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Construction and Characterization of a Single-Chain Immunoglobulin

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Abstract : We constructed a single-chain immunoglobulin in which the carboxyl end of the heavy chain variable domain is covalently joined to the amino terminus of the light chain variable domain via peptide linker and the carboxyl end of the light chain variable domain is linked to human γ1 Fc region through the hinge region. The molecule was expressed in Chinese hamster ovary cells, assembled into a dimeric molecule and secreted into the culture medium. The dimeric molecule (2E11) was purified from the culture supernatant by affinity chromatography on Protein G-Sepharose column. The size of the unreduced or reduced protein was the expected molecular weight of approximately 120 or 60 kDa, respectively, as assessed by SDS-polyacrylamide gel electrophoresis. The antigen-binding affinity of 2E11 was almost the same as that of a native antibody counterpart (CS131A), suggesting that the single-chain immunoglobulin may function like a native antibody.
Key words : antibody, immunotherapy, protein engineering, single-chain immunoglobulin

An antibody molecule consists of two heavy chains and two light chains linked together by disulfide bonds. Each chain has one variable region and one constant region. The heavy chain variable region (VH) and the light chain variable region (VL) bind to antigen and the heavy chain constant regions mediate effector functions that eliminate antigen. The domain structure of an antibody makes it particularly accessible to protein engineering because the functional domains can be used separately as fragments or swapped between antibodies (Winter and Milstein, 1991). However, expression of a functional antibody molecule is highly inefficient, since the heavy and light chains of an antibody are encoded by two separate genes.

Single-chain Fv fragments (ScFvs) in which the VH and the VL are connected via peptide linker have been produced (Bird *et al.*, 1988; Huston *et al.*, 1988). In many cases, such ScFvs retain the specificity and affinity of the antibody and thus have been considered useful agents in the development of immunotherapeutic and immunodiagnostic applications (Milenic *et al.*, 1991; Yokoda *et al.*, 1992). The ScFv molecule per se, however, will not be sufficient for therapeutic use in its native form because the cytolytic functions of antibody such as antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) are mediated by the Fc region. Therefore, fusion of the ScFvs to the Fc region to generate immunoglobulin-like single-chain immunoglobulin will be useful in the development of therapeutic antibodies. For example, this approach for the construction of a single-chain immunoglobulin may be applied to efficient generation of a bispecific antibody molecule derived from two different monoclonal antibodies, since the purification and yield of desired bispecific antibodies are notably difficult and low, due to the random association of the heavy and light chains of two different antibodies and the preferential pairing of the homodimer of heavy chain (Songsivilai and Lachmann, 1995).

In this study, we have generated a single-chain immunoglobulin from Chinese hamster ovary cells and characterized its biochemical properties.

Materials and Methods

Construction of a single-chain immunoglobulin gene

To construct the single-chain immunoglobulin, we used the VH and VL of a murine mAb H67 with specificity for the hepatitis B surface antigen and the Fc region of human γ1 (Fig. 1). The VH with the leader sequence

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or the VL was synthesized from pMHV-S or pMKV-S (Ryu *et al.*, 1994) by polymerase chain reaction (PCR) using primers 1 and 2 or primers 3 and 4, respectively. Primer 2 for the VH and primer 3 for the VL had an overlapping nucleotide sequence encoding a linker peptide $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$. The resulting VH and VL sequences were fused by recombinant PCR to yield a 900-bp DNA segment encoding the ScFv. The sequence encoding the human γ1 Fc region containing hinge, CH_2 and CH_3 was also synthesized from pCHC-S2 (Jin *et al.*, 1995) by PCR using primers 5 and 6 then fused to the 3'-end of the ScFv gene by recombinant PCR to yield the single-chain immunoglobulin gene. The sequences of the PCR primers are summarized in Table 1.

The resulting 1745-bp DNA encoding the single-chain immunoglobulin was digested with *Eco*RI and *Sal*I and subcloned into pBluescript SK(+) to yield pSGIG-S. The nucleotide sequence of the single-chain immunoglobulin gene was determined using Sequenase (USB, USA) according to the protocol suggested by the supplier.

