Production and characterization of a humanized single-chain antibody against human integrin αvβ3
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Abstract:
Anti-angiogenesis therapy is an emerging strategy for cancer treatment. This therapy has many advantages over existing treatments, such as fewer side effects, fewer resistance problems and a broader tumor type spectrum. Integrin αvβ3 is a heterodimeric transmembrane glycoprotein that has been demonstrated to play a key role in tumor angiogenesis and metastasis. We have used phage antibody display to humanize a mouse monoclonal antibody (mAb E10) against human integrin αvβ3 with a predetermined CDR3 gene. Three human phage antibodies were developed. Analysis of the humanized phage antibodies by phage ELISA revealed that the antibodies retained high antigen-binding activity and detected the same epitope as the parent mAb E10. A humanized single chain Fv (scFv) antibody was expressed in E. coli in a soluble form. Analysis of the purified scFv indicated that it has the same specificity and affinity as the original mAb. Cell viability assays and xenograft model results suggested that the humanized scFv possesses anti-tumor growth activity in vitro and in vivo. This successful production of a humanized scFv with the ability to inhibit αvβ3-mediated cancer cell growth may provide a novel candidate for integrin αvβ3 targeted therapy.

Keywords: Anti-angiogenesis; human integrin αvβ3; humanized single-chain antibody.

Introduction
Angiogenesis, the growth of new blood vessels from pre-existing vessels, is a fundamental process during cancer progression (1). Tumor angiogenesis is a complex process that depends on the balance between pro-angiogenic molecules and anti-angiogenic molecules (2). Inhibition of angiogenesis is an emerging practice for cancer treatment. Anti-angiogenesis therapy hinders tumor growth by limiting the supply of oxygen and nutrients to the tumor. This therapy has multiple advantages over other anti-cancer treatments, such as fewer side effects, fewer resistance problems and applicability to a broad spectrum of tumors (3). Two new anti-angiogenesis peptides have been shown to effectively inhibit tumor growth in mouse models (3,4). One of these, Avastin, which has been approved by the FDA, is a monoclonal antibody against vascular endothelial growth factor (VEGF), which is active during the generation of new blood vessels in tumors (5,6).

Integrins are a family of heterodimeric transmembrane glycoproteins composed of a single alpha and a single beta chain that have activated or non-activated conformations. Integrins are involved in a wide range of cell-extracellular matrix (ECM) and cell-cell interactions (7,8), and they play an important role in tumor angiogenesis and tumor metastasis. The
αvβ3 integrin, which is expressed in many tumor cells, is significantly up-regulated on endothelium during angiogenesis, but is not seen on quiescent endothelium, and is considered the most important integrin for angiogenesis (7,9,10). The αvβ3 integrin can also cooperate with several cytokines or proteinases, including matrix metalloproteinase 2, vascular endothelial growth factor receptor 2 and platelet-derived growth factor, and thereby promote tumor angiogenesis (11). This integrin is therefore a potential target for anti-angiogenic cancer therapy. Monoclonal antibodies and low-molecular weight antagonists against αvβ3 have been shown to inhibit tumor angiogenesis and tumor cell proliferation through obstructing the integrin binding to its ligand. The mAb LM609 and human monoclonal antibody Vitaxin, were found to be effective in vivo and in vitro (12,13), the RGD mimetic Cilengitide has been shown to be effective in the treatment of glioblastoma multiform (14), and the integrin αvβ3 antagonist S247 was shown to inhibit tumor angiogenesis and metastasis in a mouse model (15).

Recombinant antibody-based treatments are becoming increasingly available and are showing exciting clinical successes. Single chain Fv (scFv) antibodies consist of V\textsubscript{H} and V\textsubscript{L} regions only, thus representing the smallest fragments capable of retaining the full binding structure of a native antibody. Compared with whole antibodies, scFvs have many advantages. An scFv is composed of the variable antigen binding regions (V\textsubscript{H} and V\textsubscript{L}) without the Fc portion, which may interact with Fc receptors on normal tissues (16). It has been demonstrated that scFvs can penetrate into tumors more efficiently and facilitate faster systemic clearance (17). A humanized scFv to integrin αvβ6 has recently been shown to possess good therapeutic potential to block cancer cell invasion (18).

