Construction and characterization of a novel fusion protein consisting of anti-CD3 antibody fused to recombinant interleukin-2

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Abstract. T cells can be activated *in vitro* by monoclonal antibodies to CD3 (anti-CD3) to become non-MHC restricted killer cells (CD3-AK). Anti-CD3 activation upregulates the expression of the interleukin (IL)-2 receptors on T cells whose expansion is facilitated by IL-2. The therapeutic effect of *in vivo* administration of anti-CD3 and IL-2 has been investigated in many types of human cancers. To circumvent the toxicities posed by systemic administration of high-dose IL-2, there is interest in forming a strategy for targeting and concentrating IL-2 at the site where it is needed. This study investigates the feasibility of constructing a novel fusion protein consisting of IL-2 fused to the constant region of anti-CD3 antibody. Our results indicate that the specific IL-2 receptor-binding capability and bioactivity of the IL-2 portion as well as the CD3-binding and biological functions of anti-CD3 portion remain intact in this anti-CD3/IL-2 fusion protein. Thus, cytokines fused to anti-CD3 antibody by genetic engineering is feasible and may provide a new class of immunotherapeutics for cancer.

Introduction

Immunotherapy is a promising approach to treat cancer (1). One of the strategies that have received attention is treatment with cytokines such as interleukin (IL)-2 to enhance anti-tumor immunity. IL-2 has been shown to exert stimulatory effects on a broad range of immune cell types including T and B cells, monocytes, macrophages, lymphokine activated killer cells (LAK) and NK cells (2). Systemic administration of IL-2 has been used to treat renal cell carcinoma and malignant melanoma with moderate success (3,4); however, the effectiveness of treatment is hampered by its toxicity, the most serious manifestation of which is vascular leak syndrome (5). Thus, further efforts may be to focus on reducing the systemic toxicity while maintaining an effective dose at the site of the tumor.

Cytotoxic T-lymphocytes (CTL) are very efficient in penetrating the tumor tissue, and thus, may induce tumor lysis (6). Attempts to use CTLs in human cancer therapy have focused on the cell-surface molecules that regulate CTL function. An important molecule in CTL function is the CD3 antigen. Anti-CD3 antibody could activate resting lymphocytes to become killer cells. These anti-CD3 antibody activated killer cells (CD3-AK) are non-MHC (major histocompatibility complex) restricted CTL (7,8) and can selectively kill a variety of tumor targets (9,10). IL-2 promotes the activation of the CD3-AK cells which was found to be regulated by protein kinase C (11,12). Thus, the anti-CD3-activated and IL-2-expanded lymphocytes have emerged as an alternative treatment for many human cancers e.g. renal cancer (13), head and neck cancer (14), breast cancer (15) and brain tumor (16). The popular strategy is to infuse the activated cells intravenously along with the concomitant administration of bolus and systemic infusions of high-dose IL-2 which usually cause undesirable side effects. To circumvent the systemic toxicity posed by IL-2, targeting delivery via anti-CD3 antibody-IL-2 fusion proteins is thought to take advantage of the paracrine nature of IL-2. Because these molecules concentrate cytokine at the site of cytotoxic T cells, they may allow the cytokine to be used at lower and less toxic doses. Thus, we describe in this study the construction of a novel fusion protein anti-CD3/IL-2, consisting of anti-CD3 antibody fused to IL-2, and characterize its biological activities to test whether this genetically engineered fusion protein retains anti-CD3 antibody specificity and IL-2 bioactivity.

Materials and methods

Construction and expression of two fusion proteins, anti-CD3/IL-2 and anti-CD3/Fc. Two fusion proteins were constructed