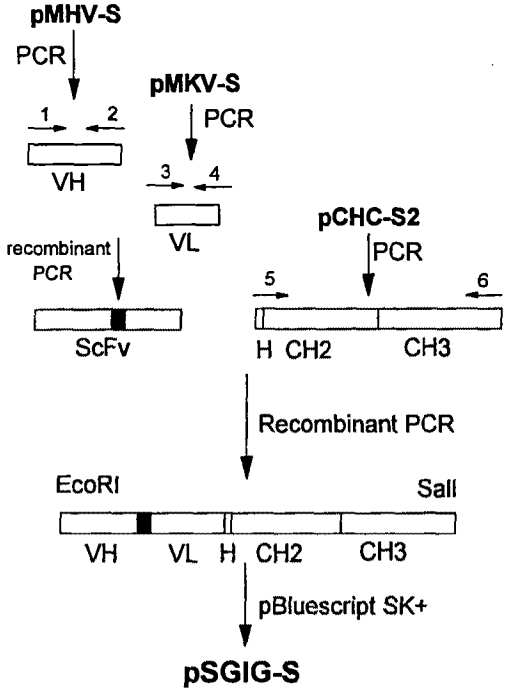

Fig. 1. Strategy for the construction of the single-chain immunoglobulin. The dark box indicates the peptide linker. The nucleotide sequences of the primers (1-6) are listed in Materials and Methods.

Construction of expression plasmid

The single-chain immunoglobulin gene in the pSGIG-S was subcloned into the *Xba*I-*Not*I sites of pCMV-dhfr (Ryu *et al.*, 1996) and thus fused to the human cytomegalovirus (HCMV) promoter. The resulting expression plasmid was named pCMV-dhfr-SG-S. This plasmid contains a dihydrofolate reductase (DHFR) expression unit with crippled SV40 promoter for the amplification of the antibody gene and a neomycin-resistant gene for the selection of transformed cells.

Transfection, selection and amplification

The expression plasmid was introduced into DHFR-deficient CHO cell line DG44. The cell line was kindly provided by Dr. Lawrence A. Chain (Columbia University). The cells were grown at 5% CO_2, 37°C in DMEM/F12 (Gibco, U.S.A.) supplemented with hypoxanthine (10 μg/ml), thymidine (10 μg/ml), glycine (50 μg/ml), glutamine (587 μg/ml), glucose (4.5 mg/ml), 10% fetal bovine serum (FBS, Gibco) and Antibiotic-Antimycotic (Gibco). The cells were transfected with 10 μg of the plasmid DNA using 30 μg of Lipofectin (Gibco), and subsequently selected in nucleosides-minus MEM α (Gibco) supplemented with G418 (550 μg/ml) and 10% dialyzed heat-inactivated FBS (Gibco). The culture supernatant of the resistant cell clones was screened for the assembled single-chain immunoglobulin by an indirect ELISA. A clone (2E11) was chosen and grown in the selection medium containing 20 nM methotrexate (MTX, Sigma) to amplify the immunoglobulin gene.

Indirect ELISA

The culture supernatant of the drug-resistant cells was analyzed in 96-well microtiter plates by an indirect ELISA (Engvall and Perlman, 1972). The antibody sample was added to each well that had been previously coated with HBsAg (1 μg, Korea Green Cross Co.) at 4°C overnight, then was incubated at 37°C for 1 h. After washing, 100 μl of goat anti-human IgG peroxidase

Table 1. Nucleotide sequences of PCR primers

Primer	Sequence
1	5'-CAGGAATTCCGCTGGACTCACAAG-3'
2	5'-TCCGGACCCACCGCCCGAGCCACCGCCACCTGCAGAGACAGTGACCAG-3'
3	5'-TCGGGCGGTGGGTCCGGAGGCGGCGGATCTGACATTGTGCTGACCCAA-3'
4	5'-AGTTTTGTCAGGAGATTTGGGCTCCCGTTTTATTTCCAGCTT-3'
5	5'-AAGCTGGAAATAAAACGGGAGCCCAAATCTCCTGACAAAACT-3'
6	5'-GGAGTCGACCTGACCAGTGGAAAGAACCATCACA-3'

conjugate (1:1000 v/v, Sigma) was added to each well and incubated at 37°C for 1 h. After washing, 100 μl of 0.2 M citrate-PO_4 buffer (pH 5.0) containing 0.04% *o*-phenylenediamine (Gibco) and 0.03% H_2O_2 was added to each well and incubated for 10 min. The reaction was stopped by the addition of 50 μl of 2.5 M H_2SO_4, and the OD was measured at 492 nm in an ELISA reader (Titertek Multiskan Plus). To determine the concentration of antibody produced by the transformed cell line, the purified human IgG was used to generate a standard curve.

Antibody purification

The amplified DG44 cells were grown in serum-free medium (CHO-S-SFM II) and the culture supernatant was subjected to affinity chromatography on Protein G-Sepharose 4B column (Pharmacia, Sweden) pre-equilibrated with 0.1 M sodium phosphate buffer (pH 8.0). The bound protein was eluted with 0.1 M glycine-HCl buffer (pH 2.7). The eluate was immediately neutralized with 1.0 M Tris (pH 9.0) and dialyzed against PBS buffer. The purified antibody (2E11), together with a chimeric antibody CS131A (Ryu *et al.*, 1996) as a native antibody control and molecular weight protein standards (Prestained SDS-PAGE standards, BioRad), was analyzed on 15% SDS-PAGE or 7.5% SDS-PAGE without β-mercaptoethanol then visualized by staining with Coomassie blue. For the estimation of the concentration of the purified antibody solution, the OD of 1.43 at 280 nm was taken for the protein concentration of 1 mg/ml (Coligan *et al.*, 1991).

