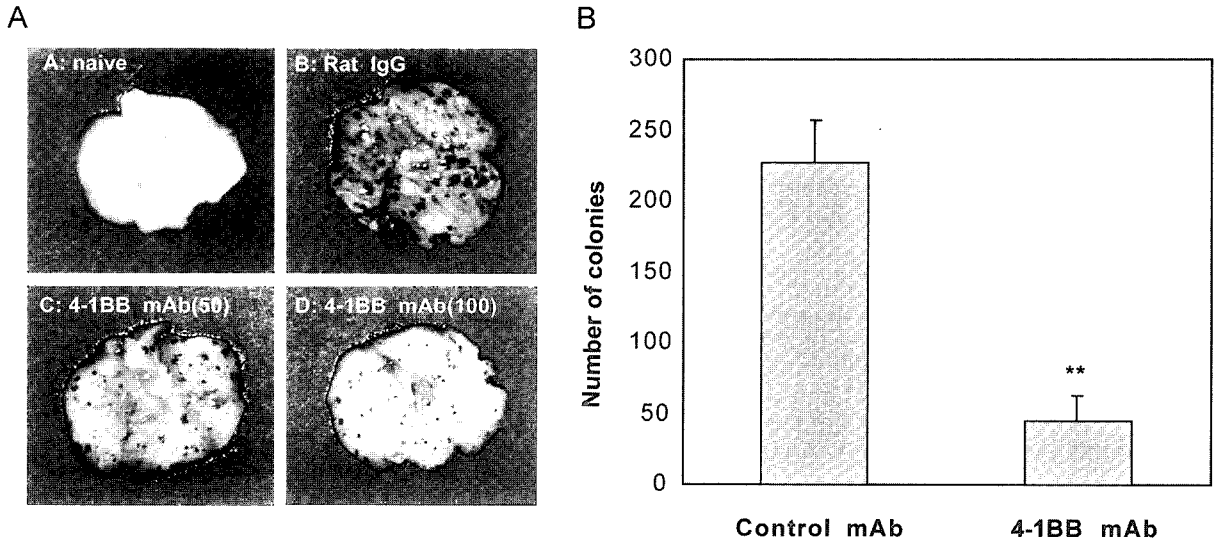

Fig. 1. Amount of pulmonary metastasis in B16F10 mice treated with anti-4-1BB mAb. A, Photographs of metastatic colonies in the lungs were taken after 14 days. 2×10^5 B16F10 melanoma cells were injected i.v. on Day 0, and various doses (0, 50, and 100 μg/mouse) of agonistic anti-4-1BB mAb (clone 3H3) were injected i.p. on Day 1, 3 and 5. As a control, rat IgG was used. B, Amount of pulmonary metastasis in B16F10 mice treated i.p. with 100 μg/mouse of anti-4-1BB mAb or rat IgG. The numbers of metastatic colonies in lungs of these mice were counted on Day 14 after the cell injection. The results are expressed as the mean±SE (** $P<0.01$ by ANOVA).

incubated on ELISA plates coated with cell extracts of B16F10 melanoma cells. Antibody production (Th2 cytokine) from sera were measured at 6, 8, 10, 12, 14 days after tumor inoculation. The results show that anti-4-1BB mAb treated mice had low levels of antibody titer compared to control IgG treated mice. To assess the effect of concentration of anti-4-1BB mAb on the generation of humoral immunity to B16F10 cells, we injected various doses of anti-4-1BB mAb into B16F10 mice. Melanoma antibody titer in sera were decreased in a manner dependent on anti-4-1BB mAb dose (Fig. 2B).

