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Antitumor immunity induced by tumor cells engineered to express a membrane-bound form of IL-2

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Abbreviation: mbIL-2, membrane-bound form of IL-2

Abstract

Transduction of cytokine gene into tumor cells is a promising method of tumor therapy, but the value is limited by accompanying side effects. To focus antitumor immune response to tumor antigen-specific CTL, we developed an antitumor vaccine by transfecting modified IL-2 gene in a membrane-bound form (mbIL-2) into B16F10 melanoma cells. The mbIL-2 clone showed reduced tumorigenicity and metastatic ability, and inhibited metastasis and prolonged the survival of mice against B16F10 cells. The inhibition of B16F10 metastasis by mbIL-2 was accompanied by the increment of $CD8^+$ T cells. The metastasis of mbIL-2 clone was significantly increased in the $CD8^+$ T cell-depleted mice, but not in $CD4^+$ T cell depleted mice. Spleen cells immunized with the mbIL-2 clone showed higher CTL activity towards B16F10 cells than those immunized with control cells. The size of $CD8^+$ T cell population in the lung of mice injected with the mbIL-2 clone was markedly greater than that of mice injected with B16F10 cells, but there was no detectible change in $CD4^+$ and $CD8^+$ T cell populations of lymph nodes and spleen. These results suggest that when the mbIL-2 clone is introduced into the blood stream, it migrates mainly to lung and activates $CD8^+$ T cells *in situ*, possibly by direct priming. Such a tumor vaccine may ameliorate the toxic side effects encountered with conventional cytokine gene therapy.

Keywords: cancer vaccines; immunotherapy, active; interleukin-2; neoplasm metastasis; T lymphocytes, cytotoxic

Introduction

Several strategies employing cytokines to enhance host immunity have been developed for use in experimental cancer therapy. Recombinant IL-2 is usually applied systemically and therapeutic effects generally are often accompanied by severe toxic side effects (Lotze MT *et al.,* 1986; Kammula *et al.,* 1998; Chen *et al.,* 2001; Yang *et al.,* 2003). The transfer of IL-2 gene into tumor cells has the advantage that it can achieve local activation of the immune system at the tumor site (Fearon *et al.,* 1990; Gansbacher *et al.,* 1990; Becker *et al.,* 1996; Jackaman *et al.,* 2003; Hillman *et al.,* 2004).

A large number of different cytokines have been shown to stimulate anti-tumor immunity in studies employing tumor cells transduced with cytokine genes as therapeutic vaccines (Blankenstein *et al.,* 1991; Dranoff *et al.,* 1993; 1995; Tepper *et al.,* 1994; Nanni *et al.,* 1999). Even tumors that are poorly immunogenic can be recognized by MHC class I-restricted $CD8^+$ cytotoxic T cells, if the tumor cells are engineered to harbor the IL-2 gene. However, tumor cells engineered to produce cytokines have unexpected side effects (Dranoff *et al.,* 1993; Lollini *et al.,* 1995; Tjuvajev *et al.,* 1995) that may be caused by activation of bystander T cells, or differentiation and expansion of T cells, without evident specificity for the tumor antigen (Rivoltini *et al.,* 1990; Colombo *et al.,* 1992). Control of the effective range, dosage, and duration of cytokines may be critical for successful cytokine gene therapy.

Several cytokines, including IL-1 (Kurt-Jones *et al.,* 1987), TNFα (Kriegler *et al.,* 1988), LTα (Browning *et al.,* 1993) and IFN-γ (Assenmacher *et al.,* 1996) are expressed as membrane-associated forms and soluble forms. Though functional differentiation between membrane-bound and soluble forms has not been clearly demonstrated, the effective range of membrane-bound form may well be narrower. Interestingly, genetically engineered membrane-bound form of TNFα on tumor cells induced anti-tumor immunity with low toxic side effect (Marr *et al.,* 1997). Such modification of cytokine gene therapy adopting membrane-bound form has been expanded to GM-CSF (el-Shami *et al.,* 1999; Soo Hoo *et al.,*1999; Yei *et al.,* 2002), FLt3 ligand (Chen *et al.,* 1997), IL-4 (Kim *et al.,* 2000), and IL-12 (Cimino *et al.,* 2004). Furthermore, anchoring of IL-2 via diphtheria toxin T domain on tumor cells induced successful anticancer immunity

(Nizard *et al.,* 2003).

In the present study, we modified B16F10 cells to express a membrane-bound form of IL-2 (mbIL-2), and tested tumorigenicity and metastatic ability and therapeutic effects. We envisaged that if the tumor cells displayed the proper tumor-associated antigen/MHC complex together with IL-2, in a membrane-bound form, tumor antigen-specific CTL precursors might be selectively activated, so minimizing side effects. Moreover, the expression of IL-2 on tumor cells might compensate the reduced function of Th cells observed in most tumor-bearing mice (Zier *et al.,* 1996).

Materials and Methods

Cells and animals

The murine melanoma B16F10 cell line was cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (all from Sigma Chemical Co., St. Louis, MI). The tumor cell line maintained regular *in vivo* passages to preserve tumorigenicity and metastatic ability. The B16F10 cells were used for experiments while in the log phase of growth. CTLL-2 cells were maintained in the same medium extra-supplemented with 10 U/ml IL-2 (R&D Systems Inc., Minneapolis, MN). GK1.5 (anti-CD4) and 3.168 (anti-CD8) were given as subcutaneous injection of 1×10^6 cells/mouse on day -1, +3, +7 prior to 3-2E5 clone injection. Depletion of T cell subsets was confirmed by FACS analysis using anti-CD4 (L3T4 clone) and anti-CD8 (Ly-2 clone) antibodies (Pharmingen, San Diego, CA). The female C57BL/6 mice were purchased from the Orient company (Gapeung, Korea) and used at 6-8 weeks of age.

