Isolation and Characterization of Human scFv Molecules Specific for Recombinant Human Heat Shock Protein (HSP) 70.1

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ABSTRACT

Background: The heat shock proteins (HSPs) play an important role in cellular protection mechanisms against physical or chemical stresses. In this study scFv antibodies specific for human HSP70.1 were isolated from a semi-synthetic human scFv library with the ultimate goal of developing anti-HSP70.1 intracellular antibody (intrabody) that may offer an attractive alternative to gene targeting to study the function of the protein in cells. **Methods:** A semi-synthetic human scFv display library (5×10^8 size) was constructed using pCANTAB-5E vector and the selection of the library against bacterially expressed recombinant human HSP70.1 was attempted by panning. **Results:** Three positive clones specific for recombinant HSP70.1 were identified. All three clones used V_H subgroup III. On the other hand, V_L of two clones belonged to the kappa light chain subgroup I, but the other utilized V_k subgroup IV Interestingly, these scFv molecules specifically reacted to the recombinant HSP70.1, yet failed to recognize native HSP70 induced in U937 human monocytic cells by heat treatment. **Conclusion:** Our results indicated that affinity selection of an scFv phage display library using recombinant antigens produced in *E. coli* might not guarantee the isolation of scFv antibody molecules specific for a native form of the antigen. Therefore, the source of target antigens needs to be chosen carefully in order to isolate biofunctional antibody molecules. **(Immune Network 2004;4(1):7-15)**

Key Words: Phage display library, scFv, heat shock protein 70, pCANTAB-5E, intrabody

Abbreviation: heat shock protein (HSP), monoclonal antibody (mAb), single chain variable fragment (scFv), 2,2'-azino-bis (3-ethylbenthiazoline-6-sulfonic acid) (ABTS)

Introduction

A large number of rodent mAbs with specificity for antigens of therapeutic interest have been generated and characterized since the development of the hybridoma approach. The fact that rodent antibodies are highly immunogenic in humans, however, severely limits their clinical applications, especially when repeated administration is required for therapy (1). As a means of circumventing this limitation, several strategies have been developed to convert rodent Ab sequences into human Ab sequences, a process termed Ab humanization. Antibody engineering has been taken to overcome the immunogenicity of mAbs of rodent origin, either the humanization of the mAbs or the direct generation of human mAbs (2). The latter route has recently gained importance with the development of a new methodology, so called combinatorial antibody library or antibody phage display library, that allows the selection of human mAbs from immune, naive, or synthetic human

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antibody repertoires displayed on a phage surface (3-5). It has now been widely accepted that phage display library is a valuable tool not only for the generation of human antibodies but also for the development of diverse intracellular antibodies or intrabodies.

Intracellular expression of antibodies in mammalian cells can be utilized to interfere in the *in vivo* function of target molecules in the modulation of cellular growth or metabolism, thereby serving as an effective alternative to gene-targeting or gene knockout (6). For example, it had been documented that expression of growth factor receptor such as IL-2Rα receptor (7) or epidermal growth factor receptor (EGFR) (8,9) could be down-regulated with intrabodies by inhibiting the transport of these receptor molecules on the plasma membrane demonstrating the potential of intrabodies as reagents for cancer therapy. In addition, inactivation of $p21^{ras}$ oncoprotein (10) and inhibition of human immunodeficiency virus (HIV-1) replication (11-13) by intrabodies had also been reported. Two requirements for intrabodies to be functional in the cells are antigen- binding specificity and appropriate folding under intracellular environment. Expression of functional intrabody is extremely difficult since the intrachain disulfide bond does not form in the cytoplasm of eukaryotic cells (14). However, scFv in which heavy and light variable domains are jointed by a flexible linker peptide as a single polypeptide is less dependent upon intrachain disulfide bond for antigen binding, and this structural feature of scFv makes a more appropriate form for intrabody compared with Fab or full antibody.

The heat shock proteins (HSPs) are important in the cellular response to stress and in cellular homeostatic functions such as protein synthesis and protein transport across membranes (15), and play a role in cellular protection mechanisms against physical or chemical stresses (16). Among them the 70 kDa heat shock proteins (HSP70) are found in bacteria and higher organisms, and their expression can be modulated by heat shock, heavy metals, metabolic inhibitors, amino acid analogs, ethanol, antibiotics, or radiation (17). In mice, there are at least seven members of HSP70 functioning as chaperons that assist protein folding and transportation in the cytoplasm, endoplasmic reticulum and mitochondria (18). Heat shock cognate protein 70 (HSC70), glucose-related protein 75 (GRP75) and 78 (GRP78) are constitutively expressed in cells, and HSP70s, HSP70.1 and HSP70.3, are induced by environmental or physiological stresses (19). Two other members, HSP70.2 and HSC70t, are expressed in tissue-specific manner (20, 21).

Although the mechanisms by which HSP70 protect cells are not fully realized, the role of HSP70.1 in *in vivo* protection mechanisms was partially understood by hsp70.1 (-/-) KO mice (22), and early protection of the brain by HSP70.1, at least after acute focal cerebral ischemia, was also observed in mice (23). Another study using mice with targeted gene disruption of the hsp70.1 or hsp70.3 gene showed that there were no obvious morphological abnormalities in both knockout mice, but inactivation of hsp70.1 or hsp70.3 resulted in deficient maintenance of acquired thermotolerance and increased sensitivity to heat-stress-induced apoptosis at the cellular level (24). Recently it had been shown that the hsp70.1 mRNA was differentially expressed due to inability of HSF1 transcription factors to bind to the hsp70.1 promoter in human hematopoietic cells, suggesting the distinct contribution of Hsp70-encoding genes against heat shock responses in cell-type-dependent manners (25).