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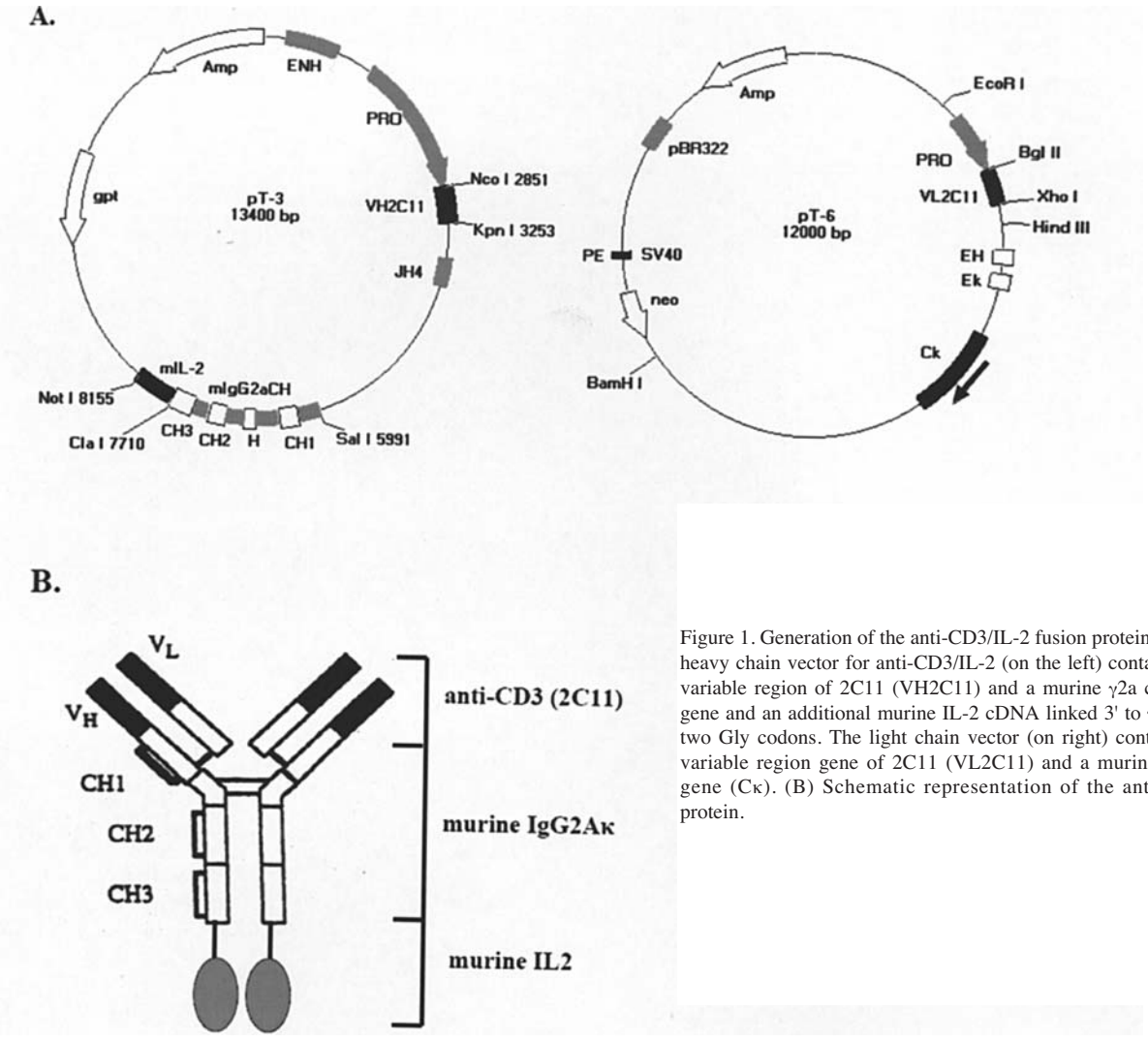

Figure 1. Generation of the anti-CD3/IL-2 fusion protein constructs. (A) The heavy chain vector for anti-CD3/IL-2 (on the left) contains the heavy chain variable region of 2C11 (VH2C11) and a murine γ2a constant region (Fc) gene and an additional murine IL-2 cDNA linked 3' to γ2a gene, spaced by two Gly codons. The light chain vector (on right) contains the light chain variable region gene of 2C11 (VL2C11) and a murine κ constant region gene (Cκ). (B) Schematic representation of the anti-CD3/IL-2 fusion protein.

