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Generation of a rabbit V_H domain antibody polyspecific to c-Met and adenoviral knob protein

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Abstract

Several types of bispecific antibodies with affinity to both adenoviral coat proteins and a targeted antigen have been developed with the aim of providing the specific delivery of adenoviral gene therapy vehicle. From a phage display library of combinatorial dAb_2s (each with an anti-adenoviral knob protein V_H fragment linked with an anti-c-Met V_H), we serendipitously enriched and isolated a clone, JS5, that has polyspecificity such that it binds both the adenoviral knob protein and c-Met, despite having only one V_H domain. Our indirect observations suggest that the polyspecificity of JS5 is developed through accumulation of antibody specificity. The method of sequential immunization of a rabbit, first with the adenoviral knob protein and then with target antigens, may provide a method by which monoclonal antibodies with stand-alone polyspecificity may be developed. Such targeted polyspecific antibodies could readily be used for redirecting adenoviral vectors to target cells.

Keywords: Bispecific antibody; Domain antibody; c-Met; Adenoviral knob protein

Various forms of antibody fragments, such as Fab, $(Fab')_2$, and variable fragment (Fv) have been devised to modify the avidity, biodistribution, and pharmacokinetics of whole immunoglobulins. Among these, Fv fragments had long been thought to be the minimal functional unit of an antibody with antigen-binding capacity [1]. However, findings confirming that species of the *tylopoda* superfamily (i.e., camels, dromedaries, and llamas) produce an antibody molecule with only a homodimeric heavy chain [2], which consists of just one variable and two constant regions [3], revealed that smaller antibody molecules do indeed exist. Soon after, it was reported that cartilaginous fishes like sharks, skates, rays, and chimeras also produce a homodimeric heavy-chain called IgNAR (immunoglobulin new antigen receptor) in one of their immunoglobulin isotypes [4,5]. In an IgNAR, each heavy chain is composed of one variable and five constant regions [5]. In light of the discovery of these natural single domain antibody forms, researchers have proceeded to develop non-natural single domain antibodies (dAbs), such as human heavy chain variable domain (V_H) dAbs [6], human light chain variable domain (V_L) dAbs [7], and mouse V_H dAbs [8].

Most dAbs can be highly expressed in bacteria or yeast [9] and have favorable biophysical properties including solubility, temperature stability, and binding affinities in the

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nanomolar range [3]. Because of the above desirable features and its small size, the dAb is regarded as an ideal subunit for the generation of bispecific antibodies [10,11]. In an effort to generate a bispecific dAb_2 that is a fusion protein composed of anti-c-Met rabbit V_H and anti-adenovirus knob protein rabbit V_H connected by a seven-amino-acid linker, we generated a dAb_2 antibody library using phage display. Through alternating rounds of biopanning with c-Met and adenoviral knob protein, we obtained antibody fragments that were reactive to both antigens. Interestingly, sequence analysis revealed that one of the selected clones encoded a dAb.

In the presently described group of experiments, we over-expressed this rabbit V_H dAb, tested its binding affinity to both antigens, and further examined the localization of the binding sites for these two antigens. The biophysical characteristics of this rabbit dAb were compared to those of other natural and non-natural dAbs. A discussion of the possible mechanisms underlying the development of polyspecific antibodies and the implications of this development is included.

Materials and methods

Reagents and kits. Recombinant human c-Met/Fc chimera was purchased from R&D Systems, (Minneapolis, MN). The Expand High Fidelity PCR System and horseradish peroxidase (HRP)-conjugated anti-influenza A virus hemagglutinin (HA) antibody (3F10) were purchased from Roche (Mannheim, Germany). BCA protein assay kits and TMB solution were purchased from Pierce (Rockford, IL). NuPAGE 4–12% Bis-Tris Gels were purchased from Invitrogen (Carlsbad, CA).

Preparation of the recombinant knob protein. A gene encoding the adenoviral knob protein was amplified from a pSP72[835]CAR ablation vector [12,13] by PCR, using the sense primer 5′-GTT TTG ACC ATA TGG GTG CCA TTA CAG TAG GA-3′ and the antisense primer 5′-TCG AGC TCA AGC TTT CAT TCT TGG GCA ATG TAT GA-3′, cloned into the pRSET vector with the *Nde*I and *Hin*dIII restriction sites, and transformed into BL21(DE3) strain of *Escherichia coli*. The transformed cells were allowed to grow in LB media containing of 50 μg/ml of carbenicillin with constant shaking at 37 °C. When the optical density at 600 nm reached 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG)-was added to the culture. After being left overnight, the culture media were centrifuged at 15,000*g* for 10 min, and the resultant cell pellet was harvested and resuspended in TE buffer (20 mM Tris 1 mM EDTA, pH 8.0). Resuspended cells were sonicated for 20 min and centrifuged for 30 min at 15,000*g*. Recombinant knob protein was then purified from the supernatant using Q-Sepharose (Amersham Biosciences, Uppsala, Sweden) as described previously [14]. The purified proteins were dialyzed against phosphate-buffered saline (PBS), divided into aliquots, and stored in the freezer (−20 °C). The purity of the recombinant knob protein in the final fraction was analyzed by 4–12% SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining.

