A Single-Step “Breeding” Generated a Diagnostic Anti-cortisol Antibody Fragment with Over 30-Fold Enhanced Affinity

Hiroyuki Oyama, Izumi Morita, Yuki Kiguchi, Tomomi Morishita, Sakiko Fukushima, Yuki Nishimori, Toshifumi Niwa, and Norihiro Kobayashi

Kobe Pharmaceutical University; 4–19–1 Motoyama-Kitamachi, Higashinada-ku, Kobe 658–8558, Japan; and Department of Medical Technology, School of Health Sciences, Tohoku University; 2–1 Seiryo-machi, Aoba-ku, Sendai 980–8575, Japan.

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Cortisol levels in bodily fluids represent a useful index for pituitary–adrenal function, and thus practical anti-cortisol antibodies are required. We have studied “antibody-breeding” approaches, which involve in vitro evolution of antibodies to improve their antigen-binding performances. Here, we produced an antibody fragment to measure serum cortisol levels with over 30-fold enhanced affinity after single mutagenesis and selection steps. A mouse anti-cortisol antibody, Ab-CS#3, with insufficient affinity for practical use, was chosen as the prototype antibody. A “wild-type” single-chain Fv fragment (wt-scFv; $K_a$ 3.4×10⁵ M⁻¹) was prepared by bacterial expression of a fusion gene combining the $V_H$ and $V_L$ genes for this antibody. Then, random point mutations were generated separately in $V_H$ or $V_L$ by error-prone PCR, and the resulting products were used to assemble scFv genes, which were displayed on filamentous phages. Repeated panning of the phage library identified a mutant scFv (scFv#m1-L10) with an over 30-fold enhanced affinity ($K_a$ 1.2×10⁻³⁹ M⁻¹). Three amino acid substitutions (Cys49Ser, Leu54Pro, and Ser63Gly) were observed in its sequence. In a competitive enzyme-linked immunosorbent assay (ELISA), the mutant scFv generated dose–response curves with measuring range ca. 0.03–0.6 ng/assay cortisol, midpoint of which (0.15 ng/assay) was 7.3-fold lower than that of wt-scFv. Although cortisone, 11-deoxycortisol, and prednisolone showed considerable cross-reactivity, the mutant scFv should enable sensitive routine cortisol assays, except for measurement after metyrapone or high-dose of prednisolone administrations. Actually, cortisol levels of control sera obtained with the scFv-based ELISA were in the reference range.

Key words antibody; single-chain Fv fragment; cortisol; in vitro evolution; affinity maturation; enzyme-linked immunosorbent assay

Immunosassays are essential tools for monitoring various biomarkers in bodily fluids because of the excellent specificity conferred by antigen–antibody reactions. Currently, most diagnostic antibodies are produced by B-cell hybridoma technology, which generates “native” antibodies (in vivo antibodies) induced in animals by immunization as cloned products ensuring constant binding abilities. However, the limited B-cell clone repertoire in mammals often prevents the generation of antibodies with practical performance. In particular, the equilibrium affinity constant ($K_a$) of antibodies against small biomarkers (i.e., haptenic compounds) rarely exceeds the 10⁻¹⁰ M⁻¹ range.

We have employed an “antibody-breeding” approach to overcome this limitation inherent to native antibodies and to enable subfemtomole detection of small molecules. Using this approach, the antigen-binding affinity can be enhanced by in vitro mutagenesis and subsequent selection of improved species (i.e., in vitro affinity maturation). Typically, the antibody of interest is converted to the corresponding single-chain Fv fragment (scFv) or Fab fragment by gene manipulation. Then, random or site-directed mutations are introduced into the genes encoding the variable heavy and light domains ($V_H$ and $V_L$) to generate a diverse “antibody library,” from which “improved binders” (fragment mutants with improved functions) are selected. Based on these strategies, we have succeeded in enhancing the affinity of anti-estradiol-17β(14,15) and anti-cotinine(15) antibody fragments by >150-fold and >40-fold, respectively. We also showed that these improved scFv molecules could be used in clinical applications.