Plasmid construction and transfection

The cDNA of mouse IL-2 was purchased from American Type Culture Collection (Rockville, MD). The cDNA of mouse TNFα was obtained from Dr. Sang-Young Chun (Chonnam National University, Korea). To construct mbIL-2 chimeric cDNA, primers specific for IL-2 (sense; 5'-CGCGAATTCATGTACAGCATG-CAGCTCGCA-3', antisense; 5'-GCGCCATGGTTGAG-GGCTTGTTGAGATGAT-3') and TNFα (sense; 5'-GCGGATCCATG-AGCACAGAA-3', antisense; 5'-CG-CGAATTCCTCCGGCCATAGAACT-3') were used to amplify the respective cDNA fragments. The 525 bp IL-2 cDNA fragment was digested with *Hin*cII/*Bam*HI to exclude IL-2 signal peptide region. The 240 bp TNFα cDNA fragment encoding transmembrane region (from -79 to -45), cytoplasmic part (from 44 to -24), and 19 extracellular amino acids (from -23 to -5) was ligated to the 525 bp IL-2 cDNA fragment. The chimeric cDNA was then subcloned in pNeoSRαII expression vector.

For electroporation, B16F10 cells were harvested and washed twice in HBSS (137 mM NaCl, 5 mM KCl, 0.7 mM H_3PO_4, 6 mM dextrose, 20 mM HEPES, pH 7.0). The cells (5×10^6 cells) were resuspended in HBSS and mixed with 20 μg of linearized plasmid DNA. After incubation on ice for 10 min, they were electroporated at 280 V, 975 μF (BioRad, Hercules, CA) and transferred to normal medium. After 48 h, cells were plated in 96-well plates in G418 (1 g/l) containing medium. The drug-resistant colonies were usually visible after 2-3 weeks.

Measurement of mbIL-2 expression

Cells were first incubated for 30 min at 4°C with anti-IL-2 mAb (S4.B6.34) appropriately diluted in staining buffer (1 × PBS containing 0.02% sodium azide and 2% FBS). Cells were then washed with staining buffer three times and incubated for an additional 30 min at 4°C with PE-goat anti-rat IgG antibody. The stained cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

Assay of IL-2 activity

Biological IL-2 activity of mbIL-2 on tumor cells was measured by using the CTLL-2 cells as described previously. Briefly, the CTLL-2 cells were harvested and washed three times with medium. The X-ray irradiated mbIL-2 tumor cells (200 Gy) were mixed with CTLL-2 cells in a final volume of 200 μl using flat-bottomed 96-well plates. As positive control, recombinant IL-2 (0.016 U/ml) was supplied. The cultures were incubated for 72 h at 37°C, pulsed for the final 16 h with 0.5 μCi of [^{3}H]thymidine (Amersham, Piscataway, NJ) per well, and samples were collected using a PHD cell harvestor (Skatron, Norway). [^{3}H]thymidine incorporation was used as an index of DNA synthesis with results expressed as mean cpm per culture.

Tumor challenge

For tumorigenicity studies, mice were inoculated subcutaneously on the neck or intraperitoneally with 1×10^5 live cells, and tumor growth was measured daily. Tumor size was estimated by measuring the longest surface diameter using a caliper. In the metastasis studies, cells were injected intravenously (2.5×10^5 live cells per mouse) through lateral tail vein. After 2 weeks, mice were sacrificed and black tumor-nodules established on lung were counted. For therapeutic effect studies, X-ray irradiated (200 Gy) tumor cells were mixed with live wild type B16F10 cells, and co-injected subcutaneously. Inoculated mice were monitored daily, and the mice were killed when they became moribund.

For inhibition effect of metastasis, X-ray irradiated (200 Gy) tumor cells (5×10^6 per mouse) were mixed with live wild type B16F10 cells (5×10^4 per mouse) and co-injected intravenously through lateral tail vein. After 12 days, mice were sacrificed and black tumor-nodules established on lung were counted.

In vivo T cell depletion

To deplete $CD4^+$ or $CD8^+$ T cells, GK1.5 hybridoma cells (1×10^6 cells/mouse) for $CD4^+$ T cell depletion and 3.168 hybridoma cells (1×10^6 cells/mouse) for $CD8^+$ T cell depletion were injected into C57BL/6 mice subcutaneously three times with 4 days interval, respectively. Depletion of the $CD4^+$ T cells or $CD8^+$ T cells was confirmed by FACS analysis of peripheral blood cells of the mice.

CTL assay

The immunized spleen cells were assessed for their cytolytic activity against B16F10 cells in triplicate in 4 h ^{51}Cr-release assays. To prepare effector cells, splenocytes were prepared from mice 2 weeks after the second immunization with X-ray irradiated (200 Gy) tumor cells. The splenocytes (1×10^7 cells) were cultured with 6.5×10^5 MMC-treated tumor cells as stimulator in 5 ml RPMI-1640 medium. After 4 days, live cells were counted and resuspended at the desired concentration for CTL assay. Target cells were labeled with 100 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear-DuPont, Bedford, MA) and suspended at a concentration of 1×10^4 cells per 100 μl in round-bottomed 96-well plates. Various numbers of effector cells suspended in 100 μl assay medium were then added to each well. After 4 h incubation and brief centrifugation, 100 μl of supernatant were collected from each well and counted in an automated gamma-counter. The percent specific ^{51}Cr release was determined using following formula: $(cpm_{experimental\ release} - cpm_{spontaneous\ release}) \times 100/(cpm_{maximum\ release} - cpm_{spontaneous\ release})$. Maximal release of ^{51}Cr was obtained by lysis of target cells with 2% Triton-X 100. Spontaneous ^{51}Cr release was measured by incubating target cells in the absence of effector cells and was less than 20% of maximum release.

