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ORIGINAL ARTICLE

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Mutated SEA-D227A-conjugated antibodies greatly enhance antitumor activity against MUC1-expressing bile duct carcinoma

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Abstract For the purpose of establishing a new adoptive immunotherapy for bile duct carcinoma (BDC), we have directed our attention to superantigens (SAgs), the most potent known activators of T lymphocytes. In our previous study, staphylococcal enterotoxin A (SEA) was conjugated chemically with MUSE11 mAb, which recognizes the MUC1 cancer-associated antigen, and shown to enhance the specific cytotoxic activity of T-LAK cells against MUC1-expressing BDC cells (TFK-1) in vitro and in vivo. However, it is probable that SEA might cause side-effects because of nonspecific binding to class II positive cells. In order to overcome these, we generated mutated SEA (mSEA) by changing Asp at position 227 of native SEA to Ala, which has reduced affinity to MHC class II molecules, but retains the potential for T cell activation. When mSEA-D227A was administered to rabbits to examine effects on blood pressure, 500 times more mSEA-D227A was tolerated than native SEA. This prompted us to construct a mSEA-D227A-conjugated mAb, reactive with MUC1. It augmented the antitumor activity of T-LAK cells significantly, and furthermore, mSEA-D227A could be conjugated to two bispecific antibodies, BsAb (anti-MUC1×anti-CD3) and BsAb (anti-MUC1×anti-CD28), which in combination had greater enhancing effects than mSEA-D227A-conjugated anti-MUC1 mAb, and combination of unconjugated BsAbs. These findings indicate a utility of mSEA-D227A-conjugated antibodies for targeted cancer immunotherapy.

Keywords Mutated SEA · MUC1 · Immunotherapy · Superantigen · Xenografted SCID mice

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Introduction

The long-term survival of patients with bile duct carcinoma (BDC) remains far from satisfactory because of the difficulty of curative resection, even with recent advances in surgical technology [33]. We have tried a multi-modal approach, incorporating irradiation and chemotherapy after surgery, but recurrence from remnant BDC still occurs in spite of aggressive therapy. To overcome these difficulties, immunotherapy has attracted a great deal of attention. In a previous study, we demonstrated that a combination of two kinds of bispecific antibodies (BsAbs), anti-MUC1×anti-CD3 and anti-MUC1×anti-CD28, enhanced tumor growth inhibition by T-LAK cells against MUC1-expressing tumor cells [16]. To refine the strategy of this specific targeting, we have directed our attention to superantigens (SAgs), the most potent known activators of T lymphocytes, which bind to MHC class II molecules and activate T cells expressing particular T cell receptor (TCR) Vβ elements [25]. Staphylococcal enterotoxin A (SEA) was therefore conjugated chemically with the MUSE11 mAb, which recognizes epithelial mucin antigen MUC1, widely expressed in adenocarcinomas such as breast cancer, pancreatic

cancer, ovarian cancer and BDC [35]. SEA binds to T-LAK cells through MHC class II molecules and thereby activates them. MUSE11 mAb, on the other hand, increases the ability of T-LAK cells to target MUC1 after introduction of an appropriate ligand. In line with expectation, we could demonstrate that the SEA-conjugated MUSE11 mAb enhances the specific cytotoxic activity of T-LAK cells against MUC1-expressing tumor cells in vitro and in vivo [32].

However, SEA might cause side-effects, such as hypotension, fever, pulmonary edema, and septic shock, because of nonspecific binding of SEA to MHC class II positive cells [10, 14]. In order to overcome these side-effects, an Ala substitution of the Asp residue at position 227 was introduced into the SEA (mSEA-D227A) to reduce binding to class II. The affinity of mSEA-D227A to class II molecules was decreased remarkably compared with native SEA, but induction of proliferation of PBMCs was restored, and mSEA-D227A-conjugated MUSE11 mAb enhanced cytotoxicity of T-LAK cells against MUC1-expressing BDC cells. Furthermore, chemically conjugated mSEA-D227A BsAb (anti-MUC1×anti-CD3) and mSEA-D227A-BsAb (anti-MUC1×anti-CD28) demonstrated particularly strong cytotoxicity against TFK-1 cells when they were given together to BDC cells. Here, we describe the effectiveness of mSEA-D227A-conjugated Abs for targeted immunotherapy.

Materials and methods

Monoclonal antibodies (mAbs)

For construction of mSEA-BsAbs, anti-CD3 (OKT-3, mouse IgG2a) and anti-CD28 (15E8, mouse IgG1), directed at effector cells, and MUSE11 mAb (mouse IgG1), reactive with MUC1 antigen on tumor cells, were used. The MUSE11 was produced by Hinoda (Sapporo Medical University) [12], and 15E8 was kindly provided by Dr. R.A. van Lier (The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). For flow cytometry analysis of SEA binding to cells, rabbit anti-SEA Ab (S-7656; Sigma, St. Louis, Mo.) was used as the first antibody (Ab), and FITC-conjugated goat anti-rabbit IgG Ab (H+L, 0833 Immunotech, Westbrook, Me.) as the second Ab.

Cell lines

Three human BDC cell lines (TFK-1, OCUCh-LM1, and HuCC-T1) were used in this work. TFK-1 [30] was established in our laboratory. OCUCh-LM1 was provided by Dr. Yamada (Osaka City University School of Medicine, Osaka, Japan) and HuCC-T1 was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Chinese ovary cell line (CHO-K1) was used as a MUC1 negative control. A Burkitt's lymphoma cell line (Raji) expressing MHC class II was used for flow cytometry. These cell lines were all cultured in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Effector cells

For preparation of T-LAK cells, peripheral blood mononuclear cells (PBMCs) isolated by density gradient (Lymphoprep) centrifugation from a healthy volunteer, were stimulated with solid phase OKT-3 mAb and 100 IU/ml recombinant human IL-2 (kindly supplied by Shionogi Pharmaceutical Co., Osaka, Japan), as reported previously [32].

