Article

Binding Activity Difference of Anti-CD20 scFv-Fc Fusion Protein Derived from Variable Domain Exchange

Shusheng Geng[1, 2, 4], Jiannan Feng[2, 3, 4], Yan Li[2, 3, 4], Yingxun Sun[2], Xin Gu[2], Ying Huang[2], Yugang Wang[2], Xianjiang Kang[1], Hong Chang[2] and Beifen Shen[2, 5]

Two novel engineered antibody fragments binding to antigen CD20 were generated by fusing a murine IgM-type anti-CD20 single-chain Fv fragment (scFv) to the human IgG1 CH_2 (i.e., $C_\gamma2$) and CH_3 (i.e., $C_\gamma3$) domains with the human IgG1 hinge (i.e. H_γ). Given the relationship between structure and function of protein, the 3-D structures of the two engineered antibody fragments were modeled using computer-aided homology modeling method. Furthermore, the relationship between 3-D conformation and their binding activity was evaluated theoretically. Due to the change of active pocket formed by CDRs, the HL23 (V_H-Linker-V_L-H_γ-$C_\gamma2$-$C_\gamma3$) remained its activity because of its preserved conformation, while the binding activity of the LH23 (V_L-Linker-V_H-H_γ-$C_\gamma2$-$C_\gamma3$) was impaired severely. Experimental studies by flow cytometry and fluorescence microscopy showed that HL23 possessed significantly superior binding activity to CD20-expressing target cells than LH23. That is to say, the order of variable regions could influence the binding activity of the fusion protein to $CD20^+$ cell lines, which was in accordance with the theoretical results. The study highlights the potential relationship between the antibody binding activity and their 3-D conformation, which appears to be worthwhile in providing direction for future antibody design of recombinant antibody. *Cellular & Molecular Immunology*. 2006;3(6):439-443.

Key Words: binding activity, scFv-Fc, variable domain exchange, molecular modeling

Introduction

Pan-B-cell monoclonal antibodies have been demonstrated to be effective antilymphoma agents (1, 2). Although the biological function of CD20 is not well understood, it has still been suggested to be a suitable antigen for monoclonal antibody (mAb) therapy of B-cell malignancies (3). Ligation of CD20 with anti-CD20 antibodies may result in intracellular alterations, including a rise in calcium, activation of serine/threonine protein tyrosine kinases, increased tyrosine phosphorylation, caspase activation, poly (ADP-ribose) polymerase (PARP) cleavage, and apoptosis (4-6).

In our previous study, we obtained a murine IgM-type anti-CD20 antibody, named as 1-28, with neutralized activity (7). In the present study, we have developed and evaluated the binding activity of recombinant single-chain variable fragments (scFv) fused to human IgG1 Fc domain. Given that the order of variable regions of antibody might affect the binding activity, two single-chain Fv (scFv)-Fc fusion proteins were designed (V_H-Linker-V_L-H_γ-$C_\gamma2$-$C_\gamma3$ named as HL23, and V_L-Linker-V_H-H_γ-$C_\gamma2$-$C_\gamma3$ named as LH23).

Firstly, distance geometry, molecular modeling and molecular dynamics trajectory analysis of HL23 and LH23 were conducted. And then, the binding activity of HL23 and LH23 to $CD20^+$ cell line, Raji cells was analyzed by flow cytometry. We observed that the binding activity depended on the order of the variable regions, which was in accordance with the theoretical results.

Materials and Methods

Molecular modeling

Three-dimensional structure of murine anti-CD20 scFv antibody, V_H-Linker-V_L and V_L-Linker-V_H, was modeled by use of computer-aided homology method. And then,

[1]College of Life Science, Hebei University, Baoding, Hebei, China;

[2]Institute of Basic Medical Sciences, Beijing, China;

[3]Lab of Cellular and Molecular Immunology, Medical School of Henan University, Kaifeng, Henan, China;

[4]These authors contributed equally to this work;

[5]Corresponding to: Dr. Beifen Shen, Institute of Basic Medical Sciences, P.O. Box 130(3), Taiping Road, Beijing 100850, China. Tel: +86-10-6693-1325, Fax: +86-10-6815-9436, E-mail: shenbf@mx.cei.gov.cn.

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computer models of the engineered antibody fragments, LH23 and HL23, were generated using the above models and human IgG1 CH_2 ($C_\gamma2$) and CH_3 ($C_\gamma3$) domain modules, which corresponded to the coordinates of the crystal structure of human IgG1 (PDB code: 1h3w). The structures of scFv and human IgG1 CH_2 ($C_\gamma2$) and CH_3 ($C_\gamma3$) domains were initially manipulated as rigid bodies with the Insight II 2000 molecular graphics package (San Diego, Accelrys Inc, 2001). Homology modeling of the antibody fragments LH23 and HL23 involved visual analysis of the protein (i.e., scFv and human IgG1 CH_2 ($C_\gamma2$) and CH_3 ($C_\gamma3$) domains) rigid model in order to find the most appropriate ligated mode between C-terminal of scFv and N-terminal of human IgG1 hinge.

