Production and characterization of a human single-chain Fv to collagenase IV *

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Abstract The over-expression of collagenase IV in tumor tissues was found to be closely related to tumor metastasis. Collagenase IV has been therefore considered as one of the novel indicative molecules for tumor diagnosis and treatment. Based on phage display antibody library technique, a single-chain Fv specific for collagenase IV was successfully cloned. This antibody, referred to as hCo4, was mainly composed of variable regions from heavy and light chains, with its molecular weight of 27 ku. The engineered antibody bound to collagenase IV specifically. The affinity of hCo4 was found to be the same as that of a single-chain antibody constructed from a monoclonal antibody to collagenase IV. Since hCo4 is the smallest among all the antibodies specific for collagenase IV and it is of human origin, it has a potential to be applied for tumor immunotherapy and for the study of the relationship between collagenase IV and tumor metastasis.

Keywords: collagenase IV, human single-chain antibody, tumor metastasis.

Invasion and metastasis are the most important characteristics of malignant tumor. The over-expression and increasing activity of collagenase IV in tumor tissues have been considered as one of the main factors involved in tumor metastasis[1]. It has been thought that the increasing collagenase IV in tumor tissues might degrade collagen IV, a main structural component of vascular basement membrane, and result in the tumor cells' penetrating through the basement membrane and then spreading with blood to distant organs to form secondary tumor.

Since a lot of inhibitors to collagenase IV, such as metalloproteinase inhibitors and specific antibodies, were found being related to tumor metastasis and effectively suppressing tumor growth[2] *in vitro*, collagenase IV inhibitors have been considered as one of the novel strategies for tumor therapy.

Antibodies to collagenase IV have played a special role in the study of the relationship between collagenase IV activity and tumor metastasis[3] as well as in tumor auxiliary diagnosis and tumor prognostic prediction[4]. However, these antibodies have limitations in their application to tumor immunotherapy because of their mouse origin and a high risk to cause human anti-mouse antibody (HAMA) reaction when they were repeatedly used in human bodies. The problem of antibody immunogenicity could be solved by making human antibody with genetically antibody engineering instead of conventional hybridoma technology. As the development of antibody engineering, human antibodies appeared increasingly. However, no human antibody to collagenase IV was

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ever reported. In this paper, we describe a human genetic-engineered single-chain Fv specific to collagenase IV derived from a phage display human antibody library. This antibody, referred to as hCo4, was found to possess three characteristics: (i) binding to collagenase IV specifically; (ii) as a human antibody, it is expected to minimize antibody immunogenicity; (iii) being the smallest antibody (MW 27 ku) to collagenase IV.

1 Materials and methods

1.1 Materials

Human semi-synthetic antibody library[5] was kindly provided by MRC center of Cambridge University in England; collagenase IV of Clostridium histolyticum origin, collagen IV and nitrocellulose membrane were purchased from Sigma Company; Recombinant Phage Antibody System (Detection Module), T7 DNA sequencing kit and sequencing primer, pCANTAB 5E vector, anti E-tag antibody were all purchased from Pharmacia Biotech Company; (α-^{32}P)-dCTP was purchased from Dupont Company, USA; Nunc-immuno tube and ELISA plates were purchased from Gibco BRL Company; Horseradish peroxidase labeled goat anti-mouse IgG was purchased from Beijing Tianxiangren Biotechnology Company; general reagents are analytically pure (A.R.).

1.2 Methods

1.2.1 Selection of human phage antibody specific to collagenase IV. 100 μL phage antibody library was inoculated in 50 mL of 2 ×TY containing 100 μg/mL ampicillin and 1% glucose and grew until the OD at 600 nm was about 0.5, and then super-infected with M13KO7 helper phage at 30 ℃ overnight. The culture supernatant containing phage antibodies was concentrated by PEG/NaCl, and then 10^{13} PFU phage was incubated with a Nunc-immunotube coated with 10—100 μg/mL collagenase IV for 2 h at room temperature. The tube was washed 20 times with PBS containing 0.05% Tween-20 and then 20 times with PBS. The bound phages were eluted by adding 1 mL of 100 mmol/L triethylamine and neutralized with 1 mol/L Tris-HCl, pH 7.4. The "adsorption-elution-amplification" procedures were repeated for three times.