Production and purification of BsAbs

BsAbs were constructed by chemical cross-linking, as described previously [16, 28]. Briefly, the OKT-3 mAb was digested to $F(ab')_2$ with pepsin in 0.1 M citrate buffer (pH 4.0) at 37°C for 2 h. Then, the $F(ab')_2$ fragments were reduced by DTT (0.5 mM), the reaction was stopped by addition of DTNB, and OKT-3 Fab′-S-NB fragments were separated from excess reagents by gel filtration. The MUSE11 mAb was digested to $F(ab')_2$ with preactivated papain in 0.1 M acetate/EDTA buffer (pH 5.5) at 37°C for 2 h. After reduction with DTT, MUSE11 Fab′-SH fragments were separated by gel filtration. Then Fab′-S-NB fragments of OKT-3 mAb and Fab′-SH fragments of MUSE11 mAb were mixed at a molar ratio of 1:1 and incubated at room temperature for 4 h. The reaction mixture was subjected to gel filtration chromatography (FPLC system) with a HiLoad 16/60 Superdex 200 prep-grade column (Pharmacia Biotech, Uppsala, Sweden) to remove the unreactive fragments, allowing the anti-MUC1×anti-CD3 BsAb to be obtained. The anti-MUC1×anti-CD28 was prepared as follows. $F(ab')_2$ fragments of 15E8 mAb were obtained by digestion with preactivated papain, and reduced with DTT. The reduction was stopped by addition of DTNB and the Fab′-S-NB fragments were separated by gel filtration chromatography. Fab′-S-NB (15E8) and Fab′-SH (MUSE11) were then mixed at a 1:1 ratio, and incubated for 4 h. The reconstituted anti-MUC1×anti-CD28 BsAb was purified with an FLPC system.

SEA and mSEA-D227A

Native staphylococcal enterotoxin A (SEA) was purchased from Toxin Technology, Inc. (Sarasota, Fla.), and recombinant SEA (reSEA) was produced as reported previously [31]. *Staphylococcus aureus* strain 129, producing SEA, was used as the source of the SEA gene. For amplification of the SEA gene by PCR, primers were designed according to the data of Betley and Mekalonos [5]. Oligonucleotide pair primers for the construction of SEA gene were as follows: 5′-ATTCCATGGCTAGCGAGAAAAGCGAAGAA A-3′ (forward) and 5′-ATCTCGAGTGAACCTCCACCTCC ACTTGTATATAAATA-3′ (reverse). For construction of mSEA-D227A, the amplified SEA gene was inserted into phagemid vector pTZ-18U. Asp in position 227 of the wild-type SEA was replaced with Ala by Kunkel's method [18] and the mSEA-D227A gene was inserted into the expression vector pUT17 (Fig. 1). *E. coli* strain BL21(DE3) was transformed by expression vector pUT-mSEA-D227A and transformed cells were grown at 28°C in LB broth, followed by addition of IPTG to induce mSEA-D227A production in the intracellular insoluble fraction as inclusion bodies. Sonicated cell pellets were dissolved in a buffer containing 6 M guanidine-HCl, 0.1 M Tris-HCl (pH 8.0), 0.2 M NaCl [31], and solubilized mSEA-D227A was purified by TALON metal affinity resin column (CLONTECH, Palo Alto, Calif.). Refolding of mSEA-D227A was performed by the phased-guanidine removing dialysis method developed by Tsumoto [34].

Production and purification of mSEA-D227A-conjugated MUSE11 mAb and BsAbs

Chemical conjugation of mSEA-D227A to MUSE11 mAb was performed as reported previously [32]. Briefly, BsAb was mixed with 64 mM SPDP (Pierce Chemical Co.) in DMSO, diluted 1:10 with 40 mM sodium phosphate/150 mM sodium chloride (pH 7.5), incubated at room temperature for 2 h and the mixture applied to a Sephadex G25-prepacked PD-10 column (Pharmacia Biotech) for purification of SPDP-BsAb. The mSEA-D227A was mixed with 2-iminothiolane HCl (Pierce Chemical Co.), prepared as a 20 mM solution, incubated at room temperature for 2 h and 2-iminothio-

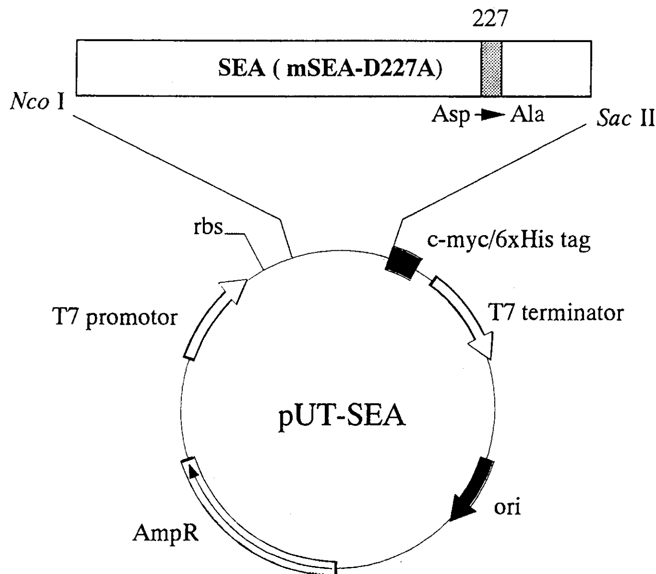


Fig. 1 Schematic representation of the SEA expression vector. *Amp*^{R} denotes the ampicillin resistance gene; *c-myc*, a sequence encoding an epitope recognized by 9E10 mAb; *ori*, origin of DNA replication; *6×His*, a sequence encoding six C-terminal histidine residues; *rbs*, a sequence encoding ribosome binding site for starting translation. *Asp* in position 227 of the wild-type SEA was replaced with *Ala* by Kunkel's method

lane-conjugated mSEA-D227A was then purified on a Sephadex G25-PD-10 column. The two fractionated reactants were mixed at a molar ratio of 1:1, and incubated for 2 h at room temperature with gentle rocking and left at 4°C overnight. The following day, the reaction mixture was subjected to gel filtration chromatography for purification of mSEA-D227A-BsAbs.

Binding of native SEA and mSEA-D227A to MHC class II positive cells

For determination of binding affinity of SEA with class II molecules, T-LAK cells and Raji cells were first incubated with 5 µg/ml of native SEA, reSEA, or mSEA-D227A. After washing twice, rabbit anti-SEA Ab (S-7656; Sigma, St. Louis, Mo.) was added as the first Ab and then FITC-conjugated goat anti-rabbit IgG (H + L, 0833, Immunotech Inc., Westbrook, Me.) as the second Ab. Stained cells were analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, Calif.).

Measurement of mean blood pressure after injection of SEA or mSEA-D227A into anesthetized Japanese white rabbits

Various concentrations of native SEA (from 0.05 to 0.1 µg/kg) or mSEA-D227A (from 0.05 to 50 µg/kg) were i.v. administered at 1.0 ml/min to Japanese white male rabbits anesthetized with diethyl ether and pentobarbital sodium solution. The mean arterial blood pressure was measured by cannulation of the femoral artery. Change in blood pressure is presented as percentage of pre-dose pressure for each animal.

