Generation of High-Affinity Fully Human Anti-Interleukin-8 Antibodies from its cDNA by Two-Hybrid Screening and Affinity Maturation in Yeast

Ling Ding,¹ Mark Azam,² Yu-Huei Lin,² James Sheridan,² Shuanghong Wei,² Gigi Gupta,² Rakesh K. Singh,³ Michelle H. Pauling,² Waihei Chu,² Antares Tran,² Nai-Xuan Yu,^{4,5} Jiefeng Hu,^{4,6} Wei Wang,⁴ Hao Long,⁴ Dong Xiang,⁵ Li Zhu,² and Shao-Bing Hua^{2,4,5,6*}

¹Department of Medical Oncology, Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China

²Genetastix Corporation, San Jose, California 95131

³Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska 68198

⁴Zhejiang California International NanoSystems Institute, Zhejiang University, Hangzhou 310029, China

⁵Avalon BioSciences, Inc., Mountain View, California 94043

⁶Hangzhou Avalon Biosciences, Ltd., Hangzhou, China

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Abstract: We have developed a technology for rapidly generating novel and fully human antibodies by simply using the antigen DNA. A human single-chain variable fragment (scFv) antibody library was constructed in a yeast two-hybrid vector with high complexity. After cloning cDNA encoding the mature sequence of human interleukin-8 (hIL8) into the yeast two-hybrid system vector, we have screened the human scFv antibody library and obtained three distinct scFv clones that could specifically bind to hIL8. One clone was chosen for further improvement by a novel affinity maturation process using the error-prone PCR of the scFv sequence followed by additional rounds of yeast two-hybrid screening. The scFv antibodies of both primary and affinity-matured scFv clones were expressed in *E. coli*. All purified scFvs showed specific binding to hIL8 in reciprocal coimmunoprecipitation and ELISA assays. All scFvs, as well as a fully human IgG antibody converted from one of the scFv clones and expressed in the mammalian cells, were able to effectively inhibit hIL8 in neutrophil chemotaxis assays. The technology described can generate fully human antibodies with high efficiency and low cost.

Keywords: two-hybrid; human scFv; affinity maturation; Interleukin-8; antibody library

Abbreviations: CDR, complementarity determining region; IL8, interleukin-8; scFv, single-chain variable fragments; V_H, variable domain of heavy chain; V_L, variable domain of light chain.

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*Correspondence to: Shao-Bing Hua, Zhejiang California International NanoSystems Institute, Zhejiang University, 268 Kaixuan Road, Hangzhou 310029, China. E-mail: bhua@zcnj.org

Introduction

Monoclonal antibodies have become a new class of medicine for treating many diseases such as cancer,^{1,2} cardiovascular disease,³ infectious disease,⁴ and inflammation diseases.⁵ This is mainly due to the establishment of several technologies to generate human or humanized monoclonal antibodies. For example, humanization of mouse monoclonal antibodies^{6,7} and transgenic mice containing human antibody genes^{8,9} have been used in generating antibodies, which lead to minimal or no human anti-

murine antibody responses. These technologies in general suffer from high cost and low efficiency in human antibody generation. An alternative strategy is phage display, which has been used in generating fully human single-chain variable fragment (scFv) or Fab antibodies against specific antigens.^{10,11} All these technologies require purified antigen proteins in the antibody screening processes. Protein expression and purification can be an expensive, time-consuming, and difficult process. Therefore, it is particularly difficult to implement a high throughput methodology based on these technologies to produce large number of antibodies for projects such as functional genomics.

The antibody–antigen interaction is a unique type of protein–protein interaction. One of the most useful approaches for studying protein–protein interactions is the yeast two-hybrid technology that was first developed by Fields and Song.¹² The technology only requires the target DNA rather than the protein to identify its binding partners. Although yeast two-hybrid system requires interacting protein partners entering the nucleus and activating transcription of the nuclear reporter genes, the technology has been successfully used for studying protein–protein interactions of cytoplasmic, nuclear, and extracellular origins.^{13–15} To apply yeast two-hybrid technology in studying antigen–antibody interaction, on the other hand, it has been suggested that expression of intracellular scFv antibodies (also known as “intrabodies”) should be confronted with the difficulties concerning the stability, solubility, and aggregation tendency,¹⁶ because of the presumed reducing environment of the cytoplasm. However, scFv antibodies derived from phage display^{17,18} can successfully bind to their cytoplasmic antigens, such as dihydroflavonol-4-reductase from *Petunia*, in the yeast two-hybrid system,^{19,20} indicating that some scFvs’ can successfully bind to their antigens. Therefore, it would be interesting to study whether scFv antibodies can be screened out from a scFv antibody library using cDNA sequences encoding antigen proteins, particularly secreted proteins or extracellular domain of membrane proteins as most of the therapeutic targets are such proteins or domains.