Preparation of cell suspensions from organs

C57BL/6 mice splenocytes were prepared using a standard protocol. Briefly, mice were killed by cervical dislocation, and splenocytes were prepared by mechanical disruption. Lung tissues were digested three times by shaking for 30 min at 37°C in RPMI-1640 medium containing 1 mg/ml collagenase VII (Sigma-Aldrich, St. Louis, Missouri) and 2% FBS (Hyclone, Logan, UT). Lung cells were passed through a 70 μm nylon filter (Becton Dickinson, Franklin Lakes, NJ), erythrocytes were lysed with an ammonium chloride potassium lysis buffer, and the total number of cells was enumerated by trypan blue exclusion.

Immunohistochemistry

Infiltration of $CD4^+$ T cells and $CD8^+$ T cells in lung was assayed by immunohistochemistry. Immunohistochemistry was performed using a Chemmate EnVision Detection Kit (DAKO, Carpinteria, California). Paraffin sections of lung were de-waxed and re-hydrated, washed three times with phosphate-buffered saline, and treated with proteinase K (DAKO ready to use kit) for 5 min at 37°C and followed by the DAKO LSAV 2 System peroxidase kit instruction. Briefly, tissue sections were washed with PBS/0.1% Tween 20 and treated with H_2O_2 for 10 min at room temperature, blocked in PBS/0.1% Tween 20/1% BSA for 20 min at room temperature. The tissue sections were washed three times with PBS/0.1% Tween 20, and followed by anti-mouse CD4 and CD8 antibodies (1:200 dilutions) for 30 min at room temperature. After incubation with secondary antibody for 30 min at RT, tissue sections were counter stained with hematoxylin and mounted. Immunostaining was visualized and pictured by a microscope. Sections without primary antibody were used as negative control.

Results

Stable expression of mbIL-2 on tumor cell surface

We transfected B16F10 cells with the pNeoSRαII vector harboring the neomycin-resistance gene and a chimeric IL-2 cDNA made up of cDNAs encoding the extracellular domain of IL-2 and the transmembrane domain of TNFα, a type II transmembrane protein (Figure 1A). The transfectants were selected with G418 and the expression of mbIL-2 in the transfectants was analyzed with FACS using IL-2 specific S4.B6.34 mAb (Figure 1B). Clone 3-2E5 was selected for further study from 7 clones carrying mbIL-2. It expressed IL-2 stably on cell surface, and growth rate in culture was indistinguishable from the parental B16F10 cells and vector-transduced clone. We also tested the biological effectiveness of mbIL-2 on the tumor cells by setting up a mixed cell culture with CTLL-2 cells, an IL-2 dependent cell line. X-ray irradiated mbIL-2 tumor cells supported proliferation of the CTLL-2 cells, whereas the B16F10 cells did not (Figure 1C). This result indicates that the mbIL-2 on tumor cells has biological function of IL-2.

Reduced tumorigenicity of mbIL-2 clone

To analyze the effect of mbIL-2 expression on tumorigenicity, B16F10 cells, vector-transduced cells, and mbIL-2 cells were injected subcutaneously into syngeneic C57BL/6 mice, and tumor growth was monitored. Tumor growth of mbIL-2 clone was much delayed compared to the other two groups of tumor cells (Figure 2A). However, the growth rate of the mbIL-2 tumors, once tumor growth started, was similar to those by other tumor cells. We also studied the tumorigenicity of the mbIL-2 cells by intraperitoneal injection. Mice injected with mbIL-2 cells survived much longer than those injected with two types of control cells (Figure 2B). All the latter died within 42 days, whereas 4 of 10 mice injected with mbIL-2 cells survived for more than 2 months. This indicates that expression of IL-2 in a membrane-bound form increases the immunogenicity of the tumor cells.