E10, a mouse monoclonal antibody against human integrin αvβ3 with the ability to inhibit tumor growth in vitro and in vivo, was previously produced in our lab (unpublished). In the study presented here, this mouse antibody was used in a humanization protocol (19) and several humanized phage Fab antibodies which recognize the same epitope as E10 were selected. We then constructed a scFv vector which expressed one of these humanized antibodies, D5, in E. coli in a soluble form. The purified scFv protein was analyzed by several methods and showed antigen-binding activity to human integrin αvβ3. Furthermore, a cell viability study demonstrated that the scFv acts to inhibit tumor cell growth in vitro, suggesting this scFv could become a candidate drug for tumor immunotherapy.

**Experimental Procedures**

**Reagents, antibodies and proteins.**
MDA-MB-435 human breast carcinoma cells were obtained from Peking Union Medical College. HT-29 cells were from our laboratory stores. Human integrin αvβ3 protein was obtained from R&D Systems (Minneapolis, USA). HRP-conjugated goat anti-mouse IgG, HRP-conjugated anti-M13 and HRP-conjugated anti-His-Tag antibodies were obtained from Merck (Shanghai, China). E. coli BL21 (DE3) (Novagen, Shanghai, China) was used as the host for expression of soluble scFv protein. Restriction enzymes were purchased from Takara (Dalian, China).

**Antibody humanization**
The mouse monoclonal antibody (E10) was humanized as previously described (19). Briefly, two sequential phage antibody displays with pre-determined CDR3 were conducted to humanize the light chain and Fd fragment of the heavy chain, respectively. First, the light chain CDR3 of mouse monoclonal antibody E10 was fused with a human light chain antibody gene library (FR1 through FR3) and this fusion library was inserted into the antibody displaying phagemid vector pComb3 between the SacI and XbaI sites. The chimeric Fd containing the E10 heavy chain variable region fused with human heavy chain constant region 1 (CH1) was inserted between the XhoI and SpeI sites. After four rounds of biopanning of this phage-displayed antibody library against human integrin αvβ3 the resultant humanized light chain antibody clones were screened and used for a second humanization step. In this second step, the humanized light chain genes were inserted into the SacI and XbaI sites of pComb3 and the heavy chain library containing the E10 heavy chain variable region CDR3 and human heavy chain variable region FR1 through FR3, as well as the human CH1 gene, were inserted into the XhoI and SpeI sites. Electro-transformation of the constructed phagemid library into the E. coli strain XL1-Blue resulted in a phage-displayed antibody library with the humanized heavy chain. After four rounds of biopanning against immobilized human integrin αvβ3 several phage antibodies with high antigen-binding activity were selected and humanized heavy chain genes were obtained.

Biopanning of phage antibody library
In the panning procedure, the human integrin αvβ3 (100 µg/ml of protein in 0.1 M sodium bicarbonate buffer pH 8.6) was coated on ELISA plates (Nunc, Roskilde, Denmark) and incubated at 4°C overnight. The plates were then blocked at 37°C for 2 h with 5 mg/ml BSA in 0.1 M sodium bicarbonate buffer and washed four times with TBST (TBS + 0.1% Tween-20). The phage library was added (10^12 pfu in 100 µl) and incubated at 37°C for 1 h. After phage binding, the wells were washed 10 times with TBST (TBS + 0.1% Tween-20) and the bound phage were eluted using 100 µl elution buffer (0.2 M Glycine-HCl, pH 2.2, 1 mg/ml BSA) per well. The eluate was immediately neutralized with 2 M Tris-base. The eluted phages were amplified as previously described (20).

Phage ELISA
Binding activity of the phage-displayed antibody was measured by phage ELISA. ELISA plates were coated with 200 ng per well of human integrin αvβ3 protein at 4°C overnight. The plates were then blocked at 37°C for 2 h with 10% non-fat milk and washed four times with PBST. The phage antibody was mixed with equal amounts non-fat milk and incubated at room temperature for 30 min. This mixture was added at 100 µl per well and incubated at 37°C for 2 h. The helper phage VCSM13 was used as a negative control. The plates were washed four times with PBST, followed by incubation with HRP-conjugated anti-M13 antibody at 37°C for 1 h. Color was developed with TAB solution (Sigma, Shanghai, China) and the plates were read in an ELISA reader at 450 nm. Non-specific reactivity of the phage antibodies was analyzed using two antigens: bovine serum albumin (BSA) and hepatitis B virus surface antigen (HBsAg) by indirect ELISA, as described above. The positive phage antibody clones were also analyzed by competitive ELISA to verify their
binding to the same epitope. In these competitive ELISAs, the human integrin αvβ3 protein was coated on ELISA plates. The mouse antibody E10 was diluted into different concentrations and was mixed with the positive phage antibody clones. The mixture was used as primary antibody and incubated at 37°C for 2 h. Other steps of the competitive ELISA were the same as for the indirect ELISA.