Immunization of c-Met/Fc chimera and recombinant knob protein. New Zealand White rabbits were immunized and given three booster injections with a 3-week inter-injection interval. For the immunization and each booster, 10 μg of either the mutated recombinant knob protein or the c-Met/Fc chimera was emulsified in 1 ml RIBI adjuvant (Sigma, St. Louis, MO) and injected. Before the initial immunization, before each booster and on the fifth day after the final booster, sera were collected from the rabbits and used to monitor the titers to antigens in an enzyme immunoassay. Recombinant knob protein and c-Met/Fc chimera were dissolved in 50 mM sodium carbonate buffer, pH 9.6 (coating buffer), at a concentration of 20 μg/ml and added to the wells of a microtiter plate. After incubation overnight at 4 °C, the plate was washed briefly with PBS. Then 3% bovine serum albumin (BSA) in PBS (PBS-B) was added to the plate to block any remaining binding sites. The polysera were diluted with PBS-B (1:5,000) and added to the individual wells of the plate. The plate was incubated for 2 h at 37 °C and washed with PBS containing 0.05% Tween 20 (PBS-T). The amount of antibody bound to the plate was detected by application of HRP-conjugated anti-rabbit IgG antibody (Pierce). After adding TMB solution, the plate was incubated for 30 min at 37 °C and the optical density was measured at 650 nm by a microtiter plate reader (Labsystems, Barcelona, Spain). Five days after the final boost, spleen and bone marrow specimens were harvested and total RNA was isolated using TRI-reagent (Molecular Research Center, Cincinnati, OH).

Construction of bispecific dAb_2 (V_H-linker-V_H) library. From total RNA, cDNA was synthesized using an oligo(dT) primer and a SUPERSCRIPT II reverse transcriptase kit (Invitrogen). The anti-c-Met rabbit V_H region was amplified while introducing an *sfi*I restriction site at the 5′ end and a linker sequence at the 3′ end using four sense primers (5′-GGG CCC AGG CGG CCC AGT CGG TGG AGG AGT CCR GG-3′, 5′-GGG CCC AGG CGG CCC AGT CGG TGA AGG AGT CCG AG-3′, 5′-GGG CCC AGG CGG CCC AGT CGY TGG AGG AGT CCG GG-3′, 5′-GGG CCC AGG CGG CCC AGS AGC AGC TGR TGG AGT CCG G-3′) and one antisense primer (5′-GGA AGA TCT AGA GGA ACC ACC ACT AGT GAC TGA YGG AGC CTT AGG TTG CCC-3′). The anti-adenoviral knob protein rabbit V_H region was amplified while introducing a linker sequence at the 5′ end and an *sfi*I *site* at the 3′ end using four sense primers (5′-GGT GGT TCC TCT AGA TCT TCC CAG TCG GTG GAG GAG TCC RGG-3′, 5′-GGT GGT TCC TCT AGA TCT TCC CAG TCG GTG AAG GAG TCC GAG-3′, 5′-GGT GGT TCC TCT AGA TCT TCC CAG TCG YTG GAG GAG TCC GGG-3′, 5′-GGT GGT TCC TCT AGA TCT TCC CAG SAG CAG CTG RTG GAG TCC GG-3′) and one antisense primer (5′-CCT GGC CGG CCT GGC CAC TAG TGA CTG AYG GAG CCT TAG GTT GCC C-3′). And these two libraries of V_H genes were combined by an overlap extension PCR with sense primer 5′-GAG GAG GAG GAG GAG GAG GCG GGG CCC AGG CGG CCG AGC TC-3′ and antisense primer 5′-GAG GAG GAG GAG GAG GAG CCT GGC CGG CCT GGC CAC TAG TG-3′. The purified PCR products were digested with *sfi*I (Roche), cloned into pComb3X, and transformed into ER2738 *E. coli* as described previously [15]. The library of phages displaying dAb_2s was prepared by infection of *E. coli* with the VCSM13 helper phage [15].