Here, we describe the successful screening and further affinity maturation of scFv antibodies from a fully human scFv library in the two-hybrid vector against cDNA encoding human interleukin-8 (hIL8), a secreted cytokine, using the yeast two-hybrid system approach. IL8 is a member of CXC chemokine family, initially identified as a major neutrophil chemotactic and activating factor.^{21,22} It has been identified now that IL8 has a wide range of activities in many physiological and pathological processes.²³ Particularly, it is evident that IL8 plays very important roles in the inflammatory process.²⁴ Anti-IL8 antibodies may have significance in clinical applications.²⁵

Results

Construction of the human single-chain antibody library

We have constructed a human scFv library in the yeast two-hybrid vector pACT2 that contains sequence encoding the Gal4 activation domain (AD). The scFv antibody fragments consist of variable regions²⁶ of heavy (V_H) and light chain (V_L) of full antibodies that are joined by a linker sequence²⁷ and flanked by sequences at each end that are homologous to the pACT2 multiple cloning sites (MCS).²⁸ Assembled PCR products were cloned into pACT2 by homologous recombination.²⁹ Such derived human scFvs are fused in-frame with the Gal4 AD. A total of 5×10^8 independent yeast colonies have been generated. To check the quality of this human scFv library, we randomly picked 51 colonies. Sequence analyses of the inserts indicated that all clones contained V_H –linker– V_L cassettes fused in-frame with the upstream Gal4 AD, and all the scFv sequences analyzed were distinct (data not shown). We used a subset of the above-described scFv library (approximately 5×10^7 clones) in the following studies. The library construction, library screening, and affinity maturation process is outlined in Figure 1.

Library screening

To determine whether human antibodies specific to extracellular proteins can be isolated from our scFv library, cDNA encoding the mature protein of hIL8 was cloned into pGBKT7, resulting in pGBK–IL8, which codes for the fusion protein of Gal4 DNA binding domain (BD) and hIL8 at the carboxy terminus. The construct was transformed into the cells of a *MAT α* -type yeast strain AH109. To screen the scFv library, the AH109 transformants were mated with *MAT α* -type yeast cells containing the library. The scFv library-harboring vector pACT2 contains the *LEU2* gene, whereas pGBK–IL8 contains the *TRP1* gene. Cells harboring both plasmids can grow in the synthetic medium lacking leucine and tryptophan (SD/–LW). Cells containing scFv/IL-8 interactions would be able to activate expression of the Gal4-dependent reporter genes *ADE2* and *HIS3* built-in to the genome of the strains, thus allowing the cells to grow on the selection medium lacking adenine, histidine, leucine, and tryptophan (SD/–AHLW). The mated diploid cells of the library were plated on SD/–AHLW plates. A total of 67 colonies grew on the selection medium. These colonies were assayed for the expression of an additional reporter gene, *lacZ*, in the β -galactosidase colony-lifting assay. Twenty-two clones from original 67 clones demonstrated β -galactosidase activity (data not shown).

We further analyzed the specificities of these *lacZ*-positive clones. Plasmid DNAs were rescued from yeast cells. They were cotransformed into yeast AH109 cells

Figure 1. Outline of procedures of yeast two-hybrid human scFv library construction, library screening, and affinity maturation.

with either pGBK-IL8, or empty vector pGBKT7, or pGBKT7-Lam that encodes an irrelevant antigen, human lamin C. The transformants were grown on selection medium lacking leucine and tryptophan (SD/-LW) and were further analyzed by the β -galactosidase colony-lifting assay. A total of 16 out of 22 clones showed specificity to human IL-8 on the β -galactosidase assay (data not shown). Sequence analysis indicated that there were three distinct scFv sequences repre-

sented by these 16 clones. The amino acid sequences of these three clones are shown in Figure 2(A). The V_H and V_L sequences of these clones are all different. The V_H sequences are derived from different V gene families. The V_L sequence of clone 4-123-157 was derived from a kappa gene, whereas those of clones 4-123-36 and 4-123-151 were from lambda genes.

To further confirm the specificity of the scFv, we performed the reverse assay. The insert of scFv from one clone 4-123-36 was cloned into pGBKT7, resulting in the plasmid that contains a fusion of Gal4 DNA-BD with the scFv anti-hIL8. Human IL8 cDNA was cloned into pACT2 in-frame with the Gal4 AD. Both plasmids were cotransformed into AH109. Such transformants were able to grow on SD/-AHLW and showed a positive result in the β -galactosidase assay (data not shown), thus confirming that the scFv clone is specific to hIL8. This clone was subjected to further analyses.