Since intrabody can be utilized to study the function of a certain protein in different cell types, we attempted to isolate scFv antibody molecules specific for human HSP70.1 from a semi-synthetic scFv phage display library.

Materials and Methods

First strand cDNA synthesis. Peripheral blood lymphocytes were obtained from 40 healthy volunteers using histopaque (Sigma Co., USA). Briefly, the blood was layered onto equal volume of histopaque and white blood cells were obtained by centrifugation for 20 min at 800 g. Total RNA isolated from 2×10^7 cells using RNeasy total RNA isolation kit (Qiagen, Germany) according to the manufacturer's protocol. First strand cDNA was synthesized from the 5μg of total RNA using oligo dT primer with the 1st strand cDNA synthesis kit (Roche Biochemicals, Germany).

Preparation of lambda DNA. The human bone marrow 5'-STRETCH PLUS cDNA library and human fatal liver 5'-STRETCH PLUS cDNA library (Clonetech, USA) were amplified by mixing the λ phages from libraries with XL-1 Blue MRF' cells (Stratagene, USA) in 10 mM $MgSO_4$ at $O.D._{600}$=1, and incubated at 37°C for 15 min. Eight ml of top agar was added into the each tube of the mixture and poured onto LB plate (150×15 mm). The LB plates were incubated at 37°C overnight and 10 ml of SM buffer was added into each plate to release the λ phages for 4 h at room temperature. Agar debris was removed from the λ phage suspension by brief centrifugation and the λ DNA was purified with the λ DNA purification kit (Qiagen) according to the manufacturer's protocol.

PCR amplification of V_H and V_L. cDNA coding for variable regions of Ig heavy chain (V_H) and light chain

(V_L kappa) were obtained by PCR using the first strand cDNA and λ DNA as PCR templates. PCR primers specific for human Ig genes (26) with slight modifications except for the random sequences varying in length from 4 to 12 residues at the V_H-CDR3 in the anti-sense primer were synthesized (Takara, Japan) (5'-TGA RGA GAC GGT GAC CRK KGT BCC TTG GCC CCA $(SNN)_{4\text{-}12}$ TCT CGC ACA ATA ATA CAC-3') and used to amplify V_H and V_L genes. Ex-*Taq* polymerase (Takara) was successfully used for PCR amplifications in this study. The resulting PCR fragments were purified with 1% low melting agarose gel (Biorad, USA).

Assembly PCR. Linker fragment [$(GGGGS)_3$] that joins V_H and V_L domains was obtained from pHEN1 (kindly provided by Dr. Greg Winter) by PCR amplification using Ex-*Taq* polymerase (Takara). PCR primers (sense: 5'-GRA CMM YGG TCA CCG TCT CYT CAG GTG G-3' and anti-sense: 5'-GGA GAC TGN GTC AWC WSR AYD TCC GAT CCG CC-3') (R=G or A, M=A or C, Y=C or T, N=A, G, C or T, W=A or T, S=G or C) were synthesized (Takara), and PCR was performed by 25 cycles of 1 min at 94°C, 2 min at 60°C, 2 min at 72°C, followed by 1 cycle of 10 min elongation step at 72°C. The resulting PCR product was purified using 2% low melting agarose gel (Biorad). For assembly PCR, 60 ng of V_H genes, 60 ng of V_L kappa genes and 20 ng of linker fragments were mixed together and PCR amplification was performed using pull-through primers (Table I) by 30 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 72°C, followed by 1 cycle of 10 min elongation step at 72°C. The resulting scFv fragments were purified with 1% low melting agarose gel (Biorad).

Cloning of scFv genes into pCANTAB-5E and phage rescue. ScFv genes were treated with *Sfi* I and Not I (Takara) and ligated with pCANTAB-5E phagemid vector recombinant phage were obtained according to manufacturer's protocols provided in Recombinant Phage Antibody System kit (Amersham Pharmacia Biotech, Sweden). The electroporated *E. coli* TG1 cells were resuspended in 15% glycerol and stored at -70°C.

Table I. Yield after 4 panning

	Input	Output	% yield
1st panning	4.6×10^{13}	2.5×10^{6}	5.0×10^{-6}
2nd panning	4.6×10^{12}	5.0×10^{6}	1.3×10^{-4}
3rd panning	1.0×10^{13}	1.2×10^{6}	12×10^{-5}
4th panning	1.2×10^{13}	1.8×10^{8}	1.5×10^{-3}

(% yield=output phage/input phage×100)

Preparation of full-length and truncated HSP70.1 protein using E. coli expression system. The HSP70.1 ΔN (N-terminal 120 amino acid deleted mutant) was constructed as described (22). The 1.5 kb of Nco I/*Bam* HI digested HSP70.1 ΔN insert was subcloned into pET28 expression vector and the ΔN mutant protein was expressed in *E. coli* BL21(DE3). Then, the recombinant protein was produced by growing cells in liquid culture for 4 h at 37°C in the presence of 1 mM isopropyl-β- D-thiogalactoside (IPTG) (USB, USA). For the preparation of full-length HSP70.1, BL21 (DE3) cells containing human hsp-70.1 cDNA in pET expression vector were grown until log phase, and expression of HSP-70.1 was induced as above, and the protein was purified using ProBond™ Resin (Invitrogen, USA).

