Construction and functional analysis of an anti-human cervical carcinoma/anti-human CD3 single-chain bispecific antibody

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Abstract. The aim of the present study was to construct a single-chain bispecific antibody (scBsAb) against cervical carcinoma and to investigate its biological activities. The scBsAb was constructed using a genetic cloning technique and antigen binding activities were detected by ELISA. The iodogen method was used to analyze the pharmacokinetics. The Rosette formation test was used to detect the binding ability between peripheral blood lymphocytes (PBLs) and Cs1213 cervical cancer cells. In addition, the MTT method was performed to detect the killing effect of PBLs. The molecular weight of the scBsAb was ~60 kDa. The antigen binding activities of scBsAbs were compared with the anti-human cervical carcinoma antibody single-chain Fv fragment (CSAs-1 scFv) and anti-cluster of differentiation (CD)3 scFv ($P>0.05$). In addition, a pharmacokinetics assay demonstrated that compared with the two corresponding scFvs, scBsAbs exhibited a significantly prolonged retention time in the body ($P<0.01$). In addition, the number of rosettes formed by PBLs and Cs1213 cells in the scBsAb group was markedly greater than that in the scFv groups or the RPMI-1640 group ($P<0.05$ and $P<0.01$, respectively). The killing activity of PBLs against scBsAb-mediated Cs1213 cells was significantly greater than that mediated by the other antibodies ($P<0.05$). When the concentration of scBsAb was 40 μg/ml, the killing rate was 64.5%. Thus, anti-human cervical carcinoma/anti-CD3 scBsAbs may possess two types of antigen binding activity, prolong the duration *in vivo* and improve the killing activity of PBLs against cancer cells.

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Introduction

Cervical carcinoma is one of the most common malignant tumors among females. Improving the survival rate of patients with cervical carcinoma is an important aim of clinical and basic research (1). With the development of genetic engineering technology (particularly the phage display technique), research and application of genetically engineered antibodies in the diagnosis and treatment of diseases has increased. Bispecific antibodies (BsAbs), prepared by chemical coupling, hybridoma cell culture and gene engineering methods, combine with associated antigens on the surface of tumor cells and T cells and produce biological effects (2). BsAbs combine with target tumor cells, as well as cytotoxic effector cells, resulting in efficient targeted killing of tumor cells, and thus present a specific and selective antibody-based treatment (3). Consequently BsAbs have become a popular topic of tumor immunotherapy research. Single-chain BsAbs (scBsAbs) link two different single-chain antibody (scAb) gene fragments at the DNA level via an inter-chain linker and is directly expressed as one scBsAb molecule. The scBsAb is of interest due to qualities, such as weak antigenicity, strong penetrability and few side effects (4).

Cytotoxic T cells are important in multiple immune mechanisms associated with the prevention of tumors (5), and the activation and mediation of the cytotoxic T cell killing effect on tumor cells is considered to be a promising therapeutic strategy. Cluster of differentiation (CD)3 is a type of membrane antigen located on the surface of mature T cells, which forms complexes with the T-cell receptor, and contributes to antigen recognition and intracellular signal transduction (6). BsAbs, formed using anti-CD3x antitumor monoclonal antibodies (mcAbs), may trigger T cell major histocompatibility complex to kill and lyse tumor cells. Numerous experiments have demonstrated that CD3-based BsAbs are an efficient type of therapeutic agent for treating tumors (7-9). In addition, the CD19 x CD13 scBsAb constructed in a previous study is currently undergoing clinical trials (10).

Currently, scBsAbs targeting different types of tumor, such as ovarian cancer (11), prostate cancer (12) and lymphoma (13), have been constructed; however, to the best

of our knowledge, an scBsAb against cervical carcinoma has not been constructed. The present study aimed to establish an anti-human cervical carcinoma/human CD3 scBsAb and detect its biological activity to provide the experimental and theoretical basis for clinical application.