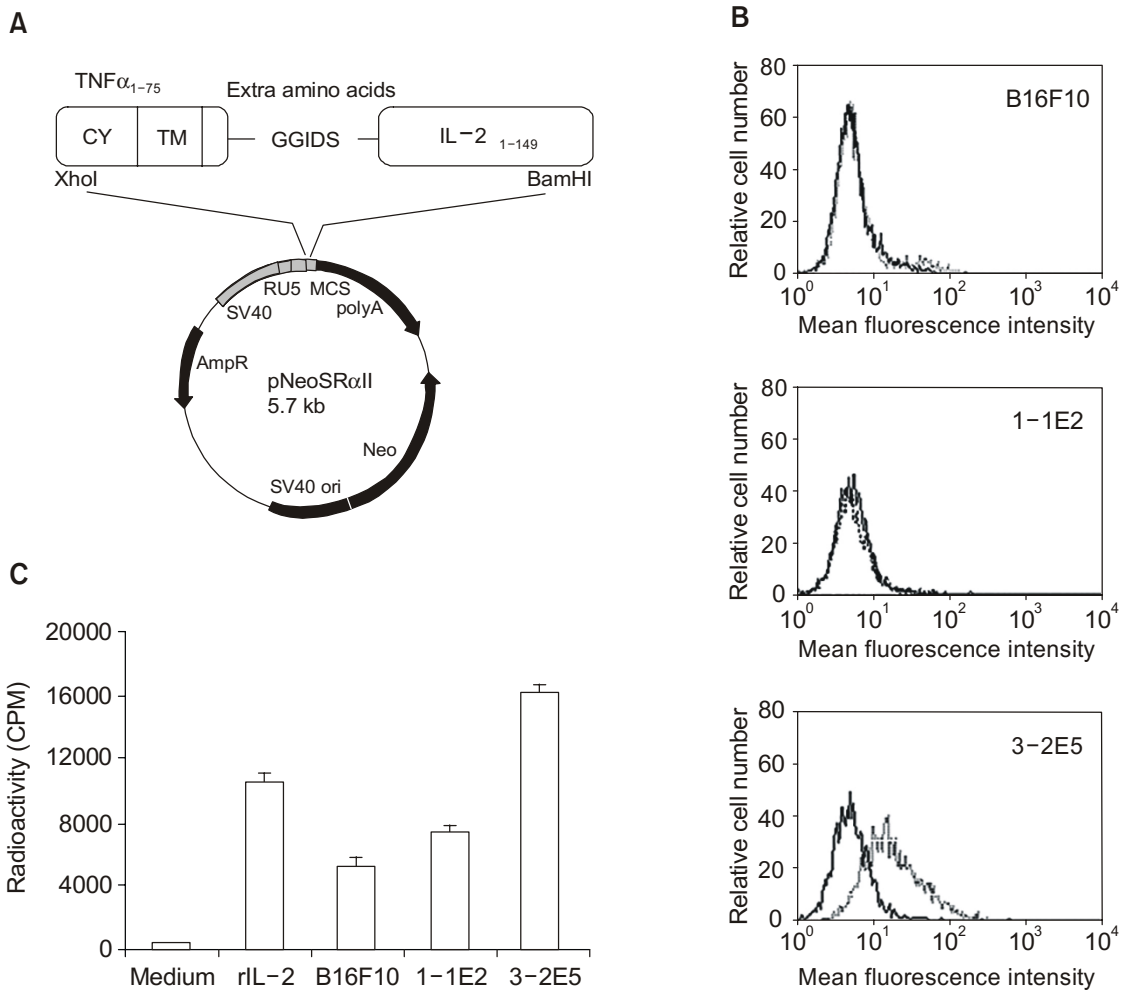

Figure 1. Structure and expression of the mbIL-2 chimera. (A) The fusion gene carried by mbIL-2 is composed of TNFα (56 amino acids encompassing the cytoplasmic, transmembrane, and 19 extracellular amino acids), and five spacer amino acids (GGIDS), and IL-2 coding sequences (from 1 to 149 amino acids without signal peptide). The chimeric cDNA was subcloned into the pNeoSRαII vector with G418 selection marker. (B) Surface expression of chimeric mbIL-2 on transfected B16F10 cells. B16F10 cells, 1-1E2, and mbIL-2 clone were stained with monoclonal antibody to IL-2 (S4.B6.34) and followed by PE-conjugated goat anti-rat IgG antibody. (C) Proliferation of CTLL-2 in response to mbIL-2 clone. CTLL-2 cells were plated at 20,000 cells per well in flat bottomed 96-well plate and cultured with irradiated B16F10, 1-1E2, and 3-2E5 cells (10,000 cells per well) for 72 h, respectively. As a control, CTLL-2 cells were treated with final concentration of 0.016 U/ml recombinant IL-2. Proliferation of CTLL-2 was measured by incorporation of [^{3}H]thymidine for last 16 h in the incubation.

To analyze the therapeutic effect of mbIL-2 cells, mice were injected with wild type B16F10 cells mixed with X-ray irradiated mbIL-2 cells (or control cells). All mice co-injected with irradiated B16F10 cells or vector-transduced cells died within 27 days, whereas the mice co-injected with inactivated mbIL-2 cells survived longer (Figure 2C). Though protection was not complete in this study, possibly because of reduced expression of MHC class I molecules on the B16F10 cells, we were able to detect a small therapeutic effect of the mbIL-2 cells.

Reduced metastatic ability of mbIL-2 clone

To analyze the ability of mbIL-2 cells to spread to intravascular locations in the lung, 2.5×10^5 cells of each type were injected through the tail vein into C57BL/6 mice. After two weeks, all the mice were killed and the black metastasis nodules in their lung were counted. Lung of the mice injected with mbIL-2 cells contained much fewer metastatic nodules than the mice injected with the control cells (Figure 3A). Thus expression of mbIL-2 on tumor cells reduced their metastatic ability. This outcome suggests that the mbIL-2 cells introduced into the blood stream may efficiently activate immune cells in the draining lymph