Cortisol is the major glucocorticoid in humans and is used as a biomarker for the functions of hypothalamic–pituitary–adrenal axis, and thus practical anti-cortisol antibodies have been in great demand as diagnostic reagents. However, only a few publications have demonstrated the production of monoclonal antibodies capable of targeting serum or urinary cortisol. We recently established several hybridoma clones producing a mouse anti-cortisol antibody. As part of our research mentioned above, we planned to generate anti-cortisol antibody fragments with practical sensitivity. To evaluate the potential of the antibody-breeding approach, we chose an antibody Ab-CS#3 as the prototype antibody, which showed somewhat insufficient affinity ($K_a$ 4.7×10⁻¹⁰ M⁻¹) for clinical use, from the panel of monoclonal anti-cortisol antibodies. This antibody was converted to the scFv form and improved to a mutant species with over 30-fold greater $K_a$, which reached 10⁻⁶ M⁻¹ range, via single step mutagenesis and subsequent selection.

MATERIALS AND METHODS

Steroids and Steroid-Protein Conjugate Steroids, including cortisol 3-(O-carboxymethyl)oxime (CS-3-CMO) (Fig. 1), were purchased from Sigma (St. Louis, MO, U.S.A.). [1,2,6,7-³²H]-cortisol (3.4 TBq/nmol) was obtained from PerkinElmer, Inc. Japan (Yokohama, Kanagawa, Japan). CS-3-CMO was linked with bovine serum albumin (BSA;
The single-stranded oligo-DNAs used for PCR amplification were synthesized and purified by Tsukuba Oligo Service (Ushiku, Ibaraki, Japan). The nucleotide sequences of the primers used are listed in Table 1.

### Table 1. Nucleotide Sequences of PCR Primers Used in This Study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′−3′; Restriction site)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS#3VH-Rev</td>
<td>CTGCGGCCCAAGCCGGCCATGGCCCAGGTCCAACTGCAGCAGCCTG (Ncol)</td>
</tr>
<tr>
<td>CS#3VL-For</td>
<td>TGAACCGCCTCCACCGCTCGAGACTGCAGAGACAGTGACCAGAGTCTGGGGCTCAACTTTCTTTGCGGCCGCAGCCCGTTTTATTTCCAGCTTG</td>
</tr>
<tr>
<td>CS#3VL-Rev</td>
<td>GGATCCGGCGGTGGCGGGTCGACGGACATTGTGCTGACACAGTCTC</td>
</tr>
<tr>
<td>CS#3VL-For</td>
<td>GGGTCAACCATTCTTTTCCGCCGCCAGCCCCTTTTATTTCCAGCTTG (NotI)</td>
</tr>
</tbody>
</table>

*Underlined in the nucleotide sequences.*
glucose, and incubated overnight at 37°C. Colonies were then scraped into 2×YT medium containing 15% glycerol (1.5 mL) and a small aliquot was used for bacteriophage rescue with the aid of VCSM13 helper phage (Agilent Technologies), as described previously.13,23,24) The resulting phages were used in the next round of selection.

Preparation of Soluble (Non-phage-Linked) scFvs
Recombinant plasmids were extracted from infected bacterial clones and digested with NcoI and NolI to cut down scFv genes therein, which were then ligated into a variant of pEXmide 7 vector with TAA TGA double stop codons after the FLAG sequence (pEXmide 7/uni vector). The resulting plasmids were transformed into XL1-Blue cells, and transformants were grown and induced with isopropyl β-D-thiogalactopyranoside and sucrose.13,24) Periplasmic extracts containing soluble scFv proteins were then prepared and used for enzyme-linked immunosorbent assays (ELISAs) without further purification.

Competitive ELISA Using scFv-Phages
Ninety six-well RIA/EIA #3590 microplates (Corning, Corning, NY, U.S.A.) were coated overnight at room temperature with CS−BSA in 0.10 M carbonate buffer (pH 8.6) (1.0 µg/mL) and blocked with M-PBS at 37°C for 2.0 h.13) Wells were then washed three times with T-PBS, and incubated at 37°C for 1.0 h with a mixture of cortisol dissolved in G-PBS (50.0 µL/well) and scFv-phages diluted with M-PBS (100 µL/well). Subsequently, wells were washed and probed using an anti-M13 antibody labeled with peroxidase (POD) (GE Healthcare UK Ltd., Amersham Place, Little Chalfont, Buckinghamshire, U.K.) diluted in PVG-PBS (0.50 µg/mL) (100 µL/well). After incubation at 37°C for 30 min, the wells were washed and captured POD activity was determined colorimetrically at 490 nm, using o-phenylenediamine as the hydrogen donor.24,25) In these assays, the concentrations of scFv-phages were adjusted to give bound enzyme activities at B0 (the reactions without unlabeled CS or analogs) of ca. 1.0–1.5 absorbance after a 30-min enzyme reaction.