Construction of expression vector
Two heavy chain primers and two light chain primers were designed based on the sequence of humanized antibody clone D5. The phagemid D5 was used as the template and two heavy chain primers (VHBam, 5'-CGG GAT CCA CCA TGA AGG TGA AAC TGC TC-3'; and LNKr, 5'-AGC CAC CTC CGC CTG AAC CGC CTC CAC CTG AGG AGA CGG TGA C-3') were used to amplify the VH coding regions, while the VL fragments were obtained by PCR using two light chain primers (VLrKpn, 5'-GGC GA T A TT TTG A TT TCC ACC TTG G-3' and LNK, 5'-CAG GCG GAG GTG GCT CTG GCG GTG GCC GAT CGG AGC TCG TGA TGA CC-3'). The scFv gene was amplified by overlapping extension PCR. The VH and VL fragments were mixed as the template, and primers VHBam and VLrKpn were used to amplify the scFv gene. The PCR products were digested with BamHI and EcoRV and inserted into the prokaryotic expression vector pQE80L (Novagen, Shanghai, China) which was digested with BamHI and SmaI, to create expression plasmid pQE80L-scFv.

Protein expression and purification
_E. coli_ BL21 (DE3) transformed with pQE80L-scFv was cultured in LB medium supplemented with 100 μg/ml ampicillin and grown at 37°C until the logarithmic phase of growth was reached (at OD600 0.5-0.6). Bacteria were induced for production of scFv protein by IPTG at a final concentration of 0.4 mM for 6 h at 25°C. Bacteria from cultures were centrifuged, and the cytoplasm was extracted after sonication. The total bacterial proteins were then partitioned into soluble and insoluble fractions by centrifugation at 10,000 × g for 20 min at 4°C. The supernatant (soluble fraction) was collected and the pellets (insoluble fraction), which contained the inclusion bodies, were resuspended in deionized water. Both fractions were analyzed in parallel by 15% SDS-PAGE to characterize the solubility of the scFv proteins.

Then the supernatant was filtered through a 0.45 μm membrane and then loaded onto a gravity-flow column packed with 3 ml Ni²⁺-NTA resin slurry (Novagen). His-tagged scFv protein was purified according to the manufacturer's instructions and the yield was quantified using a Coomassie Protein Assay Kit (Biomed, Beijing, China) (Bradford, 1976). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gels was performed to validate the identity and evaluate the purity of the target fusion proteins.

ELISA analysis
The human integrin αvβ3 protein was coated at 200 ng per well in ELISA plates at 4°C overnight. The plates were then blocked at 37°C for 2 h with 10% non-fat milk and washed four times with PBST. The scFv proteins were added as the primary antibodies at various dilutions (0.05 nM, 0.1 nM, 0.5 nM, 1.0 nM, 2.0 nM, and 5.0 nM diluted in PBST). An irrelevant protein, human secretory component (SC), which is also expressed by pQE80L vector, was used...
as a negative control. Following overnight incubation at 4°C, the plates were washed four times with PBST, followed by incubation with HRP-conjugated anti-His-Tag antibody at 37°C for 1 h. Color was developed with TAB solution and the plates were read in an ELISA reader at 450 nm.

**Western blot and Far Western blot analysis**
For western blot (WB) analysis, the human integrin αvβ3 proteins were first subjected to SDS-PAGE and then electrically transferred to a PVDF membrane. Protein scFv and mAb E10 were used as primary antibodies, with his-tagged SC as a negative control. The bound antibodies were detected using either HRP-conjugated anti-His-Tag antibody or HRP-conjugated goat anti-mouse IgG in PBST at room temperature for 90 min. The immunoreactive proteins were visualized using an ECL WB Analysis System (Pierce, USA).

