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Construction, expression and characterization of the engineered antibody against tumor surface antigen, p185$^{c\text{-}erbB\text{-}2}$

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ABSTRACT

The c-erbB-2 proto-oncogene encodes a 185kDa protein p185, which belongs to epidermal growth factor receptor family. Amplification of this gene has been shown to correlate with poor clinical prognosis for certain cancer patients. The monoclonal antibody A21 which directed against p185 specifically inhibits proliferation of tumor cells overexpressing p185, hence allows it to be a candidate for targeted therapy. In order to overcome several drawbacks of murine MAb, we cloned its VH and VL genes and constructed the single-chain Fv (scFv) through a peptide linker. The recombinant scFvA21 was expressed in Escherichia coli and purified by the affinity column. Subsequently it was characterized by ELISA, Western blot, cell immunohistochemistry and FACS. All these assays showed the binding activity to extracellular domain (ECD) of p185. Based on those properties of scFvA21, we further constructed the scFv-Fc fusion molecule with a homodimer form and the recombinant product was expressed in mammalian cells. In a series of subsequent analysis this fusion protein showed identical antigen binding site and activity with the parent antibody. These anti-p185 engineered antibodies have promised to be further modified as a tumor targeting drugs, with a view of application in the diagnosis and treatment of human breast cancer.

Key words: *p185, c-erbB-2, scFv, scFv-Fc.*

INTRODUCTION

The product p185 of c-erbB-2 oncogene correlates closely with the progress in some malignant tumors such as breast and ovarian cancer. It was demonstrated that c-erbB-2 was overexpressed in tumor tissues of 25% to 30% patients with breast cancer and part of patients with ovarian, lung, gastric, and oral cancers[1],[2]. The overexpression of c-erbB-2 has been an independent prognostic index for evaluating the malignancy of above tumors [3]. As a tumor surface antigen, p185 is a member of the class I receptor tyrosine kinase (RTK), a preferred dimerization partner of all other ErbB receptors family[4]. The activation of this receptor stimulates its intrinsic kinase activity, which leads to the phosphorylation of tyrosine residues in the intracellular domain of the receptor and finally changes the expression of a series of genes in nucleus, thus results in malignant cell proliferation[5],[6].

Overexpression of p185 in breast cancer may implies that it has the resistance to some conventional therapies such as chemotherapy or radiotherapy. Nevertheless, since it was found that monoclonal antibodies directed against p185 can inhibit the growth of the cells overexpressing erbB-2 gene and the tumor which were implanted into nude mice[7],[8], one new strategy, using monoclonal antibodies for targeting cancer was proposed. However, the application of these antibodies to the clinic is limited by some of their physical properties. At first, the rodent MAbs can induce an anti-immunoglobulin response during their application for human[9]. Secondly, the big size of MAb (155kDa)

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makes it difficult to penetrate from vasculature into the solid tumor. Bad distribution of MAb in vivo decreases its cytotoxicity and increases its side effect dramatically[10]. Thirdly, intact antibodies exhibit a prolonged residence in circulation that can lead to bone marrow exposures which are associated with unacceptable myelotoxicities when radioimmunotherapy (RAIT) methods are employed [11]. Finally, the genetic operation and produce of heterodimer composed of two chains is a laborious work. Therefore, smaller antibody fragments which combined with novel specificities and easy to reconstruct and produce were developed. Genetic engineering provides a powerful approach for redesigning antibodies for oncological applications.

In our previous reports, we have described the preparation of the hybridoma cell line A21 by surface epitope masking (SEM) which secretes the monoclonal antibody directed against the extracellular domain (ECD) of tumor surface antigen p185 and have characterized it[12]. We found that the MAb A21 can specifically inhibit the growth of cells overexpressing c-erbB-2[13]. In order to probe the possibility of modifying it for cancer therapy, we identified the inhibitory function of A21 in vivo first. Subsequently, we isolated the variable genes of this MAb and construct the scFv and the scFv-Fc fusion protein. The characteristics of these recombinant antibody fragments were then identified through a series of immunological methods.

MATERIALS AND METHODS

Cell lines

The anti-p185 monoclonal antibodies (A18&A21) expressing hybridoma were prepared as described previously[12]. They were grown in RPMI Medium 1640 (Gibco BRL) supplemented with 10% fetal bovine serum. Mouse fibroblast cell line NIH3T3 , T6-17 (NIH3T3 cells transfected with the c-erbB-2 proto-oncogene) , SKBR3 and MCF7 cells were obtained from Dr Greene MI (School of Medicine, University of Pennsylvania, Philadelphia) and cultured in Dullbecco's modified Eagle's medium (Gibco BRL) supplemented with 10% fetal bovine serum at 37℃ in a humidified atmosphere of 5% CO_2.

Experimental animals and monoclonal antibody

Inbred, congenitally athymic BALB/c nude female mice were obtained from the Experimental Animal Center of the Fourth Military Medical University. Monoclonal antibody A21, a mouse IgG1 antibody reactive with the extracellular domain of p185, has been produced from hybridoma cells and purified as described [12], [13].

Determination of the in vivo effect of the MAb A21

T6-17 cells ($6X10^6$) were released from culture dishes with trypsin and were injected s.c. into nude mice. MAb A21 was injected either from day 0 to day 21 or from day 7 to day 21 once a day with different doses. Growing tumors were measured once every two days with calipers, and tumor volume was calculated according to the formula: tumor volume equals length X width X height.

Preparation of $p185^{c\text{-}erbB\text{-}2}$ antigen

The confluent T6-17 and NIH3T3 cells in 25cm2 flask were washed three times in phosphate-buffered saline (PBS), pH7.4. Cells were then plated on ice and lysed by addition of 1 ml of PBS containing 1% Triton X-100, $5 X 10^{-4}$ M PMSF and 0.02% NaN3. After 1 h on ice, the lysates were centrifuged at 12000g for 15 min at 4℃. The supernatants were stored at -70℃ and used as the antigen and negative control for T6-17 and NIH3T3 cells respectively.

Cloning of antibody variable region genes by RT-PCR

Total RNA was extracted from $1 X 10^7$ hybridoma cells A21 using Total RNA Purification Kit (Vitagene). About 10 μg total RNA was reverse transcribed in a reaction volume of 50 μl and specific first strand V_H and V_L cDNAs were synthesized according to the manufacturer's protocol (Promega), using AMV reverse transcriptase (Promega) and the oligonucleotides Cγ (5'-GGGGCCAGTGGATAGAC-3) or Cκ (5'-GTTGGTGCAGCATCAGC-3) respectively. The cDNAs of V_H and V_L antibody fragments were subsequently amplified by PCR. The V_H gene was amplified using the degenerate primer H_L1 (5'-ATGGRATGSAGCTGKGTMATSCTCTT-3) and Cγ. The V_L gene was amplified using the degenerate primer V_L (5'-GAYATTGTGMTSACCCAAACTCCA-3') and Cκ. PCR reactions were performed in 50 μl volume, containing 2 μl of cDNA reaction, 1μM primers respectively, 2 mM Mg^{2+} concentration and reaction buffer. After 3 min denaturation at 94 ℃, 2 U of Taq DNA polymerase were added, followed by 30 cycles of 1 min at 92 ℃, 1 min at 52℃, 1 min at 72 ℃ and a final 72 ℃ for 10 min. To confirm the correction of PCR products, the V_H and V_L genes were cloned into pGEM-T vector system (Promega) and sequenced respectively. (In degenerate primers, R=A or G, S=C or G, K=G or T, M=A or C, Y=C or T, W=A or T).

