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A Novel Approach for Cancer Immunotherapy: Tumor Cells with Anchored Superantigen SEA Generate Effective Antitumor Immunity

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Murine B16 melanoma cell line is poorly immunogenic and highly aggressive. We recently reported that the transmembrane staphylococcal enterotoxin A (TM-SEA) anchors onto B16 cells and stimulates lymphocyte proliferation. The purpose of the study was to investigate whether vaccination with B16 cells bearing membrane-anchored TM-SEA fusion protein could cause tumor-specific immunity. Mice in the therapeutic vaccination group received B16 tumor inoculations, followed by treatment with B16-TM-SEA vaccine or control vaccines. Mice in the prophylactic vaccination group were given B16-TM-SEA vaccine or control vaccines, followed by challenge with wild type B16 or control EL4 cells. Significant tumor growth inhibition, prolongation of survival, and marked augmentation of NK and CTL activities were observed in mice which received B16-TM-SEA vaccine as compared to controls. Overall, our results suggest that the TM-SEA cellular vaccine is a novel and effective strategy for cancer immunotherapy.

KEY WORDS: Superantigen; transmembrane; vaccine; immune response; melanoma.

INTRODUCTION

Natural killer (NK) cells are important immune cells that have the ability to recognize and eliminate tumor- and pathogen-infected cells without prior sensitization (1). Cytotoxic T lymphocytes (CTLs) have crucial role in eliminating host cells that contain intracellular pathogens and those that have undergone malignant transformation (2). One of the major goals of tumor immunotherapy is to generate tumor-specific T-cell response that eventually contributes to the eradication of tumor. Because tumor cells often have a very poor capacity to present their own antigen to T cells (3), the recent approaches in the field of cancer vaccine development is to genetically modify tumor cells to stimulate a stronger antitumor T-cell response.

Superantigens (SAgs) are certain bacterial and viral proteins that can activate a large number of T cells irrespective of their antigen specificity, resulting in a massive release of cytokines from T cells and monocytes (4, 5). Unlike conventional antigens, SAgs bind to certain regions of major histocompatibility complex (MHC) class II molecules of antigen-presenting cells (APCs) outside the classical antigen-binding groove. Concomitantly, SAgs bind in their native form to T cells at specific motifs of the variable region of the beta chain (Vβ) of the T-cell receptor (TCR). This interaction triggers the activation and proliferation of the targeted T lymphocytes and leads to the release of significant amount of various cytokines.

SEA is a bacterial SAg produced by *Staphylococcus aureus* (6). To develop tumor-specific SAg for cancer therapy, the *SEA* gene was genetically fused to the Fab region of the C215 monoclonal antibody that is specific to human colon carcinoma (7). The Fab-SEA fusion protein has been shown to have 100-fold stronger affinity for the tumor antigen than MHC class II molecules (8). Both *in vitro* and *in vivo* studies indicated that Fab-SEA could target cytotoxic T cells against MHC class II—negative tumor cells bearing the proper tumor antigen without systemic

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side effects (9–13). However, not all malignancies express C215 antigen or any conserved antigens, and therefore, antibody-targeted SEA is limited by its applicability to only certain tumor types.

We have genetically fused the *SEA* gene to the hydrophobic TM sequence (corresponding to amino acid residues 644–687) of the *c-erb-B2* gene (14, 15) and demonstrated that the expression product of this *TM-SEA* fusion gene is capable of passively anchoring into the cell membrane of living tumor cells (16). In this study, we describe the potent immunostimulatory and antitumor effects of the B16-TM-SEA cell-based tumor vaccine.

MATERIALS AND METHODS

Cell Culture

Melanoma cell line, B16; lymphoma cell line, EL 4; derived from H-2^b C57BL/6 mice; and YAC-1, an NK-sensitive lymphoma cell line of A/S (H-2^a) origin were obtained from the American Type Culture Collection (Manassas, VA). All the tumor cells were cultured at 37°C in a humidified 5% CO_2 incubator in complete RPMI 1640 medium containing 10% fetal bovine serum (Life Technologies, Inc.), supplemented with penicillin at 100 U/mL and streptomycin at 100 μg/mL (Invitrogen, Frederick, MD).