Expression of the scFv in *E. coli* and coimmunoprecipitation analysis

The cDNA of scFv clone 4-123-36 was cloned in-frame with pelB leader sequence in pET27b(+). The construct encodes scFv anti-IL8 followed by an HSV tag and a 6xHis tag sequentially at its carboxy terminus. Expression of scFv anti-IL8 in the periplasmic space of *E. coli* was induced by isopropyl β -D-thiogalactoside (IPTG). The scFv protein was purified on a Ni-NTA column [Fig. 3(A)].

We then assayed the interaction of scFv 4-123-36 with its antigen human IL8 in coimmunoprecipitation

A

V_H

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      CDR1          CDR2          CDR3
4-123-36 EVQLVESGGGLVQPGGSLRLSCAASGFTFSS...Y.WMSWVRQAPGKGLEWVAIKQDGSEKYYVDSVKGRFTISRDNKNSLYLQMNSLRAEDTAVYYCARVSSSSWSYDSSGYYPDYWGQGLTIVTVSS
4-123-157 Q---Q---P---T-SET-S-F-NV-ASIS-...-C-S-L-PA--R---IGRVCTS-KT.Y-NP-LES-ITV-L-AS--FFS-KLT-IT-A-----Y--REAGWF.....GP-GP--L-A---
4-123-151 Q---Q---P---K-SET-S-T-TV-DSIR-GR-Y-,G-I-TP--A---IGHIYFS-ST.L-NP-FKS-VSI-L-TS--QFS-QLS-VS-A-----S--GFRQLEYGM.....DV-DQ--T-T---
  
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V_L

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      CDR1          CDR2          CDR3
4-123-36 QAVVTQEP.SLTIVSPGGTIVLTCTSSSTGFPVTTGHFSYWLQKPGQAPRTLIYDTTNKHSWTPARFSASLLGGKCALTLGARPEDAEYYCLLSYDGDG..VVGGGTKLTVL
4-123-157 ETTLT-S-S-LSA-V-DR--IT-DAS.EDIG..QYLN-Y-K--K--TL-I-ASNLGTGV-S---GTGSVTDFTF-ITSLQP--I-T---Q.Q-N.NLPITFGQ--R-EIK
4-123-151 QSVLI-.R-VSG-P-QS--IS-ETGSSDVGGYNYVS-Y--H--K--KL-M-VTKRPSV-D---GSKSDNTASL-ISGLQA--E-D---C.S-AGSYTVVFAT--K-TVL
  
```

B

V_H

```

      CDR1          CDR2          CDR3
4-123-36 EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWSWVRQAPGKGLEWVAIKQDGSEKYYVDSVKGRFTISRDNKNSLYLQMNSLRAEDTAVYYCARVSSSSWSYDSSGYYPDYWGQGLTIVTVSS
M36-8 -----
M36-11 -----D-----G-----
  
```

V_L