Construction of scFv

To obtain the scFv antibody fragments, the V_H and V_L DNA t empletes were extended at the 5' and 3' ends by PCR using prime rs encoding the linker sequence (Gly_4Ser)4. VH DNA was amplifi ed with the primers V_H Back (5'-GGCGGCGGCGGCTCCGGTG GTGGTGGATCCGAGGT- CCAGCTGCAGCAGTC-3') and V_H

For (5'-AGGATTCGC- GGCCGCTGACGAGACGGTGACTGA GGT-3'); V_L with V_L Back (5'-AGCCGGCCGAYATTGTGMTSA CCCAAAC-TCCA-3') and V_L For (5'-GGAGCCGCCGCCGCCA-GAACCACCACCACCAGAACCACCACCACCCCGTTTG- ATT TCCAGCCTGG-3'). The extended V_H and V_L products were used for assembly of the scFv by splice overlapping extension PCR (SO E-PCR)[14]. An initial denaturation step (3 min, 94℃) was follow ed by 10 cycles of 1 min at 92℃, 3 min at 72℃ in the mix of appr oximately 10 ng of each V_H and V_L extended products. After 10 c ycles the outer primers Sc Back (5'-AAGGAAGGCCCAGCCGG CCGAYATTGTG-3') and Sc For (5'-AGGATTCGCGGCCGCT G-3') was added each 1 mM and 30 cycles of 1 min at 92℃, 1 min at 66℃, 2 min at 72℃ then a final extension at 72℃ for 10 min w ere performed (the underlined nucleosides encode SfiI and NotI r ecognition site). After digestion with the SfiI and NotI, the assem bled scFv fragments were inserted into the pCANTAB 5E (Pharm acia) phagemid vector, in which scFv was fused with E-tag short peptide (5'-GGTGCGCCGGTGCCGTATCCGGATCCGCTGGA A- CCGCGT-3') in C-terminus. This tag will facilitate the immun ological assays and purification of expressed scFv. These vectors were used to transform E.coli HB2151 cells for binding activity a ssay.

Expression and purification of scFv

E.coli cells transformed with the pCANTAB 5E-scFv phagemid were grown overnight at 30℃ in 2 X YT-AG medium containing 100 μg/ml of ampicillin and 2% glucose. The 1/10 of overnight culture were added to freshly prepared 2 X YT-AG medium and grew 1 h at 30℃. Cultured cells were harvested by centrifuging at 1500 X g for 20 min and resuspended in freshly prepared 2 X YT-AI medium containing 100 μg/ml of ampicillin and 1 mM IPTG and grown 3 h at 30℃. The induced culture was centrifuged at 1500 X g for 20 min. The cell pellets were resuspended in 2% of culture volume ice-cold 1 X TES. Subsequently, 3% of culture volume ice-cold 0.2 X ES was added, and the mix was incubated on ice for 30 min to induce a mild osmotic shock. The contents were centrifuge at 12000 X g for 10 min. The supernatant which contains the soluble antibodies from the periplasm was transferred to the clean tubes and stored at -20℃.

Soluble A21 scFv from periplasmic were purified extract by affinity chromatography according to the protocols of the manufacturer. The anti-E tag Ab was covalently coupled to a Protein G column (Pharmacia) and soluble scFv were selected by binding to anti-E tag Ab. After washing with 20 mM phosphate buffer, pH 7.0, +0.05% NaN3, scFv were eluted from the column with 0.1 M glycine-HCl, pH 3.0, and neutralized immediately with 1 M Tris/HCl, pH 8.2, +0.05% NaN3. Column fractions were assayed and positive fractions were pooled and lyophilized.

ELISA analysis

ELISA plate (Nalge Nunc Inc.) was coated overnight at 4℃ with lysate of T6-17 and NIH3T3 cells respectively. All subsequent steps were 1 h incubation performed at room temperature and plate was washed three times with PBS containing 0.05% Tween 20 (PBST) between each step.. Non-specific binding was blocked using blocking buffer (2% casein in PBST). The samples containing recombinant proteins were added to plate and reacted with antigen, and then the anti-E tag second antibody labeled with horseradish peroxidase (HRP) conjugate (Pharmacia) were used respectively. After the assays were developed with o-phenylenediamine (OPD) substrate, the colour reaction was arrested by the addtion of 1 *M* H_2SO_4. Absorbance was read at 490 nm wavelength in ELx800 Universal Microplate Reader (BIO-TEK Instruments Inc.).

SDS-PAGE and Western blot

For expression product analysis, about 20 μl different portion of E.coli samples were run on 12% sodium dodecyl sulfatepolyacrylamide gel for identifying recombinant scFv. The separated proteins were transferred to Hybond ECL membranes (Pharmacia) using Towbin buffer (25 mM Tris/HCl pH 8.3, 192 mM glycine, 20% methanol). In following washing steps between each step, PBST was used three times for 5 min. Membranes were first blocked for 1 h with blocking buffer. Then the HRP-labeled anti-E tag antibody was added and incubated for another 1 h. After washing, the specific binding to expressed scFv was detected by use of an enhanced chemilumeniscence (ECL) kit (Pharmacia).

For identifying the p185 antigen binding to recombinant scFv, the lysates of T6-17 and NIH3T3 cells were separated by SDS-PAGE on 7% gel. Transferring and blocking of two membranes were described as above. Then one of them was incubated for 1 h with purified scFv, and the other was done with MAb A21. After washing, the first membranes were further incubated for 1 h with HRP-conjugated anti-E tag antibody and the latter was done with HRP-conjugated goat anti-mouse second antibody. Subsequently, two membranes were detected as above.

Immunohistochemistry

The T6-17 and NIH3T3 cells were plated on the coverslips and grew at 37℃ and 5% CO_2/air until 30-40% confluent. The coverslips were then rinsed once with PBS and drained well but not to dry. For fixing the cells, the cells were incubated in a 4% paraformaldehyde for 10 min and followed by washing twice with PBS. Subsequently the coverslips were placed on a flat surface, and the MAb A21 and purified scFv were added until volume enough to cover the desired area of the cells but not push beyond the edge of the coverslips. The coverslips were put in a humidified atmosphere at room temperature for 30 min and then washed in three changes of PBS over 5 min. After that, the HRP-coupled anti-E tag and anti-mouse second antibodies was added for scFv and MAb respectively and reacted for 30 min following another three changes washing. Finally, the specimen was achieved colour reaction with OPD substrate and taken photos on BX60 microscope (Olympus).