Selection of bispecific antibody by bead panning. Recombinant knob protein and c-Met/Fc chimera were conjugated to magnetic beads (Dynabeads M-270 Epoxy, Dynal, Oslo, Norway) according to the manufacturer's instructions. The conjugated magnetic beads were incubated with PBS-B overnight at 4 °C and washed with PBS. Then the phages expressing dAb_2s were re-suspended in 1 ml PBS-B and added to the antigen-coated magnetic beads. The beads were incubated for 2 h at room temperature and washed with PBS-T. The bound phages were eluted by incubation for 10 min with 0.1 M glycine (pH 2.2). After neutralization with 2 M Tris base, the eluted phages were infected into ER2738 *E. coli*. Then the phages were rescued for the next round of biopanning by adding VCSM13 helper phages as described previously [15]. A total of seven rounds of panning were performed on antigens on an alternating basis. That is, the first, third, fifth, and seventh rounds of biopanning were performed on the recombinant knob protein; whereas in the second, fourth, and sixth rounds of biopaning, the c-Met/Fc chimera was used for selection. The input and output phage titers in each round were monitored as described previously [15].

Selection of clones reactive to both antigens by a phage enzyme immunoassay. To select antigen binders, an enzyme immunoassay was performed using the recombinant knob protein or c-Met/Fc chimera as described previously [15]. Briefly, phages were rescued from individual carbenicillin-resistant colonies of the output titer plate of the last round of biopanning. A microtiter plate was incubated with 20 μg/ml of recombinant knob protein, c-Met/Fc chimera or human immunoglobulin in coating buffer overnight at 4 °C. After blocking with PBS-B, the culture supernatant containing phages of individual clones was mixed with an equal volume of PBS-B and applied to each well. The plate was

incubated for 1 h at 37 °C and washed with PBS-T. Bound phages were detected with HRP-conjugated anti-M13 phage antibody (Amersham Biosciences). HRP-labeled phages were then allowed to react with TMB solution for 30 min at 37 °C. The resultant optical density was measured at 650 nm. The nucleotide sequence of the insert in positive clones was determined using the following primers: 5′-AAG ACA GCT ATC GCG ATT GCA G-3′ and 5′-CCG GAA CGT CGT ACG GGT ATG CGC C-3′.

Over-expression and purification of JS5 dAb. The phagemid DNA of the JS5 dAb was transformed into HB2151 *E. coli*. The transformed cells were allowed to grow in LB media containing of 50 μg/ml of carbenicillin with constant shaking at 37 °C. When the optical density at 600 nm reached 0.6, 1 mM IPTG was added. After being left in the presence of IPTG overnight, the culture media were centrifuged at 15,000*g* for 30 min. The resultant cell pellet was harvested and resuspended in 50 mM sodium borate (pH 8.2) containing 200 μg/ml lysozyme and 200 μM PMSF. After incubation for 20 min at 37 °C, the suspension was centrifuged at 15,000*g* for 30 min. Then dAb in the supernatant was purified using a protein A column cross-linked with anti-HA antibody. Briefly after the column was equilibrated with 3 void volumes of 50 mM sodium borate (pH 8.2), the dAb-containing supernatant was applied to the column. After extensive washing with 50 mM sodium borate(pH 8.2), the bound dAb was eluted in 0.1 M glycine (pH 2.8). After neutralization with 2 M Tris base, the protein concentration was determined with a BCA protein assay kit according to the manufacturer's instructions. The purity was determined by 4–12% SDS–PAGE and Coomassie blue staining.

Western blot analysis to test the bispecificity of JS5 dAb. Recombinant knob protein and c-Met/Fc chimera were separated by 4–12% SDS–PAGE and electro-transferred onto a nitrocellulose membrane. Then the membrane was blocked with 5% skim milk in Tris-buffered saline (TBS) containing 0.2% Tween 20 (TBS-T) at room temperature for 30 min, incubated with JS5 dAb for 2 h, washed with TBS-T, and incubated with HRP-conjugated monoclonal anti-HA antibody for 1 h. After washing with TBS-T, peroxidase activity was detected using ECL supersignal chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

Dose-dependent binding of purified JS5 dAb to adenoviral knob protein, adenovirus, and c-Met in an enzyme immunoassay. Recombinant knob protein and c-Met/Fc chimera were coated onto separate microtiter plate wells and any remaining binding sites were blocked as described above. To attach the adenovirus onto the plates, each microtiter plate well was incubated with 400 ng of a monoclonal anti-adenovirus antibody (Chemicon, Temecula, CA), blocked with PBS-B, and incubated with 2×10^6 PFU of adenovirus in PBS-B. Then JS5 dAb in PBS-B was added into the wells at varying final concentrations between 46 nM and 5.88 μM. After incubation for 1 h at 37 °C, the wells were washed with PBS-T. The bound antibodies were detected by HRP-conjugated anti-HA antibody and reaction with TMB substrate. After incubation at 37 °C for 30 min, the absorbance of each well at 650 nm was determined.