```

      CDR1          CDR2          CDR3
4-123-36 QAVVTQEP.SLTIVSPGGTIVLTCTSSSTGFPVTTGHFSYWLQKPGQAPRTLIYDTTNKHSWTPARFSASLLGGKCALTLGARPEDAEYYCLLSYDGDGVVVGGGTKLTVL
M36-8 -----P-----
M36-11 ---A-----L-----
  
```

Figure 2. Alignment of amino acid sequences of V_H and V_L of the scFvs. A: Alignment of amino acid sequences of V_H and V_L of anti-IL8 scFv primary clones 4-123-36, 4-123-157, and 4-123-151. B: Comparison of primary scFv clone 4-123-36 and its affinity-matured clones (M36-8 and M36-11). Residues identical to those of original clone 4-123-36 are shown by dashes. Dots represent gaps. Kabat CDR sequences are boxed.

Figure 3. Coimmunoprecipitation of human IL-8 and its antibodies. ScFv protein was expressed in the periplasmic space of *E. coli* as a fusion with HSV and 6xHis tags at its carboxy terminus. They were used for coimmunoprecipitation experiments. Panel A: lane 1, total protein from periplasmic preparation; lane 2, scFv purified by Ni-NTA. Panel B: lane 1, western blot of total periplasmic preparation using antibody against HSV tag. Panel C: Reactigels were coated with hIL8 (lane 1), lamin C (lane 2), mouse p53 (lane 3), or no coating (lane 4) and then mixed with scFv anti-hIL8 (clone 123-36). After washing, the bound proteins were analyzed by western blot using antibody against HSV tag. Panel D: Reactigels were coated with scFvs of clones 4-123-36 (lane 1), M36-8 (lane 2), M36-11 (lane 3) or no coating (lane 4) and then mixed with hIL8 in the presence of BSA. After washing, the bound proteins were analyzed by western blot using mouse monoclonal antibody against hIL8. Open arrowhead points the scFv bands. Solid arrowhead points the hIL8 band, whereas the solid arrow points the band that is probably the dimer of the hIL8.

experiments. Human IL8 was covalently bound to Reactigel (Pierce) beads; scFv anti-IL8 was mixed with the beads in the presence of bovine serum albumin (BSA) and precipitated. The scFv was then detected on a western blot by antibody against the HSV tag [Fig. 3(C)]. A reciprocal immunoprecipitation was also performed [Fig. 3(D)]. Both coimmunoprecipitations showed that the scFv derived from clone 4-123-36 could specifically bind to hIL8. In addition, the interaction of purified scFv and IL8 was detected using enzyme-linked immunosorbent assay (ELISA) assay (Fig. 5). These results strongly indicated that anti-IL8 scFv derived from the yeast intracellular environment could specifically recognize and bind to human IL8 *in vitro*.

Affinity maturation

To increase the affinity of primary antibodies isolated from the fully human scFv library screening, DNA of scFv insert of clone 4-123-36 was subjected to mutagenesis by error-prone PCR. The error-prone PCR products were transformed into yeast and cloned into pACT2 vector by *in vivo* homologous recombination in the presence of pGBK-IL8. The yeast cells were selected on SD/-AHLW in the presence of various concentrations of 3-amino-1,2,4-triazole (3-AT), a competitive inhibitor for the *HIS3* protein.³⁰ Fast growing colonies were picked and assayed for β -galactosidase activity by filter-lift assay. The specificity of these clones was analyzed as described above. We further analyzed these clones

using a quantitative liquid assay of β -galactosidase activity using *o*-nitrophenyl β -D-galactopyranoside (ONPG) substrate.³¹ Of 45 clones analyzed, two clones showed significantly higher activities on the ONPG assays (Fig. 4). Both the clones retained their specificity (data not shown). The amino acid sequences of these clones were compared to the parental clone 4-123-36 [Fig. 2(B)]. There are two to four amino acid mutations in the affinity-maturated clones, with one amino acid change located in the complementarity determining region (CDR)3 of V_H region and another change in the CDR1 of V_L . Other changes are in the framework regions, with one change adjacent to the CDR2 of V_L . The binding of these affinity-maturated clones to hIL8 was also analyzed using ELISA assay (Fig. 5). The curves of both affinity-maturated clones shifted toward the lower concentration area, suggesting that the affinity of these clones was improved.

Converting scFv to full human antibody

Next, we tested whether the scFv anti-human IL8 could be converted to a functional full human IgG antibody. The V_H region of clone 4-123-36 was linked to the human gamma-1 constant region under the control of elongation factor-1 of human T lymphotropic virus (EF1-HTLV) promoter, whereas the V_L region of clone 4-123-36 was linked to the C-gene of human lambda constant region under the control of cytomegalovirus (CMV) promoter. Both heavy chain and light chain constructs were cotransfected into

Figure 4. ONPG assay of affinity-matured clones. Plasmids from anti-IL8 scFv clone 4-123-36 and its affinity-matured clones (M36-8 and M36-11) were cotransformed into yeast with pGBK-IL8. The β -galactosidase activities were measured by quantitative ONPG assay. Activity is displayed as the fold of increase relative to the average of the parental clone 4-123-26. Error bars represent the standard errors from three experiments.