Materials and methods

Cell lines. The CSA125 anti-cervical cancer mcAb hybridoma cell line, the Cs1213 cervical cancer cell line and the Jurkat T cell line were established and preserved in our lab according to a previous study (14). Cells were cultured in RPMI-1640 medium (Borunlaite Science & Technology Co., Ltd., Beijing, China) supplemented with penicillin (100 U/ml), streptomycin (100 mg/l) from Anpei Chemical Science and Technology Co., Ltd., (Nanjing, China) and 10% fetal calf serum (Beijing Biodee Biotechnology Co., Ltd., Beijing, China). The current study was approved by the ethics committee of the Experimental Animal Center of Zhengzhou University (Zhengzhou, China), and written informed consent was obtained prior to collection of patient samples.

Plasmids and main reagents. The pMD18-T-anti-CD3 scFv plasmid was constructed according to the methods of a previous study (15). Mouse monoclonal anti-human CD3 antibody (#BM0210) and an ELISA kit were purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Restriction enzymes and DNA ligases were provided by Promega Corporation (Madison, WI, USA) and DNA gel extraction, Mini Plasmid and MTT kits, as well as *E. coli* Top10 competent cell and *E. coli* BL21(DE3) bacterial strain were purchased from Tiangen Biotech Co., Ltd. (Beijing, China).

Construction of anti-human cervical carcinoma antibody single-chain Fv fragment (CSAs-1 scFv). Total RNA was extracted. Briefly, $1x10^6$ CSA125 anti-human cervical cancer hybridoma cells in the logarithmic growth phase were collected and added into 1.5 ml TRIzol lysate (Borunlaite Science & Technology Co., Ltd.). Subsequently, 300 μl chloroform (Yilin Chemical Company, Zhengzhou, China) was added, and the mixture was oscillated and centrifuged at 4˚C and 10,000 x g for 15 min. The solution was transferred to a fresh Eppendorf (EP) tube (Topscien Instrument Co., Ltd., Ningbo, China) and 0.5 ml Avantin (Yilin Chemical Company) was added, the EP tube was left for 10 min at room temperature and centrifuged at 4˚C and 10,000 x g for a further 15 min. The precipitates were obtained and washed using 1 ml of 75% alcohol. Centrifugation at 4˚C and 1,500 x g was conducted to remove the supernatant. The precipitates were dried, dissolved in double-distilled (dd)H_2O, processed with diethylpyrocarbonate (DEPC; Borunlaite Science & Technology Co., Ltd.) and stored at -70˚C. Amplification of VL and VH gene fragments was then performed as follows: i) Total RNA was collected to synthesize the first chain cDNA as described above. The reaction system, which contained 0.5 μl RNA, 1 μl oligo dT joint primer, 1 μl AMV reverse transcriptase, 2 μl of 2.5 mmol/l dNTP, 2 μl of 10X RNA PCR buffer solution, 4 μl $MgCl_2$ and 9.5 μl ddH_2O, was processed with DEPC. The reaction was conducted at 42˚C for 30 min, 99˚C for 5 min and 5˚C for 5 min. ii) Amplification of the VH gene fragment was conducted using the rapid amplification of cDNA ends method with the following primers (synthesized by Sangon Biotech Co., Ltd., Shanghai, China): Forward, 5'-GGTTCAGAAGTTCAACTAGTTGAC ATTGTGATGACCCAGTCTCCT-3' (F1) and reverse, 5'-GG CTCGAGTTTTATTTCCA-3' (R1). iii) Amplification of the VL gene fragment was conducted according to the conventional polymerase chain reaction method using the following primers (synthesized by Sangon Biotech Co., Ltd.): Forward, 5'-GCGAATTCCAGGTCCGCTTCAGCAGTCT-3' (F2) and reverse, 5'-AGACCCACCACCAGCGCGCTTAAGTTC TGAGGAGACGGTGACTGAGG-3' (R2). The total reaction volume was 80 μl, containing 8 μl of 10X LA buffer solution, 1 μl LA Taq DNA polymerase (Takara Biotechnology Co., Ltd., Dalian, China), 6 μl $MgCl_2$, 1 μl R2 primer and 1 μl F2 primer. The PCR reaction procedure was as follows: 98˚C for 10 sec, 55˚C for 30 sec and 72˚C for 2 min for 30 cycles. The PCR products were detected by 2% agarose gel electrophoresis and DNA extraction. The CSAs-1 scFv was then constructed. Splicing overlap extension was used to link VH to VL using a $(Gly_4Ser)_3$ linker (synthesized by Sangon Biotech Co., Ltd.). The VH, VL and $(Gly_4Ser)_3$ linker (10 μl of each) were mixed together, and 2.5 μl dNTP, 2 μl LA Taq DNA polymerase, 2 μl of 10X PCR buffer solution was added. ddH_2O was then added to form a 50-μl solution. The reaction was as follows: Initial denaturation at 94˚C for 2 min, and 20 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 2 min, with a final extension at 72˚C for 5 min. Then, 4 μl F1 primer and 4 μl R2 primer were added and the reaction was performed for 30 cycles under the above-mentioned conditions. Agarose gel (0.015 g/ml; Borunlaite Science & Technology Co., Ltd.) electrophoresis was used for identification and separation. Finally, the CSAs-1 scFv cloning vector was constructed. The CSAs-1 scFv gene was inserted into a pTHA90 vector (Biovector Co., Ltd., Beijing, China) and transformed into competent cell *E. coli* Top10. The positive clone was screened for sequencing using Sanger sequencing by Takara Biotechnology Co., Ltd.