Far Western blotting (WB) has been derived from the standard Western blotting method to allow the detection of protein–protein interactions in vitro (21). Briefly, the cell lysate of pQE80L-scFv was firstly separated by SDS-PAGE and then electrically transferred to a PVDF membrane. The cell lysate of pQE80L-SC and purified scFv protein were used as negative and positive controls respectively. The proteins in the membrane were denatured and renatured by gradually reducing the guanidine–HCl concentration. The membrane was blocked before incubation with human integrin αvβ3 protein (1 μg/ml diluted in PBST). After four washes with PBST, the membrane was incubated with mAb E10 for 2 h at RT and the bound antibodies were detected using HRP-conjugated goat anti-mouse IgG. The immunoreactive proteins were visualized using the ECL WB Analysis System.

**Immunocytochemical analysis**
The recombinant scFv protein was also analyzed by immunocytochemical assays. The human breast carcinoma cell line MDA-MB-435 was cultured and fixed on glass slides. MDA-MB-435 cells were rinsed with 0.1 M PBS and blocked with 10% non-fat milk in Tris-buffered saline-Tween solution (TBST) for 20 min, then incubated with the recombinant protein scFv at 4°C overnight. Protein SC and mAb E10 were used as control. After washing with PBS, the cell was incubated with the secondary antibody for 1 h at 37°C. The staining intensity was evaluated by microscopic observation.

**Cell viability assay (MTS assay)**
The Cell Titer 96 AQueous One Solution Cell Proliferation Assay (Promega, Beijing, China) was used to determine the number of viable cells in proliferation assays, as instructed by the manufacturer. Briefly, cells (1000 cells per well) were seeded in 96 well plates. The medium was replaced 24 h later with serum-reduced growth medium and the scFv antibody was added at a concentration range from 5 pM to 5 nM. Equimolar amounts of mAb E10 and anti-His mAb were used as controls. The cells were incubated for 24 h before the medium was replaced by MTS solution. The plates were further incubated for 1–4 h at 37°C before the absorbance was read at 490 nm.

**Anti-tumor growth study in vivo**
Female athymic BALB/c mice at 4 weeks of age were provided by the Laboratory Animal Center, Military Academy of Medical Sciences, Beijing, China. Mice
were maintained under specific pathogen-free conditions and were rested for 1 week before use. The animal study protocol was approved by the Institutional Animal Care and Use Committee. Mice received $1 \times 10^6$ MDA-MB-435 cells in 100 µl phosphate-buffered saline (PBS) per mouse, injected subcutaneously into the right flank. When subcutaneous tumors became measurable they were measured daily with a caliper in three perpendicular directions. When tumors had grown to approximately 50 mm$^3$ (about 2 weeks after implantation) tumor bearing mice were randomized into two groups that received either the scFv or SC protein. Eight mice per group were treated by intravenous injection with 30 µg scFv protein or equimolar amounts of SC protein in 100 µl PBS on days 0, 4 and 8. Mice were followed for 3 weeks and the tumor growth was measured manually using a caliper ruler.

**Statistical analysis**

Data are presented as mean ± standard deviation (mean ± SD). Statistical analyses of data were performed using Student's t-test. In all cases, P-values <0.05 were considered statistically significant.

**Results**

**Humanization of the light chain of mAb E10**

An antibody light chain library containing mAb E10 light chain CDR3 was constructed as described in the Methods. The capacity of the antibody library was about $2.1 \times 10^6$ and the titer of the antibody library was determined to be $8.96 \times 10^{14}$ pfu/ml. Twenty phage clones were randomly selected from the library and analyzed by PCR, which showed that the antibody gene insertion frequency was 80%. After four rounds of panning against immobilized human integrin αvβ3, thirty phage clones were picked randomly and analyzed by phage ELISA. Ten of the selected clones were found to bind to human integrin αvβ3. The potential cross-reactivity of these positive phage antibody clones was analyzed by indirect ELISA using two irrelevant protein antigens (BSA and HBsAg). These results indicated that all of the positive clones specifically recognize the human integrin αvβ3 with no significant non-specific reactivity (Fig. 1a). Furthermore, competitive ELISA showed that the antigen-binding activity of three of the clones (B12, B8 and A20) is inhibited by mAb E10, suggesting that these three phage antibodies target the same epitope as the mAb E10 (Fig. 1b).