Competitive ELISA Using Soluble scFv Proteins
Ninety six-well RIA/EIA #3590 microplates (Corning) were coated with CS−BSA and blocked as described above. Wells were incubated at 4°C for 2.0 h with a mixture of cortisol (or related compounds) dissolved in PVG-PBS (50.0 µL/well) and soluble scFv protein diluted with G-PBS (100 µL/well). Subsequently,
wells were washed and probed with POD-labeled anti-FLAG M2 antibody (Sigma) diluted in PVG-PBS (0.20 μg/mL) (100 μL/well). After incubation at 37°C for 30 min, wells were washed and captured POD activity was determined as described above. In these assays, concentrations of scFvs were adjusted to give bound enzyme activities at B0 of ca. 1.0–1.5 absorbance units after a 30-min enzyme reaction.

**Measurement of Serum Cortisol Levels with Improved scFv**
Commercially available control sera (Bio-Rad Lyphochek Immunoassay Plus Control, Levels 1, 2, and 3) and serum samples obtained from healthy volunteers (5 males, aged 20–52 years old and 12 females, aged 18–24 years old) were each diluted with G-PBS, and submitted to the competitive ELISA (see above) using the improved scFv (scFv#m1-L10; soluble proteins). For the measurement of serum specimens from the volunteers, protocol approval was obtained from an institutional review board.

**RESULTS AND DISCUSSION**

**Generation of the Wild-Type Anti-cortisol scFv**
Improvement of antibody molecules by constructing genetically-engineered antibody libraries requires conversion of target antibodies into the relevant smaller fragments, i.e., scFv or Fab fragments, to facilitate their bacteria expression. Thus, we first generated the wild-type scFv derived from mouse Fab fragments, to facilitate their bacteria expression. Thus, antibodies into the relevant smaller fragments, engineered antibody libraries requires conversion of target genes. DNA encoding FLAG tag was added to the 3’-end to facilitate purification and detection. This wt-scFv gene, with the structure 5’-VH-linker-VL-FLAG-3’, was expressed in E. coli cells. Typically, a 1-L culture of the transformed bacteria yielded ca. 200 μg scFv protein, the characteristics of which are described below.

**In Vitro Affinity Maturation of scFv**
Previously, we generated several improved scFvs against estradiol-17β, cotinine, and Δ9-tetrahydrocannabinol with higher affinities by the antibody breeding (in vitro molecular evolution) strategy. In these studies, the entire VH and VL genes were separately randomized by error-prone PCR, and then combined in a shuffling manner to assemble scFv genes before being expressed as phage-displayed proteins. In the present study, we randomly mutated either the VH or VL gene to investigate which domain is more effective as target of mutagenesis based on improved antigen-binding affinities in the scFvs.

Thus, the VH and VL regions of the wt-scFv gene were amplified separately by single error-prone PCR under the “dATP-diminished” condition, and the resulting VH or VL randomized genes were spliced with the wild-type gene encoding the other domain to create 2 diverse sets of scFv genes (Fig. 2A). Transformation of E. coli cells with these scFv mutants generated “VH-randomized” and “VL-randomized” bacterial libraries, each of which contained ca. 106 transformants with scFv genes. From these libraries, phage particles were separately rescued, combined, and then subjected to 3 rounds of panning against polystyrene tubes coated with CS–BSA. After the third panning, 96 phage clones were randomly selected from the bacterial libraries infected with recovered phages, and screened for cortisol-binding ability. Consequently, 3 improved clones, each displaying an scFv mutant, named scFv#m1-L7, scFv#m1-L9, and scFv#m1-L10, were isolated.

As shown below, these scFvs had amino acid substitution in the VH region, thus were generated from the VL-randomized library.