A competition enzyme immunoassay to test for competitive binding of recombinant knob protein and c-Met on JS5 dAb. A plate was coated with c-Met/Fc chimera and blocked as described above. Then 10 nM JS5 dAb was added either alone or in combination with 100 nM mutated knob protein or 100 nM c-Met/Fc chimera and incubated for 30 min at 37 °C. After the plate was washed with PBS-T, the bound dAbs were detected by incubation with HRP-conjugated anti-HA antibody as described above.

Generation of anti-cMet single-chain variable fragment library, selection of binders to c-Met/Fc chimera, and enzyme immunoassay to examine polyspecificity. Using the cDNA samples prepared from the rabbits immunized with c-Met/Fc chimera, a single-chain variable fragment (scFv) library was generated as described previously [15]. Biopanning was performed using a microtiter plate coated with c-Met/Fc chimera. After the fifth round of biopanning, a phage enzyme immunoassay was performed to select binders to c-Met. The reactivity of c-Met binders to adenoviral knob protein was also tested by conducting a phage enzyme immunoassay with a plate that was coated with recombinant knob protein.

Results

Construction of bispecific dAb_2 (V_H-linker-V_H) library

The gene encoding the knob domain of the adenovirus fiber protein was amplified from an adenoviral vector by PCR, cloned into a pRSET vector, and transfected into BL21[DE3] *E. coli* cells. The recombinant knob protein was detected as a 22 kDa polypeptide (Fig. 1). Two rabbits were immunized with the recombinant knob protein and another two rabbits were immunized with c-Met/Fc chimera. Enzyme immunoassays of rabbit sera collected throughout the immunization courses revealed that all rabbits had elevated antibody titers to the antigen to which they were immunized (Fig. 2). The elevated background in c-Met/Fc

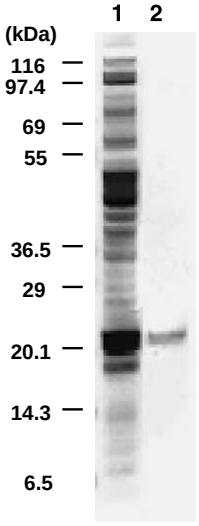

Fig. 1. Purification of recombinant knob protein. In SDS–PAGE, lanes 1 and 2 were loaded with crude cell lysate and the final purified fraction, respectively. The protein was visualized with Coomassie blue dye. Note that only recombinant knob protein band was visible in the final purified fraction.

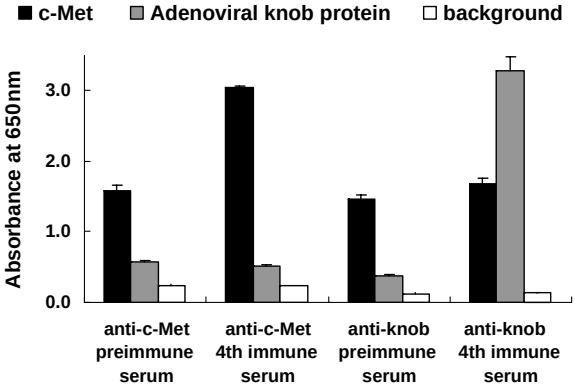

Fig. 2. Reactivity of rabbit antiserum against c-Met and adenoviral knob protein. Polysera (1:5000 in PBS-B) were added to microtiter plate wells coated with c-Met/Fc chimera or recombinant knob protein. The amount of bound antibody was determined by incubation with anti-rabbit IgG antibody conjugated with HRP. Each experiment was performed in triplicate and the mean values with standard deviations are presented.

chimera coated wells was due to cross-reactivity of HRP-conjugated anti-rabbit IgG antibody to its Fc portion of the chimera (data not shown).