Preparation of Cell-Based Vaccines

B16 cells cultured in complete RPMI 1640 medium for 3 days were harvested with 0.05% trypsin-EDTA (Invitrogen, Frederick, MD) and washed twice with sterile phosphate-buffered saline (PBS). After incubation for 4 h with either 0.3 μM TM-SEA fusion protein (16) or SEA protein at 37°C in 5% CO_2 incubator, the cells were harvested and washed twice with PBS. The cells were then incubated with mitomycin C (MMC, 100 μg/mL) at 37°C in 5% CO_2 incubator for 1 h, washed twice with PBS, and suspended in PBS for either therapeutic or prophylactic vaccination. The cells incubated with TM-SEA and inactivated with MMC were designated as B16-TM-SEA vaccine, and the cells incubated with SEA and then inactivated were designated as B16-SEA vaccine. B16 cells were also directly inactivated with MMC at 37°C in 5% CO_2 incubator for 1 h for use as a control (designated as B16 vaccine).

Animals

Female wild-type C57BL/6 mice (H-2^b), 6–8 weeks old, were purchased from Sipper BK experimental animal company (Shanghai, China), and were housed in specific pathogen-free conditions at the animal center of Zhejiang University School of Medicine in accordance with the institutional guidelines. Mice were housed 5 per cage in a 12 h light/dark cycle at an ambient temperature of 22 $\pm$ 2°C and humidity of 50 $\pm$ 10% with food and water *ad libitum*. Cages were changed twice in a week to ensure hygienic conditions. The animals were allowed to acclimate to the facility for 2 weeks before randomization into different experimental groups.

Tumor Model and Experimental Design

To establish the tumor model, B16 cells cultured for 3 days were harvested with 0.05% trypsin-EDTA and washed twice with PBS. The 1×10^5 B16 cells in 100 μL of PBS were subcutaneously injected in the right rear leg of each C57BL/6 mice. The change in tumor size over time was determined by measuring tumors at two dimensions using a digital caliper on every other day, beginning at 7 days postinoculation. Tumor size was calculated using the formula, 1/2 (length + width).

To test the effectiveness of B16-TM-SEA vaccine, the tumor-bearing mice were randomized on third day of post-tumor cell inoculation into the following groups with each group consisting of eight animals: 1) PBS (blank control), 2) B16 vaccine, 3) B16-SEA vaccine, and 4) B16-TM-SEA vaccine. On seventh day after the tumor cell inoculation, all the tumor-bearing mice in the respective groups were injected intratumorally either 100 μL of PBS or B16 vaccine cells, or B16-SEA vaccine cells, or B16-TM-SEA vaccine cells, each suspended at cell concentration of 1×10^6 in 100 μL PBS.

To examine the prophylactic effectiveness of B16-TM-SEA vaccine, the tumor-bearing mice were divided into five groups consisting of eight mice in each group: 1) PBS control, 2) B16 vaccine, 3) B16-SEA vaccine, 4) B16-TM-SEA vaccine, and 5) B16-TM-SEA vaccine (for control tumor challenge). All the cell-based vaccines (1×10^6 cells) and the PBS control were injected subcutaneously into the right groin area of the mice. Vaccination was performed at weekly interval for three consecutive weeks. Mice in the first through fourth groups were challenged with wild-type B16 cells and mice in the fifth group were challenged with syngeneic EL 4 lymphoma cells. Tumor challenge was initiated by injecting 1×10^5 tumor cells subcutaneously into the left rear leg of the mice 1 week after the last vaccination.

The final tumor volume in both the groups of mice that received the vaccine either as therapeutic or prophylactic was measured on day 24 after tumor cell inoculation (before any deaths occurred) to ensure inclusion of the data from all the mice.

Cytotoxicity Assays

To determine immune cell cytotoxicity, we used CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI), which is based on the colorimetric detection of the released enzyme, lactate dehydrogenase (LDH) (17, 18). Briefly, splenic effector cells were isolated from three of the total eight mice from each group, which were sacrificed 7 days after the last injection. Following aseptic removal, spleens were washed in PBS and the erythrocytes were lysed with 0.83% ammonium chloride. Macrophages were removed by adherence on plastic plates for 2 h, and the remaining nonadherent cells (lymphocytes) were retained and pooled. Some of the isolated lymphocytes were directly tested for the effectiveness as NK effector cells. A separate sample of splenic lymphocytes was cocultured with inactivated B16 (treated with 100 μg/mL MMC for 1 h) for 7 days in the presence of 20 U/mL recombinant murine IL-2 (Sigma, St. Louis, MO) and then collected to test for CTL activity.