Panning procedure. Affinity selection of antigen-binding recombinant phages was performed by panning as described (27). A microtiter plate was coated with 100μg/ ml of human recombinant HSP-70.1 in coating buffer (0.1 M $NaHCO_3$, pH 9.6) overnight at 4°C, and blocked with 3% bovine serum albumin (dBSA) (Sigma Co., USA) for 1 h at 37°C. Total 10^{11} recombinant phage were added, and incubated for 2 h at room temperature. The microtiter plate was washed with PBS containing 0.1% tween-20 (PBS-tween) five times with vigorous pipetting. Bound phages were eluted with elution buffer (0.1 M glycine containing 0.1% BSA, pH 2.2), and neutralized with 1 M Tris-HCl (pH 7.4). The eluted phages were amplified by infecting freshly grown TG1 cells in 2× YT media containing 100μg/ml ampicillin (Amp) followed by M13KO7 helper phage superinfection (Stratagene, USA). After overnight incubation at 30°C, recombinant phage particles were obtained by centrifugation (3,000×*g*, 15 min) followed by PEG/ NaCl precipitation, and resuspended in phosphate buffered saline (PBS). Panning was repeated 4 times.

Phage ELISA. A microtiter plate was coated with 10 μg/ ml of purified recombinant HSP70.1 or BSA as a negative control in coating buffer (0.1 M $NaHCO_3$, pH 9.6) overnight at 4°C, and blocked with 3% BSA for 1 h at 37°C. Fifty microliter of amplified phage after each round of panning was added into each well and incubated for 2 h at room temperature. Then, the microtiter plate was washed with PBS-tween five times, and anti- M13 tag antibody conjugated with horse radish peroxidase (HRPO) (Amersham Pharmacia Biotech) was added into each well. 2,2'-azino-bis (3-ethylbenthiazoline-6-sulfonic acid) (ABTS) (Sigma Co.) was used to visualize the signal. The plate was analyzed at O.D. 405 nm with ELISA reader (Biorad, USA). In order to identify positive phage clones, monoclonal phage ELISA was performed as

above except that culture supernatant containing monoclonal phage particles that obtained by overnight culture of each *E. coli* colonies at the 4th round of panning was used instead of polyclonal phages.

Soluble scFv expression. Three phage clones showing the highest positive signal in monoclonal phage ELISA were mixed with HB2151 cells (non-suppressor strain) separately for 1 h at 37°C. Then, the mixture was spread onto 2×YT agar plate containing 100μg/ml ampicillin and incubated for overnight at 30°C. Colonies were randomly picked and inoculated in 2 ml LB/amp and the expression of soluble scFv molecules was induced by adding final concentration of 1 mM IPTG for 4 h at 30°C. The culture supernatant containing soluble scFv was obtained by centrifugation at 10,000 rpm for 10 min.

Heat treatment of human U937 cell line. Human U937 cells maintained in Dulbecco's modified eagle medium (DMEM) (Sigma Co.) containing 10 mM HEPES (Sigma Co.), 100 U penicillin/streptomycin (Sigma Co.), 10% fetal bovine serum (Sigma Co.) (DMEM-10) were treated for 1 h at 42°C (28), and recovered for 1 h at 37C in the CO_2 incubator before harvesting by centrifugation. The cell pellet was resuspended in SDS sample buffer and used in Western blot analysis.

Western blot analysis. For Western blot, *E. coli* expressing recombinant HSP70.1 were resuspended in SDS sample buffer, and total *E. coli* proteins were separated by 12% SDS-PAGE followed by blotting onto nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was blocked with 3% skimmed milk solution in PBS for 1 h at room temperature. After washing the membrane with PBS-tween, the culture supernatant was incubated with the membrane for 1 h at room temperature. Binding of scFv molecules to the target protein was detected with mouse anti-E tag antibody (Amersham Pharmacia Biotech) follow by addition of goat anti-mouse IgG antibody conjugated with HRPO (Sigma Co.). ECL (Amersham Pharmacia Biotech) was used to visualize the signals followed by exposure on X-ray film. Binding reactivity of HH7-1 scFv antibody was further characterized by Western blot. Affinity-purified recombinant HSP70.1, recombinant Glutathione-S-transferase (GST), recombinant Derf II, respiratory syncytial virus (RSV) (Chemicon, USA) and recombinant mycobacterial HSP-65 proteins were separated by 12% SDS-PAGE, and the Western blot analysis was performed as above. Western blot was also performed using human U937 cellular extract prepared as above.

For epitope mapping, wild-type recombinant HSP70.1 and HSP70.1 ΔN protein were separated by 15% SDS- PAGE, respectively, and transferred into nitrocellulose membrane. Mouse anti-HSP70 monoclonal antibody (Santa Cruz) or the supernatant containing soluble scFv molecules was incubated with the membrane. Binding activity of scFv molecules was detected with anti-E tag antibody, and goat anti-mouse IgG (Alkaline phosphatase conjugated) (Sigma Co.). Nitro blue tetrazolium chloride (NBT) (Duchefa, Netherlands) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Duchefa) were used to visualize the signals.

Automatic DNA sequencing. Plasmid was isolated from HB2151 cells producing scFv molecule specific for recombinant HSP70.1 using Plasmid Miniprep kit (QIAGEN), and the presence of scFv gene was confirmed by treating the plasmid with *Sfi* I and *Not* I (Roche Biochemicals) followed by 1% agarose gel electrophoresis. For automatic DNA sequencing, scFv gene was isolated by treating pCANTAB-5E plasmid with *Hind* III and *Not* I (Roche Biochemicals), and subcloned into pBlueScript SK-vector (Stratagene). PCR was performed with Cy5™ AutoCycle™ Sequencing Kit (Amersham Pharmacia Biotech) according to manufacturer's protocol. Sequencing gel was prepared using Long Ranger TM Gel solution (FMC Corp., USA), and automatic DNA sequencing was performed using M13 forward primer and ALFexpress sequencers (Amersham Pharmacia Biotech).