Proliferation assay

Measurement of the proliferation of mitogen-activated human peripheral blood mononuclear cells (PBMCs) was performed using a Cell Proliferation ELISA system (Biotrak, Amersham Life Science, Bucks, UK) according to the manufacturer's recommendations. Briefly $1{\times}10^5$/ml freshly isolated PBMCs were plated in each well (0.2 ml) of 96-well flat-bottomed plates (Sumilon, Sumitomo Bakelite, Tokyo, Japan) in the presence or absence of varying concentrations of phytohemagglutinin (PHA), native SEA, reSEA and mSEA-D227A. For the determination of spontaneous proliferation, cells were cultured without mitogen. After incubation for 48 h at 37°C, BrdU label was added and the cells were reincubated for an additional 24 h at 37°C. Then microtiter plates were centrifuged at 300°*g* for 10 min and dried after removing the medium. The cells were incubated in 200 µl/well fixative solution for 30 min at room temperature, incubated in 200 µl/well blocking buffer for 30 min at room temperature, and after its removal further incubated in 100 µl/well peroxidase-labeled anti-BrdU working solution for 90 min at room temperature. After removing antibody conjugate, the wells were rinsed three times with 300 µl/well washing solution and 100 µl of TMB substrate was dispensed into all wells. When the required color intensity was achieved, the reaction was stopped by pipetting 25 µl of 1 M H_2SO_4 into each well. The optical density was measured using a plate reader (Bio-Rad model 3550) at 450 nm.

Tumor growth inhibition assay

Tumor growth inhibition was evaluated with a MTS assay kit (Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay, Promega Co., Madison, Wis.) as reported elsewhere [32]. Briefly, target cells, detached with 0.02% EDTA-PBS solution, were seeded (10,000 cells/well in 0.1 ml culture medium) in triplicate in a flat-bottomed half area (A/2) 96-well plate (Costar Corp., Cambridge, Mass.). Cells were cultured overnight to allow adhesion, and after removing the culture medium by aspiration, different numbers of effector cells in 0.1 ml of culture medium were added to each well, with or without various BsAbs or mSEA-D227A-conjugated BsAbs. E/T ratios tested in this study were from 1.25 to 10. After culture for 48 h at 37°C, each well was washed with 100 µl of PBS, three times, to remove effector cells and dead target cells. This was followed by addition of fresh mixture of MTS [i.e., 3-(4,5-dimethylthiazole-2-yl)-5-(3-carboxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]/phenazine methosulfate solution (Promega Co.). After incubation at 37°C for 60 min, the plates were read on a microplate reader (Bio-Rad model 3550) at 490 nm. The percentage growth inhibition was calculated as follows: [1 – (A_{490} of experiment – A_{490} of medium)/(A_{490} of control – A_{490} of medium)]×100, where target cells grown in medium alone represent the control.

Inhibition assay

To confirm the specificity of the mSEA-D227A-BsAb-enhanced antitumor activity, blocking tests using MUSE11 mAbs or anti-SEA antibody were performed. For this, T-LAK cells in the presence of mSEA-D227A-bsAbs (0.6 µg/ml) underwent 48-h MTS assay at an E/T ratio of 5, with the antibodies added for blocking.

In vivo tumor models

Severe combined immunodeficient (SCID) mice (Fox CHASE C.B.-17/ Icr-Scid Jcr), female, 6–8 weeks of age, purchased from Japan Clea (Tokyo, Japan), were inoculated with $5{\times}10^6$ TFK-1 cells s.c. into the dorsal thoracic wall on day 0. Treatment was initiated on day 10 after tumor inoculation. For therapy, T-LAK cells were preincubated with BsAbs (anti-MUC1×anti-CD3 BsAb alone, anti-MUC1×anti-CD3 plus anti-MUC1×anti-CD28 BsAbs, or mSEA-D227A-conjugated anti-MUC1×anti-CD3 plus mSEA-D227A-conjugated anti-MUC1×anti-CD28 BsAbs), 2 µg each BsAb for $2{\times}10^7$ T-LAK cells, at 4°C for 1 h, and T-LAK cells were washed once with PBS and resuspended in 0.15 ml PBS containing IL-2 (500 IU). For 4 consecutive days, the mice received i.v. $2{\times}10^7$ pretreated T-LAK cells via the tail vein, together with IL-2 (500 IU/mouse), in 0.15 ml PBS. Tumor size was measured with a caliper weekly for 10 weeks, and tumor weight (W) in mg was calculated from linear measurements of the width (A) in mm and

length (B) in mm as follows: W = $(A^2 \times B)/2$. When a mouse's tumor reached 2.0 cm in diameter, the mouse was killed according to institutional guidelines.

Results

Reactivity of native SEA, reSEA and mSEA-D227A with T-LAK cells and Raji cells

To determine the affinity of native SEA, reSEA and mSEA-D227A for class-II-expressing cells (T-LAK and Raji cells), flow cytometry was performed (Fig. 2). Both native SEA and reSEA demonstrated strong binding to T-LAK cells and Raji cells, but mSEA-D227A showed remarkably reduced reactivity with Raji cells.

Proliferative effect of reSEA and mSEA-D227A on PBMCs

PBMCs from healthy volunteers were cultivated for 72 h (final concentration 5×10^5/ml) in flat-bottomed 96-well plates with 0.1 μg/ml of reSEA, or mSEA-D227A, or PHA. After 72 h of culture, BrdU incorporation was measured by ELISA using a mAb against BrdU for measurement of cell proliferation after stimulation (Fig. 3). Very strong proliferation was induced by reSEA and PHA (1:200, 1:100), but with mSEA-D227A only moderate proliferative activity was observed (about 60% that of reSEA).

Measurement of mean blood pressure after inoculation of native SEA or mSEA-D227A into anesthetized Japanese white rabbits

Various concentrations of SEA (from 0.05 to 0.1 μg/kg) or mSEA-D227A (from 0.05 to 50 μg/kg) were i.v. administered to Japanese white male rabbits at 1.0 ml/min and mean arterial blood pressure was measured by cannulation of the femoral artery (Fig. 4). Although 0.1 μg/kg of native SEA caused remarkable hypotension, this was lacking even with 5 μg/kg of mSEA-D227A; a dose of 50 μg/kg was required to cause comparable effects.