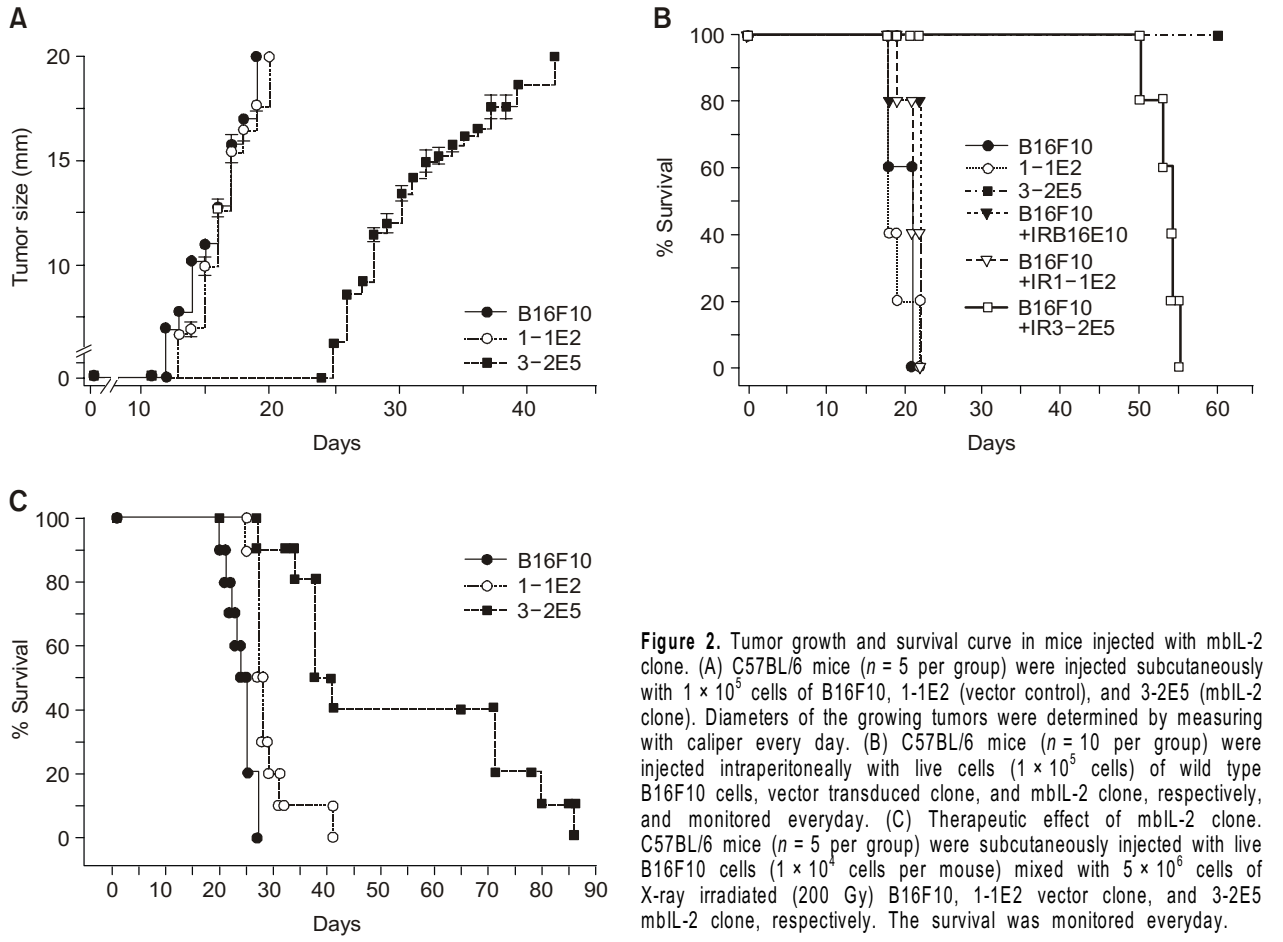

Figure 2. Tumor growth and survival curve in mice injected with mbIL-2 clone. (A) C57BL/6 mice (n = 5 per group) were injected subcutaneously with 1×10^5 cells of B16F10, 1-1E2 (vector control), and 3-2E5 (mbIL-2 clone). Diameters of the growing tumors were determined by measuring with caliper every day. (B) C57BL/6 mice (n = 10 per group) were injected intraperitoneally with live cells (1×10^5 cells) of wild type B16F10 cells, vector transduced clone, and mbIL-2 clone, respectively, and monitored everyday. (C) Therapeutic effect of mbIL-2 clone. C57BL/6 mice (n = 5 per group) were subcutaneously injected with live B16F10 cells (1×10^4 cells per mouse) mixed with 5×10^6 cells of X-ray irradiated (200 Gy) B16F10, 1-1E2 vector clone, and 3-2E5 mbIL-2 clone, respectively. The survival was monitored everyday.

nodes, or that tumor cells that have metastasized to the lung may activate immune cells *in situ*.

To examine which T cell subpopulation is responsible for the low metastatic ability of mbIL-2 clone, $CD4^+$ T cell depleted and $CD8^+$ T cell depleted mice were challenged with 3-2E5 clone. First of all, depletion of $CD4^+$ and $CD8^+$ T cell subpopulations in peripheral bloods was confirmed on day 1, and 4 after hybridoma cell injections (Figure 3B). Interestingly, the number of black nodules formed by 3-2E5 clone was significantly increased in $CD8^+$ T cell depleted mice, but not in the $CD4^+$ T cell depleted mice (Figure 3C). The results suggest that $CD8^+$ T cells are responsible for the reduced metastatic ability of 3-2E5 clone.

To analyze the immunogenicity in terms of metastasis inhibition, mice were injected intravenously with wild type B16F10 cells mixed with X-ray inactivated mbIL-2 clone. The mice co-injected with mbIL-2 clone had much fewer metastatic nodules in lung and contained prominently increased number of $CD8^+$ T cells than those co-injected with X-ray inactivated B16F10 cells (Figure 4A, 4B). This effect may result from the higher immunogenicity of mbIL-2 cells.