**Characterization of Wild-Type and Mutant scFvs**
In the ELISAs, involving competitive reactions between immobilized cortisol residues and standard cortisol, the 3 improved phage clones generated dose–response curves with >10-fold higher sensitivity based on comparison of the midpoint (i.e., the amount of standard cortisol required for 50% binding) than with the wild-type phage (Fig. 3A). The primary structures of the wild-type and improved mutant scFvs are illustrated in Fig. 2B, with the sequences of the complementarity-determining regions (CDRs) shown. The VH and VL sequences of these scFvs belonged to subgroup IIB and III, respectively, based on the Kabat definition; thus, their structures should be rather different from that of the anti-cortisol scFv derived from Abs (its VH and VL sequences belonged to IB and II, respectively) that we previously reported. We found that 5, 2, and 3 amino acids were substituted in scFv#m1-L7, scFv#m1-L9, and scFv#m1-L10, respectively. Interestingly, the Cys→Ser substitution in the second framework region in the VL domain (defined based on the Kabat definition) occurred commonly in these mutants. In the antibody variable domains, Cys residues generally appear only at fixed positions (for VH, 22 and 92; for VL, 23 and 88) and are strongly conserved to form intra-chain disulfide bonds necessary for adequate protein folding. Thus, the Cys at the position 49 in VL, where

![Fig. 3. Dose–Response Curves for Cortisol ELISAs (A) Performed Using Phages Displaying wt-scFv (●), scFv#m1-L7 (△), scFv#m1-L9 (■), and scFv#m1-L10 (○), or (B) Using Soluble wt-scFv (●) and scFv#m1-L10 (○) Proteins and the Parent Mouse Antibody Ab-CS#3 (∗)](image)
highly conserved Tyr residue appears for native antibodies, is quite an unusual residue and may have reflected an obstacle towards higher affinity with the wild-type sequence.

Because scFv#m1-L10 showed the highest sensitivity among the phage-displayed scFvs (Fig. 3A), it was then prepared as a soluble protein and further characterized in comparison with the soluble form of wt-scFv. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, affinity-purified wt-scFv and scFv#m1-L10 proteins migrated as single bands with nearly the expected relative molecular mass ($M_r$) values of 27289 and 27227 (calculated based on their primary amino acid sequences), respectively (Fig. 4A). The $K_a$ was determined at 4°C by Scatchard analysis using tritium-labeled cortisol as a tracer. The mutant exhibited a $K_a$ of $1.2 \times 10^{10} M^{-1}$, which was >30-fold greater than the $K_a$ of wt-scFv ($3.4 \times 10^8 M^{-1}$) (Fig. 4B). Protein modeling suggested that 4 CDRs, i.e., V$_H$-CDR3, V$_L$-CDR3, V$_L$-CDR1, 2, and 3 may be located within close proximity to the antigen (cortisol). Although no apparent alteration in paratope conformation was observed, cortisol docked with the paratope from different angle between the before and after the amino acid substitution (Fig. 4C).

In the ELISA experiments, soluble wt-scFv showed a dramatically more sensitive dose-response curve with a lower midpoint (1.1 ng/assay) (Fig. 3B) than the phage-displayed wt-scFv and parent antibody Ab-CS#3 (midpoints: 100 and 28 ng/assay, respectively; Figs. 3A, B). This increase in assay sensitivity, when wt-scFv was used, could be explained based on not only the greater $K_a$ of wt-scFv than the parent IgG-form antibody ($K_a$, $4.7 \times 10^7 M^{-1}$) (it is unclear why the scFv form showed stronger affinity than the relevant IgG form), but also its monovalent structure, which is less susceptible to avidity effects that should occur in ELISA systems based on the binding of multiply immobilized antigens on microplates and bivalent IgG-form antibodies. Although it is unlikely that wt-scFv-displaying phages contained a considerable fraction of virions displaying more than 2 scFvs, several virions each having a single scFv might aggregate to form multivalent complexes.

The scFv#m1-L10 mutant showed a dose-response curve with 7.3-fold higher sensitivity than that of wt-scFv based on comparison of midpoints (0.15, 1.1 ng/assay for the mutant and wild-type, respectively), measuring range of which was ca. 0.03–0.6 ng/assay (Fig. 3B). The limit of detection (0.014 ng/assay) was determined as the amount of cortisol required for a bound absorbance 2 standard deviations below the average ($n=10$) of the $B_o$ absorbance.