Antitumor activity of T-LAK cells prestimulated by mSEA-D227A

To determine the effects of mSEA-D227A on T-LAK cells, the latter were preincubated with 0.1 μg/ml or 0.5 μg/ml mSEA-D227A at 37°C for 6 h, then washed once, and resuspended in culture medium for assessment of growth inhibition of TFK-1 cells (Fig. 5). Prestimulation of T-LAK cells resulted in significant enhancement of tumor growth inhibition, indicating that mSEA-D227A had the ability to stimulate T-LAK cells regardless of the low affinity (Fig. 2). Prolongation of the prestimulation time to 12 h did not result in an increase of antitumor activity (data not shown).

Tumor growth inhibition of T-LAK cells enhanced by mSEA-D227A-conjugated MUSE11 mAb

Various concentrations of mSEA-D227A were added to the MTS assay, with TFK-1 target and T-LAK effector cells. The level of tumor growth inhibition by T-LAK cells alone was less than 20%; mSEA-D227A conjugated-MUSE11 mAb enhanced the antitumor activity of T-LAK cells against TFK-1 cells in a dose-dependent manner (Fig. 6). At an E/T ratio of 20 and a concentration of 5.0 μg/ml, tumor growth inhibition reached 60%.

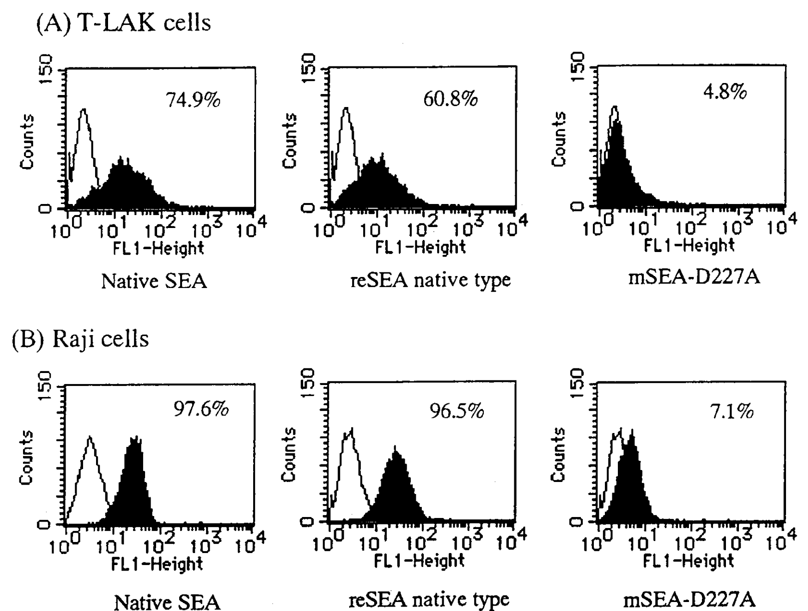


Fig. 2 Flow cytometry. To determine the binding of various SEAs to class-II-expressing cells (T-LAK and Raji cells), cells were first incubated with native SEA, reSEA or mSEA-D227A for 30 min on ice. After washing twice, rabbit anti-SEA Ab was applied as the first Ab and then FITC-conjugated goat anti-rabbit Ab as the second Ab, followed by staining. Each percentage figure indicates the frequency of positive cells

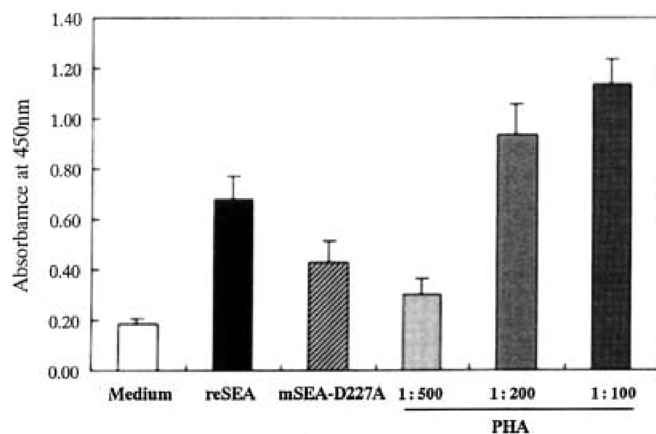


Fig. 3 Proliferative effects of two recombinant SEAs on PBMCs. PBMCs were cultured for 72 h ($1{\times}10^5$ cells/0.2 ml/well) in flat-bottomed 96-well plates in the presence of 0.1 μg/ml of reSEA, or mSEA-D227A, or PHA. After 72-h culture, BrdU incorporation was measured by ELISA. The optical density was determined with a microplate reader (Bio-Rad model 3550) at 450 nm

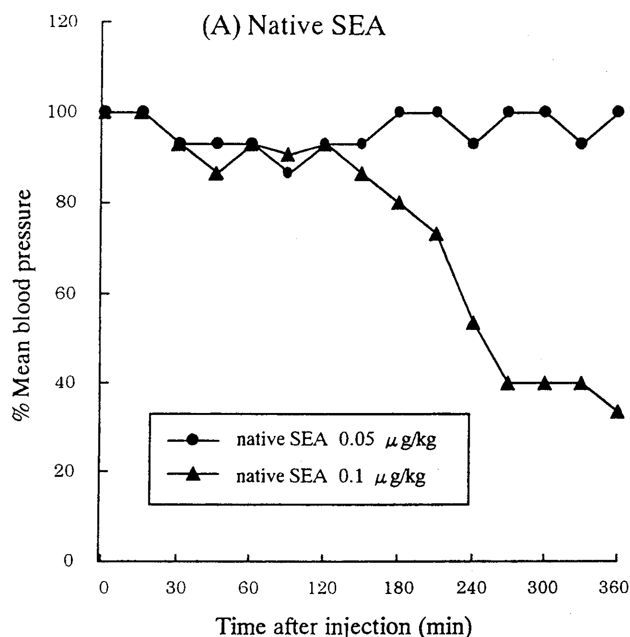
Tumor growth inhibition by BsAbs and mSEA-D227A-conjugated BsAb-mediated T-LAK cells