FACS analysis

The T6-17 and NIH3T3 cells were harvested and divided into 1 X 10^5 cells per sample. Every sample was washed with 2 ml FACS buffer (PBS containing 0.5% BSA and 0.05% NaN3) for two times. Then the 1 μg scFv and 10 μg MAb A21 was added to every sample

and incubated for 30 min on ice. After washed with 2 ml FACS buffer as above, the FITC labeled anti-E tag and anti-mouse second antibodies was added for scFv and MAb respectively and incubated on ice for another 30 min. Samples were washed and fixed by adding 100 ml of 2% paraformaldehyde resolved in PBS. All samples were analyzed using a FACScan (Becton Dickinson, San Jose, CA, USA).

pEE14-A21 plasmid construction

The Fc fragment (hinge, CH2 and CH3) of human IgG1 was amplified from a plasmid containing the gamma chain of human antibody using primers IgHinge Back (5'-ACTGCGGCCGCAGAGCCCAAATCTTGTGACAAA-3') and IgCH3 For (5'-AGTGAATTCTCATTTACCCGGAGAC- AG-3'), which append NotI and EcoRI sites (underlined) onto the 5' and 3' ends of the PCR fragments, respectively. The PCR fragments was digested by NotI and EcoRI , and linked with scFv digested by SfiI and NotI and pSecTag B (Invitrogen Inc. United States) digested by SfiI and EcoRI to generate plasmid pSecTag-A21. Plasmid pSecTag-A21 was then digested by NheI and EcoRI to get scFv-Fc insert. Subsequently, the 1.6kb scFv-Fc insert was subcloned into the expression vector pEE14 (Celltech Limited, United Kindom) treated as the insert and finally got the pEE14-A21.

Expression and purification of scFv-Fc fusion

CHO K1 cells were grown as adherent cultures in Glasgow MEM (Life Technologies), supplemented with 10% (v/v) dialyzed fetal calf serum (Hyclone). The plasmid pEE14-A21 was transfected into 1 X10^6 CHO cells per 10cm diameter plate using Lipofectamine (Life Technologies) as instructed by the manufacturer. After 24 h, fresh medium containing 25 μM methionine sulphoximine (MSX) (Sigma) was added. After two weeks, MSX resistant colonies were transferred to 16mm diameter wells with medium containing MSX. Clones expressing scFv-Fc were identified by measuring the antigen binding activity using ELISA assays. Cell lines expressing high levels of scFv-Fc were then grown in the presence of 100, 250, 500 and 1000 μM MSX to select for higher expressing clones.

The supernatant of high level expressing clones was concentrated by ultrafiltration to 1/10 volume and then dialyzed overnight against two changes of 100 X volume PBS. After dialysis, the sample was applied to a 1 ml Protein G column (Pharmacia) that had been previously equilibrated with PBS. The column was washed with 10 ml PBS, and eluted with 20 ml of 100 mM glycine (pH 3.0). 1.0 ml fractions were collected in Eppendorf tubes containing 0.1 ml of 1.0 M Tris·Cl (pH 9.0) to neutralize. Peak fractions were determined by ELISA assays as before.

About 20 μl purified samples were analyzed by SDS-PAGE on 8% gels under reducing and non-reducing conditions. The separated proteins were transferred to Hybond ECL membranes (Pharmacia) and then detected with HRP-conjugated goat anti-human second antibody as before.

Competitive binding of scFv-Fc to p185

Inhibition of binding of the scFv-Fc fusion to p185 in the presence of murine antibody A21 or A18 was measured by ELISA. Wells of an ELISA plate (Nunc-immunomodules) were coated with antigen (50 μg/ml in PBS) and incubated overnight at 4 oC. A mixture of a constant amount of fusion protein (1 μg/ml) and various amounts of A21 or A18 diluted in PBST, were then added to the wells for 1 h of incubation at room temperature. Residual binding of scFv-Fc to p185 was measured from the absorbance at 490 nm after the addition of HRP-conjugated goat-anti-human IgG, followed by OPD substrate. The percent inhibition rate was calculated as follows: percent inhibition rate = [(A-B)/A] X 100 where A = the mean absorbance of samples containing scFv-Fc only; B = the mean absorbance of samples from mixtures of monoclonal antibody and scFv-Fc.

Analysis of the binding properties of scFv-Fc

Cell immunohistochemistry and FACS were done as same as previously described. One of the difference is the addtion of SKBR3 and MCF7 cells in immunohistochemistry assay. Another is both of the anti-E tag and anti-mouse HRP/FITC second antibodies replaced by goat-anti-human HRP/FITC second antibodies.

RESULTS

The effect of MAb A21 upon tumor growth in vivo

Fig 1 demonstrates that purified MAb A21 is able to inhibit the tumorigenic growth of T6-17 cells in a dose-dependent manner. Intravenous injection of 0.2 mg of purified antibody on both day 1 and day 7 of tumor cell implantation markedly inhibited tumor growth. Treatment with 0.1 mg of antibody had a lesser but still significant effect on tumor growth when compared to that of the PBS-treated control group. Thus, antibody A21 treatment can inhibit the growth of both fresh tumor inocula and established tumors composed of p185 overexpressing cells.

Isolation of antibody variable region genes

Total cellular RNA was purified from hybridoma cells and first strand cDNAs were synthesized by priming with oligonucleotides complementary to the 5' end of the mouse CH1 and Ck domains. With a degenerate primer HL1 deduced from the signal peptide sequence of heavy chain[15], and Cγ, designed to be complementary to the N-terminus of gamma constant region, a PCR product of V_H gene including a leader peptide was obtained. Amplification of V_L gene A21 were performed using the primers VL, deduced from the framework region 1 (FR1)

sequence of the light chain, and Ck, designed to be complementary to C-terminal of the C kappa region. This product of PCR has no leader peptide. V_H and V_L PCR products were sequenced to confirm that all sequences are unique mouse immunoglobulin genes (data not shows).

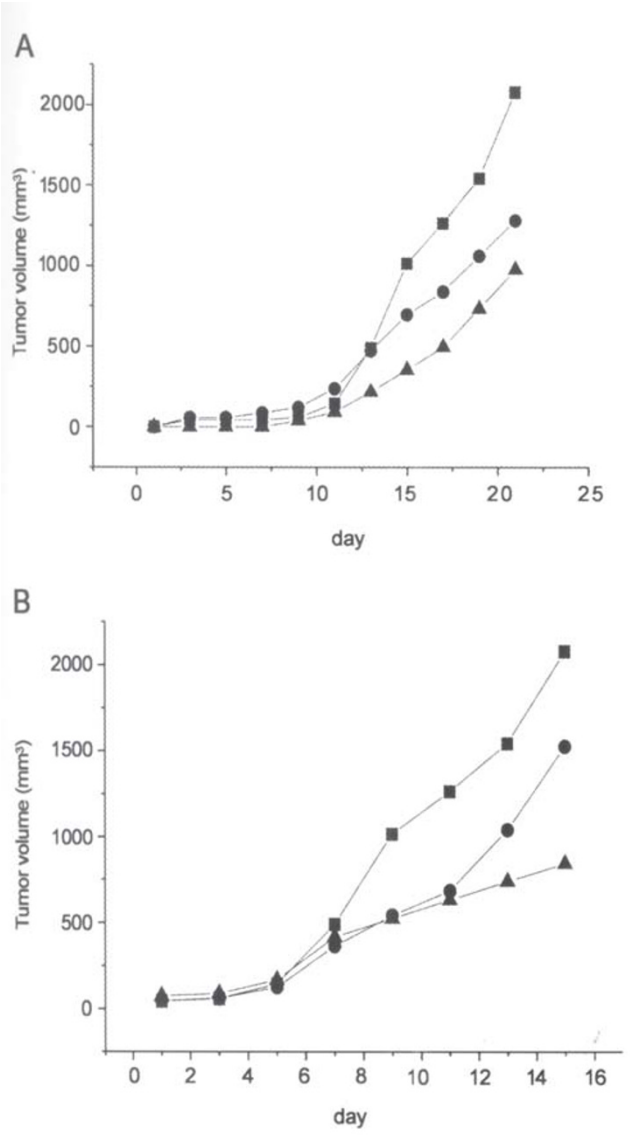


Fig 1. Purified immunoglobulin from the A21 hybridoma inhibits the tumor growth of T6-17 cells in a dose-dependent manner. Four BALB/c nude mice per group received subcutaneous injections of 6 X10^6 T6-17 cells and 0.2ml intravenous injections of either PBS (■) or PBS containing 0.1 mg (●) or 0. 2 mg (▲) of MAb A21 on day 0 (A) or day 7(B). Tumor volume was measured and calculated as described.