Results

Construction of a semi-synthetic phage display library and affinity selection with recombinant HSP70.1. A human semi-synthetic scFv library was constructed using pCANTAB-5E vector as described in the Materials &

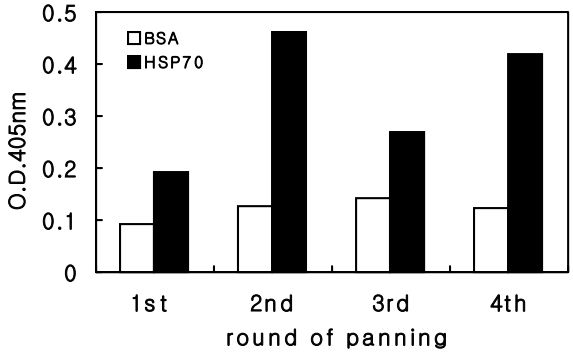

Figure 1. Polyclonal phage ELISA showing specific enrichment of recombinant phages after panning. Microtiter plates were coated with BSA (negative control antigen) or the recombinant HSP-70.1, and amplified phage particles were obtained after each round of panning added into each well. Anti-M13 antibody (HRPO conjugated) was used as a secondary antibody, and ABTS was used as a substrate. Absorbance was measured at O.D. 405 nm.

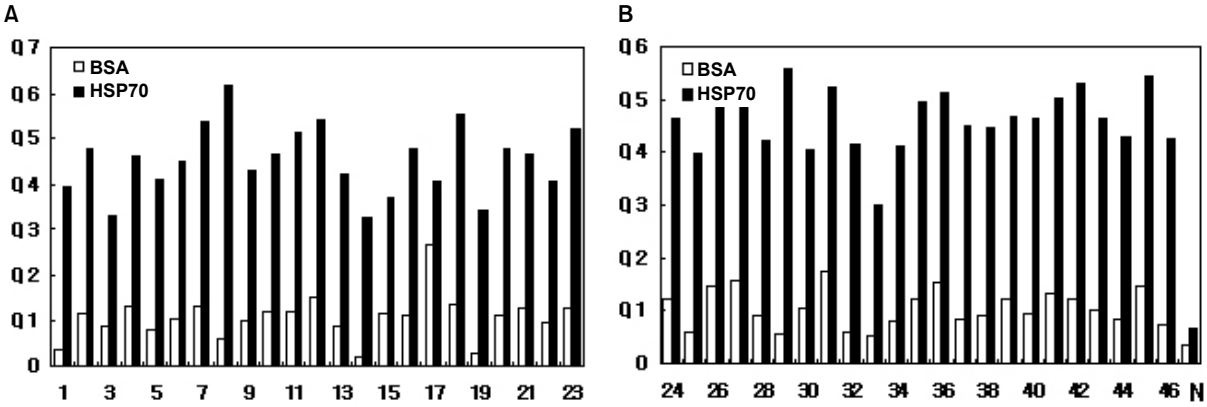

Figure 2. Determination of binding specificity of monoclonal recombinant phage clones by ELISA. After the 4th panning, the amplified phages were infected into TG1 cell strain, and 46 colonies were randomly picked. Monoclonal recombinant phages were obtained by infecting M13KO7 helper phage into each of 46 *E. coli* culture, and the culture supernatant containing phage particles were added into antigen-coated microtiter plate. Anti-M13 antibody was used as a secondary antibody and ABTS was used as a substrate. M13KO7 helper phage used as negative control. N: M13KO7 helper phage (negative control).

Methods. Total library size was 5×10^8. Analysis of 48 clones from the unselected library indicated that greater than 90% of the clones carried an scFv insert, and the library appeared to be diverse as judged by the *Mva* I restriction pattern of scFv genes (data not shown).

For the affinity selection of the library, a microtiter plate was coated with 100μg/ml of human recombinant HSP70.1 and total 10^{11} recombinant phages were used for panning. Panning was repeated 4 times. Titer of each input and output phage was calculated, and percentage yield was showed in Table 1. The yield was increased 100 folds at the 2nd and the 4th round. Enrichment of antigen-specific phage after successive rounds of panning was determined by polyclonal phage ELISA. The result of polyclonal phage ELISA (Fig. 1) showed that the recombinant phages specific for HSP70.1 were enriched especially after the 2nd and the 4th round of panning, which was in agreement with data from Table 1. In order to identify positive phage clones, monoclonal phage ELISA (Fig. 2) was performed using amplified recombinant phages from *E. coli* colonies after the 4th round of panning. As shown in Fig 2, all of recombinant phage clones except for negative phage control (M13KO7)
gave positive signal to HSP70.1 over BSA, a negative control antigen, indicating that the phage antibody clones specific for human HSP70.1 were successfully selected from a semi-synthetic scFv phage display library.

Production of soluble scFv molecules and analysis of their antigen-binding specificity. Three positive phage clones which gave the highest binding signal to human recombinant HSP70.1 at the monoclonal phage

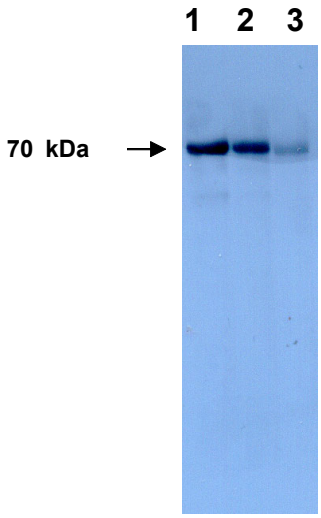

Figure 3. Western blot analysis to determine the binding specificity of soluble scFv antibodies against recombinant HSP70.1 in *E. coli* lysates. *E. coli* expressing recombinant HSP70.1 was resuspended in SDS sample buffer and separated by 12% SDS-PAGE. Then, total *E. coli* proteins were transferred into nitrocellulose membrane. After wash and blocking the membrane, culture supernatant of HH7-1 (lane 1), HH7-2 (lane 2) and HH7-3 (lane 3) clones containing soluble scFv antibodies were incubated with the membrane. Mouse anti-E tag antibody followed goat anti-mouse IgG conjugated with HRPO was incubated with the membrane to identify binding reactivity of soluble scFv antibodies. ECL was used to detect signals and visualized by exposure on X-ray film.