Anti-4-1BB mAb treatment augments IFN-γ production

To determine the daily change in Th cell responses in tumor-bearing mice, B16F10 cells were inoculated i.v. into C57BL/6 mice (B16F10 mice). IFN-γ production (Th1 cytokine) and IL-4 production (Th2 cytokine) from

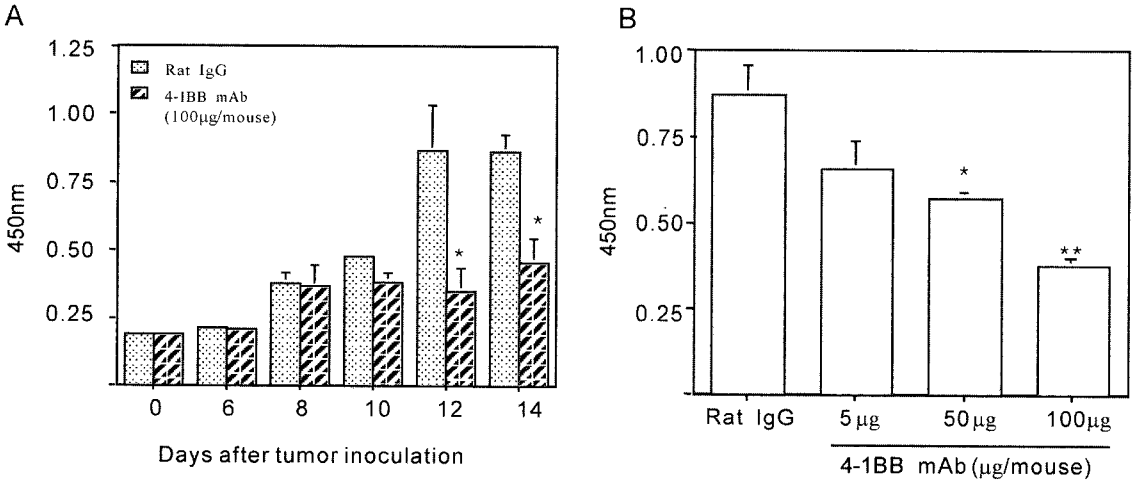

Fig. 2. Anti-4-1BB mAb treatment inhibits antibody production. A, Melanoma antibody titer at varying time points was determined by ELISA. Mice were bred on Day 0-14 of B16F10 cell challenge. At the indicated times, sera were collected from each mouse, and melanoma antibody was determined by ELISA. The antibody titers in the mouse sera are given as their relative absorbance at a wavelength of 450 nm. Each measurement was performed in triplicate. The standard deviations are indicated as error bars. B, Melanoma antibody titer in sera of various doses of 4-1BB mAb treated on B16F10 mice. 2×10^5 B16F10 melanoma cells were injected i.v. on day 0. Rat IgG or various doses of anti-4-1BB mAb were injected day 1, 3 and 5. On day 14, the sera were incubated on ELISA plates coated with cell extracts of B16F10 melanoma cells. Each measurement was performed in triplicate. The standard deviations are indicated as error bars (*,** $P<0.05$, 0.01, respectively by ANOVA).