Construction of scBsAb universal expression vector. The plasmid, pALM (Biovector Co., Ltd.) served as the primary vector. To facilitate purification and activity determination, the nucleotide sequence encoding $(His)_6$-c-Myc was synthesized and cloned between the restriction enzyme sites, *Bam*HI and *Pst*I, of pALM. The modified vector was designated, pALMm. The synthetic inter-chain linker, human serum albumin (HSA; N'-FQNALLVRYTKKVPQVSTPTL VEVS-C'; Seebio Biotech, Inc., Shanghai, China) (16) was inserted between the *Eco*RI and *Sac*I sites of pALMm to construct the scBsAb universal vector, pALMm-HSA via the sticky end connection method, which was validated by sequencing. The primers used in sequencing were as follows: 5'-TCTTACACATTCCAGCCCTG-3' and 5'-TGTAAAACG ACGGCCAGTGC-3' (synthesized by Sangon Biotech Co., Ltd.). A schematic diagram of the constructed universal vector is demonstrated in Fig. 1.

Construction and expression of anti-human cervical carcinoma/anti-CD3 scBsAb. The anti-cervical carcinoma ScAb

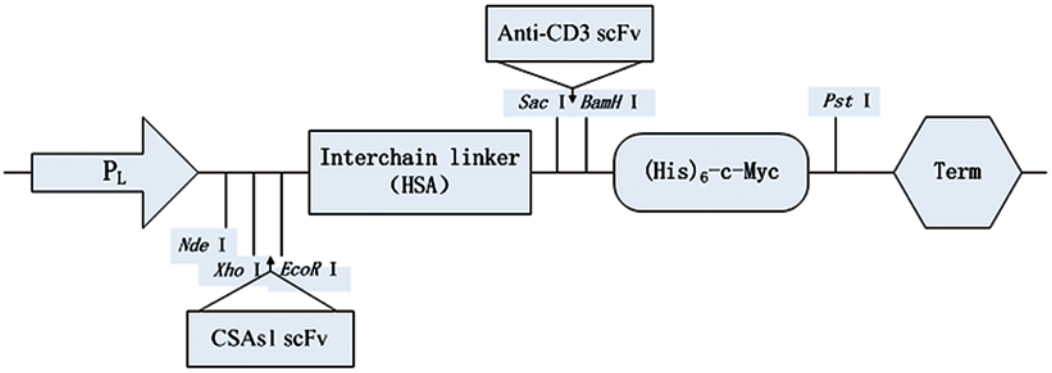

Figure 1. Schematic diagram of the scBsAb expression vector. scBsAb, single-chain bispecific antibody; P_L, PL promoter; CSAs-1 scFv, cervical carcinoma antibody single-chain Fv fragment; CD, cluster of differentiation.