Nucleotide and deduced amino acids sequences of scFv

The scFvs with an orientation V_L-linker-V_H were assembled by two-fragment hybridization, because the framework primers are overlapping in the glycine linker region and generate a $[Gly_4Ser]_4$ unit by simple hybridzation. The constructs were digested with SfiI and *NotI*, cloned into the vector pCANTAB 5E encoding the pel B leader sequence at the N-terminus and, a E-tag peptide at the C-terminus respectively[16]. After transformed into E.coli HB2151, the scFvs were expressed in the periplasm solublely. There were more than one hundred clones which were assayed for binding activity with lysate of T6-17 and NIH3T3 cells by ELISA. Three of them showed positive results clearly. We chose one with the highest activity to subclone into a modified pBluescript SK(+/-) vector (Stratagene) and sequenced it. Fig 2 shows the complete nucleotide sequences and deduced amino acid sequences for the functional scFv clone(accession numbers AY077783 for V_H and AY077781 for V_L in the GenBank). Alignment of sequences showed that the V_H and V_L gene of MAb A21 belongs to the member of the mouse H-chain VIIa and kappa chain VI subgroup respectively, defined by Kabat et al. (2001)[17].

Western blot analysis

In pCANTAB 5E, the pel B signal peptide upstream from the scFv directs the expression to the periplasmic compartment. The periplasmic extract was run through the anti-E tag affinity chromatography column and the scFv was eluted from the column as a single peak (data not show). The expressed and purified scFvs were loaded on 12% SDS-PAGE and analyzed by Western blot. This protein migrated with a molecular mass approximately 35 kDa, larger than the expected 29 kDa (Fig 3). It is because there are too many small size amino acids (twenty of Gly and Ser) inside the linker peptide between V_L and V_H. These aggregated small residues interfered the mobility of the scFv and resulted in moving slowly[18].

The specific activity of scFv was also confirmed by Western blot analysis. The lysates of the T6-17 and

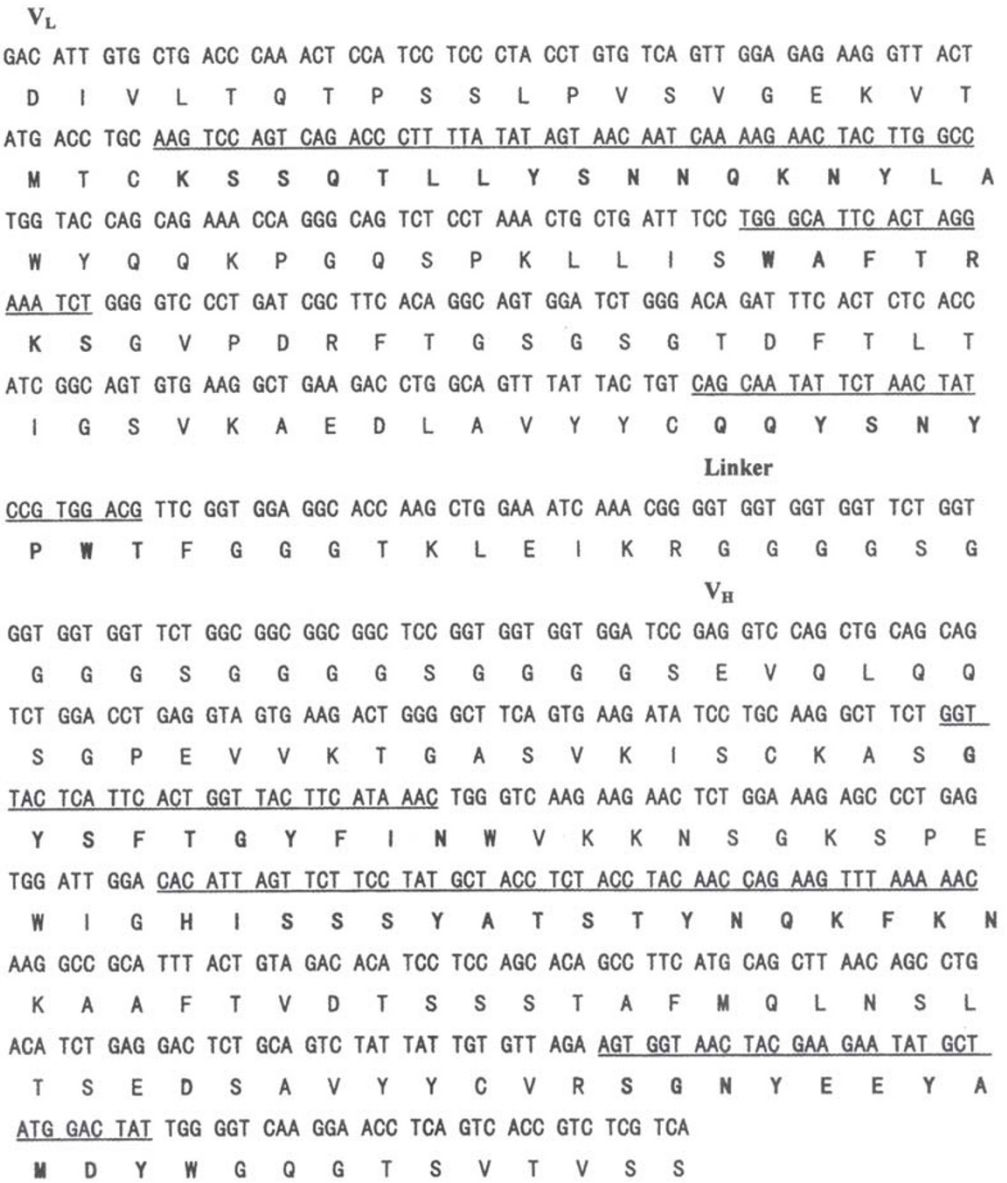


Fig 2. Nucleotide and deduced amino acid sequences of the constructed A21 scFv containing V_L, linker peptide and V_H. The CDR regions are indicated in underlining letters.