1.2.2 Expression of phage antibody to collagenase IV. As described previously[6], after three rounds of panning, the culture grew in 200 μL 2 ×TY containing 100 μg/mL ampicillin and 50 μg/mL kanamycin (without glucose) at 30 ℃ overnight. The supernatant containing selected phage antibodies was collected for immuno-activity detection.

1.2.3 Expression of soluble single chain Fv. The recombinant phagemids were extracted from the positive clones that displayed a specific binding to collagenase IV and the scFv gene was subcloned into pCANTAB 5E vector with SfiI and NotI. The pCANTAB 5E/scFv recombinant plasmids were transformed into *E. coli* HB2151. After induction with 1 mmol/L of IPTG, soluble single-chain antibodies were expressed and leaked into culture supernatant. Supernatants were collected and concentrated with 50% $(NH_4)_2SO_4$.

1.2.4 Western blot analysis. After being concentrated with 50% $(NH_4)_2SO_4$, the induced and non-induced culture supernatants were analysed with SDS-PAGE, followed by staining with Coomassie blue or electro-transferring onto nitrocellulose membrane. The membrane was blocked with 2% BSA and sequentially incubated with anti-E-tag antibody and goat anti-mouse IgG

conjugated with horseradish peroxidase (HRP). Finally substrate O-Phenylenediamine (OPD) was added for visualization.

1.2.5 Immunoactivity assay of soluble single-chain Fv

(1) Dot immunoblot. Various antigens, such as collagenase IV, collagenase I, vascular endothelial growth factor and bovine salivary mucin, were respectively dotted 1 μg each on the nitrocellulose membrane. After being blocked with 5% fat-free milk in PBS, the membrane was incubated for 2 h with soluble single-chain Fv (about 10 μg/mL), and then for 1 h with anti-E-tag antibody, followed by HRP-labeled goat anti-mouse IgG for another one hour. Finally substrate diamino benzidine (DAB) was added for color reaction.

(2) ELISA assays. Collagenase IV at the concentration of 10 μg/mL was coated on 96-well plates. After being blocked with 1% BSA, the plates were incubated for 2 h with phage antibody or soluble single-chain Fv (10 μg/mL), washed six times with 0.05% Tween 20/PBS, incubated for 1 h with HRP-conjugated anti-M13 or anti-E-tag antibody for the detection of phage antibody and soluble antibody, respectively. Finally OPD was applied for color reaction, and absorbance at 490 nm was read with a microtiter plate reader.

1.2.6 DNA sequence analysis of single-chain Fv gene. The single-chain antibody hCo4 gene was sequenced using pCANTAB5 sequencing primer set and T7 DNA sequencing kit.

2 Results

2.1 Screening human single-chain Fv to collagenase IV

Using collagenase IV as a target antigen, we have performed three rounds of "adsorption-elution-amplification" process and several positive clones were obtained. ELISA showed that they all bound to collagenase IV. Among those the one with the highest binding activity was referred to as hCo4. The affinity of hCo4 phage antibody was found not different from that of phage antibody constructed from a monoclonal antibody to collagenase IV.

2.2 DNA sequence analysis of antibody hCo4

The recombinant plasmid containing hCo4 antibody gene was detected by the digestion with SfiI and NotI. The result showed that the antibody hCo4 gene was approximately 700 bp (fig. 1), which was in accordance with the size of designed V_H-$(Gly_4Ser)_3$-V_L single-chain antibody.

DNA sequence analysis further showed that hCo4 gene was composed of 705 nucleotides, with the heavy chain variable region (V_H) 336 nucleotides, the light chain variable region (V_L) 324 nucleotides and the linker 45 nucleotides (fig. 2). In addition, there was a 39-nucleotide E-tag gene fragment at the end of the hCo4 gene which expressed E-tag peptide acting as a detection marker of soluble single chain antibody. hCo4 gene nucleotide sequence was registered in DDBJ DNA bank (accession number AB001733).