(CSAs-1) and universal vector (pALMm-HSA) were digested by *Xho*I and *Eco*RI, and ligated using T4 DNA ligase to form the pALMm-CSAs-1-HSA plasmid. Similarly, anti-CD3 scFv and pALMm-HSA were digested by *Sac*I and *Bam*HI and inserted into the pALMm-HSA-anti-CD3 scFv plasmid. The pMD18-T-anti-CD3 scFv and pALMm-CSAs-1 scFv-HSA plasmids were digested by *Sac*I and *Bam*HI, respectively and anti-CD3 scFv was inserted into pALMm-CSAs-1 scFv-HSA using a ligase to form the scBsAb expression vector, pALMm-CSAs-1 scFv-HSA-anti-CD3 scFv. pALMm-CSAs-1-HSA, pALMm-HSA-anti-CD3 scFv and pALMm-CSAs-1 scFv-HSA-anti-CD3 scFv were transferred to the *E. coli BL21(DE3)* bacterial strain and the positive clones were identified, which were then inoculated with RM medium (Shanghai Seebio Biotech, Inc.) and cultivated at 30˚C overnight. The following day, the bacterial solution was transferred to RM medium (ratio, 1:10) and grown until the culture reached an A_{600} of 0.6-0.8 at 37˚C (measured with a 759S UV-Visible Spectrophotometer; Lengguang Technology Co., Ltd., Shanghai, China). Tryptophan (Jinghai Amino Acid Co., Ltd., Wuxi, China) was added to achieve a final concentration of 100 μg/ml and cultivated for a further 4 h. The pALMm-CSAs-1 scFv-HSA and pALMm-HSA-anti-CD3 scFv plasmids served as control groups.

Purification and renaturation of antibody. The bacteria were collected by centrifugation and subsequently disrupted by ultrasonification with a JY92-II Sonifier Cell Disrupter (Scientz Biotechnology Co., Ltd., Ningbo, China) at a power of 400 W for 9 sec with intervals of 2 sec for a total of 30 min. Inclusion bodies were recycled through high-speed centrifugation at 4,000 x g and purified through a C-terminal $(His)_6$ tag using Ni-NTA Agarose under denaturation conditions. Purified antibodies were refolded by column chromatography using Sephacryl S-200 HR (1.1x90 cm), which was balanced by 3X the column volume of refolding buffer solution prior to protein loading. The purified antibodies were loaded and passed through the column. The refolding buffer solution was used to elute the antibodies, which were collected and dialyzed with phosphate-buffered saline (PBS), then stored at -80˚C. The purified and renatured proteins were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

Determination of antigen binding activity of scBsAb. The ELISA method was used to determine the antigen binding activity of scBsAb. A Cs1213 (50 μg/ml) or Jurkat (50 μg/ml) was diluted with coating buffer, then 0.5 ml was added to each 96-well plate, which was then incubated at 4˚C and subsequently blocked with 5% skimmed milk at 37˚C for 2 h; renatured scBsAb samples at 1, 10, 20, 40 and 80 μg/ml concentrations were then added. *E. coli* BL21(DE3) at the same concentrations served as a negative control and the corresponding scAb and mcAb served as positive controls, all of which were incubated at 37˚C for 2 h. Anti-c-Myc mcAb, 9E10 was added and incubated at 37˚C for 1 h. Horseradish peroxidase-labeled immunoglobulin G (IgG) was added and incubated at 37˚C for 1 h. Finally, 3,3'-diaminobenzidine substrate (Sigma-Aldrich China, Inc., Shanghai, China) was added for color development and absorbance was measured at 490 nm using a Multiskan MK3 microplate reader (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Blood pharmacokinetics assay. The iodogen method was used for radioiodination of the antibodies. BALB/c mice were provided and raised by the Animal Experimental Center of Zhengzhou University. The average daily feed and water consumption values were 5 g and 6-7 ml per 100 g body weight, respectively. Mice were caged at a temperature of 21±2˚C, with relative humidity of 30-70%, and a 12-h light/dark cycle. BALB/c mice (n=36) were divided into four groups of nine mice per group. Mice in each group were intravenously injected via the tail with ^{125}I-scBsAb, ^{125}I-CSAs-1 scFv, ^{125}I-anti-CD3 scFv and ^{125}I-anti-CD3 mcAb (0.2 ml/per mouse). Blood (10 μl) was collected from the tail veins following these injections at 0, 5, 10, and 30 min, 1, 2, 3, 6, 12 and 24 h. A blood radioactivity count value of 1 min was determined using a Hidex Automatic Gamma Counter (DL Naturegene Life Sciences, Inc., Beijing, China) and a blood elimination curve was drawn.