NIH3T3 cells were run on SDS-PAGE gel and analyzed with scFv. As seen in Fig 4, the scFv reacted specifically with the 105 and 185 kDa band of T6-17 lysates as MAb A21 and did not show any cross-reactivity with the NIH3T3 lysates. Since the band with 105 kDa size (p105) is corresponding to the extracelluar domain (ECD) of p185[19], we concluded the scFvA21 binds to ECD of p185 specifically as its parent antibody and the epitope region that the scFv recognizes is linear. The p105 was generated by post translational processing and released into the medium by T6-17 cells. The mechanism of this procession remains to be clarified.

Immunohistochemistry and FACS

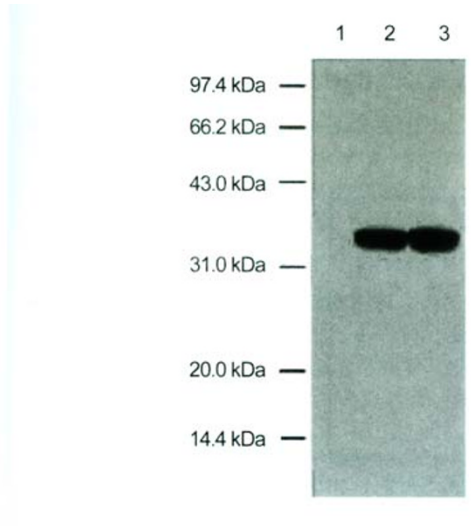


Fig 3. Analysis of recombinant proteins by Western blotting. Affinity purified recombinant scFvs were analyzed under reducing conditions on SDS-PAGE followed by transfer onto nitro-cellullose membranes. Lane 1: material from HB2151 cells transformed with phagemids without insert; Lane 2: the periplamic extract of HB2151 cells transformed with phagemids containing the scFv; Lane 3: the scFv purified by anti-E tag affinity chromatography column.

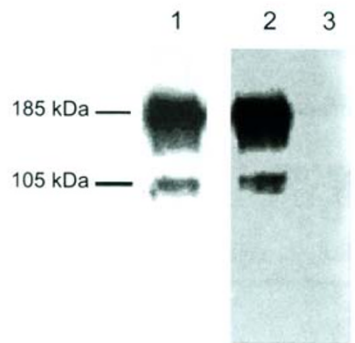


Fig 4. Western blot analysis of binding characteristic of scFv. Lane 1: the lysate of the T6-17 cells analyzed with A21 MAb; Lane 2: the lysates of the T6-17 cells analyzed with scFv; Lane 3: the lysate of the NIH3T3 cells analyzed with scFv. The membranes were incubated with HRP-conjugated, goat anti-mouse and anti-E tag secondary antibody for lane 1 and 2/3, respectively.

In order to characterize the reaction of recombinant scFv to the intact p185 overexpressing cells, we performed the immunohistochemical and fluorescence flow cytometric analysis using MAb A21 and scFvA21. Immunohistochemical analysis was performed using fixed cells of T6-17 and NIH3T3 (Fig 5). The staining pattern observed with scFvA21 was similar to that seen using its parent monoclonal antibody, with membrane of T6-17 cells stained brown strongly and scattered staining in the cytoplasm area of T6-17 cells (Fig 5A and C). But only nonspecifically weak immunoreactivity can be seen in the NIH3T3 cells (Fig 5B and D), and also in the control using anti-E tag secondary antibody alone (data not shown). Difference of staining intensity demonstrated clearly that the the scFv binds specifically to the membrane proteins of T6-17 cell. Reactivity of the scFv antibody fragment towards the ECD of p185 was also confirmed by FACS. The FACS profiles of the T6-17 and NIH3T3 cells stained with A21 whole antibody and scFvA21 have the similar distribution (Fig 6). Both MAb and scFv showed significant positive results to the T6-17 cells, whereas the NIH3T3 cells were negative. In conclusion, the

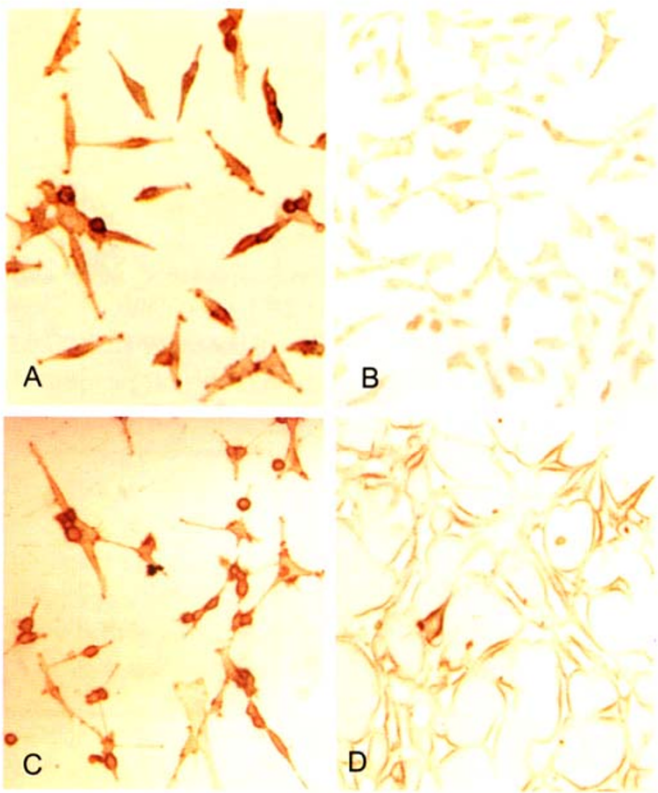


Fig 5. Analysis of antigen binding activity by cell immunohistochemistry using scFvA21. **A.** The scFv predominantly reacts with the membrane, and at lesser degree the cytoplasm, of T6-17 cells; **B.** there is no specific reactivity with the NIH3T3 cells and **C.** MAb A21 reacts with T6-17 cells; **D.** NIH3T3 cells as controls. These images were captured at 20 X magnification.

scFvA21 binds only to the surface antigen on T6-17 but not on NIH3T3 cells, which is identical to the binding characteristic of its parent MAb A21.

Construction of scFv-Fc fusion

The scFvA21 was fused to the human IgG1 Fc domain to construct a bivalent scFv-Fc fusion protein with 105 kDa molecular weight (Fig 7a). Since