Induction of CTL activity by mbIL-2 clone

The therapeutic effect of mbIL-2 cells led us to analyze their ability to induce CTL activity against B16F10 cells. Seven days after the second intraperitoneal immunization, splenocytes were prepared and stimulated *in vitro* for 4 days with X-ray irradiated mbIL-2 cells. Thereafter, ^{51}Cr-labeled B16F10 target cells were added at various effector-to-target ratios for 4 h, and significantly higher anti-B16F10 CTL activity was detected in the group stimulated with mbIL-2 cells (Figure 5). This result suggests that the therapeutic effect of mbIL-2 cells is partly due to their superior CTL induction activity.

Increase of $CD8^+$ T cells in the lung of the mice injected with mbIL-2 clone

To identify the immune cells responsible for the therapeutic effect and the reduced metastatic ability of the mbIL-2 cells, we analyzed the $CD4^+$ and $CD8^+$ T cell subpopulations of the lung, spleen and lymph nodes, two weeks after the intravenous injection of mbIL-2 cells. Increased numbers of $CD8^+$ T cells were detected only in the lung of mice injected with mbIL-2 cells (Table 1). Interestingly enough, there was no

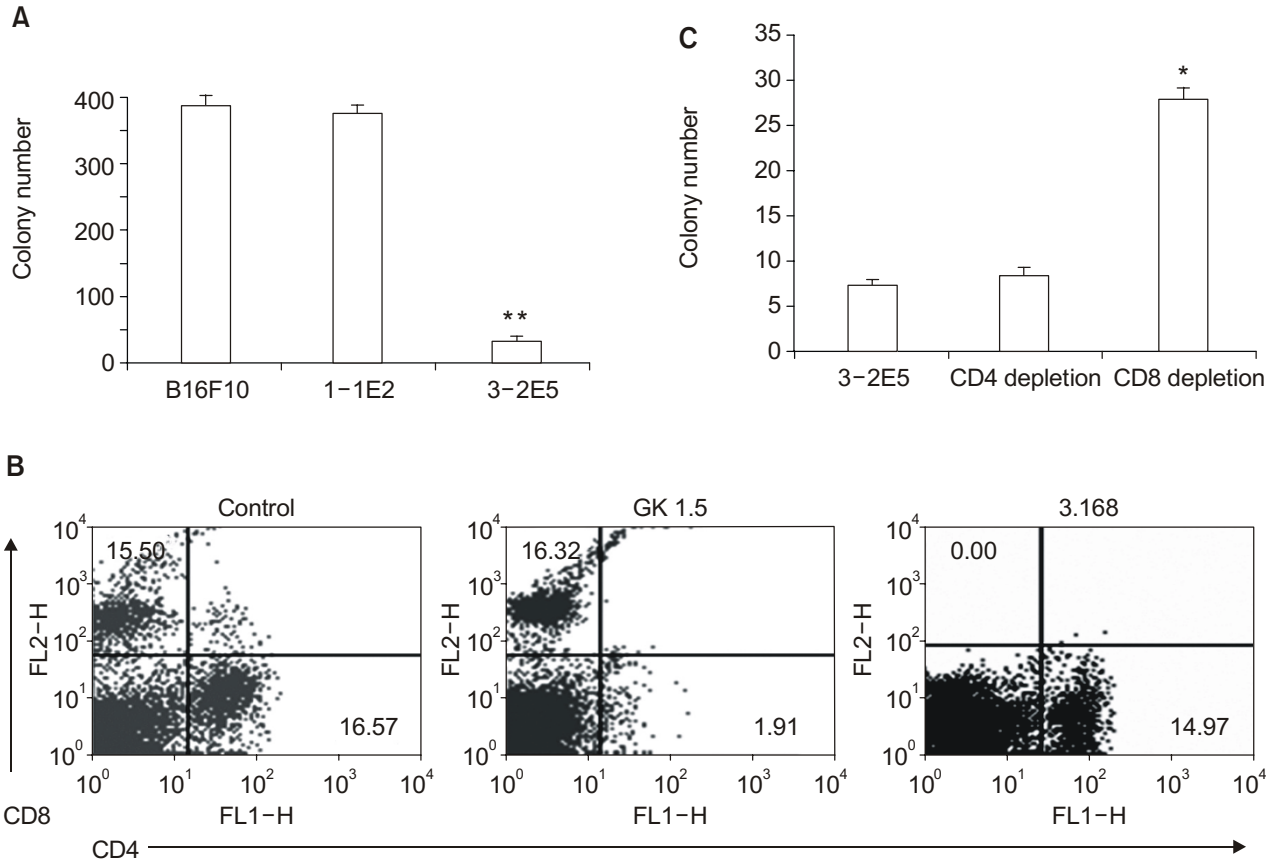

Figure 3. Comparison of metastatic ability. (A) C57BL/6 mice were injected with mbIL-2 clone (2.5×10^5 cells per mouse intravenously) through tail vein, and executed on 14th day. The black metastatic nodules were counted with naked eyes. The experiment was repeated three times and the data presented as mean ± SE. (B) The percentage of $CD4^+$ T cells and $CD8^+$ T cells in peripheral blood cells were determined 4 days after subcutaneous injection GK1.5 hybridoma and 3.168 hybridoma (1×10^6 cells/mouse). 3-2E5 cells (1×10^6 cells/mouse) were injected subcutaneously as control. (C) To deplete the $CD4^+$ T cells or $CD8^+$ T cells subpopulation, C57BL/6 mice were injected with GK1.5 hybridoma cells or 3.168 hybridoma cells subcutaneously three times with 4 days interval, respectively. After 24 h of final injection, mice were challenged with 3-2E5 cells 1×10^6 intravenously. After 10 days, mice were sacrificed, and the black metastatic nodules in lung were counted. The experiment was repeated at least 3 times and a representative result was presented (2 mice per group). $^{**}P < 0.05$ compared to B16F10 group (A), and $^{*}P < 0.01$ compared with 3-2E5 group (C).

significant change in the $CD4^+$ T cell population in the lung. There was no significant change in $CD4^+$ and $CD8^+$ T cell populations of the lymph nodes and spleen. These results suggest that the mbIL-2 cells migrate into lung and activate selectively $CD8^+$ T cells *in situ*, possibly by the direct priming mode.