Antigen-binding assay

Various amounts (0~30 ng) of each purified antibody were separately added to each well that had been coated with 1 μg of the HBsAg and then an indirect ELISA was done, as described above.

Affinity determination

Three ng of the purified antibody and various amounts of the HBsAg (10^{-12}-10^{-6} M) were first incubated in 100 μl PBS at 37°C for 2 h and added to each well that was previously coated with 1 μg of HBsAg. The concentrations of free antibody were determined by an indirect ELISA. Apparent affinity was determined as the reciprocal of the antigen concentration required to inhibit 50% maximal binding in this competitive ELISA. This is a close approximation to the affinity determined by Friguet *et al.* (1985).

Results and Discussion

We constructed a murine/human chimeric single-chain immunoglobulin with specificity for the S surface antigen of HBV. The carboxyl end of the murine VH with leader sequence was linked to the amino terminus of the murine VL through a linker peptide $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$, and this VH-linker-VL was fused to the hinge region of human γ1 Fc region (Fig. 1). In this construct, Cys^{220} in the hinge region, which forms a disulfide bridge with Cys^{214} of human κ light chain in a native antibody molecule, was replaced with a proline residue. Otherwise, the spare cysteine residue may interfere with proper assembly of a dimeric antibody molecule. The other Cys^{226} and Cys^{229} were retained for interchain disulfide bonds. Thus, the single-chain immunoglobulin expressed in animal cells is expected to form a homodimeric antibody-like molecule (Fig. 2).

The resulting single-chain immunoglobulin gene was linked to the HCMV promoter in pCMV-dhfr (Ryu *et al.*, 1996) to yield expression plasmid pCMV-dhfr-SG-S (Fig. 3). The plasmid contains the selectable/amplifiable *dhfr* gene and neomycin-resistant gene. The single-chain immunoglobulin gene was expressed in DHFR-deficient CHO cells and subsequently amplified by MTX selection. Finally, a cell clone (2E11) with the productivity of 2.5 $\mu g/10^6$ cells/day was chosen for further study.

The 2E11 cells were grown in serum-free medium and the antibody was purified from the culture supernatant by affinity chromatography on Protein G-Sepharose. The size and purity of the purified antibody was confirmed by SDS-PAGE analysis. For comparison, the chimeric antibody CS131A (Ryu *et al.*, 1996), which consists of the H67 variable regions and human γ1 and κ constant regions, was included in this analysis. As shown in Fig. 4, the unreduced and reduced 2E11 protein have molecular weights of approximately 120 and 60 kDa, respectively, indicating that the produced single-chain immunoglobulin formed a dimeric molecule.

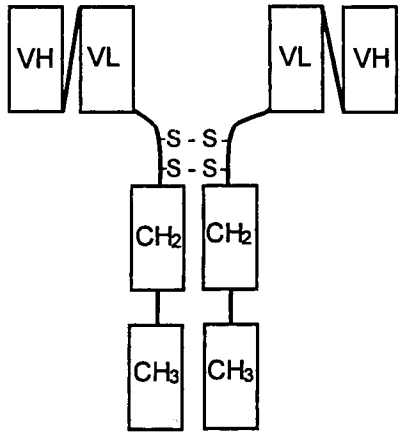

Fig. 2. Schematic diagram of the dimeric single-chain immunoglobulin. The VH and VL were linked by peptide linker $(Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser)_3$.

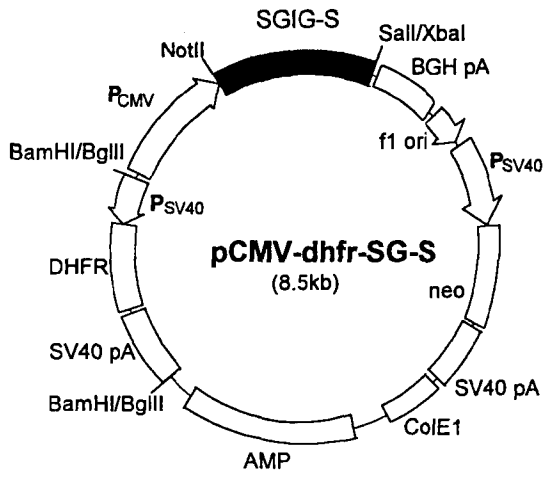

Fig. 3. Expression plasmid of single-chain immunoglobulin. The DNA sequence encoding the single-chain immunoglobulin is represented by the shaded box. The arrows indicate the orientaton of the transcription.