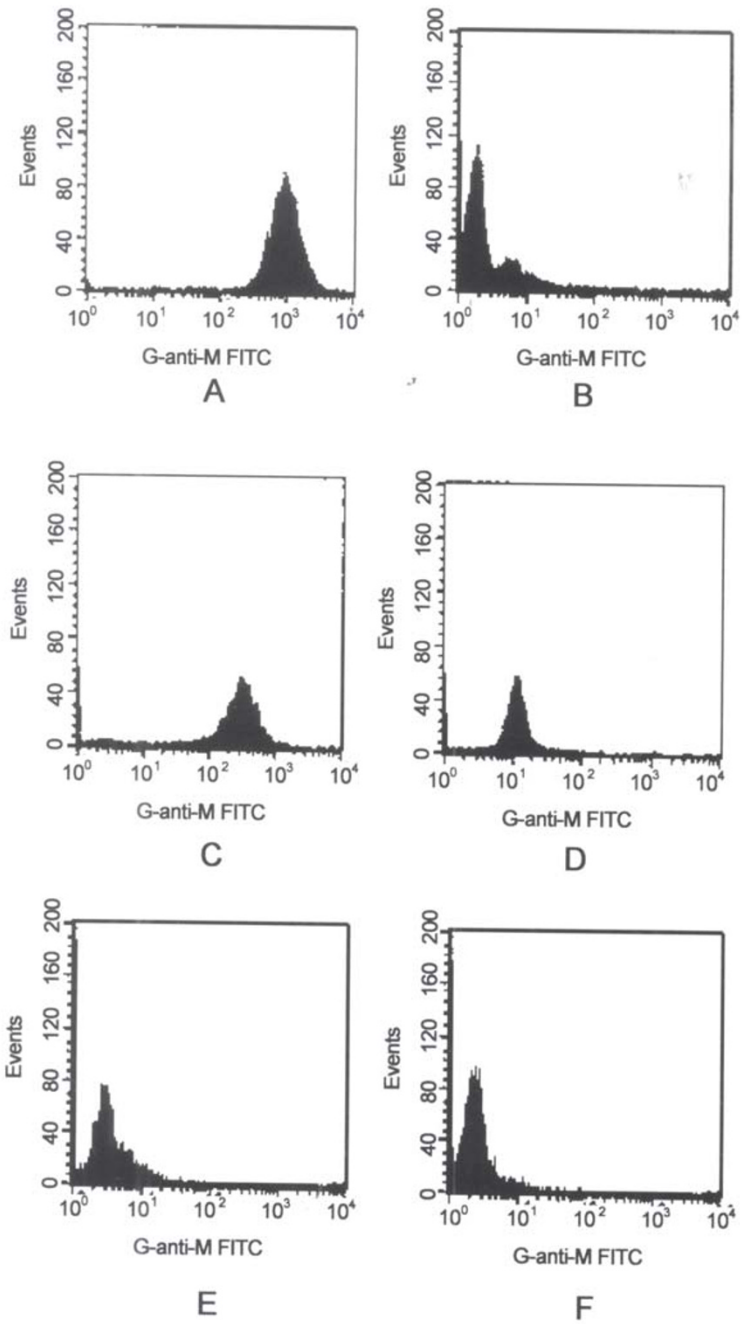
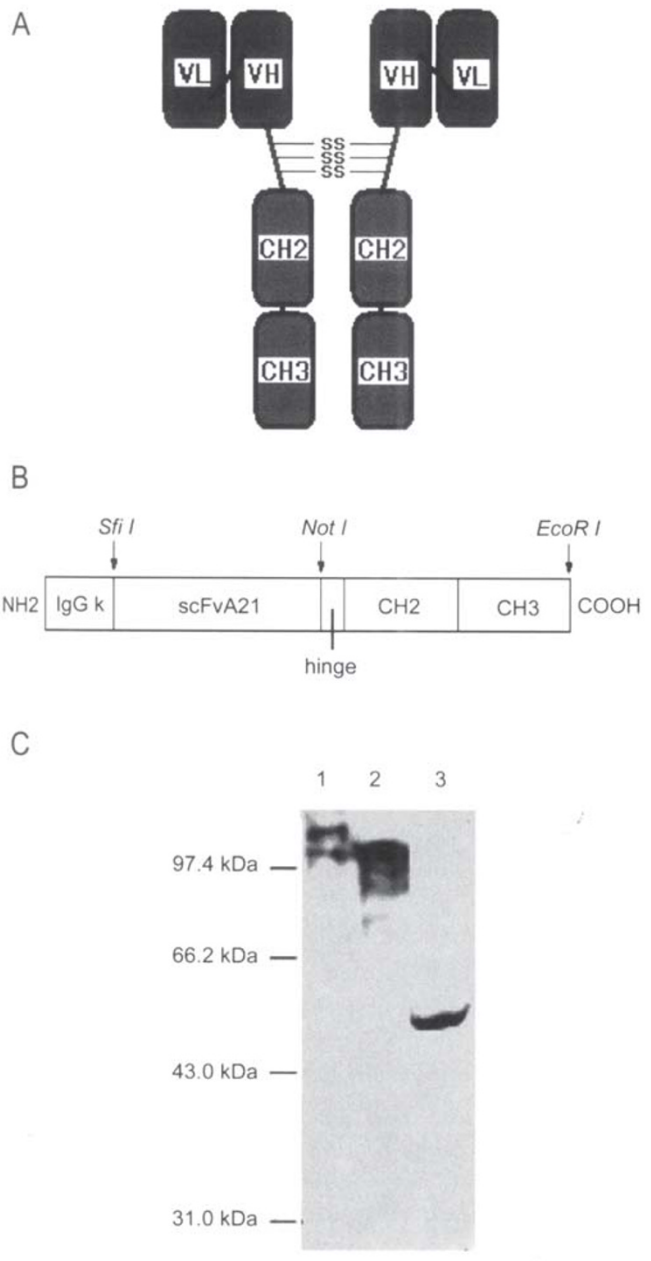


Fig 6. Detection of antigen binding activity by FACS. The results of immunofluorescence reaction of the T6-17 cells (A) and the NIH3T3 cells (B) with MAb A21, or the T6-17 cells (C) and the NIH3T3 cells (D) with scFv intensity demonstrated different labeling activities of same antibody fragment to different cells. The reaction between the T6-17 cells with anti-TPA monoclonal antibody (E) and only second antibody (F) were done as negative controls. The similarity between A21 MAb and scFv showed that they own same binding characristic.

glycosylation of the Fc region is a characteristic of the eukaryotic system, an antibody for therapeutic application must be produced in eukaryotic cells. Plasmid PEE-14 for the expression of the scFv-Fc is based on the mammalian expression plasmid pSecTag B. As shown in Fig 7b, proteins are fused at the N-terminus to the murine Ig kappa-chain leader sequence for secretion, and the human hinge, CH2 and CH3 domains of IgG1 linked to scFvA21 through the NotI site. A stop codon was included following the C-terminus of CH3. This scFv-Fc was subcloned into a gene-amplification system utilizing the glutamine synthetase (GS) which was inserted into the plasmid pEE14. The GS gene expressed from the plasmid confers resistance to a low level of the GS inhibitor methionine sulfoximine (MSX). The cDNA of scFv-Fc was expressed from the powerful hCMV promoter-enhancer. CHO cells transformed with the vector are selected for lines containing increased numbers of copies of the vector using increased levels of MSX in a single round of amplification. The clone at the highest MSX concentration was isolated and the yield of its scFv-Fc were used for assaying binding properties.



The scFv-Fc fusion protein which was expressed in CHO cells forms a disulfide-linked homodimer form, as shown by Western blot analysis. It has a molecular weight of about 55 and 106 kDa respectively under reducing and non-reducing conditions (Fig 7c).