by genetic engineering. The anti-CD3/IL-2 fusion protein contains both anti-CD3 and the IL-2 molecules conjugated at the C-terminal of constant region (Fc), while the anti-CD3/Fc contains anti-CD3 only. Briefly, the variable region of anti-CD3 (2C11) heavy chain gene (VH2C11) was amplified by reverse transcription (RT-PCR) from the hybridoma 145-2C11 (American Type Culture Collection, Rockville, MD, USA) using an upstream primer containing an NcoI site overlapping the translational start codon ATG (5'-CTTGCCATGGACTC AGGACTCCAATTG-3') and a downstream primer containing a KpnI site in the J segment (5'-CATGGTACCTTGGCCCC AGTA-3'). Similarly, the variable region of 2C11 light chain gene (VL2C11) was obtained with an upstream primer containing a BglII site (5'-GGAAGATCTATGAGGGCCCC TACTGTG-3') and a downstream primer containing an XhoI site (5'-GATCTCGAGCTTGGTGCCAGGTCCG-3'). The NcoI-KpnI amplified fragment (VH2C11) and BglII-XhoI amplified fragment (VL2C11) were cloned, respectively, into the heavy and light chain expression vectors (a gift from Dr Mi-Hua Tao, Academia Sinica, Taipei, Taiwan) that contain the murine γ2a constant region (mIgG2aCH) gene and murine Cκ constant region, respectively (Fig. 1A). Murine IL-2 cDNA was cloned by RT-PCR from C57BL/6J splenocytes with two flanking primers 5'-CTTATCGATGGCGGCGCACCC ACTTCAAG-3' and 5'-GGGCGGCCGCAGTTATTGAGGG CTTG-3', and cloned 3' to murine (2a gene with a two-amino-acid (GlyGly) linker between IL-2 and CH genes. Fusion proteins were made by cotransfecting a heavy chain gene with a light chain gene into the mouse myeloma cell line, FO (obtained from the American Type Culture Collection). The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (Sigma Chemical Co., St. Louis, MO), 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10 mM HEPES. Transfectomas were selected by resistance to G418 and screened by sandwich ELISA with rat anti-mouse IgG2a as coating antibody and HRP-rat anti-mouse Igκ as detection antibody.

Purification of recombinant anti-CD3/IL-2 and anti-CD3/Fc fusion proteins. Secreted fusion proteins from transfectomas were purified by protein A column. Bound antibodies were eluted from the column under acidic condition with a buffer containing 0.1 mol/l glycine, 0.15 mol/l NaCl, pH 2.4, and brought to neutral pH with 0.5 mol/l sodium phosphate pH 8.0. The purified fusion proteins were dialyzed against PBS and sterilized by filtration and determined for concentration by BCA assay (Pierce). The purified anti-CD3/IL-2 protein and anti-CD3/Fc protein were analyzed by SDS-PAGE under reducing conditions using 4-12% polyacrylamide gels. SDS-PAGE gels were stained with Coomassie blue. Proteins were

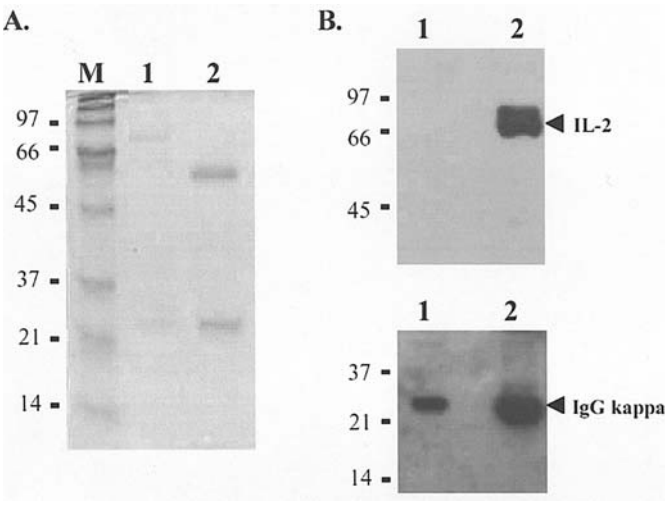

Figure 2. SDS-PAGE and Western blot analysis of the purified anti-CD3/IL-2 and anti-CD3/Fc fusion proteins. (A) The secreted proteins in transfected FO cells were purified by immnoaffinity chromatography and subjected to SDS-PAGE. Lane 1, molecular marker; lane 2, purified anti-CD3/IL-2 protein; and lane 3, purified anti-CD3/Fc protein. (B) The proteins were transferred into nitrocellulose and probed with mAb specific for murine IL-2 (upper) and IgG kappa (bottom), respectively. Lane 1, anti-CD3/Fc protein; lane 2, anti-CD3/IL-2 protein.

