論文の内容の要旨
Abstract of Dissertation

論文題目：Affinity and specificity maturation of anti-hapten antibodies by Open-sandwich selection

（オープンサンドイッチ選択による抗ハプテン抗体の親和性・特異性成熟）

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During the past time, accurate detection of haptens are widely required because of their use in clinical in vitro diagnostics and environmental or food monitoring. However, all the conventional methods have their own drawbacks. To overcome these demerits, open-sandwich immunoassay (OS-IA) was established for the noncompetitive detection of small molecules. The principle of OS-IA is based on the association of the antibody variable region (VH and VL) in the presence of the corresponding antigen. In order to obtain highly specific and sensitive binders to haptens, here we introduced a novel selection method Open-Sandwich (OS) selection. In this study we performed OS selection to select better binder for 17β-estradiol (E2) from a randomized library (Chapter 2) and binders for analogous steroids progesterone, estriol, and cortisol from a binary code library (Chapter 3). These targets are the most commonly measured steroid compounds.

On the first chapter of PhD thesis, I overviewed this area and made introduction to the following two topics: 1) Affinity and specificity maturation of anti-E2 antibody by OS selection from a randomized library, and 2) Selection of anti-steroid antibodies by OS selection from a binary code library.

In Chapter two, the affinity and specificity maturation of anti-E2 antibody by OS selection from a randomized library is described. The first step was to construct a randomized library. The VH domain was diversified using in vitro mutagenesis called error-prone PCR, and the randomized fragments were ligated to a phagemid vector and transformed to *E. coli*. After V_H-displaying phages were produced from the bacteria, three rounds of OS biopanning were performed, and a mutant that showed improved signal was obtained. To estimate the affinity and specificity of the mutant, OS phage ELISA with immobilized MBP-VL was performed. According to the dose-dependency for E2, the mutant exhibited increased E2-dependent signal compared with the wild type, suggesting its

higher affinity and suitability to OS-IA. However, for other E2 analogs of testosterone, estriol, cortisol and progesterone, the mutant showed much lower cross-reactivity than the wild type.

To further confirm the affinity and specificity of the clones, chemiluminescence-based (CL) OS- and competitive ELISAs were performed. For CL OS-ELISA, the result was consistent with the result of phage OS ELISA, suggesting the mutant's increased affinity for E2 and selectivity against the analogs. Due to higher sensitivity of the CL assay than the chromogenic assay, the detection sensitivity for E2 was improved compared with that of phage OS ELISA, as judged by their EC_{50}s. In the case of competitive ELISA with single-chain Fv (scFv) fragments, free E2 exhibited stronger inhibition to the binding of the mutant scFv to E2-BSA, than the wild-type scFv. However, the rest of analogs exhibited much weaker inhibition for the mutant than the wild-type, also confirming the increased affinity and specificity of the mutant. The calculated cross reactivity suggested that the mutant was specific enough for clinical diagnostics for E2.

After nucleotide sequencing of the mutant and wild-type, we found that the mutant had one mutation at site 85 compared with the wild type, changing from E (glutamine) to K (lysine). Assuming the similar property of amino acid R (arginine) to K (lysine), the residue was substituted by amino acid R (arg) using site directed mutagenesis by SOE PCR to construct the mutant E85R. The result of OS phage ELISA showed that the affinity and specificity of E85R are both decreased compared with the original mutant, demonstrating the importance of the original mutation (E to K).

In the third chapter of this thesis, an attempt to select novel anti-steroid antibodies by OS selection from a binary code library was described. The recognition of antigen by antibody variable region is mainly mediated by the complementarity determining regions (CDRs), because each CDR region gives a large surface to contact with the corresponding antigen. Several studies revealed that the CDR regions of the known antibodies are rich in the amino acids Y (tyrosine) and S (serine), maybe because these two kinds of amino acids are suitable for molecular recognition. As the most contacts with antigen were mediated by Y and S, it has been shown possible to develop a library that can be used as an universal source of antigen binders. However, it has not been clear that the above strategy is also applicable to anti-hapten antibodies. Hence, here I chose estrone, estriol, progesterone, cortisol, and testosterone as the target haptens. To begin with the construction of the library, the CDR region of VH domain was introduced by a binary Y/S diversity through SOE PCR. Using the diversified VH fragments, a VH-displaying phage library with a size of about 107 was constructed. For the selection, MBP-fused VL was immobilized, and VH-phage and target haptens were incubated to select for specific antigen binders. For efficient selection, increasing selection stringency was adopted through reducing the amount of the corresponding antigen from one round

to another. After three rounds of biopanning, polyclonal OS phage ELISA with the same concentration of phage was performed to estimate the efficacy of the selection. In case of estriol, the signal was increased from round 1 to round 3, but in cases of the rest of four targets, round two showed the highest signal.

In the fourth chapter, I used the combinatorial mutagenesis to try to improve the affinity and specificity of the anti-estradiol antibody. Because the mutant M2 we obtained in the previous study showed a much higher signal than the wild type and the rest mutants, and the mutant m6 also showed increased signal compared with other mutants, we decided the mutation position 63 at M2 and mutation position 85 at m6 as the mutation positions in this combinatorial mutagenesis library construction. In this combinatorial mutation chapter, three libraries were constructed, namely, H63, H85, and H63+85 respectively. H63 is originally Phe in the wild type, which was mutated to Tyr in M2, and this position is randomized to 8 mainly hydrophobic residues F/Y/L/H/I/N/V/D. Also, H85 is randomized to 12 hydrophilic residues using codon (CAG)(CAG)N. After one round of OS selection, 96 colonies were picked up for monoclonal ELISA. The result demonstrates that the affinity and specificity of the mutant E85D are improved at the same time, however, the specificity improvement is more obvious.

In the fifth chapter of the thesis, I described the conclusion and perspective. Compared with the conventional antibody selection methods, antigen conjugation is not necessary in OS selection, and the selected V_H mutants could be directly applicable for OS-IAs. This system will be applied to develop sensitive detection systems for many small molecules.