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Original article

Optimization and modification of anti-rhTNF-α single chain variable fragment antibody: Effective in vitro affinity maturation and functional expression of chimeric Fab

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ABSTRACT

Aims: Single chain variable fragment (scFv) is one of the most popular recombinant antibody (rAb) formats. However, sometimes scFv with the most favorable specificity profile lack sufficient affinity or acceptable pharmacokinetics for clinical applications. To address these problems, we described a method to modify recombinant anti-rhTNF-α scFv-F6D2E7.

Results: Random mutations were inserted into CDR-H3 by performing PCR with tailored degenerate primers. After construction of a mutated antibody gene library, affinity selection was performed. Meanwhile the scFv (scFv-G10) selected from the library exhibited the most improved affinity to rhTNF-α (2.9-fold higher than the parental scFv-F6D2E7). The scFv-G10 sequence and human constant (CH1 & CL) regions were used to construct a novel vector for developing an expression system that allows the production of a completely functional antigen-binding fragment (Fab) in *Escherichia coli*. The bioactivity of the Fab was determined by L929 cell cytotoxicity assay. Fab-G10 could neutralize rhTNF-α-induced cytotoxicity to L929 cells, and the calculated 50% inhibition rate (IC50) was 5.0×10^{-7} M.

Conclusion: We generated an artificial antibody fragment (scFv-G10) that had improved affinity and desirable specificity. Further, the Fab-G10 was constructed and expressed in *E. coli*, where the bioactivity was further detected.

1. Instruction

Recently, small recombinant antibody (rAb) fragments are increasingly being used as alternatives to monoclonal antibodies (mAb) in medical diagnostic and therapeutic applications. One of the most popular types of rAbs is single-chain variable fragment antibodies (scFvs) as they have been successfully modified into a number of antibody formats and are easily expressed by various expression systems [1]. To generate a desired scFv fragment, phage display-based strategy is widely used because it can present scFvs on bacteriophage particles [2–5]. Further binding selections can be used to enrich for clones that bind to antigen of interest and protein sequences can be decoded from the DNA, respectively. However, the affinity of antibodies derived from phage display is usually not high enough for lacking antigen-driven affinity maturation process. On the other hand, high affinity is always required for clinical application of genetic engineered antibodies. Another weakness of scFv is that small molecular weight results in short half-time and non-acceptable pharmacokinetics for clinical applications.

Complete antibodies are made up of two regions: functional antigen-binding fragment (Fab) and fragment crystallizable region (Fc). The Fab fragments consist of variable regions including antigen-binding site. The Fc fragments allow the antibodies to recruit additional components from the immune system with the Immunoglobulin G Fc Receptor II (FcgR) [6]. Fab alone couldn't induce antibody-dependent cell-mediated cytotoxicity (ADCC) in human body. In current clinical studies, Fab fragments have been proved to be safer and less immunogenic than the whole antibody, which sometimes causes side effects by FcgR activation [7]. In addition, comparing to scFv, Fab is more functionally stable and has better toxin neutralization capacity in vivo [8].

In this study, an affinity maturation method named "CDR-H3 shuffling" was utilized to improve the affinity of a well-characterized scfv-F6D2E7 selected from *Griffin 1* phage library in previous studies [9]. The scFv fragment showed a practical specificity but relatively lower affinity to recombinant human tumor necrosis factor α (rhTNF-α). A library in which heavy chain complementarity determining region 3 (CDR-H3) of each scFv member had been randomly mutated was generated. From the mutant library, a mutated scFv clone (scFv-G10) was selected

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Table 1
Nucleotide sequences of primers used for the PCR to construct the mutated scFv libraries and expression vector and co-express vector.

Primer	Sequence (5′ → 3′)
For construction of mutated scFv libraries	
P_{VHS}[a]	GGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGG
P_{VHA}	CTGACCCCAGTAGTCC NNN NNN NNN NNN NNN NNN NNN NNN NNN CGAGCGCAGTAGT
P_{FRS}	TGGGGTCAGGGTACTCTCGTTACCGTCTCGAGTGGTGG
P_{FRA}	CGGCCCCGTGATGGTGATGATGATGTGCGGCCGCACG
P_{EXPS}[b]	GGCAGCCGCTGGATTGTTATTACTCGCGGCCCAGCCGGCCATGG
P_{EXPA}[c]	CGGCCCCGTGATGGTGATGATGATGTGCGGCCGCACG
For construction of Fab co-express vector	
P_{VHsFab}	GGATATCGGAATTAATTCGGCTCAGGTTCAGCTGCTGC
P_{VHaFab}	GAGCCAGAGGGAAGACGGAGACGGTAACGAGAGTACCC
P_{VLsFab}[d]	TGTGGATCCGAGTGCACTTGCCATCC
P_{VLaFab}	TCAGGACGGTCAGCTGGGTGCCCGCACGTTTGATTTCCACCTTG
P_{CLsFab}	CAAGGTGGAAATCAAACGTGCGGGCACCCAGCTGACCGTCCTGA
P_{CLaFab}[e]	TGTGAATTCTTGCTGCACTCAGCAGG
$P_{CH1sFab}$	GGGTACTCTCGTTACCGTCTCCGTCTTCCCTCTGGCTC
$P_{CH1aFab}$[f]	CCGCTCGAGAACCTTCTTATCCACCTT
$P_{StopsFab}$[e]	CCGGAATTCGTGAGAAGGAGATATACATATG
$P_{pelBaFab}$	GCAGCAGCTGAACCTGAGCCGAATTAATTCCGATATCC