transferred into nitrocellulose by semidry electroblotting and probed with mAb specific for murine IL-2 and IgG kappa (PharMingen, San Diego, CA), respectively.

Cell lines and mice. The 38C13 cell is a carcinogen-induced B lymphoma cell of C3H/HeN mice origin (17). Cells were maintained in RPMI-1640, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Sigma) at 37˚C with 5% CO_2 in a humidified incubator. Female C3H/HeN mice, 10 weeks old, were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan).

Generation of anti-CD3 activated killer cells, CD3-AK. Murine splenocytes from normal mouse were cultured in the medium consisting of RPMI-1640 with 10% heat-inactivated fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10 mM HEPES, 50 μM 2-mercaptoethanol, and 2 mM glutamine. The hamster IgG monoclonal anti-CD3 antibody (145-2C11) was obtained from American Type Culture Collection (ATCC) and was added to cell culture. After 1-2 days of anti-CD3 activation, the CD3-AK cells were cultured in 50 U/ml of murine recombinant IL-2 (Genzyme Co., MA, USA) for another 5-7 days with a change of medium every 2 days.

Flow cytometry. To examine whether the fusion protein anti-CD3/IL-2 or anti-CD3/Fc can bind to CD3 molecule or IL-2 receptor on the surface of activated T lymphocytes, flow cytometric analysis was performed. C3H/HeN splenocytes were treated with 2C11 supernatant (1:100) and IL-2 (50 U/ml) for 2 days to differentiate to activated CD3-AK cells. The cells were washed and cultured for 6 days, respectively, in the medium containing indicated concentrations of 2C11 supernatant and exogenous IL-2 (50 U/ml), or anti-CD3/Fc and exogenous IL-2 (50 U/ml), or anti-CD3/IL-2 alone. After washing, cells were incubated with a combination of the fluorochrome-conjugated monoclonal antibodies, rat anti-CD3-FITC or anti-IL-2α-receptor-PE (PharMingen, San Diego, CA), at 4˚C for 30 min and cytofluorometric analysis was determined by flow cytometry. Viable cells ($1x10^4$) in the lymphocyte gate, based on their sideways and forward light-scattering properties, were analyzed.

Proliferation assay. Normal splenocytes ($2x10^5$) were cultured in 96-well flat-bottom plate for 2 days with indicated concentrations of fusion proteins and then, pulsed with 0.04 μCi/ml [^{3}H]-thymidine (^{3}H-TdR) for 6 h. Cells were then harvested on a 96-well plate (Packard, Meriden, CT) and ^{3}H-TdR incorporation measured using a beta plate reader (Packard).

Cell-mediated cytotoxicity assay. The ^{51}Cr release assay was used to detect the cell-mediated cytotoxicity. The target cells ($2x10^6$ of 38C13 cells) were harvested in log phase of growth and resuspended in 0.5 ml media and radio-labelled by 150 μCi sodium ^{51}chromate (Amersham Pharmacia Biotech) for 1 h incubation at 37˚C, with regular and gentle mixing. After chromium labeling, target cells were thoroughly washed in RPMI-1640 media, and resuspended in fresh media to a final concentration of $5x10^3$ cells/100 μl. The effector cells were prepared by incubating C3H splenocytes with fusion proteins at 37˚C for 1-2 days, followed by subculturing in RPMI-1640 with or without IL-2 (50 U/ml) for 6 days. Dilutions of effector cells (C3H/HeN splenocytes were added to 96-well round-bottomed tissue culture plates in 200 μl final volume to produce a range of effector/target (E/T) ratios. The cells were incubated for 4 h at 37˚C, pelleted by spin, and the radio-activity of 100 μl supernatant was determined using a gamma-counter (LKB). The results are expressed as percentage of lysis according to the following formula: lysis % = (sample cpm - spontaneous cpm)/(total cpm - spontaneous cpm) x100%. The spontaneous release usually ranged from 5 to 15% of the total cpm. The standard error obtained with total % lysis was usually between 0.5 and 5% of the mean. One lytic unit (LU) was defined as the number of effectors required to give 30% lysis for $5x10^3$ target cells.