Competitive binding of scFv-Fc

Competition assays were performed to compare the binding of MAb A21, A18 and scFv-Fc to the cell lysates of p185 overexpressing cells. A21 and A18 were used to compete with the chimeric scFv-Fc fusion for binding to antigen. It was demonstrated previously that both of MAb A21 and MAb A18 recognize the epitopes localized at the ECD of p185 [12]. In another experiment from ELISA identifying the binding of A18 and A21 with different subdomain of ECD of p185 showed that MAb A18 and A21 bind the sites in the subdomain III-IV and subdomain I-II of ECD respectively (data not shown). The recombinant scFv-Fc construction we describe here mimics the antigenic specificity of the native antibody since its binding to ECD of p185 was inhibited by MAb A21 in a dose-dependent manner (Fig 8). Approximately the same

◁**Fig 7.** Schematic representation of scFv-Fc fusion protein. **A**. The dimeric scFv-Fc fusion. V_H and V_L: heavy and light chain variable domain respectively; CH2 and CH3: heavy chain constant domains 2 and 3; -SS-: disulfide bonds between the two hinge domains. **B**. The scFv-Fc fusion contains IgG kappa leader sequence fused to N-terminus of the scFvA21. The scFv was linked to the hinge region of human Fc fragment to generate the fusion protein. **C**. Western blotting analysis of the expressed scFv-Fc fusion under reducing and non-reducing conditions. Lanes 1 and 2 are Human IgG1 and scFv-Fc under non-reducing conditions; lane 3 is scFv-Fc under reducing conditions.

concentration A21 were required for 50% inhibition of the binding of scFv-Fc fusion to ECD of p185. As a control antibody, MAb A18 shows no competitive inhibition with scFv-Fc.

The binding properties of scFv-Fc

The results in Fig 9 showed a very similar result to those got from the antigen binding activity analysis of the scFv. Both of the SKBR3 and MCF7 cells belong to human breast carcinoma cell lines. The difference between them is that the cell surface of former one has overexpressed p185, and the latter without. The results in Fig 9C and 9D are very similar to those in Fig 9A and 9B. In the same way, the results in Fig 10 are also very similar to those got from FACS assay of MAb A21. In summary,

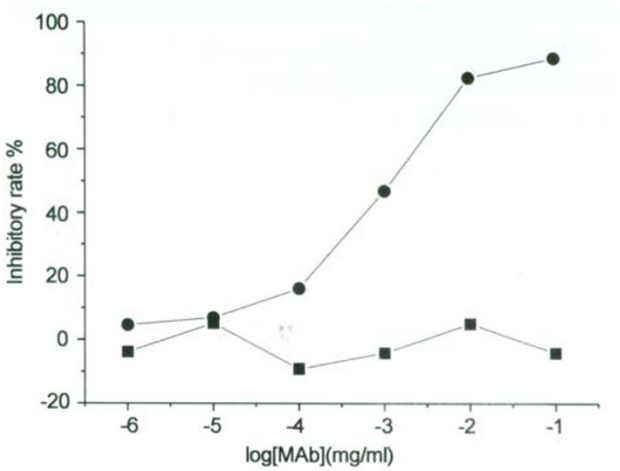


Fig 8. Competitive binding assay of MAb A18 (■) and A21 (●) with the scFv-Fc fusion.

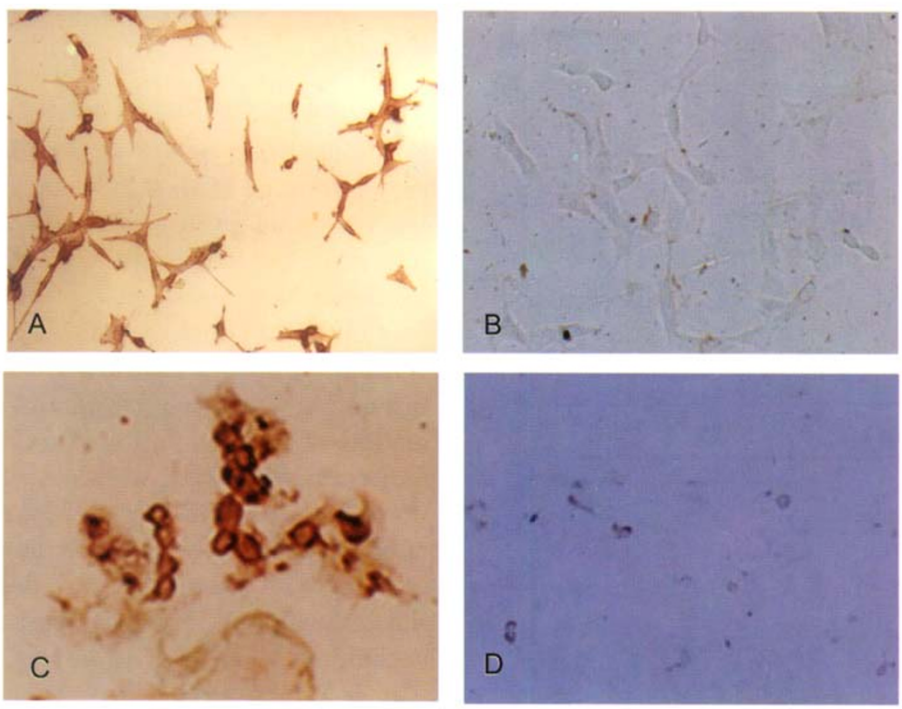


Fig 9. Immunohistochemistry assay of binding activity of scFv-Fc fusion. The result of T6-17 cells (A), NIH3T3 cells (B), SKBR3 cells (C) and MCF7 cells (D) were stained by scFv-Fc fusion.

mmunohistochemistry and FACS assays of scFv-Fc fusion demonstrated that this new engineered antibody molecule have the same antigen binding specificity to its parent antibody MAb A21.

DISCUSSION

Since Kohler and Milstein first described making monoclonal antibodies, it has spent more than 25 years to develop applied reagents to treat human disease. These "magic bullets" with high specificity and high affinity for their target antigens are required to address several shortcomings of murine MAbs, including high immunogenicity, sub-optimal targeting and pharmacokinetic properties, and un-

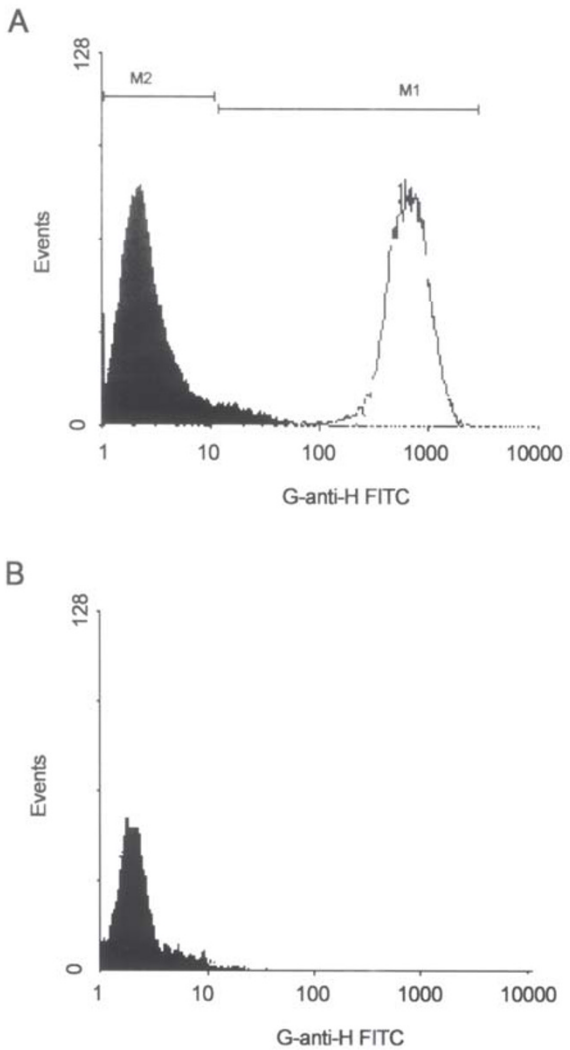


Fig 10. FACS assay of binding activity of scFv-Fc fusion (A). M1: positive region of T6-17 cells; M2: negative region of NIH3T3 cells. The reaction of the T6-17 cells to only goat-anti-human FITC antibody was done as negative control (B).