[a] The Sfi I recognition site is underlined.
[b] The Nco I recognition site is underlined.
[c] The Not I recognition site is underlined.
[d] The BamH I recognition site is underlined.
[e] The EcoR I recognition site is underlined.
[f] The Xho I recognition site is underlined.

which showed a higher affinity than scFv-F6D2E7. Then, the sequences of G10-scFv and human constant (CH1 & CL) regions were used to generate a novel construct for developing an expression system that produces completely functional antigen-binding fragment of Fab-G10 in *Escherichia coli*. The bioactivity of the Fab was determined in L929 cells through cytotoxicity assay. To our knowledge, this is the first study of anti-rhTNF-α mAb Fab. Our high affinity and specificity Fab-G10 could be potentially used in medical diagnosis and clinical treatment in the future.

2. Materials and methods

2.1. Materials

2.1.1. Bacterial strains

Bacterial strains TG1 (supE hsdΔ5 thi Δ [lac-proAB] F' [*traD36 proAB+ lacIqlacZΔM15*]) was used for phage antibody production. The HB2151 bacterial host strain (*nalr thi-1 ara Δ* [*lac-proAB*] F' [*proAB+ lacIq lacZΔM15*]) was used for soluble antibody production. The BL21 (*DE3*) (*F-ompT hsdSB* [*rB-mB-*] *gal dcm* [*DE3*]) bacterial host was used for large amount expression of the Fab fragment.

2.1.2. Oligo-DNAs

Single-stranded oligo-DNAs used for PCR primers were synthesized and purified by Invitrogen. DNA degeneracies are represented in the IUB code (N = A/C/G/T, R = A/G, Y = C/T). Degenerate codons are shown in bold text. The following mutagenic oligonucleotides were used for library and co-expression vector constructions (Table 1).

2.2. Methods

2.2.1. Construction of the scFv library by CDR-H3 shuffling and isolation of scFv clones with improved affinity from the library

The library of scFv mutation was constructed by CDR-H3 shuffling as below (Fig. 1). Briefly, primers were designed to introduce random mutations to CDR3 of VH. PCR was carried out in 50 μl of the following buffer solution: 200 mM Tris-HCl (pH 8.0), 200 mM KCl, 200 mM $(NH4)_2SO_4$, 20 mM $MgSO_4$. The pHEN2 vector containing scFv-F6D2E7 gene fragment (1ng) was mixed with P_{VHS} and P_{VHA} primers (or P_{FRS} and P_{FRA}) (0.1nmol each), EasyTaq DNA polymerase (TransGen Biotech) (5U), and dNTPs. This mixture was amplified for 30 cycles at 94 °C (30s), 55 °C (30s), 72 °C (30s), followed by a 7 min extension at 72 °C. Equal amounts of the resulting two PCR products were combined and used as the template for the following PCR. Two resulting PCR fragments were assembled by 10 cycles of PCR without primers and then amplified with external primers P_{VHS} and P_{FRA}. The final PCR product was agarose-purified, digested with restriction enzymes *Sfi*I and *Not*I, and cloned into pHEN2. A ligation mixture was used to transform the TG1-competent cells to get a mutant library with a complexity of 10^6 members.

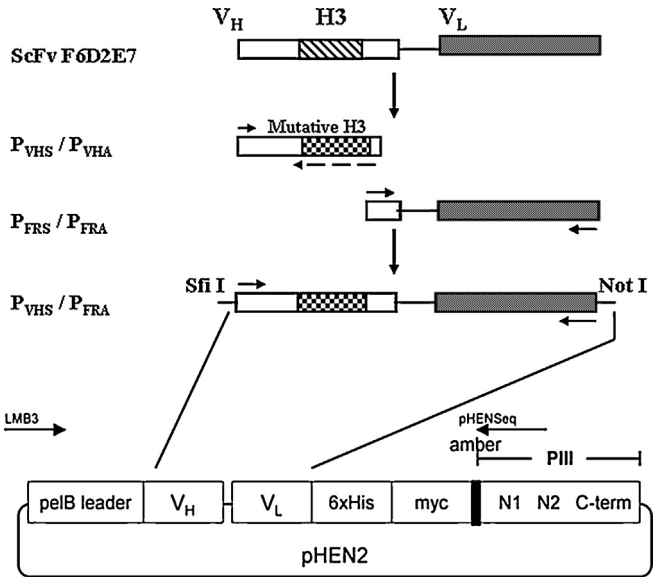

Fig. 1. Construction of phage display library vector with mutative CDR-H3.