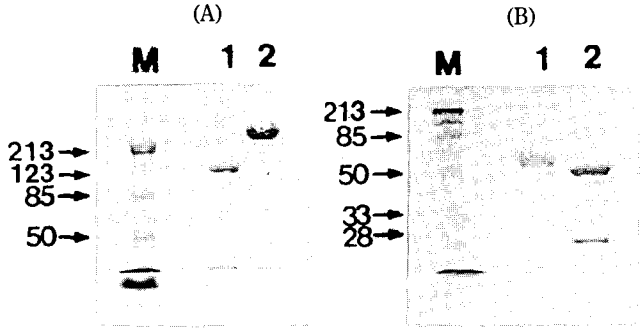

Fig. 4. SDS-PAGE analysis of purified single-chain immunoglobulin. (A) 7.5% SDS-PAGE of unreduced proteins. (B) 15% SDS-PAGE of reduced proteins. Lane M, molecular weight standards; lanes 1, single-chain immunoglobulin 2E11; lanes 2, chimeric antibody CS131A.

The reduced CS131A generated the 50 and 25 kDa of heavy and light chain protein bands, respectively, although the unreduced CS131A migrated more slowly than expected 150 kDa protein band. The slower migration of the unreduced CS131A on this gel may be due to the tetrameric structure of a native antibody molecule in which two heavy chains and two light chains are linked through interchain disulfide bonds and assembled into a tetramer.

The affinity of the purified 2E11 antibody to the HBsAg was determined and compared with that of the purified chimeric CS131A antibody. The result showed that the affinity (1.6×10^8 /M) of 2E11 was almost the same as that (3.3×10^8 /M) of CS131A (Fig. 5). This suggests that the two VH-linker-VL of this dimeric single-chain immunoglobulin were folded into functional antigen-binding domains. In fact, in many cases investigated, the affinities of ScFvs were almost identical

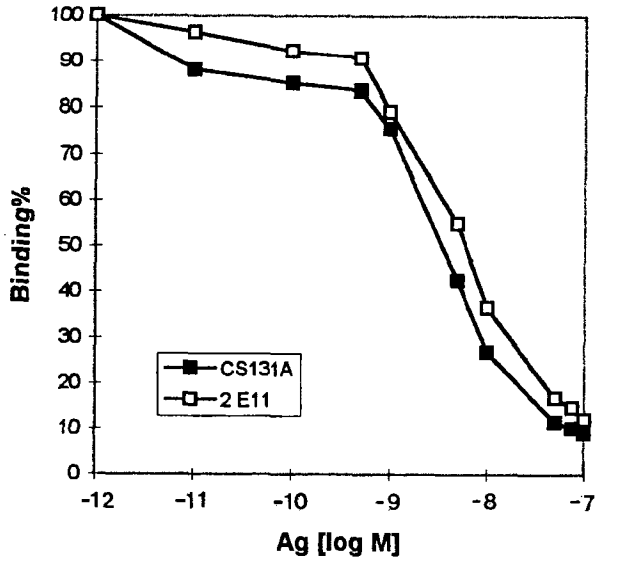

Fig. 5. Affinity determination of single-chain immunoglobulin 2E11 and chimeric antibody CS131A. Various concentration (10-12-10-6 M) of HBsAg and 3 ng of 2E11 (□) or CS131A (■) were incubated in 100 μl PBS solution at 37°C for 2 h then the mixture was added to each well coated with the antigen and incubated at 37°C for 1 h for an indirect ELISA.

to those of complete antibody and thus have been considered useful agents in the development of immunotherapeutic and immunodiagnostic applications (Bird *et al.*, 1988; Huston *et al.*, 1988; Glockshuber *et al.*, 1990; Milenic *et al.*, 1991; Yokoda *et al.*, 1992).

Since 2E11 contains human γ1 Fc region, it is expected to mediate cytolytic functions such as ADCC and CDC most effectively (Bruggemann *et al.*, 1987). We have not tested the effector functions of 2E11 but we confirmed that the same Fc region in a chimeric antibody (H69K) binds to C1q molecule (Jin *et al.*, 1995), which is the first antibody-binding molecule in the activation of the classical complement cascade (Reid, 1983). Since the antigen-binding variable domains and the effector function-triggering constant domains of an antibody molecule can be separable (Winter and Milstein, 1991), the Fc region of this single-chain immunoglobulin is expected to function as an effector molecule.

In conclusion, we successfully constructed a single-chain immunoglobulin which forms a dimeric molecule and has almost the same antigen-binding affinity as the native antibody counterpart. We expect that this approach for the construction of a single-chain immunoglobulin will be useful in efficiently generating a bispecific antibody molecule and other multivalent therapeutic antibodies.

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