YAC-1 lymphoma cells or B16 melanoma cells (2×10^6) in 0.5 mL RPMI 1640 medium with 10% FBS were used as the targets for the NK and CTL assays, respectively. Targets (2×10^5 cells/well) were mixed with splenocytes at effector:target (E:T) ratios of 100:1, 50:1, and 25:1, and incubated for 4 h in a humidified incubator at 37°C, 5% CO_2. Lysis solution (10×) was added to a portion of the target cells, prior to centrifugation, as a maximum LDH release control. Supernatant (50 μL) was transferred to the enzymatic assay plate after centrifugation, 50 μL of the substrate mix was added to each well, the plate was covered to protect it from light, and incubated for 30 min at room temperature. Stop solution (50 μL) was added to each well and the absorbance was recorded at 490 nm. The percentage of specific lysis was determined according to the following formula: 100 × (Experimental – Effector Spontaneous – Target Spontaneous)/(Target Maximum – Target Spontaneous).

Statistical Analysis

Analysis of survival rate was conducted by a log-rank statistical test, based on the Kaplan-Meier method. For tumor measurement data, mean values of measured tumor sizes and 95% confidence intervals are reported. p values were determined using a two-tailed Mann–Whitney nonparametric test, and a p value of less than 0.05 was considered to be statistically significant.

RESULTS

To test our hypothesis that the tumor cell-based superantigen vaccine would augment the antitumor response of the poorly immunogenic B16 melanoma, we tested the efficacy of B16-TM-SEA vaccine either prior to or after inoculation with live B16 tumor cells in C57BL/6 mice to determine its prophylactic and therapeutic potentials.

Antitumor Effects of the Tumor Cell-Based Vaccine

To determine the therapeutic efficacy of the vaccine, tumor-bearing mice (eight in each group) were injected intratumorally with either PBS, B16 vaccine, B16-SEA vaccine, or B16-TM-SEA vaccine, and the tumor size was monitored every other day. B16-TM-SEA vaccine exhibited a notable antitumor effect as compared to control vaccines. Specifically, the average tumor size in the B16-TM-SEA vaccine group was significantly reduced, while B16 vaccine and B16-SEA vaccine each had a slight antitumor effect as compared to PBS control (Fig. 1a).

The animals which were tested for the prophylactic use of B16-TM-SEA vaccine exhibited significant antitumor effect against challenge with B16 melanoma cells but had no protective effect against the challenge with EL 4 lymphoma cells. In the same experiment, B16 vaccine and B16-SEA vaccine demonstrated marginal antitumor effect as compared to PBS control. Administration of B16-TM-SEA vaccine resulted in a significant antitumor response compared to that with other control vaccines. The average tumor size in the B16-TM-SEA vaccine group was lower and the tumor development was delayed (Fig. 2a).

NK Activity of Splenic Lymphocytes from the Mice in the Therapeutic Groups

Splenocytes derived from the tumor-bearing mice after various therapies were used in a cytotoxicity assay against YAC-1 cells at E:T ratios of 25:1, 50:1, and 100:1, using a Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay kit. It was demonstrated that the NK activity of lymphocytes from the control tumor-bearing mice was very low, but it was significantly higher in the mice which were treated with B16-TM-SEA vaccine (Fig. 3, $p < 0.05$). The NK activity in the mice treated with B16 and B16-SEA vaccines was slightly higher than that in the mice treated with PBS, but difference was not statistically significant ($p > 0.05$). These results show that B16-TM-SEA vaccine elicits a nonspecific immune effector response, detectable against a highly NK-sensitive target cell line.