COS-7 cells. Transient expression of the recombinant full antibody in the culture medium was analyzed using ELISA. Plates coated with goat anti-human IgG γ were used to capture the recombinant full antibody, and the bound antibody was detected using horse radish peroxidase (HRP)-conjugated goat anti-human λ . Culture medium from the cell line expressing empty vectors was used as a negative control. The ELISA results showed that full antibody containing IgG γ heavy chain and λ light chain was successfully expressed (data not shown). Similarly, the recombinant full human IgG antibody can also

Figure 5. ELISA analysis of primary and affinity-matured scFvs against human IL-8. Human IL8 was coated on 96-well plates. Serially diluted scFv proteins were incubated in the IL8-coated wells in quadruplicates of each experiment. Binding was detected by HSV tag monoclonal antibody followed by anti-mouse IgG-HRP conjugate. The interaction was detected with TMB liquid substrates. Average readings of OD₄₅₀ in the figure are after subtraction of the background. Solid diamond (◆): the primary clone (4-123-36); solid square (■): affinity matured clone M36-8; solid triangle (▲): affinity matured clone M36-11. Each point is the average of four experiments.

Figure 6. Coimmunoprecipitation of full human IgG antibody anti-human IL-8 by western blot. V_H and V_L regions of scFv clone 4-123-36 were cloned to the N-termini of human Ig γ 1 heavy chain constant region and λ constant regions, respectively. The full human antibody constructs were transfected into COS cells. Full human IgG antibody was precipitated from the medium using beads coated with human IL-8 (lane 1) or uncoated beads (lane 2). Similarly, the medium of COS cells transfected with mock vectors was precipitated with IL-8-coated beads (lane 3). The blot was detected using HRP-conjugated goat anti-human IgG antibody.

bind to human IL8-coated wells using ELISA analysis (data not shown).

The expressed full human anti-hIL8 antibody IgG was also analyzed by coimmunoprecipitation. Full human IgG antibody was precipitated from the culture medium using hIL8-coated beads. The precipitates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected on a western blot using rabbit anti-human IgG antibody (Fig. 6). The results demonstrated that hIL8 could effectively precipitate the full human IgG antibody converted from anti-IL8 scFv antibody.

Inhibition of neutrophil migration by anti-hIL8 human scFv and its IgG antibodies

To measure the biological activities of the anti-hIL8 scFvs and full IgG antibody, we analyzed inhibition of IL8-induced neutrophil chemotaxis as described in Materials and Methods section. Both primary and affinity-matured scFv anti-IL8 showed significant inhibition of IL8-induced chemotaxis activities at both concentrations (1 and 10 μ g/mL) tested [Fig. 7(A)]. More interestingly, two affinity-matured clones (M36-8 and M36-11) showed higher inhibition than that of the primary clone at 1 μ g/mL, although all three clones showed similar inhibition at the

Figure 7. Inhibition of IL8-induced neutrophil chemotaxis by anti-IL8 antibodies. A: Purified scFv proteins were mixed with human IL8 and loaded at the bottom wells of Boyden chambers. Isolated neutrophil suspensions were added to upper chamber in each well. The chemotaxis was measured by counting neutrophil cells that migrated from the upper chamber to the low chamber. Percentages of inhibition are calculated based on the relative IL8-induced chemotaxis in the absence of scFv antibodies. Solid diamond (◆, fourth curve from the top): the primary clone (4-123-36); solid square (■, second curve from the top): M36-8; solid triangle (▲, third curve from the top): M36-11; open triangle (△, top curve): mouse anti-hIL8 monoclonal antibody (positive control); and cross (×, bottom curve): scFv anti-lamin (negative control). B: The V_H and V_L regions of primary clone were converted to full human IgG antibody (full Ab). The full Ab was transiently expressed in COS cells. Similarly to A, the culture medium (after fourfold concentration) was assayed for the inhibition of IL8-induced neutrophil chemotaxis. The positive control is mouse anti-hIL8 monoclonal antibody (mouse mAb) at 10 µg/mL, and the negative control is the culture medium of COS cells transfected with the empty vectors.

concentration of 10 µg/mL [Fig. 7(A)], suggesting that it has reached saturation at 10 µg/mL in the chemotaxis inhibition assay.

We then used anti-hIL8 full IgG antibody derived from primary scFv clone (4-123-36) in an inhibition of hIL8-induced neutrophil chemotaxis assay. Both anti-hIL8 full human IgG antibody and the mouse monoclonal antibody showed similar inhibitory effects with over 80% inhibition [Fig. 7(B)]. Therefore, both scFv (primary and affinity-matured clones) and full human IgG antibodies derived from our yeast two-hybrid technology can effectively block the biological function of human IL8 in the neutrophil chemotaxis assay.

Discussion

