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Optimization of anti-tumor necrosis factor-α single chain Fv displayed on phages for creation of functional antibodies

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In this study, we converted the immunoglobulin-type anti-human tumor necrosis factor-α (TNF-α) monoclonal antibody (Mab) to a scFv-type antibody in order to assess its basic properties. The immunoglobulin VH and VL genes were isolated from the hybridoma that produced an anti-TNF-α neutralizing Mab, and they were then linked together to create scFvs of the VL-VH or VH-VL-form. The binding affinity to TNF-α was retained in both scFvs. Interestingly, the VL-VH-type scFv effectively inhibited the TNF-α-mediated cytotoxicity, while this neutralization activity was dramatically decreased in the VH-VL-type scFv. These results suggest that the VL-VH-type scFv is a suitable template to create improved versions of the anti-TNF-α antibody using a phage display system, and they also show that the structural format must be taken into account in manufacturing scFvs.

Recently, targeting therapy and cytokine neutralization therapy using Mabs such as trastuzumab and infliximab have been applied to treat cancer and various inflammatory diseases. However, there are several issues that should be resolved in order to establish effective Mab-based therapies for various diseases. The first issue is that the molecular weight of the immunoglobulin (Ig)-form of a Mab (approximately 150 kDa) is too high to allow it to diffuse and reach target antigens that exist deep in the tissue (Batra et al. 2002). The second issue is that it is difficult to carry out large-scale preparations of antibodies using mammalian cell culture. A single chain Fv (scFv) is a genetically engineered antibody manufactured by conjugating the VH and VL domains of immunoglobulin with a flexible linker sequence. Due to their small molecular size (approximately 25 kDa), scFvs can diffuse into tissues more efficiently than immunoglobulin-form antibodies (Batra et al. 2002). In addition, the generation and large scale production of genetically modified scFvs such as humanized antibodies and immunotoxins are relatively easy, so clinical applications of scFvs and their modified versions are highly anticipated (Chen et al. 2005; Onda et al. 2004). However, because the binding affinity and *in vivo* stability of scFvs are generally lower than those of immunoglobulin-form Mabs, frequent administrations and high dosages are necessary for clinical efficacy. Therefore, improvements in the binding affinity and stability of scFvs are important challenges for research.

Recently, attention has been focused on the phage display system as a method to construct protein libraries having huge diversity on the phage surface (Clackson et al. 1991; Kolonin et al. 2004; Smith 1985). Using this method, peptides or protein libraries can be intensively explored to identify high-affinity targets. In particular, phage display scFv antibody libraries allow researchers to isolate antibodies to various antigens *in vitro* to produce antibodies modified by genetic engineering (Ho et al. 2005).

We report here the conversion of an anti-human TNF-α neutralizing antibody to the scFv-form using a phage display system. The VL and VH genes were isolated from a hybridoma producing an anti-TNF-α neutralizing antibody. The scFv-form antibodies were prepared as both VL-VH and VH-VL types connected by a flexible linker peptide. It is necessary to determine whether or not the binding and neutralizing activities of scFvs are maintained similar to the parent immunoglobulin type. We compared the properties of the VL-VH and VH-VL types of anti-TNF-α scFvs and found unexpected differences in their binding properties.

The scFv-type antibodies have been utilized in many research fields to date. Two types of scFvs, VL-VH and VH-VL, may be prepared, but there is not any kind of standard formula to decide which type of construct is more suitable for a given application. Because we could not determine in advance which type would be more suitable for our application, we investigated the binding and neutralizing activities of both VL-VH and VH-VL type scFvs.

In the M13 phage display system, scFv molecules are expressed as fusions with the N-terminal region of the gene III protein (g3p), which is a phage minor coat protein. The C-terminus of the VH or VL is connected with g3p in the VL-VH-type or VH-VL-type scFv, respectively. It is generally assumed that connecting of scFvs to g3p will not affect the scFv function, because X-ray crystallography has revealed that the C terminal region of the V domain is localized far from the antigen-binding site (Kaufmann et al. 2002). In fact, we were able to confirm that both types of phage-displayed anti-TNF-α scFvs bound to TNF-α in a dose dependent manner, with only small differences in their binding properties (Fig. 1).

Effective anti-TNF-α neutralization therapy requires that the antibody has both high binding affinity to TNF-α and TNF-α neutralizing activity. We constructed anti-TNF-α scFvs from the hybridoma producing the neutralizing antibody, and examined whether the scFvs maintained their TNF-α neutralizing activity. We prepared the soluble forms of the C-terminal FLAG-tagged anti-TNF-α scFvs from the supernatants of *E. coli* HB2151 and estimated their inhibitory effect on TNF-α-mediated cytotoxicity towards L-M cells (Fig. 2). Interestingly, neutralizing activity against TNF-α-mediated cytotoxicity was observed only for VL-VH-type scFvs, while VH-VL-type scFvs showed no inhibitory effect. The concentrations of anti-TNF-α scFvs in the culture supernatants were shown to be comparable by ELISA using TNF-α for the solid phase