Rosette formation test of peripheral blood lymphocytes (PBLs) and Cs1213 cells mediated by scBsAb. Peripheral blood mononuclear cells (PBMCs) and PBLs were separated according to the standard method of Ficoll-Paque density gradient centrifugation (17). Briefly, Ficoll lymphocyte separation liquid was used to separate peripheral blood of three

healthy donors (3 ml fasting blood from each patient), who all provided written informed consent. PBMCs were obtained following the removal of platelets by centrifugation at 150 x g. Fresh PBLs were obtained after the removal of macrophages and monocytes subsequent to PBMC adherent growth for 2 h. RPMI-1640 complete medium was added and the solution was placed in an incubator with 5% CO_2 at 37˚C. PBLs ($1x10^6$/ml) were pre-stimulated with 20 μg/ml phytohemagglutinin and 100 U/ml interleukin (IL)-2 (Shanghai Yuanye Biotechnology Co., Ltd., Shanghai, China) for 2 days. On the third day the cell concentration was adjusted to $0.5x10^6$/ml, cultivated in complete medium containing 100 U/ml IL-2 and passaged every 2-3 days to maintain the same density of lymphocytes.

The rosette formation test was performed on the following groups: i) scBsAb [scBsAb (40 μg/ml) + Cs1213 + PBLs]; ii) CSAs-1 ScFv [CSAs-1 ScFv (20 μg/ml) + Cs1213 + PBLs]; iii) Anti-CD3 ScFv [anti-CD3 ScFv (20 μg/ml) + Cs1213 + PBLs]; iv) CSAs-1 ScFv + anti-CD3 ScFv [CSAs-1 ScFv (20 μg/ml) + Anti-CD3 ScFv (20 μg/ml) + Cs1213 + PBLs]; and v) medium (RPMI-1640 perfect medium + Cs1213 + PBLs). Cs1213 cells ($1x10^5$/ml) were collected and resuspended in PBS and the antibodies at corresponding concentrations were added according to the above groups until the final volume of each group was 1 ml. The solutions were incubated at 4˚C for 2 h and centrifuged at 150 x g, and the supernatant was discarded. The precipitates were washed with PBS three times and 1 ml fresh PBLs ($1x10^6$/ml) was added and incubated at 4˚C for a further 2 h. Rosette formation was observed under an IX73 inverted microscope [Olympus Corporation (China) Co., Ltd., Beijing, China]. The number of Cs1213 cells (out of 200 Cs1213 cells) that combined with at least three lymphocytes were counted to give the rosette formation rate, and the number of lymphocytes that combined with 100 Cs1213 cells was counted to give the conjugate rate.

Determination of killing effect of scBsAb-mediated PBLs on tumor cells. Cs1213 cells in the logarithmic growth phase were seeded on 96-well plates ($1x10^4$/well). The plate was incubated with 5% CO_2 and cultivated overnight at 37˚C to grow Cs1213 adherent cells. The Cs1213 cells were designated as the target cells and the PBLs were added at a ratio of 10:1 effector:target cells; in addition, antibodies at different concentrations were added. The concentrations of scBsAbs were 1, 10, 20, 40 and 80 μg/ml and the corresponding controlled concentrations of scAb were 0.5, 5, 10, 20 and 40 μg/ml. The target Cs1213 cells and the RPMI-1640 complete medium group served as controls. Following cultivation for 48-72 h, the effector cells were washed away and MTT was added for further incubation for 4 h. The supernatant was discarded and 100 μl dimethyl sulfoxide was added. After standing at 37˚C for 30 min, the absorbance at 570 nm was measured using a microplate reader and the killing rate was calculated according to the following formula: Killing rate = $(A_{target}-A_{experiment})/(A_{target}-A_{control})$ x 100.