Using human IL8 cDNA, we have successfully isolated novel scFv antibody clones from a fully human scFv library constructed in the yeast two-hybrid system. This represents the first case that antibodies against an extracellular (secreted) protein can be isolated through the yeast two-hybrid system. The affinity of the scFv antibody has also been successfully improved using our novel affinity maturation process by incorporating error-prone PCR followed by the yeast two-hybrid analyses. Furthermore, both scFv (primary and affinity-matured clones) and its converted full human IgG antibody demonstrated biological inhibitory activities in hIL8-induced neutrophil chemotaxis assays. Thus, the process described here represents a new approach in generating novel fully human antibodies against extracellular antigens using the antigen coding sequences. This approach may provide a fast, efficient, and low cost

path to develop human therapeutic antibodies. In addition, no purified antigen is required in the initial antibody library screening. Furthermore, two-hybrid screening can be performed in a high throughput fashion.^{32,33} Therefore, this strategy may be used to obtain a large number of antibodies for studies at the genomic scale.

Yeast two-hybrid systems have been successfully used in identifying numerous protein-protein interactions of both cytoplasmic and nuclear origins,^{13,14} but fewer examples exist for protein interactions of extracellular origin.^{34,35} The cytoplasm and nucleus of eukaryotic cells are thought to be the reducing environments.³⁶ The redox potential in cells is assumed to be unfavorable to disulfide bonds formation. On the other hand, antibodies are secreted into the extracellular nonreducing environment. Here we report the successful isolation of interactions of human IL8, an extracellular chemokine, with novel scFv antibody clones in the yeast intracellular environment by the two-hybrid screening. Similarly, other investigators confirmed that scFv antibodies derived from phage display^{17,18} can bind to their cytoplasmic antigens, such as dihydroflavonol-4-reductase from *Petunia*, in the yeast two-hybrid system,^{19,20} indicating that scFv antibodies can successfully bind to their antigens. However, certain scFv clones showed lower binding or loss of the binding in the yeast cells, indicating that certain scFv clones derived from other approaches may reduce or lose their affinities to their antigens in the yeast two-hybrid system.

IL8 contains four cysteine residues, whereas the scFvs reported here contain five cysteine residues

each. Our observations, as well as protein–protein interaction studies using regular yeast two-hybrid system by others,^{34,35} suggest that the interior of yeast cells may well be suitable for the formation of protein structural motifs stabilized by disulfide bonds. Distinct from many other eukaryotes, yeast cells prefer a more acidic environment (pH 4.0–4.3) for optimal growth, yet its cytosol is maintained at neutral pH via the action of a membrane proton ATPase.³⁷ The large efflux of protons across the yeast cell membrane might result in the capacity for disulfide bond formation through localized variations in redox potential. Alternatively, it is possible that the conformation of human IL8 expressed in yeast cells was different from that expressed in the extracellular environment. The scFv we isolated could recognize IL8 epitopes that remained the same in both environments. One such scFv clone and its derived full human IgG antibody could successfully neutralize the IL8 biological activity in the extracellular assay conditions.

In human and rodents, the host immune system is capable of increasing the affinity of antibodies against specific antigens by a process called affinity maturation. Mammalian affinity maturation involves somatic mutations, clonal expansion and selection process. B cell clones with high affinity are selected in germinal centers and thought to mount the highly efficient secondary immune reactions.^{38,39} We adopted the natural process of antibody affinity maturation by creating a mutagenized scFv pool using an error-prone PCR that is followed by further selection in the yeast two-hybrid system at higher stringencies. Such a yeast-based process may be considered as mimicking the mammalian affinity maturation in two aspects: a mutation pool derived from a single progenitor clone and selection by the same original antigen. Two affinity-maturated scFv clones showed both higher binding affinities and higher inhibitory activities in the IL8 bioassay. Limited number of amino acid changes occurred in both the CDR and framework regions of the affinity-matured clones.

In conclusion, we developed a new approach for generating novel fully human antibodies using the yeast two-hybrid screening and affinity maturation. The method can be successfully applied in generating novel and biologically functional fully human antibodies against extracellular ligands, such as human IL-8. We believe that such method can be used to produce fully human antibodies for therapeutics, as well as for gene therapy and functional genomics studies, among other applications.

Materials and Methods