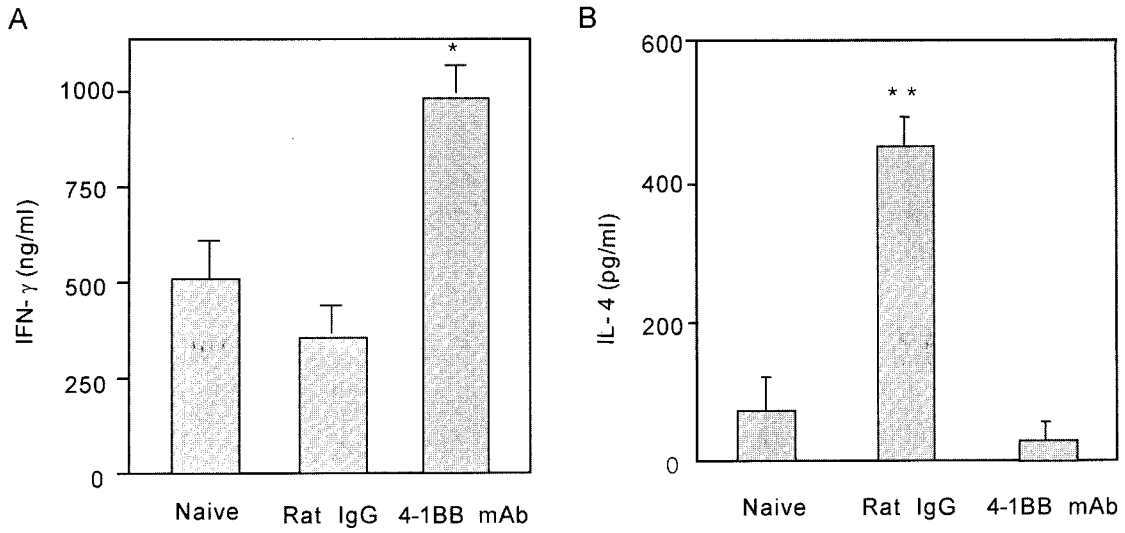

Fig. 3. Effect of anti-4-1BB mAb treatment on IFN-γ and IL-4 production. 2×10^5 B16F10 cells were injected i.v. on day 0. Rat IgG or anti-4-1BB mAb were injected on Day 1, 3 and 5. On day 14, splenocytes were isolated, and cultured in the presence of anti-CD3 mAb for 48 h. IFN-γ (A) or IL-4 (B) production in culture supernatants were analysed by ELISA (*$p<0.05$ **$p<0.01$ by ANOVA).

splenocytes stimulated by anti-CD3 mAb (1 μg/ml) *in vitro* were measured at 3, 7 10, 14 days after tumor inoculation. IFN-γ production was significantly decreased in a time dependent manner after tumor inoculation. IL-4 production was increased at 7, 10, 14 days. This result indicated that Th cell responses in B16F10 mice tend to be toward Th2 dominance (data not shown). On the other hand, to determine the relationship between Th1 cell