Results

Generation of anti-CD3/IL-2 and anti-CD3/Fc fusion protein constructs. We have constructed the light chain and heavy chain expression plasmids for these two fusion proteins. The light chain vector used is the same for anti-CD3/IL-2 and anti-CD3/Fc, containing the light chain variable region (VL) gene of 2C11 and a murine κ constant region gene (Cκ) (Fig. 1A, right). The heavy chain vector for anti-CD3/Fc contains the heavy chain variable region (VH) of 2C11 and a murine γ2a constant region (Fc) gene, while the heavy chain vector for anti-CD3/IL-2 has an additional murine IL-2 cDNA linked 3' to γ2a gene, spaced by two Gly codons (Fig. 1A, left). The expression plasmids after construction were completely sequenced to confirm there was no mutation introduced during the construction. Fusion proteins were made by cotransfecting

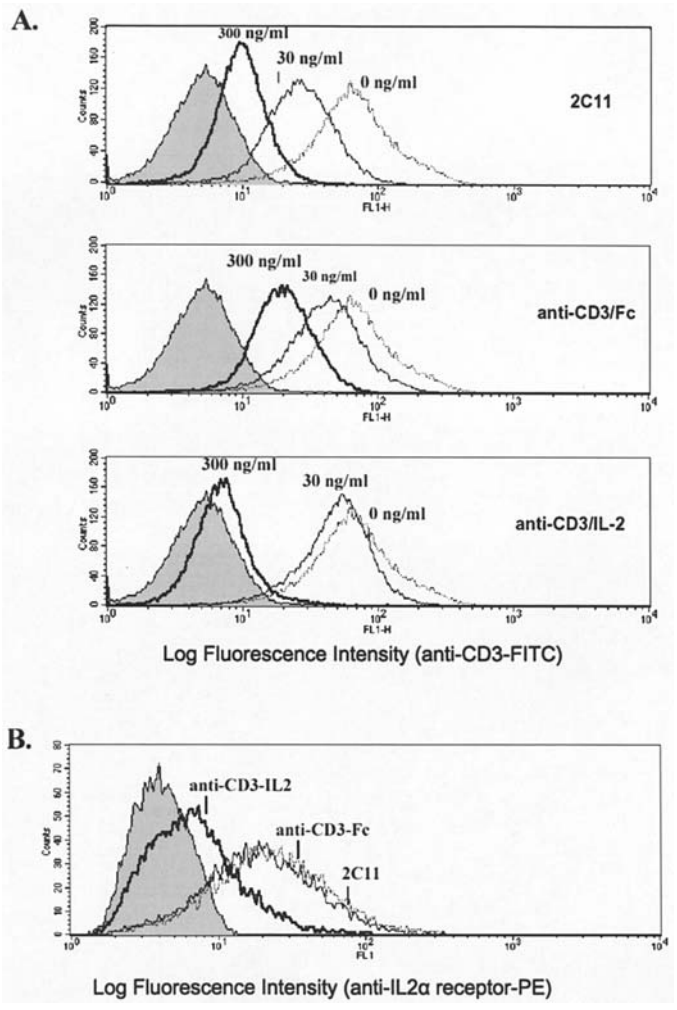

Figure 3. Analysis of CD3-binding ability of the anti-CD3 portion and IL-2 receptor-binding ability of the IL-2 portion of the fusion proteins by flow cytometry. C3H/HeN splenocytes were stimulated with parental anti-CD3 (2C11) supernatant (1:100) and IL-2 (50 U/ml) for 2 days and incubated in the presence or absence of indicated concentrations of the 2C11 supernatant, anti-CD3/Fc, or anti-CD3/IL-2 fusion proteins. The cells were stained with either (A) FITC-labeled anti-CD3 mAb or (B) PE labeled anti-IL-2α-receptor mAb. The binding curve of FITC-conjugated anti-CD3 was shifted by anti-CD3/IL-2, anti-CD3/Fc and 2C11 at 30 ng/ml and 300 ng/ml. The binding curve of PE-conjugated anti-IL-2α receptor was shifted by anti-CD3/IL-2 only, not anti-CD3/Fc and 2C11. The shaded peak represents unstained cells.