ELISA were chosen and used to infect HB2151 (non-suppressor *E. coli* strain) cells for soluble scFv expression. The culture supernatant containing soluble scFv molecules was obtained as described in the Materials & Methods, and the binding specificity of

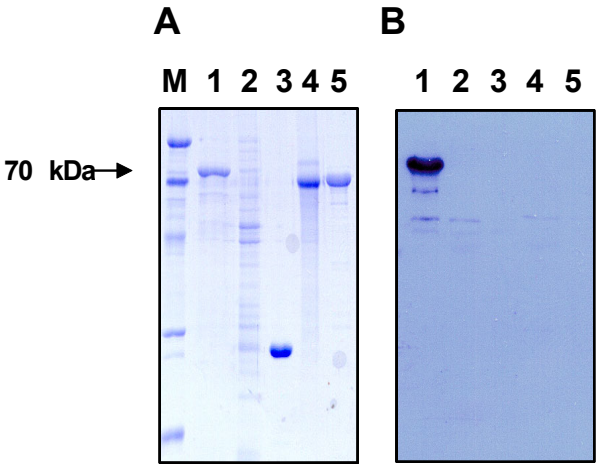

Figure 4. Western blot analysis of to determine binding reactivity of HH7-1 scFv antibody to various antigens. Recombinant HSP-70.1 (lane1), recombinant Derf II (lane 2), recombinant GST (lane 3), RSV (lane 4) and recombinant mycobacterial HSP65 (lane 5) proteins were separated in 12% SDS-PAGE and stained with Coomassie blue dye (A); or transferred onto nitrocellulose membrane for Western blot (B). The membrane was incubated with culture supernatant of HH7-1 scFv clone. Mouse anti-E tag antibody followed by incubation with goat anti-mouse IgG conjugated with HRP was used to identify binding specificity. ECL was used to detect signals and visualized by exposure on X-ray film.

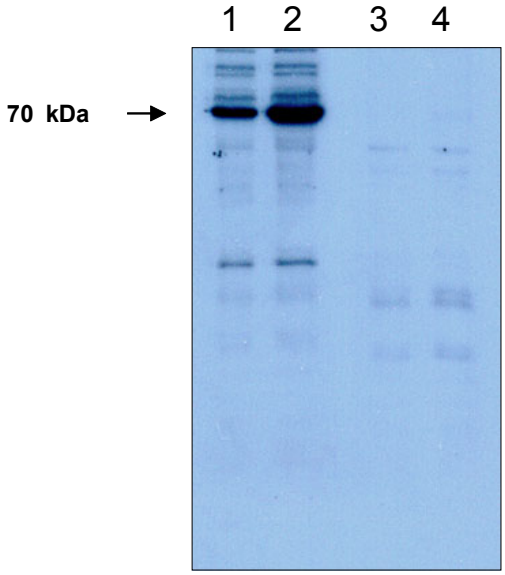

Figure 5. Western blot analysis showing the binding specificity of HH7-1 scFv clone to native HSP70 protein. Total cellular extracts from control (37°C, land 1 and 3) and heat-treated (42°C for 1 h, lane 2 and 4) U937 cells were separated by 15% SDS-PAGE, and transferred onto membrane. After blocking, mouse anti-HSP70 mAb (lane 1 and 2) or supernatant of soluble scFv (HH7-1) (lane 3 and 4) were incubated with the membrane strips. Then the signal detected anti-mouse IgG (HRPO conjugated) for mouse anti-HSP70 mAb, or anti-E tag antibody for HH7-1 scFv antibody. ECL was used to detect signals and visualized by exposure on X-ray film.

three scFv antibody clones to HSP70.1, named HH7-1, HH7-2 and HH7-3, respectively, was determined by Western blot (Fig. 3). All three clones reacted with recombinant human HSP70.1, and HH7-1 was chosen for further characterization to determine the possible cross-reactivity of the scFv antibody molecule to other irrelevant antigens since it showed the strongest positive binding signal in Western blot. Fig. 4 (A) showed the Coomassie blue staining of recombinant HSP-70.1 (lane 1), recombinant glutathione S-transferase (GST) (lane 2), recombinant Der f II (lane 3), respiratory syncytial virus (RSV) (lane 4) and recombinant mycobacterial HSP-65 (lane 5) separated in 15% SDS-PAGE. These proteins were transferred to nitrocellulose membrane and Western blot analysis was performed using HH7-1 antibody (Fig. 4B). The blot data clearly showed that human recombinant scFv antibody (HH7-1) recognized only HSP70.1 (lane 1), but not reacted to other antigens. The binding specificity of HH7-1 also confirmed by ELISA in that the scFv molecule specifically reacted to recombinant HSP70.1 but not to recombinant GST, recombinant Der f II, RSV nor recombinant mycobacterial HSP-65 protein (data not shown), and it has a medium binding affinity (Kd$\approx 5\times10^{-7}$ M) to recombinant HSP70.1 determined by competitive ELISA (data not shown).