The cross-reactivities of wt-scFv and scFv#m1-L10 were determined for 8 endogenous and 3 synthetic steroids (Fig. 5). Although entire recognition patterns were similar for both
scFvs, the mutant exhibited higher specificity as shown with decreased cross-reactivities with cortisone, prednisolone, and prednisone. Almost negligible cross-reactivity with corticosterone (<0.2%) and aldosterone (<0.01%) indicated that both scFvs recognized modifications at the C-17 and C-18 positions of cortisol. Regarding the C-11 position, however, these scFvs lacked satisfactory recognition. 11-Deoxycortisol, the biosynthetic precursor of cortisol lacking the 11-hydroxy group, showed rather stronger reactivity than cortisol (>100%). Cortisone, an active metabolite with a ketone group at the C-11 position, also cross-reacted for wt-scFv (45%), but this was significantly improved after the mutation (17%). Among the exogenous steroids, prednisolone with an additional carbon–carbon double bond exhibited 18% cross-reactivity for wt-scFv, which was dramatically improved for the mutant scFv (3.3%).

Considering these recognition patterns, we expected that the mutant scFv could be applicable for clinical use, except in case where patients were under metyrapone-stimulation tests or administered high-dose of prednisolone: in the former conditions, serum 11-deoxycortisol levels dramatically increase. In normal subjects, however, the 11-deoxycortisol levels are usually much lower (<10 ng/mL) than cortisol levels (10–250 ng/mL) and thus this precursor should not cause substantial overestimation. Recently, the average cortisone/cortisol concentration ratio in serum was reported to be 0.225. Taking into account of the cross-reactivity of the mutant scFv (see above), overestimation due to cortisone, if any, would not cause serious problem.

In fact, for commercially available control sera, we obtained acceptable assay values with a reasonable parallelism between the sample dilution rates (Fig. 6). Average serum levels for healthy male and female subjects were 23±5.5 (n=5) and 23±5.6 (n=12) µg/dL (total levels, 23±5.4 µg/dL), respectively, which agreed with the reference ranges.

CONCLUSION

Previously, we generated a mutant scFv clone against the nicotine metabolite, cotinine, with >40-fold enhanced

Fig. 5. Percent Cross-Reactivity of scFvs in ELISA Determined by the 50% Displacement Method According to the Following Equation

\[
\text{Cross reactivity (\%) = (X/Y) \times 100; where X is the midpoint (ng/assay) of cortisol, and Y is the midpoint (ng/assay) of a related steroid cross-reactivity of which is to be determined.}\]

The competitive reactions in ELISA were performed at 4°C for 2.0 h.

Fig. 6. Serial Dilution Study for Measuring Serum Specimens

Control sera (levels 1, 2, 3; see text) were serially diluted and analyzed by ELISA using scFv#m1-L10. The competitive reactions were performed at 4°C for 2.0 h. The vertical bars indicate the standard deviation (n=4).
antigen-binding affinity (calculated as the $K_a$). This was achieved via “1-shot” in vitro evolution (i.e., via single-step mutagenesis and subsequent selection) and resulted in 5 amino acid substitutions. Here, we succeeded again in improving the $K_a$ by >30-fold by single-step mutagenesis and selection with an scFv targeting another small biomarker, cortisol, with only 3 amino acid substitutions concentrated on the $V_L$ domain. In this study, we selected a mouse anti-cortisol antibody (Ab-CS#3) with a practically insufficient affinity as the prototype. As expected, the antibody-breeding approach improved the affinity to produce a novel species with practical binding performance.

A limitation of this study is that cannot explain why the $V_L$-randomized library did not generate any improved scFv clones. We are unsure whether no improved clones were actually generated, or whether this outcome simply depended on inadequate experimental parameters. Next, we will explore affinity-mutation of the present $V_L$-substituted mutants by randomizing its $V_H$ domain. The phagemid vector pEXmide7 was used in this study, which allows for independent subcloning of randomized $V_H$ and $V_L$ genes, is suited for such a chain-shuffling strategy. 

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**Conflict of Interest**

The authors declare no conflict of interest.

**REFERENCES**