After the fifth booster injection, total RNA was extracted from spleen and bone marrow of the immunized rabbits and subjected to cDNA synthesis. Genes encoding the V_H region of the antibody were amplified from both sets of prepared cDNA by using a set of degenerate primers. To construct a library of genes encoding dAb_2, two libraries of the V_H gene were connected to each other by linker PCR with a short linker sequence encoding GGSSRSS. The *sfi*I restriction sites were also introduced at both 5′- and 3′-ends of the dAb_2 gene. The libraries of genes encoding dAb_2s were cloned into phagemid pComb3X vectors [16,17], using two asymmetric *sfi*I sites and subsequently transformed into *E. coli.* (ER2738 strain). This procedure yielded a dAb_2 library with a complexity of 8.6×10^9. Plasmid DNA samples of eight clones randomly selected from the library were prepared and used to determine the nucleotide sequences of inserted DNA. Six clones encoded dAb_2 genes and their sequences were heterologous, confirming the complexity of the dAb_2 library (data not shown).

Selection of dAb bispecific to c-Met and recombinant knob protein

From the output titer plate of the last round of biopanning, eight clones were randomly selected, rescued by infection of helper phage, and tested for their reactivity to both antigens in phage enzyme immunoassay using a plate coated with c-Met/Fc chimera or recombinant knob protein and HRP-conjugate anti-M13 antibody (Figs. 3A and B). Five of the eight selected clones showed selective reactivity to recombinant knob protein, whereas three clones showed bispecificity to both antigens. Because the c-Met/Fc chimera was used for immunization and selection of binders, the reactivity of clones to the Fc region was also checked using a plate coated with human immunoglobulin. As none of the clones reacted with human immunoglobulin, their reactivity to c-Met was confirmed to be specific (Fig. 3A). Plasmid DNA samples of the three clones that showed bispecificity were prepared and used to determine the nucleotide sequence of the gene encoding the antibody fragments. Interestingly, two clones had only one V_H domain with exactly the same sequence, which was later named JS5 (Fig. 4). The third bispecific clone encoded an Fd gene that was comprised of a JS5 V_H domain and a human C_{H1} domain, which might have been incorporated due to contamination during the PCR procedure.

Over-expression, purification, and characterization of dAb JS5

Following over-expression in *E. coli* and purification by anti-HA affinity column chromatography, a 5 mg sample of JS5 dAb was finally obtained from 1 L of a shaking cul-

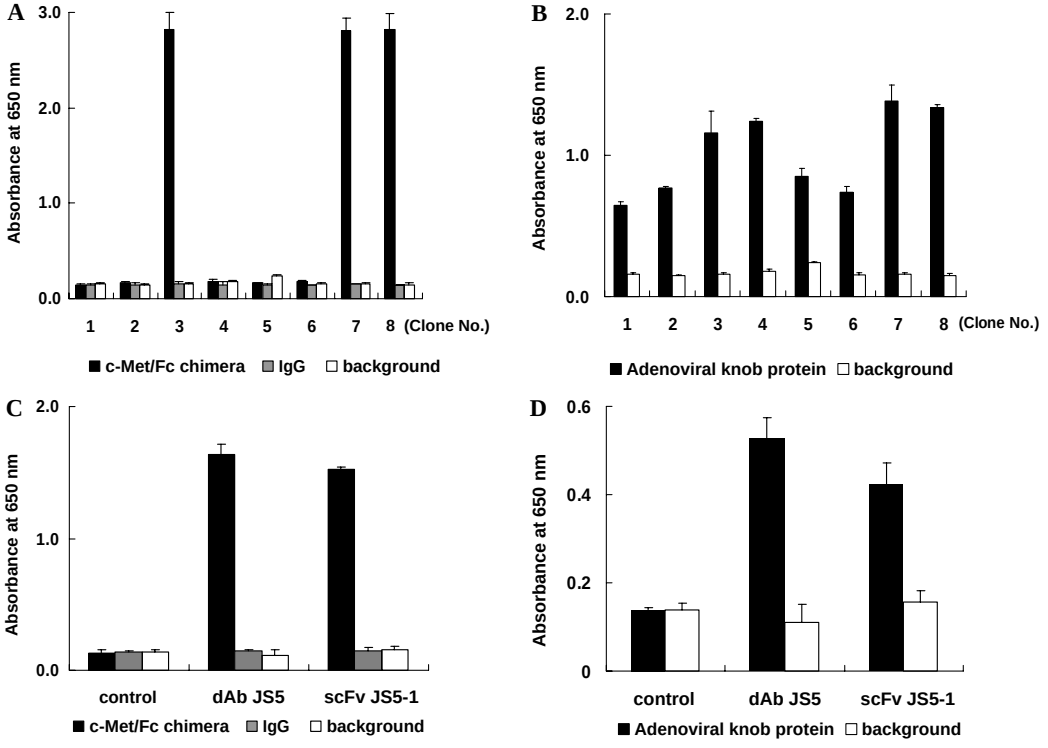

Fig. 3. Selection of dAb and scFv clones bispecific to c-Met and adenoviral knob protein. The reactivity of eight dAb clones retrieved following the seventh round of biopanning to c-Met (A) and recombinant knob protein (B) was investigated by an enzyme immunoassay using a plate coated with each antigen and HRP-conjugated anti-M13 antibody. As the c-Met/Fc chimera was used as an immunogen, the reactivity of clones to human IgG was also tested (A). The polyspecificity of JS5 dAb and JS5-1 scFv to c-Met (C) and adenoviral knob protein (D) was confirmed by parallel phage enzyme immunoassays. VCSM13 helpher phages were used as a negative control. Each experiment was performed in triplicate. The mean values are shown with standard deviations.