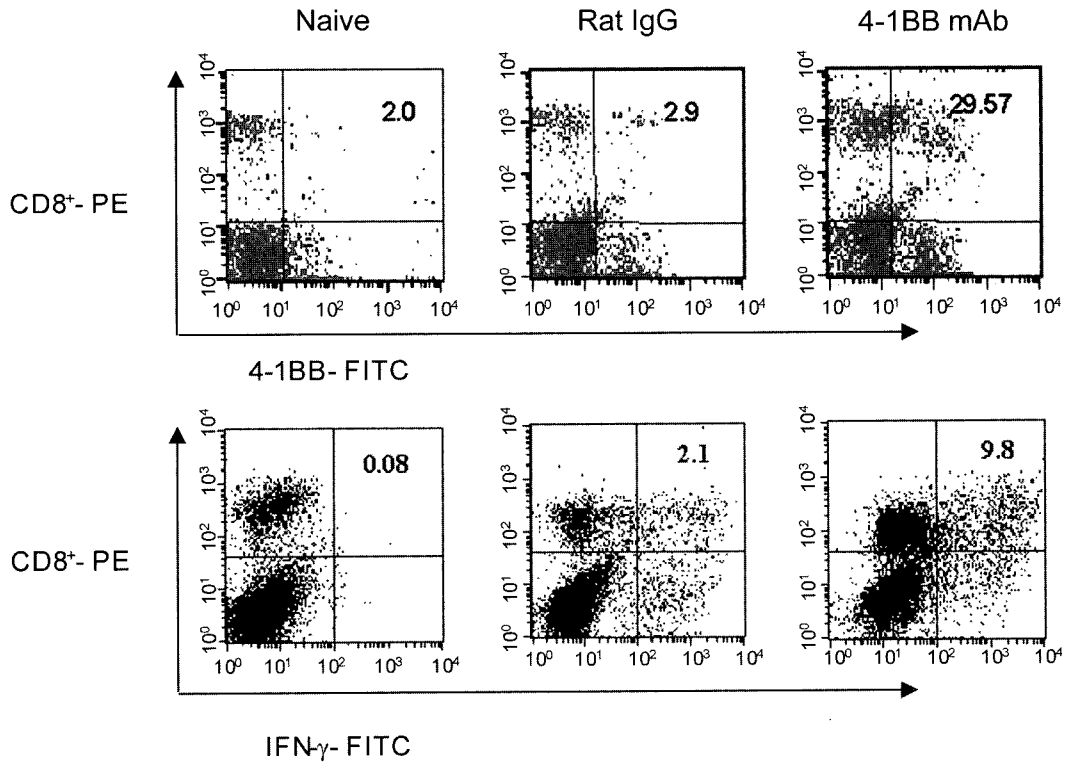

Fig. 4. Anti-4-1BB mAb treatment increases the number of $CD8^+4\text{-}1BB^+$ T cell and $CD8^+$ IFN-γ^+ T cell population. A, Lymphocytes were isolated from anti-4-1BB mAb treated or rat IgG treated on day 10 after tumor injection. Cells were double stained with PE-conjugated anti-CD8 and FITC conjugated anti-4-1BB and analyzed by FACS. B, Lymphocytes were isolated and cultured 48 h with PMA (200 ng/ml) and ionomycin (500 ng/ml). Cytokine release was blocked by treatment with bredfeldin A. Cells were doubly stained with PE conjugated anti-CD8 and FITC-conjugated anti-IFN-γ.

responses and 4-1BB, we measured IFN-γ and IL-4 production in cultures splenocytes from anti-4-1BB mAb treated B16F10 mice. Fig. 3 shows IFN-γ and IL-4 production in cultures of splenocytes of anti-4-1BB mAb treated or control IgG treated groups. The culture supernatant of anti-4-1BB mAb treated group allows high IFN-γ production and low IL-4 production compared with control group. Taken together, our data suggest that anti-4-1BB mAb can be switched to a Th1 response.

Anti-4-1BB mAb treatment increases the number of CD8⁺4-1BB⁺T cell and CD8⁺ IFN-γ⁺ cell population

To understand the mechanisms underlying the inhibition of metastasis by anti-4-1BB mAb, we examined the phenotypic change of lymphocytes in the B16F10 mice. Stimulation of 4-1BB increased the populations of CD4, CD8, CD11b, and NK1.1 (data not shown). Among of them, the most of highly increased population was CD8⁺T cell population. Fig. 4A shows a 10 fold increase in CD8⁺4-1BB⁺ T cell population. In order to examine whether this increase functionally effective, we performed immunofluorescent staining assay by flow cytometry analysis. IFN-γ, in particular, has been considered as a key cytokine in tumor immunity. On the basis of our observation, we analysed IFN-γ producing CD8⁺ cell population in anti-4-1BB mAb or control IgG treated B16F10 mice. As shown in Fig. 4B, IFN-γ producing CD8⁺ T cell population was 4.7 fold greater than control antibody treated mice.

Anti-4-1BB mAb treatment enhances CTL activities of B16F10 tumor bearing-mice

To determine the effects of IFN-γ on B16F10 cells, we measured surface expression of MHC molecules on mouse rIFN-γ treated B16F10 cells. The expression levels of MHC class I on malignant cells are important for determining the malignant and metastatic capacities of tumor cells (Kawano et al., 1986). MHC class I-expressing melanoma cells contain antigens that can activate cytotoxic CD8⁺ T cells, although they are weakly immunogenic (Bellone et al., 1994; Bloom et al., 1997; Harada et al., 1998). B16 melanoma cell lines cultured in culture media expressed only very low levels of MHC-I molecules on cell surface. When the cells were treated with IFN-γ, the surface expression of class I antigens were enhanced significantly (Fig. 5A).

To further analyze the mechanisms behind the anti-tumor immunity of anti-4-1BB mAb treatments, we evaluated cytotoxic activities of splenocytes against B16 target cells. ^{51}Cr release cytotoxicity assay for 4 h was performed with B16 cells preincubated with IFN-γ to increase MHC expression. The cytotoxic activity of CD8⁺ T cells against IFN-γ-treated B16 melanoma cells was significantly enhanced with the cells from anti-4-1BB mAb-treated mice (Fig. 5B).