Single-chain antibody library construction

Poly A⁺ RNA from human bone marrow, human fetal liver, human spleen, and human peripheral blood

leukocytes were purchased from Clontech (Palo Alto, CA). First strand cDNAs were made from the poly A⁺ RNA using the Reverse transcriptase kit (Clontech). To amplify the cDNA of variable regions of all heavy chain and light chain genes of human antibodies by PCR, we used a set of degenerated oligonucleotides, according to Sblattero and Bradbury,²⁶ that recognize all functional V genes. At the 5' end of cDNAs encoding V_H, 21 nucleotides (CCATACGATGTTCCAGATTAC) corresponding the upstream side sequence of pACT2 vector MCS were added, whereas a short linker sequence²⁷ (GCCGCCTGATCCACCACCGCC) was added at the 3' end of V_H into the primer designs. Likewise, at the 5' end of V_L, we added linker sequence (GGCAGCGGTGGTGGAGGCAGT), and at the 3' end of V_L, the downstream sequence of pACT2 vector MCS site (GGGGTTT TTCAGTATCTACGA). Individually amplified V_H and V_L PCR products were joined by the short linker sequence via secondary PCR amplifications using longer nucleotide sequences (5'-ACCCACCAAACCC AAAAAAAGAGATCTGTATGGCTCCATACGATGTTCCAGATTAC) and (5'-ACTGCCTCCACCACCGCTGC CACCTCCGCCAGATCCTCCGGGGTTTTTCAG-TATCTACGA) that correspond the upstream and downstream of MCS, respectively. In this secondary assembly PCR process, recombination between V_H and V_L sequences occurred. Such assembled sequences have the structure of V_H–linker–V_L cassettes (i.e., scFv) flanked by sequences homologous to those at the both sides of the MCS of the yeast two-hybrid vector pACT2.^{28,29}

The above-assembled PCR products containing scFv cDNA sequences were cotransformed with Eco RI- and Bam HI-linearized pACT2 DNA²⁹ into yeast strains Y187 cells (*MAT α* , *ura3-52*, *his3-200*, *ade2-101*, *lys2-801*, *trp1-901*, *leu2-3,112*, *gal4 Δ* , *gal80 Δ* , *URA3::GAL1_{UAS}-GAL1_{TATA}-lacZ*) or MaV203 (*MAT α* , *ura3-52*, *his3 Δ 200*, *ade2-101*, *trp1-901*, *leu2-3,112*, *cyh2^R*, *can1^R*, *gal4 Δ* , *gal80 Δ* , *GAL1::lacZ*, *HIS3_{UAS}-GAL1::HIS3@LYS2*, *SPAL10::URA3*),⁴⁰ and the transformants were plated on synthetic medium lacking leucine (SD/–L). Homologous recombination²⁹ between the linearized pACT2 vector and assembled scFv sequences occurs inside the yeast cells efficiently so that the scFv sequences are integrated into the vector, and the yeast cells can grow on the selection medium. Such generated scFv library in yeast cells were harvested and stored frozen until use for library screening.

Yeast two-hybrid scFv library screening

We used hIL8 as the antigen for the library screening. Human IL8 cDNA was purchased from InvivoGen (San Diego, CA). The cDNA encoding the mature sequence of hIL8⁴¹ was cloned into vector pGBKT7 (Clontech), resulting in pGBK–IL8. pGBK–IL8 encodes the fusion of Gal4 DNA-BD with hIL8

at its carboxy terminus. After confirmation of IL8 sequence, pGBK-IL8 was transformed into the yeast strain AH109 (*MAT α* , *ura3-52*, *his3-200*, *ade2-101*, *trp1-901*, *leu2-3,112*, *gal4 Δ* , *gal80 Δ* , *LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3*, *GAL2_{UAS}-GAL2_{TATA}-ADE2*, *URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ*; Clontech). The transformants were selected on synthetic medium lacking tryptophan (SD/-W). AH109 cells containing pGBK-IL8 were mated with *MAT α* -type yeast cells (Y187 or MaV203 strain) containing the scFv library following the protocols from Clontech. Colonies able to grow on the yeast synthetic medium lacking adenine, leucine, histidine and tryptophan (SD/-ALHW) were picked and assayed for *lacZ* expression using β -galactosidase filter-lifting assay as described in the instruction manual from Clontech. Plasmid DNA of pACT2 containing the scFv fragment was retrieved from the yeast cells. The sequences of scFv fragments were determined with an Applied Biosystems, Inc. (ABI) automatic sequencer. Plasmid pGBKT7-Lam (Clontech), which contains sequence encoding fusion protein of Gal4 DNA-BD with human lamin C, was used in the specificity analyses.

Mutagenesis of scFv cDNA and affinity maturation screening

The scFv fragment in pACT2 was mutagenized by using the DiversifyTM PCR random mutagenesis kit from Clontech according to the instructions. The mutagenized PCR products were cotransformed with linearized pACT2 and pGBK-IL8 into yeast MaV203 cells. The transformants were selected on SD/-ALHW with various concentrations of 3-AT. Fast growing clones were selected, and the β -galactosidase activity was quantitatively measured in a liquid assay using ONPG as the substrate³¹ according to the instructions from Clontech.

Protein expression in E. coli

DNA of anti-IL8 scFv clone was cloned in-frame with the leader sequence of pelB at its amino terminus and with the HSV tag and His tag at its carboxy terminus of the expression vector pET27b(+) (Novagen, Madison, WI). The resulting plasmid was used to transform *E. coli* BL21 DE3 (Novagen, Madison, WI). Protein expression of scFv in BL21 DE3 cells was performed according to the instructions from the manufacturer (Novagen). The scFv protein was purified using Ni-NTA beads following instructions from the manufacturer (Qiagen, Valencia, CA). The purified scFv protein was evaluated by SDS-PAGE and immuno-blotting with HSV tag antibody (Novagen).