**Humanization of the heavy chain of mAb E10**

Following generation of the light chain antibody library, a heavy chain antibody library was also generated from mAb E10. This pComb3-based heavy chain library expressed the humanized light chain gene B12 and contained a human Fd gene library with E10 heavy chain grafted to HCDR3. Electrotransformation of these heavy chain constructs into *E. coli* strain XL1-Blue resulted in a phage displayed antibody library with a capacity of $2.0 \times 10^7$. Twenty phage clones were randomly selected from this library and PCR analysis results suggested that the human antibody heavy chain insertion frequency was 50%. After four rounds of panning against immobilized human integrin αvβ3, twenty phage clones were picked randomly and the antigen-binding activity and cross-reactivity of these clones were analyzed by phage ELISA. Eight clones were found to have
high antigen-binding capacity with human integrin αvβ3 with no significant cross-reactivity with irrelevant antigens (Fig. 2a). Competitive ELISA showed that the antigen-binding activity of three highly reactive phage clones (C16, D5 and D10) was inhibited by mAb E10, suggested that these antibodies detected the same epitope as mAb E10 (Fig. 2b). The heavy chain and the light chain sequences of the clone with strongest antigen-binding activity, D5, were determined. Homology analysis and germline gene analysis indicated that the light chain had a typical human immunoglobulin structure and belonged to the VKIII family and the heavy chain belonged to the V_H gene family (GenBank Nucleotide Sequence Database accession no.AY489290 and DQ192641).

Construction and purification of the scFv
This newly generated scFv construct was cloned into the prokaryotic expression vector pQE80L to create the expression plasmid pQE80L-scFv, as described in the Methods. pQE80L-scFv was transformed into the E. coli host strain BL21 (DE3) and the recombinant protein scFv was produced. The recombinant protein can be expressed in E. coli as both soluble and insoluble forms of approximately 28 kDa, with recombinant proteins comprising about 28% of the total soluble protein extract (Fig. 3). Recombinant proteins were purified from cell lysates as described in the Methods.

Recombinant mAb E10 scFv specifically recognizes target antigen and retains antigen-binding activity
To investigate the antigen-specificity of the recombinant scFv, an ELISA assay was performed using human integrin αvβ3 as a capture antigen. Increasing concentrations of scFv corresponded to enhanced colorimetric signals, but no significant background was detected in wells where irrelevant proteins were used as negative controls (Fig. 4a). The recombinant scFv protein sample was also analyzed by WB. The human integrin αvβ3 protein was first subjected to SDS-PAGE. The scFv detected a polypeptide band corresponding to the human integrin αvβ3 at a fragment size similar to that of mAb E10, while no background staining was detected with the negative protein SC (Fig. 4b).

Far WB analysis demonstrated that human integrin αvβ3 protein can bind to the recombinant scFv but is unable to recognize SC protein. These results also imply that the recombinant scFv retains the desired antigen-binding activity (Fig. 4c).

Immunocytochemical assays
To verify whether the purified scFv was able to bind integrin αvβ3 on the cell membrane, cells of the human breast carcinoma cell line MDA-MB-435, which express αvβ3, were incubated with the purified scFv and examined by immunocytochemical assay. These assays showed that the scFv bound to integrin αvβ3 expressed on the membrane of MDA-MB-435 cells to a similar extent to that observed with mAb E10, while SC protein did not react with MDA-MB-435 cells (Fig. 5).

Recombinant scFv can inhibit tumor growth in vitro and in vivo
To test whether the scFv was able to inhibit tumor cell growth in vitro, MDA-MB-435 cells and HT-29 cells, which have been reported to be integrin αvβ3-negative (22), were incubated with purified scFv or control antibody. Then cell viability was examined by MTS assay. The results suggested that the humanized scFv has good anti-tumor
growth activity in vitro. The mAb E10 also inhibited MDA-MB-435 cell growth, but the negative antibody had no effect on the MDA-MB-435 cell growth (Fig. 6a). Another result showed that the humanized scFv cannot influence HT-29 cell growth (Fig. 6b). These results suggest that the scFv can specially bind to the integrin αvβ3 to inhibit tumor cell growth. Neither mAb E10 nor anti-His mAb had any effect on the HT-29 cell growth (data not shown).