Results

Purification and detection of antibodies. scFvs and scBsAbs produced by induced expression were purified by Ni-NTA metal affinity chromatography and denaturation, and target proteins with >90% purity were obtained. Following refolding by gel filtration chromatography, the purified antibodies became active proteins. Detection of SDS-PAGE demonstrated that the molecular weight of scBsAb was ~60 kDa, compared with CSAs-1 scFv and anti-CD3 scFv, which were ~30 kDa (Fig. 2), which was consistent with the hypothesis.

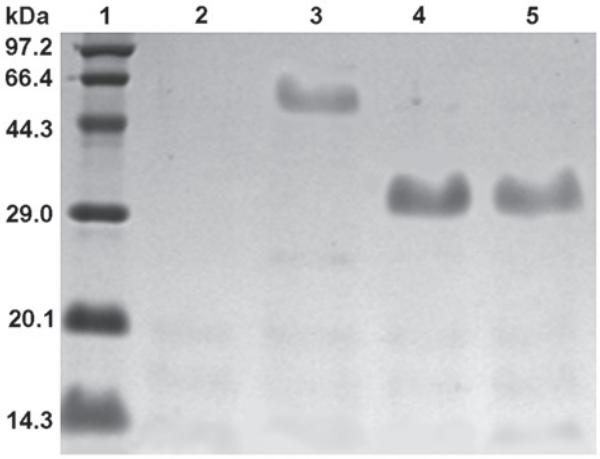

Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified antibodies. Lane 1, protein marker; lane 2, blank control; lane 3, anti-cervical carcinoma/anti-CD3 scFv; lane 4, CSAs-1 scFv; lane 5, anti-CD3 scFv. CD, cluster of differentiation; CSAs-1 scFv, cervical carcinoma antibody single-chain Fv fragment.

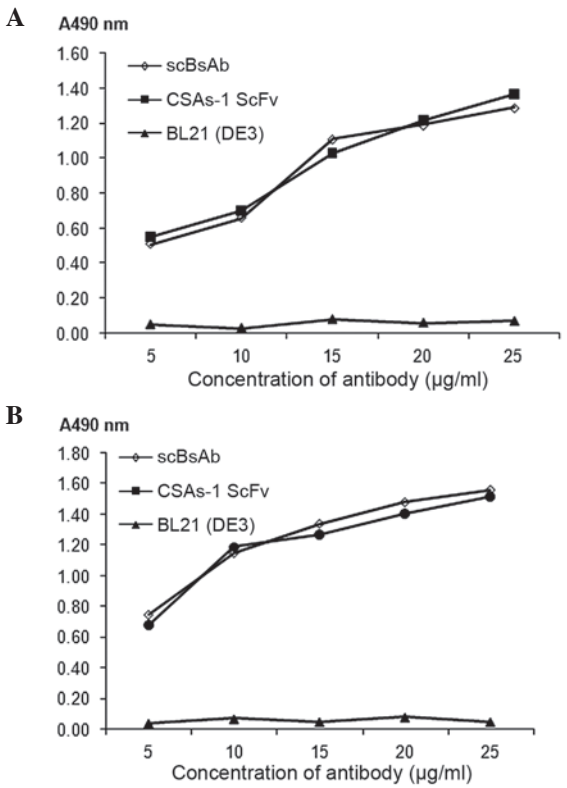

Figure 3. Detection of antigen binding activity by ELISA. Antigen binding ability to (A) Cs1213 and (B) Jurkat membrane antigens. scBsAb, single-chain bispecific antibody; CSAs-1 scFv, cervical carcinoma antibody single-chain Fv fragment.

Table I. Analysis of pharmacokinetic parameters.

Antibody type	$T_1/_{2\alpha}$ (min)	$T_1/_{2\beta}$ (h)
scBsAb	17.8±0.12	4.4±0.05
CSAs-1 scFv	5.5±0.08[a]	2.6±0.09[a]
Anti-CD3 scFv	5.7±0.11[a]	2.3±0.04[a]
Anti-CD3 mcAb	60.3±0.10[a]	18.1±0.11[a]

[a]P<0.01 vs. scBsAb. $T_{1/2\alpha}$, distribution half-life; $T_{1/2\beta}$, elimination half-life; scBsAb, single-chain bispecific antibody; CSAs-1 scFv, cervical carcinoma antibody single-chain Fv fragment; CD, cluster of differentiation; mcAb, monoclonal antibody.