Fig. 1. Analysis of recombinant pHEN1/scFv with restriction enzymes. a, DNA molecular weight marker SppI/EcoRI; b, the digested products with SfiI and NotI.

2.3 Expression of soluble single-chain antibody

In order to obtain soluble form of antibody, hCo4 gene was subcloned into pCANTAB5E expression vector and transformed into *E. coli* HB2151, a

V_H

GAG GTG CAG CTG GTG GAG TCT GGA GGA GGC TTG ATC CAG CCT GGG GGG TCC CTG
E V Q L V E S G G G L I Q P G G S L
AGA CTC TCC TGT GCA GCC TCT GGG TTC ACC GTC AGT AGC AAC TAC ATG AGC TGG
R L S C A A S G F T V S S N Y M S W
CDR1
GTC CGC CAG GCT CCA GGG AAG GGG CTG GAG TGG GTC TCA GTT ATT TAT AGC GGT
V R Q A P G K G L E W V S V I Y S G
GGT AGC ACA TAC TAC GCA GAC TCC GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC
G S T Y Y A D S V K G R F T I S R D
CDR2
AAT TCC AAG AAC ACG CTG TAT CTT CAA ATG AAC AGC CTG AGA GCC GAG GAC ACG
N S K N T L Y L Q M N S L R A E D T
GCC GTA TTA CTG TGC AAG AGA GAC TTG TGT GAT TGG GGC CAA GGT ACC CTG GTC
A V L L C K R D L C D W G Q G T L V
CDR3
ACC GTG TCG AGA GGT GGC GGT GGC TCG GGC GGT GGT GGG TCG GGT GGC GGC GGA
T V S R G G G G S G G G G S G G G G
V_L Linker
TCT TCT GAG CTG ACT CAG GAC CCT GCT GTG TCT GTG GCC TTG GGA CAG ACA GTC
S S E L T Q D P A V S V A L G Q T V
AGG ATC ACA TGC CAA GGA GAC AGC CTC AGA AGC TAT TAT GCA AGC TGG TAC CAG
R I T C Q G D S L R S Y Y A S W Y Q
CDR1
CAG AAG CCA GGA CAG GCC CCT GTA CTT GTC ATC TAT GGT AAA AAC AAC CGG CCC
Q K P G Q A P V L V I Y G K N N R P
CDR2
TCA GGG ATC CCA GAC CGA TTC TCT GGC TCC AGC TCA GGA AAC ACA GCT TCC TTG
S G I P D R F S G S S S G N T A S L
ACC ATC ACT GGG GCT CAG GCG GAA GAT GAG GCT GAC TAT TAC TGT AAC TCC CGG
T I T G A Q A E D E A D Y Y C N S R
GAC AGC AGT GGT AAC CAT GTG GTA TTC GGC GGA GGG ACC AAG CTG ACC GTC CTA
D S S G N H V V F G G G T K L T V L
CDR3
GGT
G

Fig. 2. The nucleotide sequence of antibody hCo4 and the deduced amino acid sequence. The complementary determining regions (CDRs) are underlined.

non-suppressor strain. In this strain hCo4 antibody was predominantly expressed as soluble form after induction with IPTG and secreted into the culture supernatant. Western blot showed that the induced culture supernatant contained single-chain antibody with the MW of 27 ku (fig. 3). The expression level of soluble hCo4 was estimated at 2 mg/L according to SDS-PAGE stained by Coomassie blue.

2.4 Immunoactivity analysis of soluble single-chain antibody

Soluble antibody was detected by using anti-E-tag antibody. ELISA (fig. 4) and immunodot blot (fig. 5) showed that single-chain antibody hCo4 bound to collagenase IV, but not to other

proteins, e.g. collagenase I, vascular endothelial growth factor and bovine salivary mucin.