ELISA analysis

Recombinant human IL8 protein (Pierce) was coated on 96-well Maxisorp plates (Nunc) diluted to 1 μ g/

mL in 50 mM carbonate buffer, pH 9.6. Wells were blocked with SuperBlock (Pierce) for 30 min. Purified scFv proteins were serially diluted in phosphate buffered saline (PBS) containing 0.02% BSA. Binding was detected by HSV tag monoclonal antibody (Novagen) followed by anti-mouse IgG-HRP conjugate (100 pg/mL). After the final washing, the interaction was detected with TMB liquid substrate (Sigma). The reaction was stopped with sulfuric acid, and the optical density was read at 450 nm.

Immunoprecipitation

Recombinant IL8 (50 μ g) was mixed in bicarbonate buffer with 50 μ L of Reactigel. The remaining reactive sites were inactivated by washing with 1M Tris-HCl buffer, pH 8. To 10 μ L of beads from above, single-chain antibodies diluted in PBS containing 0.02% BSA were added, and the beads were rotated overnight at 4°C. The beads were washed three times with 1 mL of PBS containing 0.1% Tween-20. The beads were boiled in SDS-PAGE buffer, resolved on 4–20% gradient gel, and blotted to nitrocellulose membrane. The blot was probed with mouse anti-HSV tag monoclonal antibody (Novagen), followed by alkaline phosphate (AP)-conjugated goat anti-mouse antibody and developed with AP substrate kit from Bio-Rad. In the reverse coimmunoprecipitation experiment, 50 μ g of scFv preparations were coupled to the beads. The remainder of the protocol was essentially the same. The blot was probed with an IL8 monoclonal antibody purchased from Sigma.

Mammalian expression

The human gamma-1 heavy chain expression cassette was assembled which contains a leader sequence, an intron with the C-gene splice acceptor site, followed by the cDNA of human gamma-1 constant region using the standard cloning techniques. The cDNA encoding human gamma-1 constant region was cloned from the cDNA of human leukocytes (Clontech). The human gamma-1 expression cassette was then assembled and cloned downstream of the hEF1-HTLV promoter in pGT60 (Invivogen). DNA sequence of the V_H domain of anti-hIL8 scFv clone #4-123-36 was PCR-amplified from the scFv clone. It was cloned in-frame downstream of the leader and contained the V-gene splice donor site at the 3' end.

The human lambda light chain expression cassette for the V_L domain of 4-123-36 consists of the leader sequence, the V_L, and the C-gene of human lambda light chain cloned from human spleen cDNA (Clontech). The cassette was cloned into the PstI-SmaI locus downstream of the hCMV-1A promoter of pGT60 and replaced the HSV-tk gene. Transient transfections of COS-7 cells were carried out using Lipofectamine transfection reagent according to the manufacturer's descriptions (Invitrogen).

ELISA for detecting antibody expression was performed by coating the Maxisorp (Nunc, Denmark) plates with goat anti-human IgG γ (Biosource International) antibody. The binding of test samples to the coated plates was detected using HRP-conjugated goat anti-human λ (Biosource International) antibody. HRP was detected using the tetramethylbenzidine (TMB) peroxidase enzyme immunoassay (EIA) substrate (Bio-Rad). Immunoprecipitation of the full human antibody from culture medium was performed similar to the procedures described above, except that the western blot was probed with rabbit anti-human IgG antibody (Sigma) and detected using the alkaline phosphatase goat anti-rabbit immuno-blot assay kit (Bio-Rad). For the neutrophil chemotaxis assay, the culture medium was concentrated by approximately fourfold using a Centricon with a molecular weight cutoff of 30 kDa.

Neutrophil chemotaxis assay

The effect of anti-IL8 antibodies was assayed using inhibition of neutrophil chemotaxis in response to recombinant human IL8. The neutrophil chemotaxis assay was performed as described previously.^{42,43} Hank's buffered salt solution (HBSS) containing IL8 (10 ng/mL) alone or with anti-IL8 antibody (Pharmingen) or scFvs were added to the bottom wells of the blind well modified Boyden chamber (New Probe, Cabin John, MD) and incubated for 10 min at room temperature. A 3.0- μ m pore size polycarbonate filter was positioned between the upper and lower chamber, and 50 μ L of isolated neutrophils (2×10^5) suspension was added to upper chamber in each well. The chemotaxis chamber was incubated for 1 hr at 37°C in a CO₂ incubator. The filter was removed, and then cells from upper chamber were fixed in methanol and stained using Diff-Quick. Neutrophils that migrated to the bottom of the filter were counted in three high power fields (400 $\times$). Results are expressed relative to positive control (IL-8 alone).

Accession numbers

The GenBank accession numbers for clones 4-123-36, 4-123-157, 4-123-151, M36-8 and M36-11 are AY059374 to AY059378, respectively.

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