Augmentation of CTL Activity in the Mice from Both the Therapeutic and Prophylactic Vaccine Test Groups

The results in Fig. 4a demonstrate that the relatively greater CTL activity was induced in the mice which were

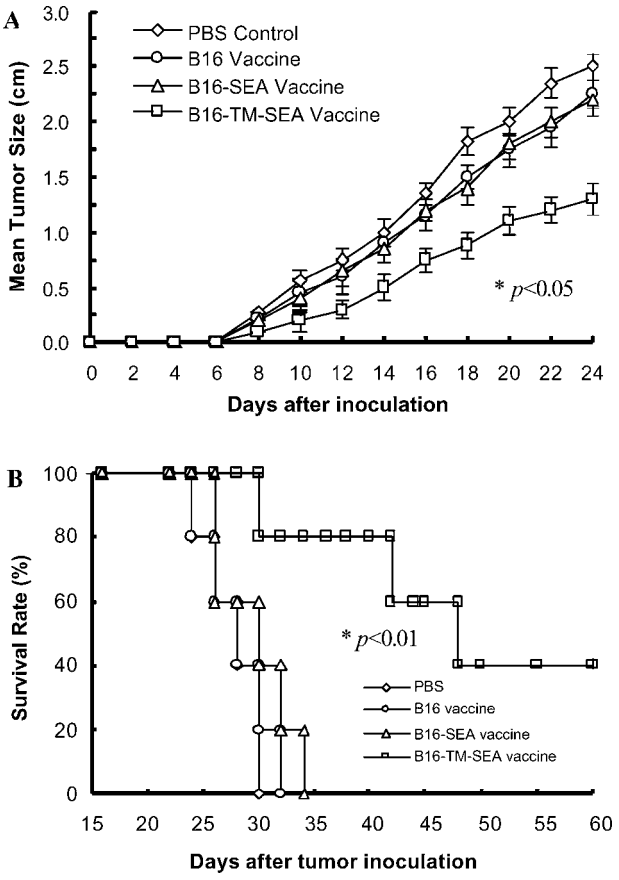

Fig. 1. Tumor size (A) and survival (B) analysis of tumor-bearing mice after intratumoral injection of PBS (open diamonds), B16 vaccine (open circles), B16-SEA vaccine (open triangles), and B16-TM-SEA vaccine (open squares). C57BL/6 mice were inoculated s.c. with 1×10^5 B16 cells on day 0, randomized into four groups (eight mice in each group), and treated with PBS, B16 vaccine, B16-SEA vaccine or B16-TM-SEA vaccine (1×10^6cells) on the third day. The tumor dimensions were monitored with digital calipers every other day, the mean tumor size was calculated as 1/2 (length + width), and the survival of the mice was monitored daily. $^*p < 0.05$ in panel A and $p < 0.01$ in panel B for B16-TM-SEA vaccine vs. the control vaccines.