Discussion

In most experimental approaches to cytokine gene tumor therapy, different effector cells are involved, depending on the type of cytokine and the origin of tumor (Blankenstein *et al.,* 1991; Colombo *et al.,* 1992; Dranoff *et al.,* 1993; Tepper *et al.,* 1994; Dranoff *et al.,* 1995; Nanni *et al.,* 1999). GM-CSF-transduced tumor cells activated dendritic cells and led to activation of CTL as well as Th cells (Dranoff *et al.,* 1993). Tumor cells transduced with IL-2 gene activated CTL effectively in various tumor models (Fearon *et al.,* 1990; Gansbacher *et al.,* 1990; Blankenstein *et al.,* 1991; Becker *et al.,* 1996; Jackaman *et al.,* 2003; Hillman *et al.,* 2004). Though there have been many positive results with cytokine gene transfer approach for tumor therapy, unexpected side effects became obstacles. More thorough *in vivo* studies in animal system revealed that the cells infiltrated the tumor injection sites were heterogeneous and only a limited numbers of CTL were specific for the tumor antigens (Rivoltini *et al.,* 1990; Colombo *et al.,* 1992). The activated Th cells may secrete a variety of cytokines and cause massive immune cell activation not relevant to tumor antigens. The effective rage of cytokine and non-specific activation of immune cells may contribute to the side effects.

To focus anti-tumor immunity on tumor antigen-specific CTL, we developed a strategy based on di-

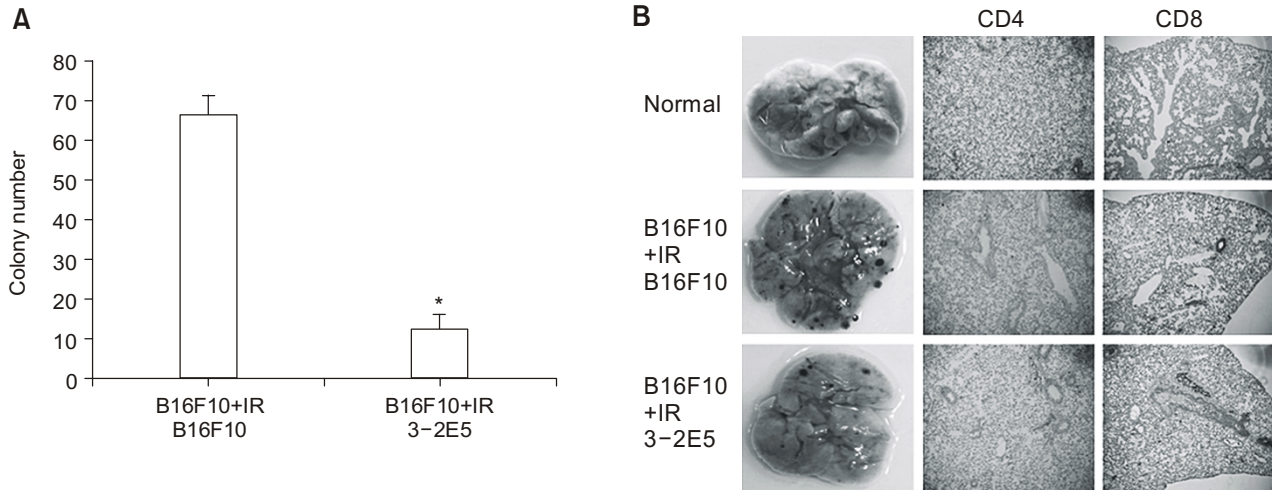

Figure 4. Inhibition of metastasis by mbIL-2 clone. (A) C57BL/6 mice (n = 5 per group) were injected intravenously with live B16F10 cells (5 × 10^4 cells per mouse) mixed with 5 × 10^6 cells of X-ray irradiated (200 Gy) B16F10 and 3-2E5 mbIL-2 clone, and executed on 12th day. *P < 0.01 compared with B16F10 group. (B) Each lung paraffin sections was stained with anti-mouse CD4 antibody and anti-mouse CD8 antibodies. Immunostaining was visualized and pictured by a microscope.

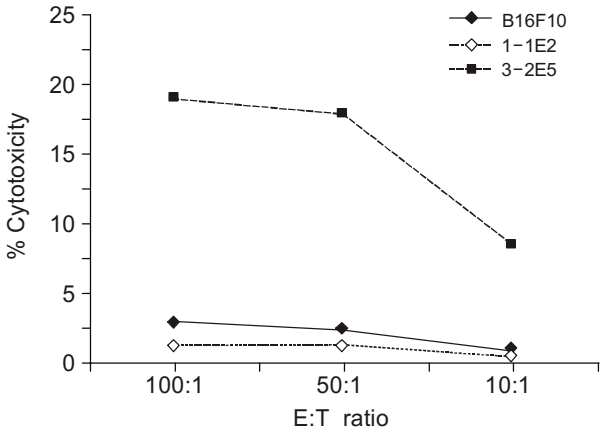

Figure 5. Induction of CTL activity by mbIL-2 clone. Spleen cells were prepared from the mice *in vivo* immunized twice with 1 × 10^6 cells of the irradiated (200 Gy) B16F10 cells, 1-1E2 vector clone, and 3-2E5 mbIL-2 clone, respectively. Two weeks after the second immunization, the spleen cells (1 × 10^7 cells) from each group of mice (n = 3 per group) were mixed-cultured with each corresponding stimulator cells (6.5 × 10^5 cells) after MMC-inactivation. After 4 days, the effector cells were harvested and ^{51}Cr-labeled B16F10 cells were used as target for the CTL assay.