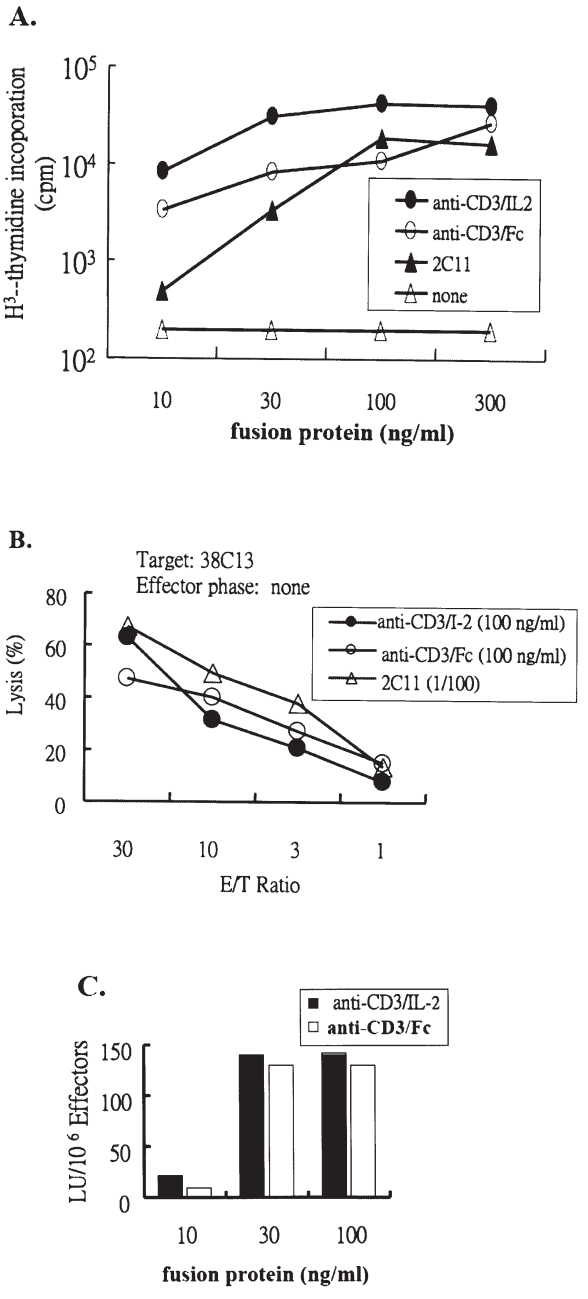

Figure 4. Biological activity of the anti-CD3 portion of the fusion proteins. (A) Proliferation assay using 3H-TdR incorporation showed that anti-CD3/IL-2 is able to activate splenic T cells to proliferate, and the effect is similar to anti-CD3/Fc or parental 2C11. (B) Cytotoxicity assay showed that the anti-CD3/IL-2, as well as anti-CD3/Fc could induce T cells to CD3-AK cells to give the same killing effect as those induced by parental 2C11. (C) When anti-CD3/IL-2 and anti-CD3/Fc were added, respectively, at graded doses in the effector phase of the cytolytic reaction of the CD3-AK cells against 38C13 as target, both anti-CD3/IL-2 (black) and anti-CD3/Fc (white) fusion proteins were shown to augment the killing activity of CD3-AK. All experiments were repeated at least 3 times.

a heavy chain vector with a light chain vector into FO mouse myeloma cells (non-immunoglobulin-secreting myeloma cell line) using electroporation. After G418 selection and limiting dilution, clones with high production of fusion proteins were screened by sandwich ELISA. The resulting fusion proteins are illustrated in Fig. 1B. The genetically engineered fusion protein anti-CD3/IL-2 contains both anti-CD3 and the IL-2 molecules conjugated at the C-terminal of Fc region, while anti-CD3/Fc contains anti-CD3 only, similar to the parental 2C11 antibody except that it was genetically engineered.