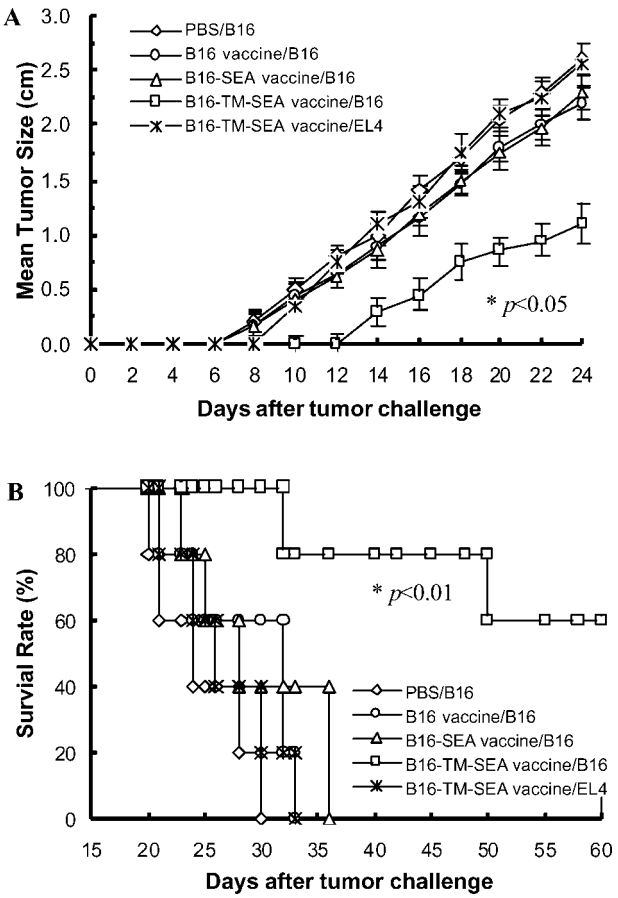

Fig. 2. Elicitation of protective immunity in mice receiving prophylactic vaccinations. C57BL/6 mice were randomized into five groups (each group contained eight mice): 1) the PBS/B16 group (open diamonds) was injected with PBS and challenged with 1×10^5 B16 melanoma cells each; 2) the B16 vaccine/B16 group (open circles) received the B16 vaccine and was challenged with 1×10^5 wild-type B16 cells; 3) the B16-SEA vaccine/B16 mice (open triangles) were vaccinated with B16-SEA vaccine and challenged with 1×10^5 wild-type B16 cells; 4) the B16-TM-SEA/B16 mice (open squares) were vaccinated with B16-TM-SEA vaccine and challenged with 1×10^5 wild-type B16 melanoma cells; and 5) the B16-TM-SEA vaccine/EL 4 mice (open cross) were given the B16-TM-SEA vaccine and challenged with 1×10^5 EL 4 lymphoma cells. The vaccinations were performed at weekly intervals for 3 consecutive weeks. Tumor challenge was initiated by injecting 1×10^5 tumor cells subcutaneously on the left rear leg of the mice in all the groups 1 week after the last vaccination. The length and width of the tumor mass were measured with digital calipers every other day after tumor challenge. Tumor size was expressed as 1/2 (length + width). (A) Tumor growth in vaccinated or unvaccinated C57BL/6 mice challenged with wild type B16 melanoma cells or EL 4 lymphoma cells. (B) Survival period of the vaccinated or unvaccinated C57BL/6 mice challenged with wild-type B16 melanoma cells or EL 4 lymphoma cells. $^*p < 0.05$ in panel A and $p < 0.01$ in panel B for B16-TM-SEA vaccine vs. the control vaccines.

treated with B16-TM-SEA vaccine ($p < 0.01$). The CTL activity of re-stimulated splenic lymphocytes from the mice treated with B16 vaccine or B16-SEA vaccine was slightly higher than that in the PBS-treated mice, but the difference was not significant ($p > 0.05$). As shown in Fig. 4b, the splenic CTL activity was markedly enhanced in the mice that were prophylactically immunized with B16-TM-SEA vaccine and subsequently challenged with wild-type B16 cells as compared to that in the mice which were treated with control vaccines ($p < 0.01$). The CTL activity in the mice that received B16 vaccine or B16-SEA vaccine prior to tumor cell

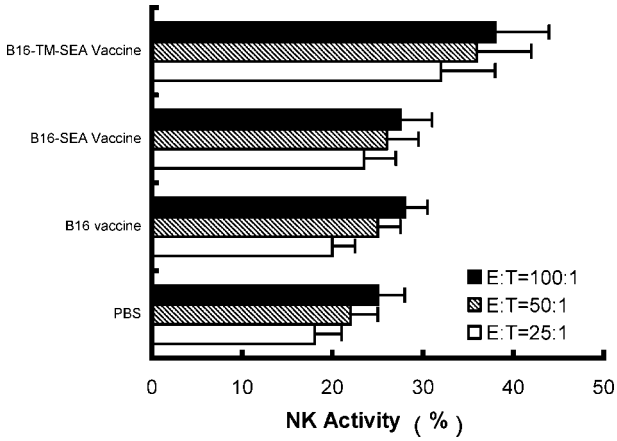

Fig. 3. Splenic NK activity of the mice in the therapeutic test groups. Nonadherent splenocytes isolated from the tumor-bearing mice of different groups 7 days after the last injection of PBS or vaccine were used in a CytoTox 96® Non-Radioactive Cytotoxicity Assay against YAC-1 cell targets at E:T ratios of 25:1, 50:1, and 100:1.

inoculation was also higher than that in the PBS-treated mice ($p < 0.05$). The data thus suggest that B16-TM-SEA vaccine induces a stronger T cell-mediated antitumor immunity

Survival of Mice Vaccinated After or Before Tumor Inoculation

The results in Fig. 1b demonstrate that tumor-bearing mice treated with B16-TM-SEA vaccine survived for a longer period of time than those treated either with PBS, B16 vaccine or B16-SEA vaccine ($p < 0.01$). The survival rate in different groups was 80% after 35 days for B16-TM-SEA vaccine group, whereas on animals survived in other treatment groups.