Effects of the humanized scFv on tumor growth were tested in a MDA-MB-435 cell murine xenograft model. When tumors had grown to approximately 50 mm$^3$, mice were treated by intravenous injection with scFv protein or SC protein which had also been expressed by pQE80L vector. Mice were followed for 3 weeks and the tumor growth was measured manually using a caliper ruler. The results suggest that tumor growth was delayed significantly by multiple injections of humanized scFv protein (Fig. 7). The SC protein had nearly no effect on the tumor growth, similar to the control group.

Discussion

Integrin αvβ3 is a heterodimeric transmembrane glycoprotein and is believed to be involved in tumor angiogenesis due to its increased expression in proliferating vascular endothelial cells (8). Integrin αvβ3 can bind to extracellular matrix protein through the RGD (Arg-Gly-Asp) sequence and plays an important role in tumor angiogenesis and the migration of cancer (23). Therefore, if it is possible to interfere with αvβ3 expression at the endothelial surface of tumor cells, tumor angiogenesis may be inhibited and tumor growth may be slowed due to a lack of cellular nutrition. The mouse monoclonal antibody LM609, specific for the human integrin αvβ3, has been shown to have anti-tumor activity in vivo (13). However, mouse antibodies may cause human anti-mouse antibody responses, resulting in a short serum half-life of these antibodies, which severely limits their clinical applicability. Human antibodies are much less immunogenic in humans, and are therefore more desirable candidates for therapy. Vitaxin, the humanized version of the mAb LM609, has recently been approved by the FDA for clinical trials and has passed phase 1 safety trials (24, 25).

In this study, we generated a humanized Fab antibody, D5, against human integrin αvβ3 from a phage-displayed antibody library. Human(ized) single chain Fv antibodies can be produced in two ways using phage display library technology. One way is to humanize a mouse monoclonal antibody that is known to have high antigen-binding affinity and specificity. Another method is via de novo panning of a human phage display library with the target antigen. The latter method is of limited use due to the tendency to lose high affinity binders (26). In this study, we used the former method to engineer a humanized scFv from the mouse monoclonal antibody against human integrin αvβ3 by phage antibody display using a predetermined CDR3 gene.

The scFv of this humanized antibody was expressed in E. coli in a soluble form. Fab and scFv antibodies have a lower molecular weight than full-length antibodies, but can retain the same antigen affinity as the parent antibody. As such, these antibody fragments have been widely studied for targeted therapy and drug delivery purposes.

scFvs can be expressed in mammalian, plant and bacterial cells (27-29). While each of these systems has its own advantage, the
bacterial expression system is the most universally used, as it is relatively inexpensive and easy to manipulate, and has a rapid growth rate (30). However, inclusion bodies of scFv proteins are often produced during bacterial expression, which requires the incorrectly folded proteins to be denatured and renatured before further use. It is preferable for the antibody fragment to be directly expressed in a soluble and active form in *E. coli*. The use of a low culture temperature, low IPTG concentrations and the co-expression of molecular chaperones are typical methods that can promote the soluble expression of recombinant proteins (31). Expressing fusion proteins in the soluble fraction allows proper folding of the protein and disulfide bond formation in the cytoplasm, thereby increasing the likelihood of obtaining soluble proteins with preserved biological activity (32). In this study, the scFv against human integrin αvβ3 was expressed at a low temperature (25°C) and low IPTG concentration (0.4 mM). To further increase the recombinant protein solubility, we chose *E. coli* BL21 (DE3) as the host strain, as it has been previously demonstrated to be appropriate for soluble protein expression (31). By adopting these strategies, the scFv against integrin αvβ3 was expressed in soluble form and easily purified for further use.

The humanized scFv protein purified by this method retained a high binding activity to human integrin αvβ3 and acted to inhibit tumor cell growth *in vitro* and *in vivo*. These results suggest that this humanized scFv antibody represents a potential novel tool in cancer therapeutics. This humanized scFv may also be useful for delivery of other therapeutic molecules via the construction of bifunctional antagonists, since tumor angiogenesis does not exclusively depend on integrin αvβ3.