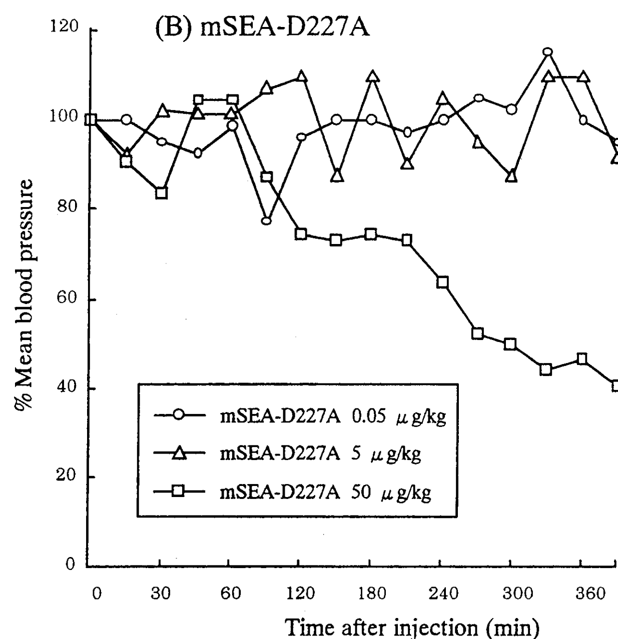
In order to enhance the antitumor activity further, we constructed two mSEA-D227A-conjugated BsAbs: anti-MUC1×anti-CD3 and anti-MUC1×anti-CD28. Various BsAbs, together with T-LAK cells, were examined for antitumor activity. At a concentration of 0.1 μg/ml, anti-MUC1×anti-CD3 BsAb alone and combination of two unconjugated BsAbs (anti-MUC1×anti-CD3, plus anti-MUC1×anti-CD28) showed similar levels of tumor growth inhibition (Fig. 7). When combinations of mSEA-D227A-conjugated BsAb anti-MUC1×anti-CD3 and BsAb anti-MUC1×anti-CD28 were applied, tumor growth inhibition was about 60%. The highest antitumor activity (about 80% tumor growth inhibition) was obtained with addition of mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) and mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD28) together. At a concentration of 0.5 μg/ml BsAbs, enhancement of antitumor activity was more remarkable than for 0.1 μg/ml, with similar tendencies for each BsAb. Maximum tumor growth inhibition (95%) was obtained by the combination of mSEA-D227A-conjugated BsAb(anti-MUC1×anti-CD3) and mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD28).

Specificity of antitumor activity

To verify the specificity of the antitumor activity in this study, two BDC lines that had previously been shown to express MUC1 [32] and the CHO-K1 cell line were used as target cells in the MTS assay. T-LAK cells in the presence of combinations of either the two BsAbs (anti-MUC1×anti-CD3 plus anti-MUC1×anti-CD28) or the two mSEA-D227A-conjugated BsAbs [mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) and mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD28)] demonstrated antitumor activity against OCUCh-LM1 cells (MUC1-positive) in a BsAb-dose-dependent manner, though the latter combination was

Fig. 4 Measurement of mean blood pressure after inoculation of native SEA or mSEA-D227A into anesthetized Japanese white rabbits. Various concentrations of SEA (from 0.05 to 0.1 μg/kg) or mSEA-D227A (from 0.05 to 50 μg/kg) were i.v. administered to Japanese white male rabbits at 1.0 ml/min and mean arterial blood pressure was measured by cannulation of the femoral artery. Change in blood pressure is presented as the percentage of the predose pressure for each individual animal





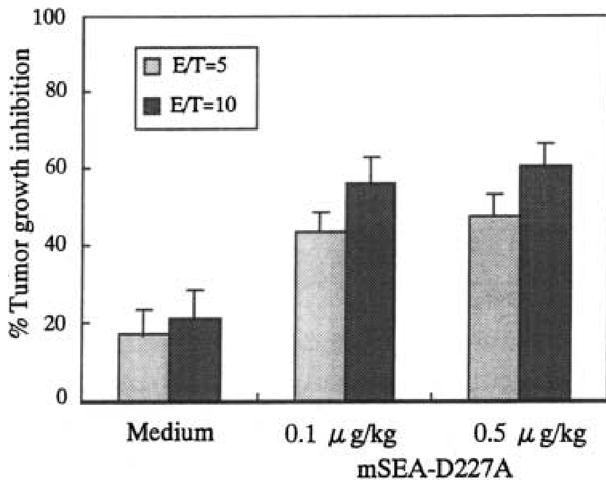


Fig. 5 Tumor growth inhibition by T-LAK cells prestimulated by mSEA-D227A. T-LAK cells were preincubated at 37°C for 6 h with 0.1 μg/ml or 0.5 μg/ml mSEA-D227A, washed once, resuspended in culture medium and utilized in the growth inhibition assay against TFK-1 cells. Percentage tumor growth inhibition was determined by 48-h MTS assay, as described in Materials and methods. The *columns* show the results (mean values, with *bars* indicating SD) of triplicate determinations

more effective than the former (Fig. 8A). When Hu-CCT1 cells, with marginal MUC1-expression, served as MTS targets in the same assay system, the antitumor activity of BsAbs was negligible at concentrations of less than 1 μg/ml with either combination of BsAbs, though slight antitumor activity was observed with greater doses (Fig. 8B). When MUC1-negative CHO-K1 cells served as the target cells, the two mSEA-D227A-conjugated BsAbs did not enhance antitumor activity to this cell line even at 1.5 μg/ml (data not shown), indicating that antitumor activity of these BsAbs was dependent on the expression of MUC1 on the target cells.

Antibody blocking tests

In order to confirm the MUC1-specificity of BsAbs, various concentrations of anti-SEA Ab or anti-MUSE11 $F(ab')_2$ mAb were added to the MTS assay, with T-LAK effector cells and the combination of mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) and mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD28). Significant dose-dependent reduction of antitumor activity was observed (Fig. 9), indicating the mSEA-D227A-conjugated BsAb dependent antitumor activity to be MUC1-specific.

Experimental therapy of xenografted SCID mice

Xenografted SCID mice were divided into five groups (10 mice/group), which received injections of T-LAK cells sensitized with various BsAbs, together with IL-2, according to the following protocols: group A, injection of PBS; group B, T-LAK cells alone; group C, T-LAK cells sensitized with unconjugated BsAb (anti-MUC1×anti-CD3); group D, T-LAK cells sensitized with unconjugated BsAbs (anti-MUC1×anti-CD3 plus anti-MUC1×anti-CD28); group E, T-LAK cells sensitized with mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) plus mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD28). Group A and group B showed continuous similar tumor growth, and group C showed slight retardation of growth compared with the former two groups (Fig. 10). Group D showed slower tumor growth compared with the former 3 groups up to week 7, but tumor growth was gradual thereafter. Group E showed the best results, though tumor size differed from mouse-to-mouse; the average inhibition being about 70% compared with the groups A and B, and complete disappearance of tumors was observed in 3 out of 10 mice.