The findings shown in Fig. 2b indicate that the mice immunized with B16-TM-SEA vaccine and then challenged with wild-type B16 cells survived for longer period than those challenged with EL 4 cells ($p < 0.01$) and 60% of the mice that survived were tumor-free for 2 months. Mice immunized with B16 vaccine or B16-SEA vaccine survived slightly longer than those that received PBS after they were challenged with wild-type B16 cells, but there was no significant difference between the two groups ($p > 0.05$). These results show the specificity of the protective immunity in the mice immunized with B16-TM-SEA vaccine and challenged with wild-type B16 tumor cells, suggesting the important role of T cells-mediated immune response. Thus, the relative survival data, tumor growth rate, and NK and CTL activities, suggest that B16-TM-SEA vaccine can elicit a potent immune response with specific antitumor efficacy.

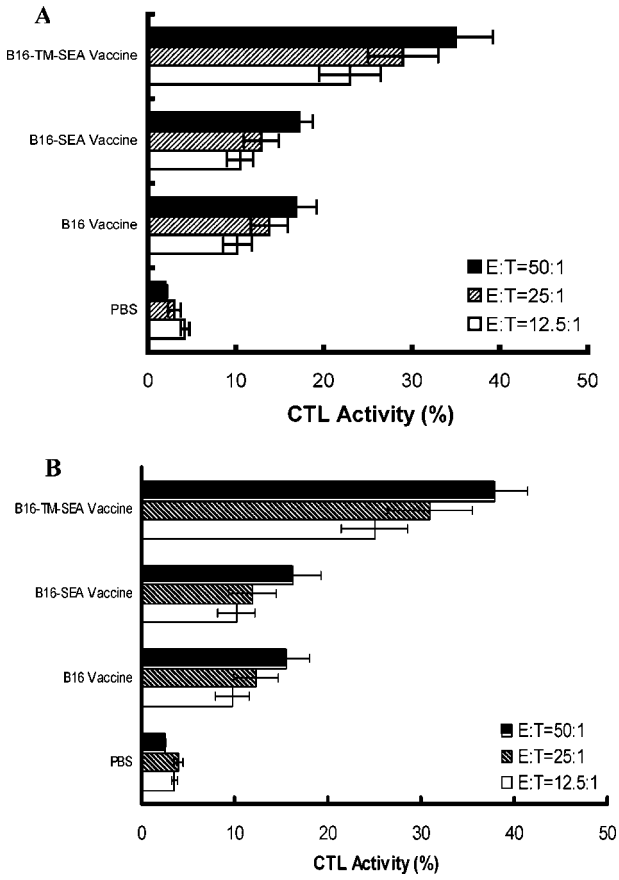

Fig. 4. Induction of CTL activity in the tumor-bearing mice treated with B16-TM-SEA vaccine, B16-SEA vaccine or B16 vaccine (A) and the mice receiving pre-tumor inoculation vaccinations with B16-TM-SEA vaccine, B16-SEA vaccine or B16 vaccine (B). Splenic lymphocytes were isolated from the vaccinated or unvaccinated mice 7 days after the last injection. The splenocytes were cocultured with inactivated B16 (MMC 100 μg/mL) for 7 days in the presence of recombinant murine IL-2 (20 U/mL) and then collected as CTL effector cells at E:T ratios of 12.5:1, 25:1, and 50:1. Data are presented as mean specific lysis of triplicate values $\pm$ standard deviation. The CTL activity was determined by a standard CytoTox 96® Non-Radioactive Cytotoxicity Assay.

DISCUSSION

NK cells often play an important role in immune defense against tumors (19–22) whereas CTLs play a crucial role in eliminating host cells that contain intracellular pathogens and those that have undergone malignant transformation (2). However, the efficiency of T cell-based active immunotherapy (23, 24) has been limited due to low expression of either MHC molecules (25, 26) or their associated antigenic peptides by tumor cells (27). In contrast, monoclonal antibodies binding with high affinity to cell surface molecules abundantly expressed on cancer cells

have been shown to localize specifically to tumor tissues. Therefore, the strategy of using mAbs reacting with tumor cells to target CTL-defined antigens in the form of Fab' fragments coupled to a soluble MHC/peptide complex represents an attractive immunotherapeutic strategy. However, the capacity of antibodies to kill carcinoma cells is much weaker than that of cytotoxic T lymphocytes, and the results of clinical trials based on the use of antibodies alone have shown less than 10% complete tumor remissions (28, 29). Thus, there is a strong rationale for developing antitumor strategies that are focused on cellular immunity and that demonstrate a capacity to overcome even poorly immunogenic tumors.