Antigen binding activity assay of scBsAb. The ELISA assay indicated that there was no significant difference between scBsAb and CSAs-1 ScFv in their ability to bind to Cs1213 membrane antigens (P>0.05; Fig. 3A). Furthermore, there was no significant difference between scBsAb and anti-CD3 scFv in their ability to bind to Jurkat membrane antigens (P>0.05; Fig. 3B). No binding was observed between *E. Coli* BL21(DE3) and the two antigens. This indicated that the binding ability of scBsAb with the two antigens was comparable to the original scFv. In addition, the A490 nm value increased with the increase in antibody concentration within a range of 5~25 μg/ml.

Blood pharmacokinetics. The results of pharmacokinetics demonstrated that the blood elimination curve of scBsAb was consistent with the two compartment model, the distribution half-life ($T_1/_{2\alpha}$) was 17.8 min and the elimination half-life ($T_1/_{2\beta}$) was 4.4 h. Compared with complete mcAb, scBsAb demonstrated rapid distribution and quick excretion (P<0.01). However, when compared with CSAs-1 scFv and anti-CD3 scFv, scBsAb exhibited a prolonged retention time in the body (P<0.01; Table I). Additionally, the conjugate rate mediated by scBsAb was significantly higher than that mediated by scFv (P<0.05) or RPMI-1640 medium (P<0.01).

Rosette formation detection. The number of rosettes formed by PBLs and Cs1213 cells following stimulation with scBsAb at 4˚C for 2 h was significantly higher than those that were stimulated with scFv (P<0.05) or RPMI-1640 medium (P<0.01). However, there was no significant difference between the scFv and the RPMI-1640 groups (P>0.05; Table II). This indicated that scBsAbs mediate the combination of CD^{3+} T cells and Cs1213 cells.

In vitro cytotoxic activity mediated by scBsAbs. When the ratio of effector cells to target cells was 10:1 and the action time was 48 h, the strength of cytotoxic activity increased in an antibody concentration-dependent manner ≤40 μg/ml. At a concentration of 40 μg/ml antibody, the cytotoxic activity reached 64.5%. The killing rate of Cs1213 by PBL in the scBsAb group was significantly higher than that in the CSAs-1 scFv, anti-CD3 scFv and CSAs-1 scFv + anti-CD3 scFv groups (P<0.05). Thus, the killing rate of target cells by PBLs was associated with the dose of antibodies (Fig. 4).

Table II. Rosette formation and conjugate rates induced by scBsAb.

Group	Rosette formation rate (%)	Conjugate rate (%)
scBsAb	15.5±1.12	82.6±3.24
RPMI-1640	10.9±1.45[a]	54.8±1.89[a]
CSAs-1 scFv	12.3±0.77[b]	61.7±1.55[b,c]
Anti-CD3 scFv	13.1±1.02[b]	62.3±2.12[b,c]
CSAs-1 + anti-CD3 scFv	12.6±1.14[b]	51.9±1.87[a]

[a]P<0.01, [b]P<0.05 vs. scBsAb; [c]P<0.05 vs. RPMI-1640. scBsAb, single-chain bispecific antibody; CSAs-1 scFv, cervical carcinoma antibody single-chain Fv fragment; CD, cluster of differentiation.

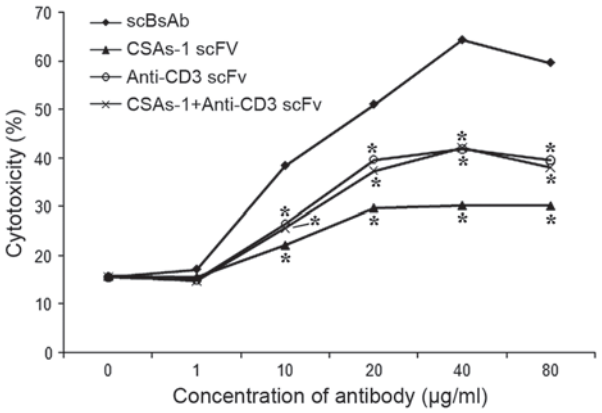

Figure 4. Association between cytotoxic activity of peripheral blood lymphocytes and antibody concentration. *P<0.05 vs. scBsAb. scBsAb, single-chain bispecific antibody; CSAs-1 scFv, cervical carcinoma antibody single-chain Fv fragment; CD, cluster of differentiation.