V_H sequence

```
                  FR1              CDR1       FR2            CDR2                    FR3                   CDR3          FR4

JS5 dAb     QSLEESGGDLVQPEGSLTLTCKASGFDFS SHWIY WVRQAPGKGLEWIG TIYTGNGNTYYATWAKG RSTISRTSSTTVTLQMTSLTAADTATYFCAR DLGGSSSTSYISDL WGPG TRVTVSS
JS5-1 scFv  QSVEESGGGLVQPEGSLTLTCKASGFDFS SHWIY WVRQAPGKGLEWIG TIYTGSDSTYYATWAKG RETISKTSSTTVTLQMTSLTVADTATYFCAR DLGGSSSTSYISDL WGPGTLVTVSS
JS5-2 scFv  QSLVESGGDLVQPEGSLTLTCTASGFDFS SHWIY WVRQAPGKGLEWIG TIYTGSDSTYYATWAKG RETISKTSSTTVTLQMTSLTVADTATYFCAR DLGGSSSTSYISDL WGPGTRVTVSS
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V_L sequence

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                  FR1              CDR1          FR2           CDR2                    FR3                   CDR3         FR4

JS5-1 scFv  ELVLTQSPSASAALGASAKLTC  TLSSAHKTYSIA  WYQQQQGEAPRFLMQ  LKSDGSYTKGT  GVPDRFSGSSSGADRYLFIASVQPADEADYYC  STDYATGYY  VFGGGTQLTVT
JS5-2 scFv  ELVLTQSPSASAALGASAKLTC  TLSSAHKTYSIA  WYQQQQGEAPRYLMQ  LKSDGSYTKGT  GVPDRFSGSSSGADRYLFIASVQAADEADYYC  STDYATGYY  VFGGGTQLTVT
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Fig. 4. The amino acid sequence of JS5 dAb and JS5-1 and JS5-2 scFv.

ture. Its purity was confirmed by SDS–PAGE and Coomassie blue staining (Fig. 5A). Under non-reducing conditions, the dAb presented a molecular size of 17 kDa, as calculated from the gene sequence. Importantly, in the final purified fraction, only the JS5 dAb band was present.

Western blot analysis using the purified dAb demonstrated that the purified JS5 dAb successfully reacted to recombinant knob protein and c-Met/Fc chimera (Fig. 5B). Furthermore, enzyme immunoassay experiments revealed that the JS5 dAb bound to recombinant knob protein, adenovirus, and c-Met dose-dependently in the range from 46 nM to 5.88 μM (Fig. 5C). These findings provided clear evidence that the JS5 dAb has polyspecificity.

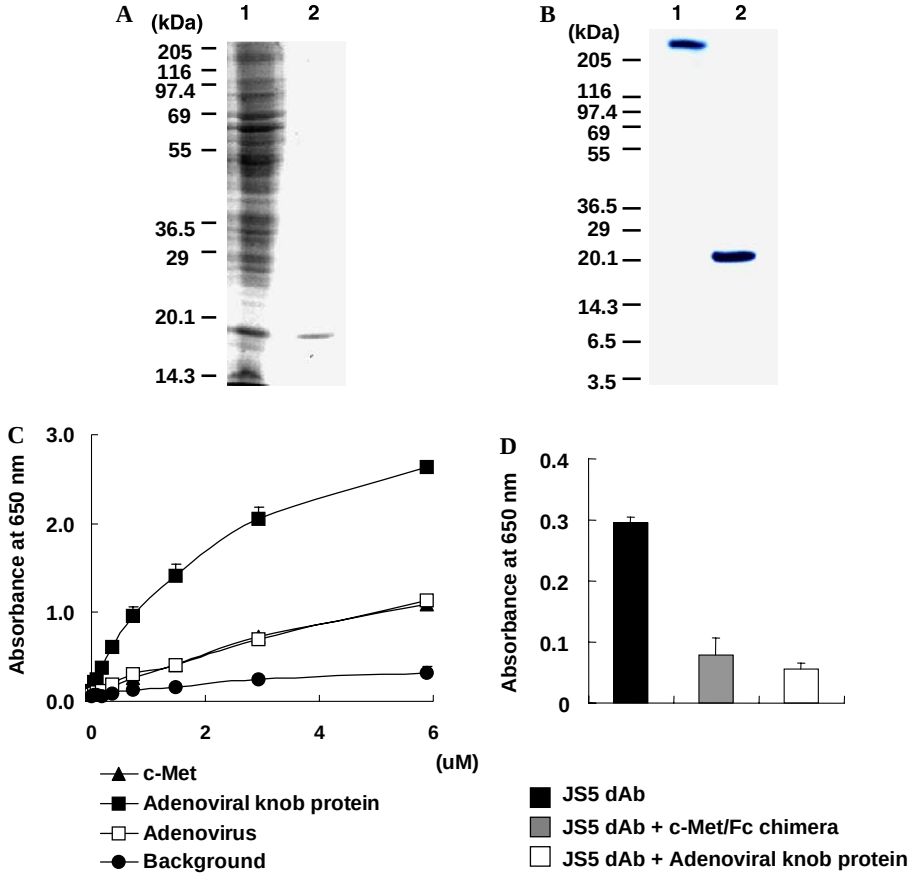

Fig. 5. Bispecific binding of JS5 Ab to c-Met and adenoviral knob protein. (A) The purity of the final JS5 dAb product was confirmed by SDS–PAGE and Coomassie blue staining. Lanes 1 and 2 were loaded with crude cell lysate and the final purified fraction, respectively. Only the JS5 dAb band was visible in the final purified fraction. (B) Reactivity of JS5 dAb to c-Met and adenoviral knob protein was confirmed by Western blot analysis. The c-Met/Fc chimera and recombinant knob protein were loaded into lanes 1 and 2 of 4–12% SDS–PAGE, respectively. Proteins transferred to nitrocellulose membranes were probed with JS5 dAb and bound dAb was visualized by HRP-conjugated anti-HA antibody and an electrochemiluminescent kit. (C) An enzyme immunoassay using a plate coated with the antigens and HRP-conjugated anti-HA antibody revealed that JS5 dAb bound to c-Met, adenoviral knob protein, and adenovirus in a dose dependent manner. (D) Competition enzyme immunoassay in microtiter plate wells coated with c-Met/Fc chimera revealed that binding of JS5 dAb to the chimera protein was inhibited in the presence of soluble c-Met/Fc chimera or adenoviral knob protein ($P < 0.05$). All experiments in (C,D) were performed in triplicate. Mean values are shown with standard deviations.

Competition enzyme immunoassays performed in triplicate with c-Met/Fc chimera-coated wells revealed that inclusion of recombinant knob protein (100 nM) inhibited binding of JS5 (10 nM) to c-Met ($p < 0.05$). Soluble c-Met/Fc chimera was used as a positive control for the competitor. This inhibition of binding indicated that the binding sites of c-Met and adenoviral knob protein are co-localized on JS5 dAb (Fig. 5D).