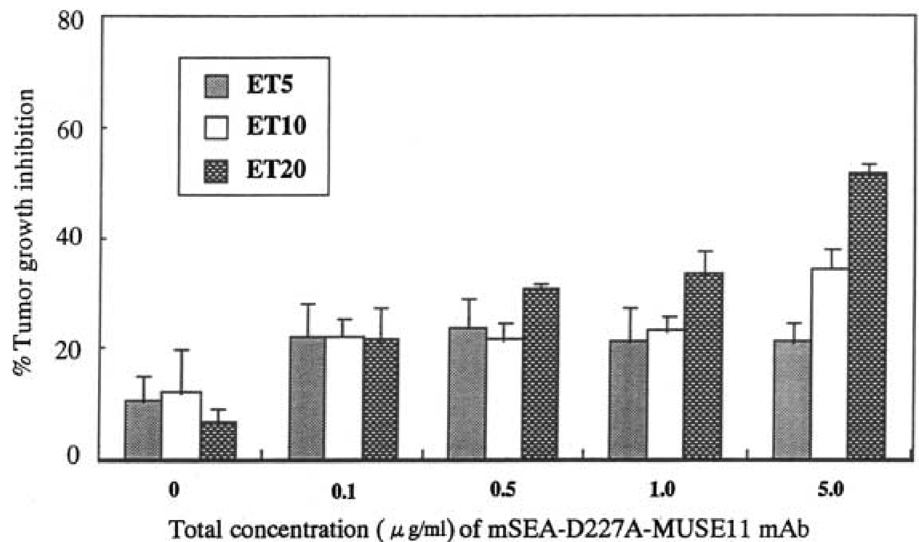


Fig. 6 Enhancement of T-LAK cell antitumor activity by mSEA-D227A-conjugated MUSE11 mAb. Various concentrations of mSEA-D227A-MUSE11 mAb were added to MTS assay, in the presence of TFK-1 target and T-LAK effector cells

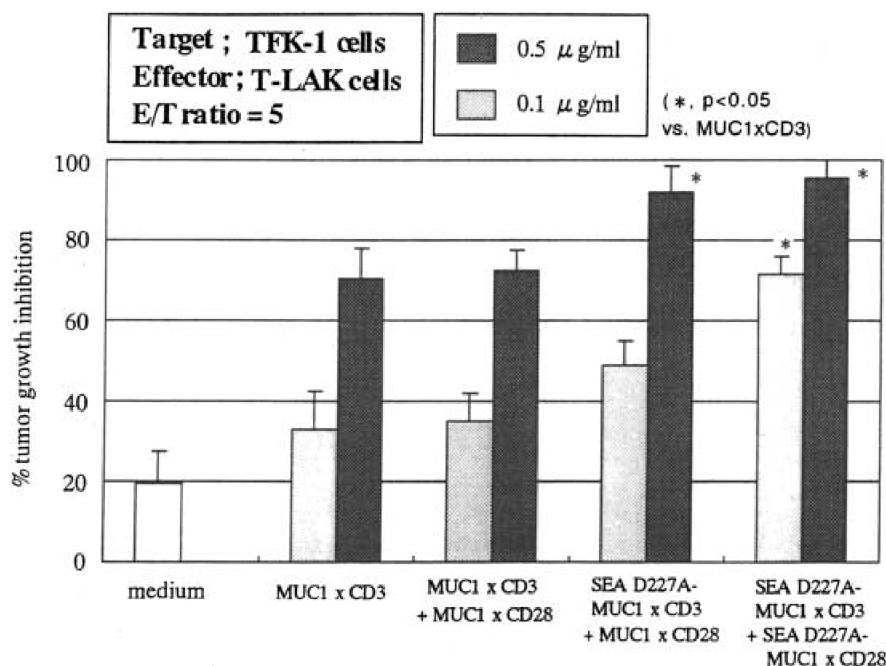


Fig. 7 Enhancement of T-LAK cell antitumor activity by unconjugated BsAbs or mSEA-D227A-conjugated BsAbs. Various BsAbs, together with T-LAK cells, were examined for antitumor activity against TFK-1 cells (48-h MTS assay). BsAbs used were: (1) unconjugated BsAb (anti-MUC1×anti-CD3); (2) unconjugated BsAb (anti-MUC1×anti-CD3) plus unconjugated BsAb (anti-MUC1×anti-CD28); (3) mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) plus unconjugated BsAb (anti-MUC1×anti-CD28); (4) mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) plus mSEA-D227A-conjugated BsAb (anti-MUC1 plus anti-CD28)

Discussion

In our previous study [32], SEA-conjugated MUSE11 mAb enhanced remarkably the specific cytotoxic activity of T-LAK cells against MUC1-expressing BDC cells (TFK-1), both in vitro and in vivo. A very low concentration (about 0.01 μg/ml of SEA-MUSE11 mAb) increased growth inhibition. Furthermore, a SEA-antibody fusion protein (SEA-MUSE11 Ab), namely, SEA-single chain Fv (SEA-scFv), constructed by bacterial expression system, also enhanced T-LAK cell cytotoxicity in vitro [31]. Since SEA is one of the most potent known activators of T lymphocytes, the results were expected. However, it binds nonspecifically to MHC class II molecules [25] and activates T cells expressing particular T cell receptor (TCR) Vβ elements [15, 24, 26], these proliferating and producing cytokines, such as tumor necrosis factor (TNF)-α, INF-γ, IL-2, IL-6 and IL-12 [6, 9, 20, 21]. Therefore, SEA can cause serious side-effects, such as hypotension, fever, pulmonary edema, and septic shock [10]. In order to overcome this problem, we have generated mSEA-D227A by changing Asp at 227 of native SEA to Ala, which is reported to reduce affinity for MHC class II molecules [1, 11, 13, 23]. Indeed, the mSEA-D227A constructed in this study demonstrated clear reduction of the affinity for MHC class II-expressing Raji cells (Fig. 2). In contrast to native SEA, mSEA-D227A also did not cause a drop in blood pressure at 5 μg/kg, and the rabbits tolerated about 500 times greater quantities. The appearance of side-effects with SEA has been reported to be inversely related to the serum antibody activity to SEA [2]; patients with Abs to SEA showing only slight side-