Purification of anti-CD3/IL-2 and anti-CD3/Fc fusion proteins. The fusion proteins were purified from cell supernatants by immunoaffinity chromatography. Purified proteins were subjected to SDS-PAGE under reducing conditions and Coomassie blue staining. The anti-CD3/IL-2 fusion protein had two predominant stained bands migrated at ~70 kDa (heavy chain) and 25 kDa (light chain), while the anti-CD3/Fc fusion protein had two bands at 50 kDa (heavy chain) and 25 kDa (light chain) (Fig. 2A), which is consistent with the predicted molecular mass for the corresponding heavy chain and the light chain, respectively. The proteins were transferred into nitrocellulose and probed with mAb specific for murine

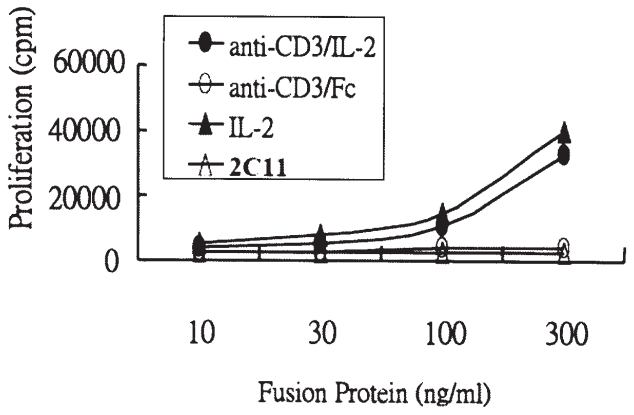

Figure 5. IL-2 activity of the anti-CD3/IL-2 fusion protein. Activated CD3-AK cells were cultured with graded doses of anti-CD3/IL-2, anti-CD3/Fc, 2C11, or recombinant murine IL-2 protein and then pulsed with 2 μCi/ml of ^{3}H-thymidine to determine cell growth. Anti-CD3/IL-2 was able to maintain the growth of activated T cells, similar to recombinant IL-2. This effect was not seen with anti-CD3/Fc or parental 2C11.

IL-2 (Fig. 2B, upper) and IgG kappa (Fig. 2B, bottom), respectively. Western blots confirm the observation that the IL-2 molecule is intact in anti-CD3/IL-2 fusion protein without dissociation (Fig. 2B).

CD3-binding ability of the anti-CD3 portion and IL-2 receptor-binding ability of the IL-2 portion in anti-CD3/IL-2 fusion proteins. The binding ability of the anti-CD3/IL-2 and anti-CD3/Fc fusion proteins to CD3 molecules or IL-2 receptors on the surface of activated T lymphocytes was determined by flow cytometry. C3H/HeN splenocytes were treated with 2C11 supernatant (1:100) and IL-2 (50 U/ml) for 2 days to differentiate to activated CD3-AK cells. The stimulated cells were washed and cultured for 6 days, respectively, in the medium containing indicated concentrations of 2C11 supernatant plus exogenous IL-2 (50 U/ml), or anti-CD3/Fc plus exogenous IL-2 (50 U/ml), or anti-CD3/IL-2 alone. The cells were stained with FITC-labeled anti-CD3 mAb (Fig. 3A) and PE-labeled anti-IL-2α-receptor mAb (Fig. 3B), respectively. The data showed that anti-CD3/IL-2 blocked the binding of detection antibodies to both CD3 and IL-2Rα on the activated T cells, while anti-CD3/Fc as well as 2C11 only blocked the binding to CD3, suggesting that the anti-CD3 portion and the IL-2 portion of the anti-CD3/IL-2 fusion protein is capable of binding to CD3 and IL-2 receptor, respectively.