Selection of scFvs bispecific to c-Met and recombinant knob protein

To determine whether scFvs generated from rabbits immunized with c-Met/Fc chimera had polyspecificity, biopanning and phage enzyme immunoassay were performed. Of the eight clones randomly selected from the output plate of the fifth round of biopanning, two showed polyspecificity (Figs. 3C and D). Plasmid DNA samples of these two clones were prepared and used for determination of the nucleotide sequence of the gene encoding the antibody fragments. Both polyspecific clones encoded a V_H gene that was nearly identical to that of the JS5 dAb (Fig. 4).

Discussion

In an effort to generate bispecific antibodies that can be used to re-direct adenoviral vectors to target cells, we set out to produce a dAb_2 consisting of two rabbit V_H domains connected by a flexible linker. This idea was developed from our previous observations. First, we had serendipitously obtained an anti-adenoviral knob protein rabbit V_H dAb from a phage display of a combinatorial scFv library. As this single domain antibody was highly over-expressed in *E. coli*, very stable, and of an affinity comparable to those of other scFvs, the rabbit dAb was expected to share characteristics common to those of other natural and non-natural single domain antibodies [9,10].

We noticed that the affinities of numerous scFv or Fab clones selected from the phage display of the combinatorial antibody library that shared the same V_H genes, but had different sets of light chains, were nearly equivalent to one another [18]. In addition, we frequently observed that recombinant Fab or scFv clones that shared the same light chain had distinct antigen specificities in the nanomolar range (data not shown). These observations provided compelling evidence that the specificity and affinity of recombinant antibodies are much more dependent upon the V_H than the V_L. Therefore, we hypothesized that dAb_2s would have the ability to concurrently bind two different antigens and have benefits in overproduction afforded by their small size compared to other bispecific antibody forms.

From the phage display of the combinatorial dAb_2 library, we enriched two phage clones that encoded the JS5 dAb (Fig. 4), and one clone that encoded the JS5 V_H connected with a human C_{H1} region (JS5 Fd). All three of these showed polyspecificity in phage enzyme immunoassay (Figs. 3A and B). It seemed that one clone encoding the JS5 Fd had been contaminated from a rabbit/human chimeric Fab library that was concomitantly constructed using the cDNA prepared from a rabbit immunized with c-Met. Clones encoding a dAb_2 with polyspecificity were not enriched by biopannning. However, six out of the eight clones selected from the constructed dAb_2 library contained a dAb_2 gene. Currently, we do not know the reason for the failures. It may be that the structures of those dAb_2s were not suitable for binding to both antigens.