practical production. All of these let the progress unsuccessfully slow until the emergence of antibody engineering. The development of this new technology has allowed the conversion of existing mouse MAbs into recombinant fragments that retain high affinity and show a favourable distribution in normal tissues and solid tumors. An important first step to do this, was the construction of the recombinant smallest antibody fragment with antigen binding specificity, single-chain Fv (scFv). The scFv was composed of antibody heavy and light chain variable domains joined via a synthetic linker peptide[[20], [21]. With a size of approximately 25kDa, it owns lots of advantages over intact antibody. The scFv shows a rapid penetration into solid tumor and a faster clearance from the circulation, so the tumor:normal tissues activity ratio is improved highly[22],[23]. This can result in an overall better targeting index and lower toxicity towards the patient. The scFv also shows a negligible immunogenicity which makes it optimized for tumor imaging[24]. Furthermore, the scFv can be manipulated more easily than the bivalent parent antibody and engineered into other formats (Fab, (Fab')2, IgG or other fusion proteins). As minimal antigen binding fragment, the scFv is favored for phage display techniques and phage antibody library construction[25], and can be produced on a large scale in bacterial expression system. These characteristics render the scFv an ideal vector for delivery of agents such as radionuclides, enzymes, drugs or toxins *in vivo*.

In some situation the scFv itself is the desired molecule for uses. However, it will not be sufficient for therapeutic use in its unconjugated form. As discussed above, it would be desirable to transfer the scFv to other molecules as therapeutic agents. In this paper, we combined the scFv with a human Fc fragment and construct the fusion protein with a scFv-Fc form. The combination of Fc fragment takes a lot of virtues into the scFv[26]. Firstly, the disulfide bonds between two hinge regions transfer the monovalency of scFv into bivalency of scFv-Fc. This will increase the functional affinity-avidity intensively and hence improved the specificity of antibody fragment. Secondly, the effector functions of a complete immunoglobulin reside in the Fc region were remained. The Fc fragment will restore the cytolytic functions such as comlement-dependent cytotoxicity (CDC) and antibody-dependent cell cytotoxicity (ADCC). It will enhances the tumor killing effect of engineered antibody markedly. Thirdly, the increasing molecular size prolongs serum half-life of scFv and therefore improves its in vivo pharmacokinetics efficiently. It will be more suitable for modifying into a drug. Fourthly, because the molecular weight of scFv-Fc is about two third of intact antibody, this molecule will penetrate into the center of solid tumor more rapidly and get increased tumor distribution compared to the whole Ig without fast renal clearance. Thereby it will exert greater effect on tu-

mor tissue[27]. Moreover, because of it single-gene structure, it will be very easy to conjugate this molecule with other effectors such as cytokines and immunotoxin by fused with their genes. And to operate in single-gene form also saves lots of works in the course of reengineering it. Finally, the Fc fragment provides an excellent tag for convenient detection and purification of recombinant antibody. Altogether, the construction of the scFv-Fc fusion offered a potential candidate molecule for cancer therapy.

Our previous data demonstrated that all three of our monoclonal antibodies can inhibit growth of the cells overexpressing p185. Now we showed obvious in vivo tumor growth inhibitory activity of MAb A21. These data made it be worthy of modifying as a potential cancer therapy drug. Therefore, we decided to reengineer the MAb A21 to the candidate molecules for cancer therapy.

The main problem to clone the variable region genes of MAb is to get the accurate sequence of their N-terminus. In the course of cloning the genes of MAb A21, the heavy chain was amplified successfully by PCR with the signal peptide primers, so its variable region gene was cloned perfectly. However, only the aberrant kappa transcript was gained with the corresponding signal peptide primers (data not shown) when we tried to amplify the light chain[28]. The aberrant transcript is derived from mouse myeloma cell line using for constructing hybridoma. In this case, it is arising from myeloma Sp2/0. Referring to the immunoglobulin sequences of Kabat Database and conception reported previously[17],[29], we redesigned the degenerate 5'-primers and acquired the light chain genes. However, the N-terminus of light chain may be important because these regions can be contiguous with the surface of complementary determining region (CDR). Therefore, the N-terminus alternatives of the variable regions introduced by PCR primers may affect binding activity of the antibody seriously[30],[31]. In order to identify the antigen binding capacity of different variation, we assayed a great number of colonies by ELISA after expressing these recombinant scFvs with different N-terminus.

Among more than a hundred expressing colonies, three colonies with specific binding activity to T6-17 but not NIH3T3 cell were selected out by ELISA. We chose the best one of them to sequence and undergo the following assays. In order to compare the cell binding activity of recombinant scFv with parent MAb, we performed the immunohistochemical and fluorescence flow cytometric analysis of A21 and scFv. It showed that the scFv binds only to the surface antigen expressing highly in T6-17 but not NIH3T3 cells. Therefore, we concluded that scFvA21 maintains the binding activity of parent MAb.

After the characterization of the scFvA21 by a series of immunological methods, we further constructed the scFv-Fc fusion and identified its binding activity primarily. The expression, purification and characterization of the fusion presented here demonstrate that a recombinant single gene can be expressed in a mammalian cell to result in subsequent secretion of a functional immunoglobulin-like molecule. The molecule, generated by a convenient single-step transfection of the CHO cells, showed fidelity to the antigen-binding specificity of the parental antibody.

Furthermore, the results of competition assays showed that the scFv-Fc fusion recognizes the same site of the ECD of p185 as its parent antibody. Similarly, it can also be deduced that the scFvA21 binds to the same site as MAb A21 either. This assays also compared the binding affinity of scFv-Fc and MAb A21 to p185 antigen primarily. Because approximately the same concentration of A21 was required for 50% inhibition of the binding of scFv-Fc to p185, the relative antigen binding affinity between A21 and scFv-Fc should be in a comparative degree.

In conclusion, we constructed two engineered antibodies and identified their binding properties completely. In all assays, these engineered antibodies show the same antigen-binding specificity as native antibody. The scFv-Fc fusion, a single gene immunoglobulin-like molecule, not only includes part of human antibody sequence but also avoids the low efficiency of delivering two genes of an intact antibody into a mammalian cell and assembling them into a functional protein. It would facilitate ex vivo transfection of cells for gene-therapy purpose and may induce little or no human anti-mouse antibody (HAMA) response in human patients because of virtue of chimeric structure. Taking into account of the fact that MAb A21 shows a favorable growth inhibitory activity to tumor cells overexpressing the sur-

face antigen p185[13] and tumor established by implanting T6-17 cells into nude mice, we considered that the scFv-Fc fusion may be deserved to develop as a potential anti-tumor agent. In addition, the scFvA21 can also be modified to other engineered antibody fragments for tumor targeting in future.

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