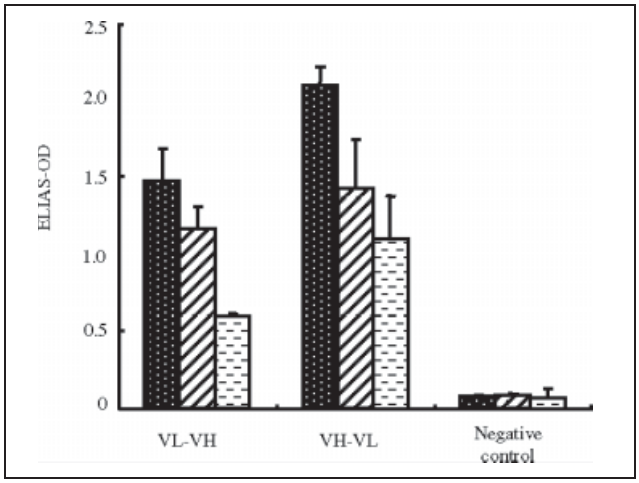

Fig. 1: Binding activities of anti-TNF-α scFvs. Phages displaying anti-TNF-α scFvs were added to immobilized TNF-α, and their binding activities were detected by an anti-M13 HRP conjugate. ■; 3×10^{10} CFU, ▨; 6×10^9 CFU, ▧; 1.2×10^9 CFU. The negative control phage displayed an anti-CD25 scFv. This experiment was performed three times and each value is given as the Mean ± SD

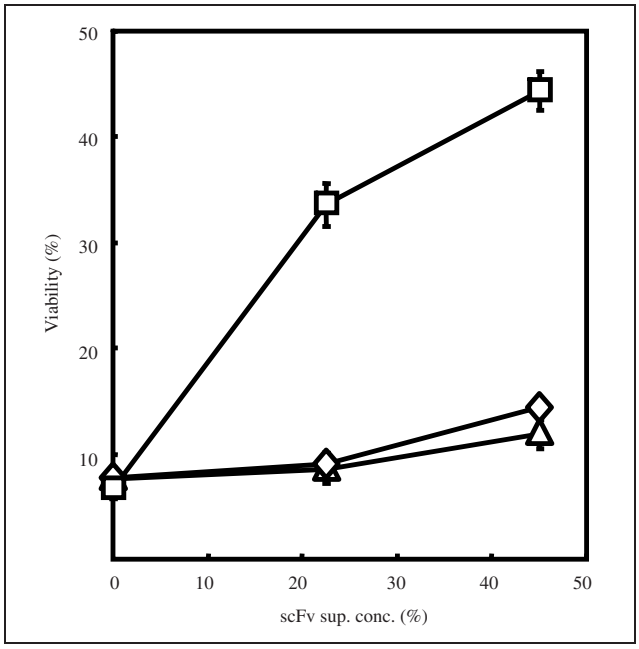

Fig. 2: Neutralizing activities of anti-TNF scFv constructs. Neutralization activities were measured using an inhibition assay against the L-M cell line in the presence of TNF-α and actinomycin D. L-M cells were seeded in a 96-well tissue culture plate at $5 \times 10_4$ cells/well. Anti-TNF-α scFvs were produced in *E. coli* HB21 51 supernatant following the protocol of Recombinant Phage Antibody System (GE Healthcare Bio-Sciences Corp.). They were incubated with 0.05ng of TNF-α for 30min at 37 °C and then added to the L-M cells. After 24 h incubation, cellular viability was measured by a methylene blue assay. □; VL-VH scFv, ◇; VH-VL scFv, △; anti-CD25 scFv. This experiment was performed three times and each value is given as the Mean ± SD

and an anti-FLAG antibody for detection (data not shown). Therefore, the loss of neutralizing activity in the VH-VL-type scFvs suggested that some conformational changes occurred upon conversion of the immunoglobulin-form antibody to the VH-VL-type scFv. Antibodies bind to antigens at complementarity determining regions (CDRs) formed by both the VL and VH domains. Recently, it was reported that some antibody clones were able to recognize their antigens through either the VL or VH alone (Tanaka et al. 2003). Therefore, it is possible that in this case, although both scFv types were able to bind TNF-α, the VH-VL type scFv may have undergone a slight conformational change so that it no longer blocked binding of the scFv-TNF-α complex to the TNF-α receptor, and therefore it lost its neutralizing activity. These results indicate that even if the antibodies used are Mabs from hybridomas whose binding properties have been confirmed, it is necessary to determine the properties again after converting from the immunoglobulin-type to the scFv type.

We concluded that the VL-VH type TNF-α scFv was a suitable antibody for functional modification using the phage display system because both the binding affinity and neutralizing activity of the original immunoglobulin were maintained. Though scFvs are generally thought to have lower binding affinities, a current report (Ho et al. 2005) indicates that affinities may be improved using the phage display system. We have previously established that this methodology that can be used to create functional mutant proteins such as cytokines with modified functions (Yamamoto et al. 2003). Furthermore, the *in vivo* stabilities of cytokines were improved using this technique (Shibata et al. 2004). We expect that various antibody therapies will be developed by applying this type of methodology to production of scFv antibodies in the future.

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