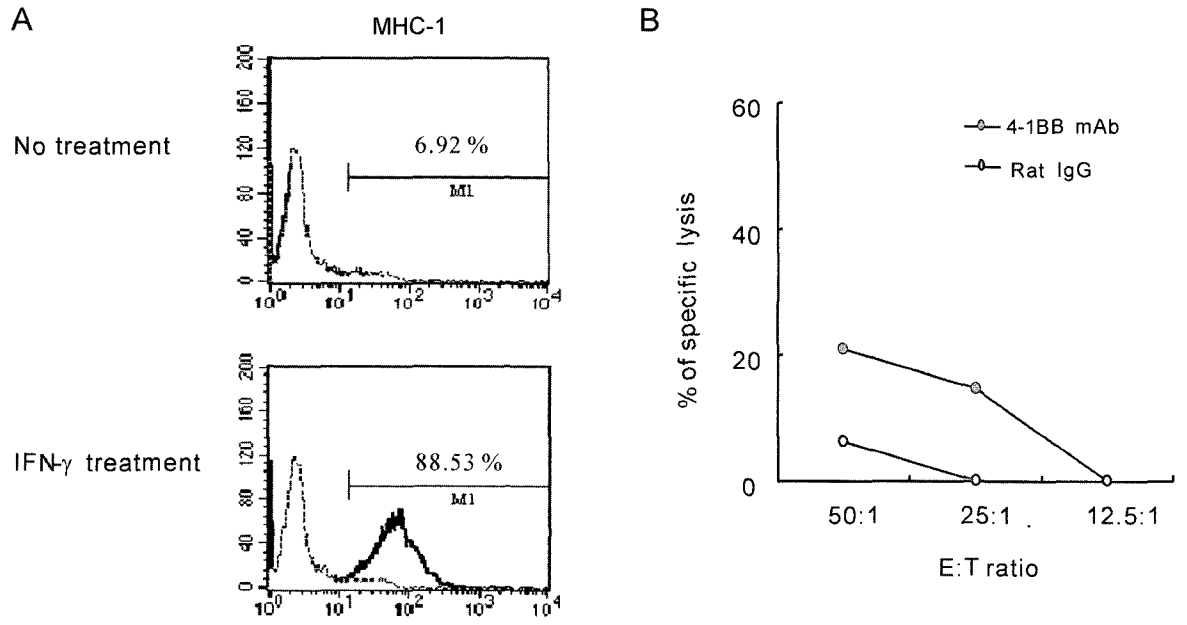

Fig. 5. Enhanced CTL activity by anti-4-1BB mAb treatments. A, Surface expression of MHC-I on B16F10 cells. B16F10 cells cultured in 10% FBS contained RPMI medium were unstimulated or stimulated for 48 h with 200 U/ml IFN-γ. Surface expression of MHC-I was measured using FACS analysis. B, Cytolytic activities of lymphocytes from control antibody or anti-4-1BB mAb treated mice. CTL activity was set up using lymphocyte as effectors and IFN-γ-pretreated and ^{51}Cr-labeled B16F10 cells as targets at the indicated effector/target ratios. Cells were incubated for 4 h and radioactivities released were counted.

Discussion

Interferon γ (IFN-γ) is a pleiotropic cytokine that plays a central role in promoting innate and adaptive mechanisms of host defense (Boehm et al., 1997). It is known that this cytokine plays a key role in promoting antigen processing and presentation via both the MHC class I and Π pathways (Dighe et al., 1994; Webber et al., 1998). Thus it can be speculated that IFN-γ, by inducing in tumor cells specific cellular proteins involved in antigen processing/presentation, may force the transformed cell to up-regulate expression of the appropriate antigenic peptide(s), leading to its recognition and elimination by the immune system. IFN-γ, therefore, plays a central role in promoting tumor surveillance, and this system may in fact be the basis of immune surveillance (Kaplan et al., 1998).