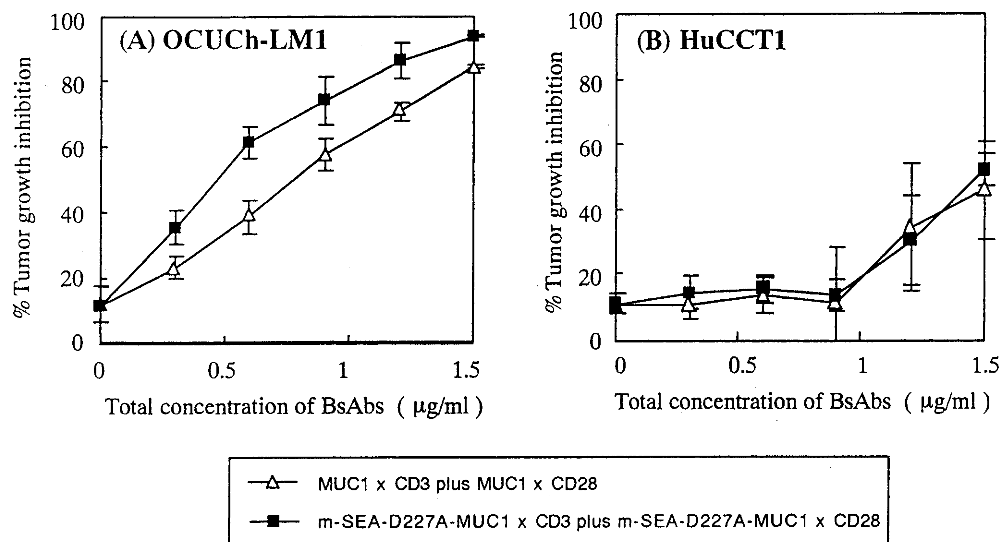
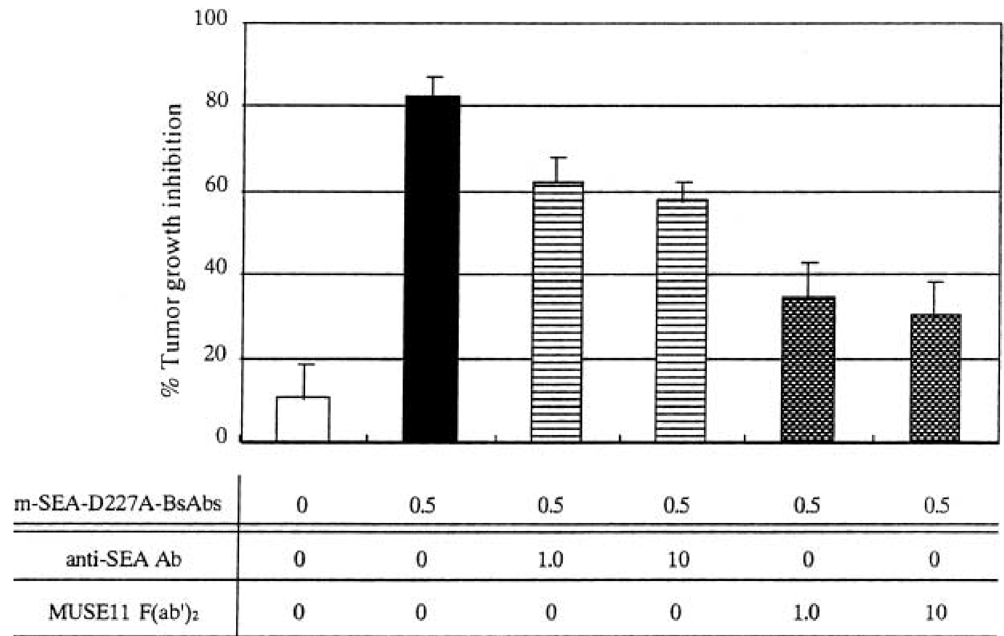


Fig. 8A, B mSEA-D227A-conjugated BsAb-mediated antitumor activity to two BDC cell lines. To verify the specificity of antitumor activity with combinations of unconjugated BsAb (anti-MUC1×anti-CD3) and unconjugated BsAb (anti-MUC1×anti-CD28) (△) or mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) and mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD28) (■), MUC1-positive OCUCh-LM1 (**A**) and MUC1-faint HuCCT1 cells (**B**) were used as targets in a 48-h MTS assay, with T-LAK effector cells



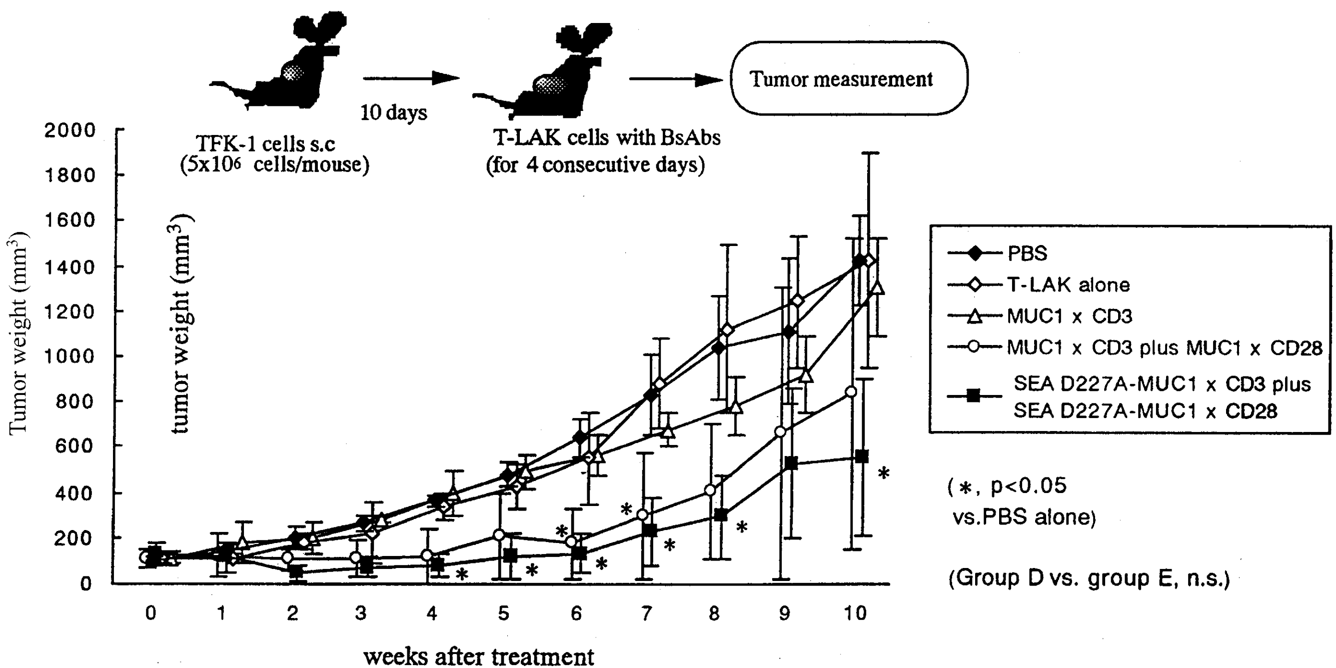
m-SEA-D227A-BsAbs	0	0.5	0.5	0.5	0.5	0.5
anti-SEA Ab	0	0	1.0	10	0	0
MUSE11 F(ab')₂	0	0	0	0	1.0	10