The bacterial superantigen SEA exhibits highly potent lymphocyte-transforming (mitogenic) activity towards human and other mammalian T lymphocytes. Unlike conventional antigens, SEA can bind to certain regions of MHC class II molecules of APCs outside the classical antigen-binding groove, and simultaneously binds in native form to T cells at specific motifs of the variable region of the beta chain (Vβ) of the TCR. This interaction triggers the activation and proliferation of the targeted T lymphocytes and leads to the release of high levels of various cytokines and other effectors by immune cells. In contrast to conventional peptide antigens, SEA has a capacity to activate both subsets of T cells, and it has been demonstrated that both subsets of T cells were recruited to the tumor area in response to antibody-targeted SEA therapy (30, 31). In addition, C215Fab-SEA can trigger cytotoxic T cells against C215 antigen-positive tumor cells and induce tumor-suppressive cytokines (32, 33). Unfortunately, not all malignancies express a common antigen, and therefore, these types of SEA-based therapies are limited by their applicability to only certain tumor types.

It has been previously demonstrated that tumor cells transduced with SAg staphylococcal enterotoxin genes show increased immunogenicity and decreased tumorigenicity, and that the tumor vaccines prepared from tumor cells transduced with staphylococcal enterotoxin genes can induce potent antitumor cellular immunity (34–36). As a further step to improve the antitumor effect of SEA immunotherapy, we have shown that a transmembrane SEA (i.e., TM-SEA) anchored onto tumor cells stimulates lymphocyte proliferation (16). In the present study, we have extended our earlier results with TM-SEA, and have demonstrated a novel and effective vaccination method where SAg SEA was incorporated on to the surface of tumor cells and used them to elicit a potent antitumor immune response *in vivo*.

Tumor growth inhibition was observed not only in the mice immunized with B16-TM-SEA vaccine and then challenged with wild-type B16 cells, but also in the mice that were inoculated with tumor cells and subsequently treated with B16-TM-SEA vaccine. The tumor growth in control mice was faster relative to that in the mice which were treated with B16-TM-SEA vaccine or in those which received B16-TM-SEA vaccine and then challenged with wild-type B16 tumor cells. The animal survival was also prolonged in the above treatment groups as compared controls. These findings indicate that B16-TM-SEA vaccine is highly effective for treating established B16 melanoma and also has significant protective effect against a challenge with wild-type B16 cells.

Efficient immune effector cell activities were induced in the mice of therapeutic as well as prophylactic vaccination groups. Splenic lymphocytes from the tumor-bearing mice treated with B16-TM-SEA vaccine showed greater NK activity as compared that in controls. In addition, re-stimulated splenic lymphocytes both from the tumor-bearing mice treated with B16-TM-SEA vaccine and from the mice immunized with B16-TM-SEA vaccine and challenged later exhibited greater CTL activity as compared that in controls. Furthermore, the mice that received B16-TM-SEA vaccine, the inhibition of tumor growth and prolongation of survival was specific to challenge with B16 tumor because no inhibitory effect was observed when the animals were challenged with EL 4 lymphoma cells. Together, these data suggest that there was augmentation of nonspecific, and more importantly tumor-specific immunity induced by B16-TM-SEA tumor vaccine. Although the NK response reactive against B16 was also enhanced by B16-TM-SEA tumor vaccine, the predominant antitumor effect was apparently T cell-mediated.

CONCLUSIONS

The current study utilized a novel strategy for anchoring superantigen onto the surface of tumor cells, without any genetic manipulation, for the development of an anti-melanoma vaccine. In our study, significant tumor inhibition and prolonged survival were observed in mice receiving B16-TM-SEA vaccine before or after B16 inoculation. Significantly augmented immune effector cell activities existed in tumor-bearing mice either treated with B16-TM-SEA vaccine or immunized with B16-TM-SEA vaccine and then challenged with wild-type B16 cells. Our findings that the prophylactic immunization with B16-TM-SEA vaccine was effective against B16 but not against a control tumor cell line are consistent with the dominant role of tumor-specific T lymphocytes. In summary, our observations suggest that the tumor cell vaccine with membrane-anchored TM-SEA have the potential for cancer immunotherapy that can be used for eradication of

nonresectable tumors, for elimination of micrometastases, or for prevention of tumor recurrence.

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