4-1BB (CD137) is known as a costimulatory molecule expressed in activated T cells, natural killer cells, and dendritic cells (Hurtado et al., 1997; Sica et al., 2000; Wilcox et al., 2002). Cell signaling via 4-1BB pathways can promote T-cell proliferation and cell survival (Takahashi et al., 1999; Cannons et al., 2000). The use of 4-1BB mAb as a therapeutic agent for established cancer has been explored previously by several investigators. The *in vivo* administration of anti-4-1BB mAb was first discovered by Melero et al. to have antitumor efficacy against weakly immunogenic s.c. tumors that was mediated by both $CD4^+$ and $CD8^+$ T cells. On the other hand, Kim et al. reported that poorly immunogenic s.c. and pulmonary tumors were refractory to anti-4-1BB mAb. In such cases, the use of 4-1BB mAbs to enhance antitumor immune responses has shown mixed results, depending upon the immunogenicity of the tumor and animal strain, and degree of T cell activation *in vivo* (Melero et al., 1997; Kim et al., 2001).

The purpose of this study was to analyse inhibitory mechanisms of melanoma metastasis through costimulation of anti-4-1BB mAb. In the present study, using the B16 melanoma, a poorly immunogenic experimental tumor that expresses low levels of MHC class I products, we assessed the effects of anti-4-1BB mAb on production of IFN-γ by murine leukocytes in B16 melanoma mice. Malignant melanoma is the prototype of an extremely aggressive tumor for which many immunotherapeutic approaches have been designed. In this study, we have used an agonistic anti-4-1BB mAb as the stimulus to examine its effects on melanoma therapy. Using an agonistic anti-4-1BB mAb, we were able to demonstrate inhibition of melanoma metastasis. Fig. 3 shows IFN-γ and IL-4 production in cultures of splenocytes of anti-4-1BB mAb treated or rat IgG treated groups. The culture supernatant of anti-4-1BB mAb treated group allows high IFN-γ production. This may represent an important mechanism accounting for the potent therapeutic efficacy of anti-4-1BB mAb. When 4-1BB is stimulated with an agonistic mAb, an highly increased $CD8^+$ T cell population was observed. Fig. 4A shows 10 fold increase in $CD8^+4$-$1BB^+$ T cell population. In order to examine whether the increased $CD8^+$ T cell population has effective function, we performed immunofluorescent staining assay by flow cytometry analysis. As shown in Fig. 4B, IFN-γ producing $CD8^+$ T cell population was 4.7 fold greater than the control antibody treated mice. Our study suggests that the augmented antitumor effect due to the use of anti-4-1BB mAb was dependent on the enhanced IFN-γ secretion and the number of $CD8^+$ T cell population. Poorly immunogenic tumors, which often lack surface expression of MHC class I (Kawang et al., 1986). B16F10 also lack surface expression of MHC class I. The decrease in class I MHC expression can be accompanied by progressive tumor growth, and the absence of MHC molecules on a tumor is generally an indication of a poor prognosis. Surface expression of MHC-I molecules by B16F10 cells was low, but was strikingly up-regulated by IFN-γ treatment (Fig. 5A). The mechanisms of anti-4-1BB mAb in enhancing tumor immunity remain to be fully elucidated (Ryan et al., 2002). Using an agonistic 4-1BB mAb, we demonstrate inhibition of melanoma metastasis *in vivo*, which may explain this effect, at least in part.

In summary, the increase in number of $CD8^+$ T cells and enhanced MHC-I molecules on the B16F10 cells by augmented IFN-γ production in response to anti-4-1BB mAb may result in suppression of tumor growth and metastasis.

Acknowledgements

This work was supported by a Korea Research Foundation grant (KRF-2002-075-C00017).

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[Received April 19, 2004; accepted May 24, 2004]