Biological activity of the anti-CD3/IL-2 fusion proteins. We further demonstrate biological activity of the anti-CD3 portion of the anti-CD3/IL-2. Fusion protein anti-CD3/IL-2 and anti-CD3/Fc as well as the parental 2C11 antibody, at graded doses, were added to 2x10^{6} cells/well of C3H/HeN splenocytes. After culture for 2 days, the proliferative activity was determined by pulsing with ^{3}H-thymidine at 2 μCi/ml for 5 h. Anti-CD3/IL-2 was shown to activate splenic T cells to proliferate. Similar effect was also seen by anti-CD3/Fc and parental 2C11 (Fig. 4A). In cytotoxicity assay, 38C13 was used as target cells for effector T cells treated by anti-CD3/IL-2, anti-CD3/Fc and 2C11, respectively. All antibodies were able to induce CD3-AK cells to give the killing effect (Fig. 4B). To test the arming effect of these fusion proteins, anti-CD3/IL-2 and anti-CD3/Fc were added, respectively, at graded doses in the effector phase of the cytolytic reaction of the CD3-AK cells against 38C13 as target. Both fusion proteins were shown to augment the killing activity of CD3-AK (Fig. 4C), suggesting that these two fusion proteins could arm the CD3-AK cells to kill the tumor target. To demonstrate the biological function of the IL-2 portion of the anti-CD3/IL-2 fusion protein, two-day activated CD3-AK cells at 3x10^{5} cells/well were cultured with graded doses of anti-CD3/IL-2, anti-CD3/Fc, 2C11 or recombinant murine IL-2 protein for another 2 days, then pulsed with 2 μCi/ml of ^{3}H-thymidine for 5 h to determine cell growth. Anti-CD3/IL-2 was able to maintain the growth of activated T cells, similar to recombinant IL-2, because of its IL-2 activity. This effect was not seen with anti-CD3/Fc or parental 2C11 (Fig. 5). Taken together, these data indicate the IL-2 portion of anti-CD3/IL-2 fusion protein has similar biological activity to recombinant IL-2 *in vitro* and the conjugation of IL-2 at the C-terminal of constant region (Fc) does not affect the activity of the anti-CD3 portion.

Discussion

Genetically engineered antibody offers a new method for the delivery of cytokines for therapy. We demonstrated that it is feasible to construct a biologically active fusion protein comprised of an anti-CD3 antibody and a cytokine. This fusion protein is capable of binding to CD3 molecules and IL-2 receptors on the surface of activated T cells, mediating the functions by: 1) augmenting the T cell function by activation through CD3; 2) targeting the CD3-AK cells to B lymphoma cells to augment the specific killing of tumor cells; 3) maintaining T cell growth and augmenting T cell anti-tumor activity by IL-2. Thus, it retains the two essential biological activities as expected, anti-CD3 and IL-2. To our knowledge, this fusion protein has never been reported in literature.

We have selected IL-2 for our fusion protein because IL-2 has been shown to exert pleiotropic roles in the modulation of antigen-specific immune responses. Targeted delivery of the cytokine IL-2 has been used by fusing it to a tumor antigen-specific antibody. The fusion proteins consisting of IL-2 fused to anti-HER2/neu antibodies can mediate killing of HER2/neu positive tumor cells (18) However, many tumors do not display specific antigens like breast cancer. Anti-CD3 is a potentially powerful agent to enhance non-MHC restricted cellular cytotoxicity *in vitro* in normal individuals and cancer patients (19). Activation with the combination of anti-CD3 mAb and IL-2 produces rapidly expanding cultures of cytolytic cells with demonstrated *in vivo* antitumor efficacy (20). The construction of this fusion protein may offer a strategy for circumventing the systemic toxicity posed by the need of exogenous administration of high-dose IL-2. The results suggest that this fusion protein can arm the AK cells and, thus, may bypass the requirement for further systemic administration of IL-2. Fusion to the immunostimulatory cytokine IL-2 does not alter the antibody specificity of anti-CD3.

In light of these findings, anti-CD3/IL-2 fusion protein may be used to enhance an anti-tumor response with less major systemic toxic effects, suggesting that it has potential as a new agent for immunomodulatory cancer therapy.

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