Fine binding specificity of HH7-1 scFv antibody to HSP70 determined by Western blot. Western blot was performed to determine whether HH7-1 scFv antibody reacts to native human HSP70 (Fig. 5). Expression of native HSP70 was induced by treating human U937 monocytic cells at 42°C as described in the Materials & Methods. The normal U937 cells and the heat-treated U937 cells were harvested from the tissue culture flasks and total cellular proteins were separated in 12% SDS-PAGE. After blotting cellular proteins from normal U937 (lane 1 and 3) and heat-treated U937 (lane 2 and 4) onto nitrocellulose membrane, immunoblot was performed using mouse anti-HSP70 monoclonal antibody (mAb) (lane 1 and 2) or soluble HH7-1 scFv antibody (lane 3 and 4). The data showed that mouse mouse anti-HSP70 mAb recognized both cognate HSP70 (lane 1) and heat-induced HSP70 (lane 2). However, HH7-1 scFv antibody did not react to any of native HSP70 produced in U937 human monocytic cell line suggesting that HH7-1 binds to the epitope present only in the recombinant form of HSP70.1. This peculiar phenomenon was also observed by HH7-2 and HH7-3 (data not shown)

In order to shed light to on this puzzling result, Western blot analysis was performed using truncated HSP70.1 mutant protein as shown in Figure 6 (A) and

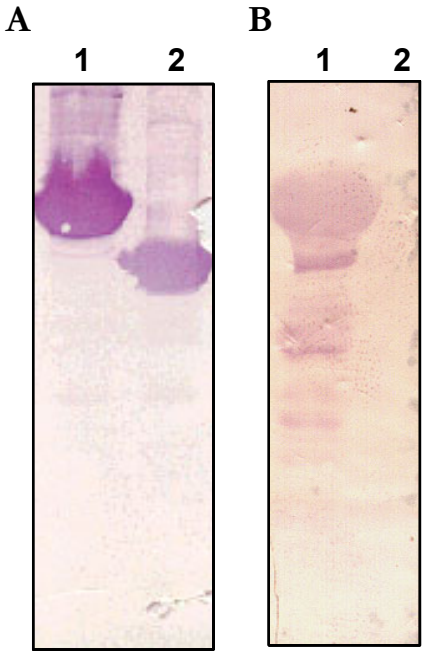

Figure 6. Epitope mapping of HH7-1 scFv clone on the recombinant HSP70.1 recognized by Western blot. *E. coli* extracts expressing recombinant HSP70.1 (lane 1) or recombinant HSP70 ΔN (lane 2) were separated by 15% SDS-PAGE. The immunoblotting was performed using either mouse anti-HSP70 mAb (A) or soluble HH7-1 scFv antibody (B) as a primary antibody. Anti-mouse IgG antibody conjugated with AP for monoclonal HSP70 antibody or mouse anti-E tag antibody followed by incubation with goat anti-mouse IgG conjugated with HRP for HH7-1 scFv antibody were used to identify binding specificity, respectively. ECL was used to detect signals and visualized by exposure on X-ray film.

(B). A recombinant full-length HSP70.1 (lane 1) and a recombinant HSP70.1 ΔN which has deletion of 120 amino acids at the N-terminus (lane 2) were blotted with either mouse anti-HSP70 mAb (Fig. 6A) or HH7-1 scFv antibody (Fig. 6B). The blotting results showed that epitope recognition pattern of mouse anti-HSP70 mAb and HH7-1 scFv antibody differed in that mouse anti- HSP70 mAb bound to the C-terminal region of the recombinant HSP70.1, whereas HH7-1 scFv antibody reacted to the N-terminal region of the recombinant HSP70.1.

V_H and V_L gene usage of the scFv antibody clones. The three positive scFv clones, HH7-1, HH7-2 and HH7-3 were subcloned into a SK- vector and the nucleotide sequences of V_H and V_L were determined using automatic DNA sequencer (GeneBank accession number AF521009, AF521010, and AF521011). Deduced amino acid sequences were showed in Fig. 7. All three clones used V_H subgroup III. On the other hand, V_L of HH7-1 and HH7-2 belonged to the kappa light chain subgroup I, but HH7-3 utilized V_k subgroup IV.

Discussion

Phage display technology has been considered as one of the major platform technologies for the generation of human mAbs, since it has several distinct advantages. For example, no immunization protocol is needed and diverse human mAbs specific for any target antigens can be obtained from a single pot of antibody display library in which a large array of immunoglobulin fragments, usually either scFv or

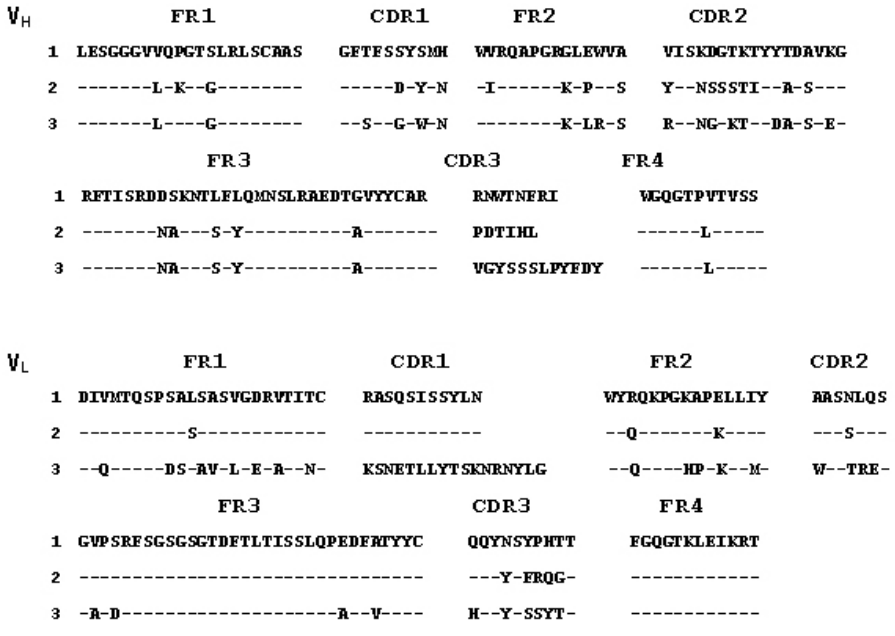

Figure 7. Deduced amino acid sequences V_H and V_L of HH7-1, HH7-2 and HH7-3 clones. Frame work (FW 1, 2, 3, 4) and complementarity determining regions (CDR 1, 2, 3) were shown.