Hints of the mechanism mediating the development of polyspecificity on a single V_H were provided when we made the surprising observation that the sera obtained from rabbits before and after immunization with c-Met/Fc chimera showed somewhat elevated antibody titers to recombinant knob protein (Fig. 2). This observation led us to investigate the presence of the JS5 V_H gene in the c-Met binders selected from the scFv antibody library generated from rabbits immunized with c-Met/Fc chimera. Interestingly, two scFv clones encoded V_Hs that were nearly identical to the JS5 V_H (Fig. 4), and these clones were polyspecific to both c-Met and recombinant knob protein (Figs. 3C and D). As the antibody titer to c-Met/Fc chimera was not elevated after immunization of recombinant knob protein and the titer to recombinant knob protein was not elevated after immunization of c-Met/Fc chimera, it was clear that these two proteins do not have cross-reacting epitopes. Consistent with this conclusion were the observations that any clone selected by biopanning with recombinant knob protein from the scFv library generated from rabbits immunized with recombinant knob protein did not show affinity to c-Met/Fc chimera and did not have the JS5 sequence (data not shown). These findings strongly suggest that the JS5 dAb originated from rabbits immunized with c-Met/Fc chimera. It is possible that the rabbits immunized with c-Met/Fc chimera had been previously infected with wild type adenovirus and that the B cell encoding JS5 dAb had originated from B cells that had previously been reactive to adenoviral knob protein.

It was recently proven that the most broadly neutralizing anti-HIV human monoclonal antibodies, 2F5 and 4E10, are polyspecific auto-antibodies that are also reactive with phospholipid cardiolipin [19]. Auto-antibodies reactive to cardiolipin are commonly found in patients with autoimmune diseases, such as systemic lupus erythematosus (SLE) [20,21], and are reported to be induced by syphilis, leprosy, leishmaniasis, Epstein–Barr virus, and HIV-1 [22–25]. There are two lines of indirect evidence suggesting that these anti-HIV antibodies are developed from B cells that originally expressed antibodies to cardiolipin. First, as autoreactive B cell clones that are reactive to cardiolipin are normally deleted from the repertoire or made tolerant [26], neutralizing antibodies against 2F5 and 4E10 epitopes are rarely made in HIV-1-infected humans [27] or in immunization settings [28]. Second, HIV-1 infection rarely occurs in SLE patients, who may be unable to delete these self-reactive clones [21,29]. And interestingly, just like the JS5 dAb, the polyspecific binding abilities of 2F5 and

4E10 are very dependent upon the V_H domain. V_H domain-dependence was evidenced by findings demonstrating that the HCDR3 region, which is quite long and contains many hydrophobic residues, is essential for 2F5 and 4E10 polyspecificity.

It is not yet known how common the occurrence of cumulative development of specificity resulting in polyspecific antibodies is in the generation of antigen-reactive B cells following immunization. But our routine preference for the use of specific pathogen-free (SPF) animals in antibody production might be re-considered in light of these new observations. The life-long diversification and accumulation of the B cell repertoire driven by infectious agents might actually increase the chance that animals may develop antibodies to certain antigens, although the polyspecificity of the antibody clones must be carefully monitored. The generation of polyspecific antibodies by the sequential immunization of antigens, for example immunizing first against adenoviral knob protein and subsequently against a mixture of cancer cell membranes, might enable monoclonal antibodies with stand-alone bispecificity to be developed. Such bispecific antibodies could be used for re-directing adenoviral vectors to cancer cells and thereby represent a method for targeted anti-cancer therapies.

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