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Footnotes

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Figure Legends:

Figure 1. ELISA analysis of humanized light chain antibodies. (a) Competitive ELISA analysis of humanized light chain antibody clones. Plates were coated with αvβ3 (or BSA or HBsAg for cross-reactivity assays) and then incubated with phage antibodies. All phage antibodies reacted with the target antigen, while no cross-reactivity with other antigens was observed. (b) Competitive ELISA analysis of humanized light chain antibody clones. Mouse mAb E10 was diluted and mixed with the humanized light chain antibody at the indicated concentrations, and these mixtures were used as the primary antibody. Antigen-binding activity of three clones (B12, B8 and A20) was inhibited by mAb E10.

Figure 2. ELISA analysis of humanized heavy chain antibodies. (a) Competitive ELISA analysis of humanized heavy chain antibody clones. Plates were coated with αvβ3 (or BSA or HBsAg for cross-reactivity assays) and then incubated with phage antibodies. All phage antibodies reacted with the target antigen, while no cross-reactivity with other antigens was observed. (b) Competitive ELISA analysis of humanized heavy chain antibody clones. Mouse mAb E10 was diluted and mixed with the humanized heavy chain antibody at the indicated concentrations, and these mixtures were used as the primary antibody. Antigen-binding activity of three clones (C16, D5 and D10) was inhibited by mAb E10.

Figure 3. Expression and purification analysis. E. coli BL21 (DE3) transformed with pQE80L-scFv. After induction, cytoplasmic proteins were extracted following sonication. The total bacterial proteins were then partitioned into supernatants and pellet fractions by centrifugation. The protein sample was separated on a 15% SDS-PAGE gel and stained with Coomassie blue. Lane M, protein molecular mass markers (kDa); lane 1, the sediments after sonication of the induced bacterial cells; lane 2, the supernatants after sonication of the induced bacterial cells; lane 3, the purified scFv protein.

Figure 4. Antigen-specific binding activity of the recombinant scFv. The antigen-specificity of
the recombinant scFv was analyzed by ELISA, WB and Far WB. (a) ELISA analysis of the recombinant scFv showing that the recombinant scFv binds to target antigen in a concentration dependent manner. (b) WB analysis of the recombinant scFv. The scFv detected a polypeptide band similar to that observed with mAb E10, while no specific staining was observed with the negative control His-tagged SC. (c) Far WB analysis of the recombinant scFv. The human integrin αvβ3 protein bound to the recombinant scFv but was unable to recognize His-tagged SC.

Figure 5. Immunocytochemical analysis of the recombinant scFv. Cells of the human breast carcinoma cell line MDA-MB-435 were incubated with the purified scFv and examined with an immunocytochemical protocol. The purified scFv protein concentration was 0.2 mg/ml and the SC protein concentration was 0.5 mg/ml. (a) scFv at 1:5 dilution, (b) scFv at 1:20 dilution, (c) mAb E10 at 1:200 dilution, (d) SC protein at 1:20 dilution.

Figure 6. Inhibitory activity of the recombinant scFv on tumor cell growth. MDA-MB-435 cells and HT-29 cells were incubated with the purified scFv or control antibody and examined by MTS assay. (a) Human breast carcinoma cell line MDA-MB-435 cells were incubated with the purified scFv and examined by MTS assay. mAb E10 and anti-His mAb were used as positive and negative controls respectively. The scFv protein and mAb E10 had an inhibitory effect on the MDA-MB-435 cells growth but the anti-His mAb had no influence on the MDA-MB-435 cells growth. (b) HT-29 cells, which have been reported to be negative for integrin αvβ3, were incubated with the purified scFv and examined by MTS assay. The scFv protein had no effect on the HT-29 cells growth.

Figure 7. Inhibitory effect of scFv protein on in vivo tumor growth. Effects of the humanized scFv on tumor growth were tested in a MDA-MB-435 cell murine xenograft model. Mice were treated by intravenous injection with scFv protein or SC protein which had also been expressed from the pQE80L vector. The tumor growth was measured manually using a caliper ruler. Results shown are mean ± SD (n=8).
Figure 4

(a) Absorbance at 450 nm vs. scFv concentration (nM/mL)

(b) Western blot images:
- mAb E10
- scFv
- SC

(c) Immunoblot images:
- Supematant scFv
- Purified scFv
- Supematant SC
Figure 5

(a) scFv at 1:5 dilution

(b) scFv at 1:20 dilution

(c) mAb E10 at 1:200 dilution

(d) SC protein at 1:20 dilution
Figure 6

(a) MDA-MB-435 cells group

(b) HT-29 cells group
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