Fab form, are displayed on the surface of filamentous bacteriophage. Immunoglobulin gene used for the construction of an antibody display library can be obtained from a naïve or artificial (semi-synthetic) source. If the diversity of antibody fragments encoded by either nave or semi-synthetic, especially, in the phage display library is large enough, recombinant human mAbs specific for self-antigens can be isolated. This is important in the development of antibody drugs, since most target antigens are of eventually human origin as in the case of cancer.

In this study, we constructed a semi-synthetic antibody display library containing 5×10^8 independent clones using peripheral blood mononuclear lymphocytes (PBML) from 40 human volunteers, the human bone marrow (BM) cDNA library and the human fetal liver cDNA library as diverse sources of immunoglobulin genes. The resulting library was screened with recombinant human HSP70.1 produced by using *E. coli* expression system and three positive scFv antibody clones were isolated. All these clones reacted specifically with recombinant human HSP70.1 produced by using an *E. coli* expression system, but interestingly not to native HSP70 from mammalian origin, whereas mouse anti-HSP70 mAb that commercially available recognized both recombinant human HSP70.1 and native HSP70, suggesting that our recombinant human scFv antibody clones recognized somewhat different epitopes that present only in recombinant HSP70. Epitope mapping data indeed showed that the epitopes recognized by our scFv antibody clones reside at the N-terminal region, the part of ATP binding domain, of recombinant HSP70.1.

It is not understood yet why screening our semi-synthetic scFv library with the recombinant HSP70.1 preferentially directed to this particular region but not to the C-terminal region of the protein as seen in mouse anti-HSP70 mAb. Since the antibody fragments isolated from the library are highly specific to the antigen used in panning, it is possible that the epitope recognized by our scFv clones may be located in the cleft of HSP70.1 in natural conformation, and exposed to the surface only when the protein is produced in a prokaryotic expression system because of unnatural conformation of the protein due to the lack of post-translational modification. It is not yet certain whether isolation of peculiar scFv clones specific for bacterially expressed HSP70.1 but not for native HSP70 from a semi-synthetic scFv display library is unique only in our experimental setup. Interestingly, however, it was found that mice immunized with bacterially expressed antigen such as mesothelin or epidermal growth factor receptor (EGFR) may produce antibodies reacting only with the recombinant protein, but not with the antigen in its native conformation on cells (29-31), suggesting that use of recombinant antigens produced in *E. coli* does not always generate immunological effectors against antigens with native conformation.

In addition, the chances of finding a phage with specific binding activity depend on its affinity and the efficiency and number of rounds of selection. Therefore, the design of an appropriate selection strategy is just as important as V repertoire construction. For example, selection with a microtiter plate or an immunotube can be performed very simply and effectively by panning recombinant phage on antigen-coated plates, yet gives slightly different results (32). It has also been well noted that purification and immobilization of antigens directly on the solid matrix causes significant changes in the antigen conformation due to denaturation and/or aggregation of the antigens, and biotinylated antigens have been immobilized on the streptavidin-coated matrix as an alternative (33). For instance, some scFv clones obtained from human nave scFv display libraries by selecting them with potato leafroll virus (PLRV) in PBS failed to react with PLRV coat protein in immunoblots or ELISA coated with PLRV in carbonate coating buffer, suggesting that the configuration of antigens on the matrix greatly influences the fine specificity of scFv clones after panning (33). Another possibility is that our semi- synthetic scFv library does not have enough diversity to contain scFv clones that may recognize other regions of the recombinant HSP70.1.

In conclusion, isolation of functional scFvs from an antibody display library is largely dependent upon the conformation of target antigens; therefore, source and configuration of target antigens should be carefully taken into consideration before the enrichment of recombinant antibody clones specific for the antigens, especially in the development of intrabodies that have to have specific binding reactivity to the native form of target molecules.

References

1. Khazaeli MB, Conry RM, LoBuglio AF: Human immune response to monoclonal antibodies. J Immunother 15;42-52, 1994
2. Baca M, Presta LG, O'Connor SJ, Wells JA: Antibody humanization using monovalent phage display. J Biol Chem 272;10678-10684, 1997
3. Barbas CF, Burton DR: Selection and evolution of high-affinity human anti-viral antibodies. Trends Biotech 14; 230-234, 1996
4. Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G: By-passing immunization: Human antibodies from V-gene libraries displayed on phage. J Mol Biol 222;581-597, 1991
5. Hoogenboom HR, Winter G: By-passing immunization: Hu-

man antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. J Mol Biol 227; 381-388, 1992
6. Richardson JH, Marasco WA: Intracellular antibodies: development and therapeutic potential. Trends in Biotechnol 13; 306-310, 1995
7. Richardson JH, Sodroski JG, Waldmann TA, Marasco WA: Phenotypic knockout of the high-affinity human interleukin 2 receptor by intracellular single-chain antibodies against the alpha subunit of the receptor. Proc Natl Acad Sci USA 92; 3137-3141, 1995
8. Beerli RR, Wels W, Hynes NE: Intracellular expression of single chain antibodies reverts ErbB-2 transformation. J Biol Chem 269;23931-23936, 1994
9. Alvarez RD, Barnes MN, Gomez-Navarro J, Wang M, Strong TV, Arafat W, Arani RB, Johnson MR, Roberts BL, Siegal GP, Curiel DT: A cancer gene therapy approach utilizing an anti-erbB-2 single-chain antibody-encoding adenovirus (AD21): a phase I trial. Clin. Cancer Res 6;3081-3087, 2000
10. Biocca S, Pierandrei-Amaldi P, Cattaneo A: Intracellular expression of anti-p21ras single chain Fv fragments inhibits meiotic maturation of xenopus oocytes. Biochem Biophys Res Commun 197;422-427, 1993
11. Chen SY, Khouri Y, Bagley J, Marasco WA: Combined intra- and extracellular immunization against human immunodeficiency virus type 1 infection with a human anti-gp120 antibody. Proc Natl Acad Sci USA 91;5932-5936, 1994
12. Mhashilkar AM, Biswas DK, LaVecchio J, Pardee AB, Marasco WA: Inhibition of human immunodeficiency virus type 1 replication in vitro by a novel combination of anti-Tat single-chain intrabodies and NF-kappa B antagonists. J Virol 71;6486-6494, 1997
13. Marasco WA, LaVecchio J, Winkler A: Human anti- HIV-1 tat sFv intrabodies for gene therapy of advanced HIV-1-infection and AIDS. J Immunol Meth 231;223-238, 1999
14. Biocca S, Ruberti F, Tafani M, Pierandrei-Amaldi P, Cattaneo A: Redox state of single chain Fv fragments targeted to the endoplasmic reticulum, cytosol and mitochondria. Biotechnology (NY) 13;1110-1115, 1995
15. Moseley PL: Heat shock proteins and the inflammatory response. Ann NY Acad Sci 856;206-213, 1993
16. Samali A, Cotte TG: Heat shock proteins increase resistance to apoptosis. Exp Cell Res 223;63-70, 1996
17. Kiang JG and Tsokos GC: Heat shock protein 70 kDa: Molecular biology, biochemistry, and physiology. Pharmacol Ther 80;183-201, 1998
18. Georgopoulos C, Welch WJ: Role of the major heat shock proteins as molecular chaperones. Annu Rev Cell Biol 9;601-634, 1993
19. Hunt CR, Gasser DL, Chaplin DD, Pierce JC, Kozak CA: Chromosomal localization of five murine HSP70 gene family members: Hsp70-1, Hsp70-2, Hsp70-3, Hsc70t, and Grp78. Genomics 16;193-198, 1993
20. Allen RL, O'Brien DA, Jones CC, Rockett DL, Eddy EM: Expression of heat shock proteins by isolated mouse spermatogenic cells. Mol Cell Biol 8;3260-3266, 1988
21. Zakeri ZF, Wolgemuth DJ, Hunt CR: Identification and sequence analysis of a new member of the mouse HSP70 gene family and characterization of its unique cellular and developmental pattern of expression in the male germ line. Mol Cell Biol 8;2925-2932, 1988
22. Kwon S, Young C, Kim D, Choi H, Kim K, Chung J, Eun H, Park K, Oh C, Seo J: Impaired repair ability of hsp70.1 KO mouse after UVB irradiation. J Dermatol Sci 28;144-151, 2002
23. Lee S, Kim M, Yoon B, Kim Y, Ma S, Roh J, Lee J, Seo J: Targeted hsp70.1 disruption increases infarction volume after focal cerebral ischemia in mice. Stroke 32;2905-2912, 2001
24. Huang L, Mivechi NF, Moskophidis D: Insights into regulation and function of the major stress-induced hsp70 molecular chaperone in vivo: analysis of mice with targeted gene disruption of the hsp70.1 or hsp70.3 gene. Mol Cell Biol 21;8575-8591, 2001
25. Leppa S, Kajanne R, Arminen L, Sistonen L: Differential induction of Hsp70-encoding genes in human hematopoietic cells. J Biol Chem 276;31713-31719, 2001
26. McCafferty J and Johnson KS: Construction and screening of antibody display libraries. In: Kay BK, Winter J, McCafferty J eds.: Phage display of peptides and proteins, p79-111, Academic Press, 1996
27. McCafferty J, Griffiths AD, Winter G, Chiswell DJ: Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348;552-554, 1990
28. Li CY, Lee JS, Ko YG, Kim JI, Seo JS: Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. J Biol Chem 275; 25665-25671, 2000
29. Chowdhury PS, Chang K, Pastan I: Isolation of anti- mesothelin antibodies from a phage display library. Mol Immunol 34;9-20, 1997
30. Huls GA, Heijnen IA, Cuomo ME, Koningsberger JC, Wiegman L, Boel E, van der Vuurst de Vries AR, Loyson SA, Helfrich W, van Berge Henegouwe GP, van Meijer M, de Kruif J, Logtenberg TA: recombinant, fully human monoclonal antibody with antitumor activity constructed from phage-displayed antibody fragments. Nat Biotechnol 17;276-281, 1999
31. Kettleborough CA, Ansell KH, Allen RW, Rosell-Vives E, Gussow DH, Bendig MM: Isolation of tumor cell-specific single-chain Fv from immunized mice using phage-antibody libraries and the re-construction of whole antibodies from these antibody fragments. Eur J Immunol 24;952-958, 1994
32. Barbara K, Robert R, Silke R, Christine R, Michael T, Elisabeth T, Manqiu C, Torsten D, David F, Adolf H, Landon I, Achim K, Matthias M, Peter P, Xian-Qin M, Pobert S, Peter S, Jill W, Joachim W, Titus K: High-throughput generation and engineering of recombinant human antibodies. J Immunol Meth 254;67-84, 2001
33. Harper K, Toth RL, Mayo MA, Torrance L: Properties of a panel of single chain variable fragments against potato leafroll virus obtained from two phage display libraries. J Virol Meth 81;159-168, 1999