Discussion

With the developments in biology, immunology and molecular genetics, cancer biotherapy has become a key area of research and BsAbs are a prominent focus (18). The aim of the present study was to construct an scBsAb against cervical carcinoma. The hybridoma cell strain, CSA125, which secretes highly specific anti-human cervical carcinoma mcAb was used to construct the scAb gene, CSAs-1. The modified plasmid, pLAM served as the universal expression vector, and scBsAbs were constructed and successfully expressed. scBsAb was demonstrated to combine with the Cs1213 cervical cancer and Jurkat cell membrane antigens, and maintain the binding activities in a similar manner to the original scAb. This indicated that scBsAb was able to combine with the membrane antigens of tumor cells and triggering molecules on effector cells to activate effector cells, enabling them to accumulate on, and subsequently kill, the tumor cells (19). Sharkey *et al* (20) created CEA/HSG bispecific antibodies combining PET and SPECT applied in the molecular imaging of nude mice with colon cancer, which demonstrated that the antibody had high target ability and was able to locate the tumor accurately. Melanoma-associated

chondroitin sulfate proteoglycan x CD3 bsAb constructed by Gary *et al* (21) produced $CD4^+$ and $CD8^+$ T cells in order to kill melanoma cells. Therefore, this may serve as a novel immunological method of treating melanoma. Another study demonstrated that CD3xCD20 bispecific antibodies could be applied in the oncotherapy following umbilical cord blood stem cell transplantation (22). In the present study, the constructed anti-human cervical carcinoma x anti-CD3 scBsAb mediated the combination of PBLs with Cs1213 cells and activated the specific killing effect of PBLs. This indicated that anti-human cervical carcinoma x anti-CD3 scBsAb has potential clinical application in the therapy of human cervical cancer.

Pharmacokinetics demonstrated that the retention time of scBsAb was longer than scFv, although markedly shorter when compared with the complete antibody, anti-CD3 mcAb. Compared with complete IgG, small genetically engineered antibodies are able to effectively permeate into tumors. However, due to their short half-life, various genetically engineered antibodies have short retention times and, therefore, low absolute intake by tumors. Adams *et al* (23) demonstrated that the tumor absorption peak of BsAb against HER2/neu marked by ^{125}I was 10.1%, and the $T_1/_{2\beta}$ was 6.42 h; markedly lower when compared with a complete antibody. Thus, the differences in size and affinity affect the pharmacokinetic features of BsAbs. Increasing the molecular weight of the antibody reduces the elimination rate whilst weakening the tumor infiltrability. However, the molecular size is just one of the factors that determines the metabolism in the body. Other factors include conformation of the antibody, charge characteristics and arrangement of domains, which may influence the pharmacokinetic features of BsAb (24).

Certain studies demonstrated that sequences of interchain linkers affect the antigen binding activity of the antibody, as well as its stability within body (25). The development of a technique, which uses HSA to prolong the half-life of polypeptides and protein therapeutics, has become attractive. As a stable and natural vector, HSA has been used successfully to improve certain metabolic parameters of recombined molecules (26,27).

In conclusion, in the present study, the fragment of HSA, which contained no Cys and numerous polar amino acids, was regarded as an interchain linker. Consequently, it was identified that the interchain linker, HSA was able to significantly prolong the half-life of scBsAb in the body and enhance the therapeutic effect of this small molecular antibody. The targeting of BsAbs serves an important guiding function in the diagnosis and treatment of disease. However, certain aspects remain unknown, including which specific types of BsAbs produce the strongest targeting, the longest residence time and the fewest side effects to normal tissues and organs, and how the BsAbs being used from animal experiment to clinical application.

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