Table 1. Change in the T cell subpopulations in the lung, spleen, and lymph node after mbIL-2 clone injection.

T cell population		B16F10	1-1E2	3-2E5
Lung	$CD4^+$ T cell	17.79	15.06	15.25
	$CD8^+$ T cell	23.57	20.51	34.52
Spleen	$CD4^+$ T cell	14.63	19.26	16.07
	$CD8^+$ T cell	13.34	15.80	14.83
Lymph node	$CD4^+$ T cell	19.00	19.21	21.00
	$CD8^+$ T cell	26.22	25.91	27.48

C57BL/6 mice were injected with 1x105 cells of B16F10, 1-1E2 (vector) and 3-2E5 (mbIL-2) cells intravenously. After two weeks, cell suspensions from lung, spleen and lymph node were prepared and stained with FITC-conjugated anti-mouse CD4 mAb and PE-conjugated anti-mouse CD8 mAb.

rect priming. Several evidences for direct priming of CTL have been already reported (Wang *et al.,* 1995; Wolkers *et al.,* 2001). Expression of co-stimulatory molecules on tumor cells also provides strong signal for CTL activation (Huang *et al.,* 1994; Bai *et al.,* 2001; Vesosky *et al.,* 2003). These results indicate that the tumor cells contact physically to CTL precursors and the co-stimulatory molecules on tumor cells act on corresponding receptors on T cells. Tumor cells engineered to express membrane-bound form of TNFα induced antitumor effect with lower toxic side effect compared with those expressing secretory form of TNFα (Marr *et al.,* 1997). GM-CSF (el-Shami *et al.,* 1999; Soo Hoo *et al.,* 1999; Yei *et al.,* 2002) and Flt3 ligand (Chen *et al.,* 1997) were also engineered to be expressed as membrane-bound forms on tumor cells with intention to prepare potent stimulators for antigen presenting cells. The tumor cells expressing GPI-anchored IL-12 induced antitumor immunity comparable to the effects of secretory IL-12 (Cimino *et al.,* 2004). Nizard group developed anticancer vaccine anchoring recombinant IL-2 via diphtheria toxin T domain to avoid using viral vectors, which may cause toxic side effects (Nizard *et al.,* 2003). The membrane-anchored IL-2 on tumor cells

induced tumor specific CTL activity. These all suggest that membrane-bound form of cytokines acts on target cells by cell-to-cell contact.

In a previous study, we reported that the tumor cells expressing IL-4 in membrane-bound forms lost tumorigenicity and induced systemic anti-tumor immunity (Kim *et al.,* 2000). These cells were effective in activating $CD8^+$ T cells without noticeable effect on $CD4^+$ T cells. In this study, we expanded our observations, by introducing IL-2 gene in a membrane-bound form into the spontaneous metastatic tumor cell line, B16F10 cells. Expression of mbIL-2 on tumor cells was effective in inducing CTL activity and in prolonging the survival of mice co-challenged with viable wild type B16F10 cells. Furthermore, the mbIL-2 clone inhibited metastasis of B16F10 cells effectively. Though both mbIL-2 and mbIL-4 on tumor cells induced anti-tumor immunity in different tumor models respectively, the two cytokines would affect CTL precursors in different ways. It will be interesting to differentiate the effect of the cytokines on CTL precursors.

The induction of tumor-specific T cell immunity is thought to involve two pathways. First, it is induced as a result of direct priming of naive $CD8^+$ T cells by tumor cells that have migrated from the tumor injection site to the secondary lymphoid tissues (Wang *et al.,* 1995). Second, the T cell response may result from cross-presentation of tumor cell-derived antigens by professional antigen presenting cells (Huang *et al.,* 1994; Muller *et al.,* 2001; van Mierlo GJ *et al.,* 2004). Our results suggest that the mbIL-2 cells mainly migrate into lung and stimulate $CD8^+$ T cells by direct priming *in situ*, because there was no significant change in the number of $CD4^+$ and $CD8^+$ T cells in the spleen and lymph nodes (Table 1). Alternatively, mbIL-2 cells in the draining lymph nodes may activate CTL by direct priming and the CTL primed by mbIL-2 cells may migrate to the tumor-growing site, mainly to lung. Metastasis of breast cancer cells shares relevant similarities with leukocyte trafficking and seems to be mediated through CXCR4 or CCR7 signaling (Cardones *et al.,* 2003). We also found that the mbIL-2 increased the expression of CXCR4 on B16F10 cells, but not the ICAM-1 (data not shown). The lung was known to express SDF-1, a CXCR4 ligand, so that the mbIL-2 clone might migrate to lung effectively.

On the basis of these *in vivo* and *in vitro* results, we propose that mbIL-2 on tumor cells may act as a selective stimulatory molecule for the activation of tumor antigen-specific $CD8^+$ T cells, possibly by direct priming. Our approach, expressing IL-2 as a membrane-bound form on tumor cells would be an efficient way to focus antitumor immune responses to CTL in conventional cytokine gene therapy

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