Fig. 9 Blocking tests by anti-SEA Ab and anti-MUSE11 mAb. To confirm the specificity of the mSEA-D227A-BsAb-enhanced antitumor activity, blocking tests were performed using MUSE11 mAb or anti-SEA Ab. T-LAK cells (effector cells) and TFK-1 cells (target cells) were cultured in the presence of combination of mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) plus mSEA-D227A-conjugated BsAb (anti-MUC1 plus anti-CD28) at the concentration of 0.6 μg/ml (48-h MTS assay at an E/T ratio of 5), with either MUSE11 mAb or anti-SEA Ab added for blocking

effects. Thus, mSEA-D227A might be particularly applicable in individuals with serum Ab to SEA.

To determine the effects of mSEA-D227A on T-LAK cells, their antitumor activity was measured (Fig. 5). Prestimulation of T-LAK cells with mSEA-D227A at 37°C for 6 h resulted in significant enhancement of antitumor activity, regardless of the low binding affinity. Prolongation (12 h) of the preincubation time resulted in a significant decrease in antitumor activity (data not shown), as reported for native SEA with resting T cells [7, 28]. However, this anergy appears to be canceled by the addition of IL-2, which enhances and prolongs the survival of SEA-reactive T cells after exposure to SEA [19]. This is the reason why mSEA-D227A-BsAb-sensitized T-LAK cells were given together with IL-2 to xenotransplanted SCID mice (Fig. 10).

To apply the mSEA-D227A for specific targeting therapy, mSEA-D227A was conjugated to MUSE11 mAb chemically. The conjugated mAb significantly enhanced antitumor activity of T-LAK cells (Fig. 5), but quite a high E/T ratio of 20 and a high concentration (5 μg/ml) were required to obtain the same levels (about 60% tumor growth inhibition) as with native SEA-conjugated MUSE11 mAb (0.05 μg/ml) found previously [32]. In our preliminary experiment, reSEA showed remarkable enhancement of antitumor activity,



almost equivalent to native SEA, as reported previously [32], and reSEA-conjugated MUSE11 mAb demonstrated antitumor activity and antibody specificity almost similar to native SEA-conjugated MUSE11 mAb (data not shown). In order to avoid harmful effects caused by contamination of reSEA, we omitted the production of reSEA-conjugated BsAbs and experiments with them.

To enhance the relatively low antitumor activity of the mSEA-D227A-conjugated MUSE11 mAb, we constructed mSEA-D227A-conjugated BsAbs by chemical cross-linking [17, 27]. A combination of two mSEA-D227A-conjugated BsAbs (anti-MUC1×anti-CD3 plus anti-MUC1×anti-CD28) was very effective. The present experiments with MUC1-positive (TFK-1, OCUch-LM1), marginal (HuCC-T1) and MUC1-negative (CHO-K1) target cells, and with blocking antibodies, clearly showed the MUC1 specificity of the combination of two mSEA-D227A conjugated BsAbs.

Significant reduction of tumor growth was also observed in the tumor-xenografted mice, which received mSEA-D227A-conjugated BsAbs (group E), with a 30% cure rate. While the antitumor activity with mSEA-D227A-conjugated BsAbs was stronger than with the two unconjugated BsAbs in vitro, no significant difference was observed in the in vivo results, when two groups (D and E) were compared in respect of tumor weight. This may be partly due to the lack of recruitment of human T cells reactive with mSEA-D227A in SCID mice, though they received T-LAK cells at the beginning of therapy. Another possibility might be that partial inactivation of mSEA-D227A due to conformational change could have taken place during chemical conjugation. Construction of mSEA-D227A-conjugated BsAbs as fusion proteins by gene engineering methods might be favorable for this purpose, since massive production of SEA-MUSE11 Ab fusion protein is relatively easy in bacterial expression systems [31]. In order to facilitate the application of mSEA-D227A-conjugated BsAbs to clinical trials, production of a mSEA-D227A-bispecific single chain Fv (Bs-scFv) fusion protein by genetic engineering warrants attention, because such fragments offer several advantages, such as better penetration of solid tissues, lower toxicity/immunogenicity, and reduction of costs compared with the BsAbs produced by tetradoma technology or chemical cross-linking [8, 22].

In our in vivo study, exogenous IL-2 probably prevented the reduction in antitumor activity of T-LAK cells after repeated injections with mSEA-D227A, as in the case with SEA [3, 4]. When mSEA-D227A-conjugated BsAb-sensitized T-LAK cells are given to patients, cytokine production takes place not only by these injected cells but also by local T cells in the tumor area, which might bring about amplification of antitumor activity.

In conclusion, mSEA-D227A-conjugated MUSE11 mAb or BsAbs are good candidates for specific targeting therapy. Further research should make it possible for us to apply SAgs with low toxicity to clinical trials for patients with BDC or other MUC1-positive carcinomas.

Fig. 10 Experimental therapy of BDC-xenografted SCID mice. SCID mice were inoculated with 5×10^6 TFK-1 cells s.c. into the dorsal thoracic wall on day 0, and treatments initiated at day 10 thereafter. For therapy, five groups of mice (10 mice/group) were treated according to the following protocols: *A* (◆), injection of PBS; *B* (◇), T-LAK cells alone; *C* (△), T-LAK cells sensitized with unconjugated BsAb (anti-MUC1×anti-CD3); *D* (○), T-LAK cells sensitized with combination of two unconjugated BsAbs (anti-MUC1×anti-CD3 plus anti-MUC1×anti-CD28); *E* (■), T-LAK cells sensitized with a combination of mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD3) and mSEA-D227A-conjugated BsAb (anti-MUC1×anti-CD28). On day 10, T-LAK cells (2×10^7 cells) were preincubated with various combinations of BsAbs (2 μg for each BsAb), at 4°C for 1 h, washed once with PBS, resuspended in 0.15 ml PBS containing IL-2 (500 IU), and inoculated (2×10^7 cells) with IL-2 (500 IU/mouse) i.v., via the tail vein. The therapy was repeated daily for 4 consecutive days. Tumor size was measured with a caliper weekly for 10 weeks, and tumor weight (W) in mg was calculated from linear measurements of the width (A) in mm and length (B) in mm as follows : $W = (A^2\times B)/2$

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