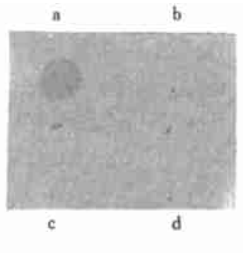

Fig. 3. Immuno-dot blot assay showing the reactivity of antibody hCo4 with collagenase IV (a), collagenase I (b), vascular endothelial growth factor (c), and bovine salivary mucin (d).

3 Discussion

We have successfully cloned a human single-chain antibody to collagenase IV from a semi-synthetic human antibody library based on phage antibody display technique[7]. The antibody hCo4 was composed of heavy and light chain variable regions linked by 15 amino acids [$(Gly_4Ser)_3$]. The complementary determining region CDR1 and CDR2 of heavy chain were derived from human antibody genome and CDR3 was synthetic. Variable region of light chain was derived from $V_{\lambda 3}$ family. Comparing the nucleotide sequence of hCo4 heavy chain variable region with human antibody germline gene sequences[8], we found that the hCo4 heavy chain variable region belonged to V_{H3} family, homologous to DP-42 and 8-1B[3] in V_{H3} family. This observation suggested that antibody hCo4 can be purified by affinity chromatography with Protein A column because it has been demonstrated that V_{H3} family antibody bound to protein A specifically[5].

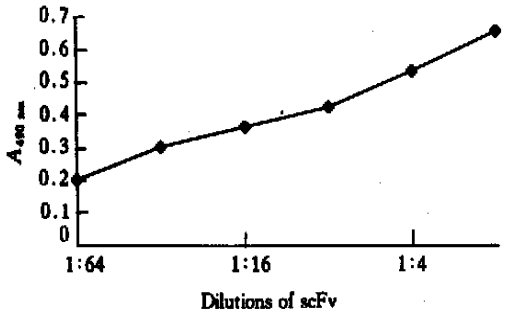

Fig. 4. ELISA assay showing the binding of antibody hCo4 to collagenase IV. Vertical axis shows the absorption at $A_{490\ nm}$; horizontal axis shows various dilutions of antibody hCo4.

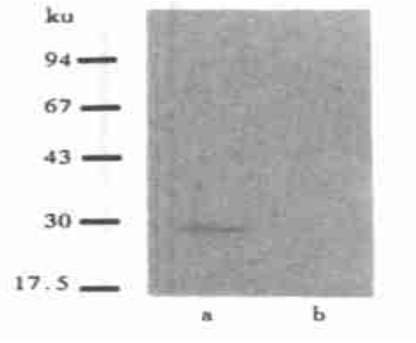

Fig. 5. Western blot analysis of soluble single-chain antibody in culture supernatants. a, Induced culture supernatant; b, non-induced culture supernatant. Standard protein marker is shown on the left.

There are several expression vectors which can express soluble single-chain antibody, for instance pHEN1 and pCANTAB 5E. Each of them has a tag peptide at the end of the soluble antibody that serves as a marker. The pHEN1 vector has c-myc tag, whereas pCANTAB 5E vector has E-tag. In this work we subcloned collagenase IV antibody gene from pHEN1 to pCANTAB 5E expression vector just for a simple reason that it was more convenient to detect E-tag in our laboratory.

The affinity of antibody hCo4 displayed was the same as that of a single-chain antibody constructed from a monoclonal antibody to collagenase IV, but slightly lower than that of the parent monoclonal antibody. There are several strategies to improve single-chain antibody affinity[9]: (i) the quality of antibody library should be improved in affinity and diversity; (ii) the V_L and V_H gene fragment can be substituted by chain shuffling; (iii) variable regions of antibody can be randomly or site-directedly mutagenized.

We described here two forms of antibodies to collagenase IV. One was phage antibody and the other was soluble form antibody. The phage antibody can be used as a research tool in the study of the relationship between collagenase IV and tumor metastasis. The soluble form antibody has a potential to be applied as a tumor drug carrier. Combining hCo4 with an anti-tumor antibiotic (C1027) (recombinant immunotoxin) is under the way.

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