The original structures were optimized to possess their approximate location and orientation on the molecule surface where the side chain rotamers could make favorable interactions. Finally, the structures were again initially optimized for 3,000 iterations using the Steepest Descent method to relax the geometry of the side chains and thus remove any reasonless contacts. Furthermore, with all hydrogen atoms removed, the structure was optimized for 20000 steps using the Conjugate Gradient method with a cutoff at 0.02 kcal/mol. All minimization was manipulated under CVFF forcefield. The Homology module and Discover of the Insight II 2000 software package (San Diego, Accelrys Inc, 2001) were used for modeling, manipulating and minimizing the antibody fragments.

Cell culture

Chinese hamster ovary (CHO)/DG44 cells (ATCC, USA), $dhfr^-$, were grown in pyy003 (TGS Biotech Co., China) supplemented with HT (Sigma, USA). $CD20^+$ human non-Hodgkin lymphoma cell lines, Raji (Burkitt lymphoma), and negative cell Jurkat (T-leukemia cell line) were maintained in RPMI 1640 (GIBCO, USA).

Genetic construction

Variable region genes were amplified from murine anti-CD20 monoclonal antibody 1-28 (IgM) by RT-PCR and transferred into immunoglobulin expression vectors (8). The plasmid was used as template for cloning the variable heavy (V_H) and variable light (V_L) chain domains of anti-CD20 antibody by polymerase chain reaction. Appropriate restriction enzyme sites and the linker sequences $(Gly_4Ser)_3$ were introduced by splice overlap extension. The primers of LH23 were: upstream primers 5'-GGT GGA AGC GGT GGT GGC GGT TCC GGA GGC GGA GGA TCA CAG CTG ACT CCG AGG-3' and 5'-ACT CTC TCG AGG TCT CCT CTG GCG GAG GTG GAA GCG GTG GTG G-3', downstream primers 5'-GAC ACC CTC CCT CCC TGT GCT GGC CTC TCA CCA GAA TCT GGG CAC GGT TCT GG-3' and 5'-AGG CAG GAG CGC TGA GCC TGG CTT CCA GCA GAC ACC CTC CCT CCC TGT GCT-3'. The primers of HL23 were: upstream primers 5'-GGT GGA AGC GGT GGT GGC GGT TCC GGA GGC GGA GGA TCA GAT ATC GTT CTC ACC CAG TC-3' and 5'-CAA GGG ACT CTG GTC ACC GTC TCC TCT GGC GGA GGT GGA AGC GGT GGT GG-3', and downstream primers 5'-CCT CCC TCC CTG TGC TGG CCT CTC ACC TTT GAT CTC GAG CTT GGT C-3' and 5'-AGG CAG GAG CGC TGA GCC TGG CTT CCA GCA GAC ACC CTC CCT CCC TGT GCT-3'. DNA fragments encoding V_H-Linker-V_L and V_L-Linker-V_H were inserted into the pCMV-c4 vector (TGS Biotech Co., China) containing the DNA sequence of -H_γ-$C_\gamma2$-$C_\gamma3$ portion of human IgG1 to be expressed. All of the constructions were confirmed by restriction site analyses and sequence. Restriction enzymes used for DNA manipulation were obtained from New England Biolabs.

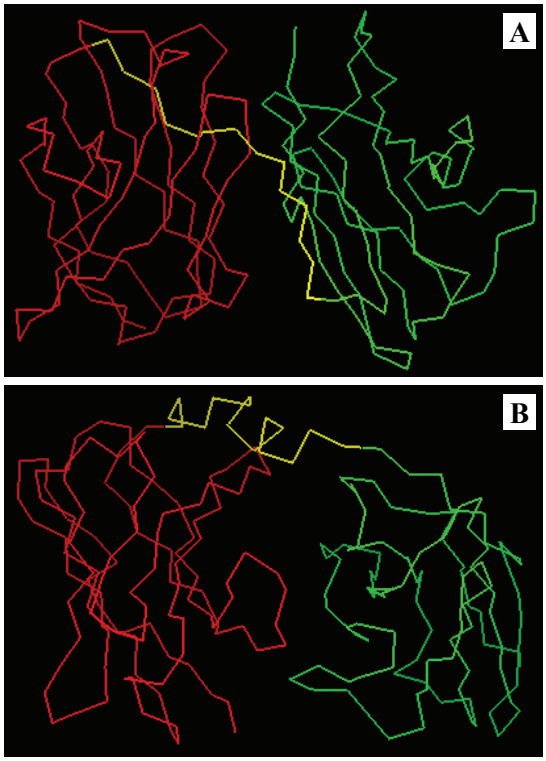

Figure 1. The optimized 3-D structure of V_H-Linker-V_L (A) and V_L-Linker-V_H (B). The red denoted the α-carbon atom orientation of V_H, the green denoted the α-carbon atom orientation of V_L and the yellow denoted the α-carbon atom orientation of Linker.

Mammalian expression

Transient transfections were performed by electroporation using a GenPulser (Bio-Rad, USA). Culture supernatants of transformed cells were screened for antibody secretion by sandwich ELISA. Cellular debris was removed by low speed centrifugation and clarified supernatants were ultrafiltrated with a 30-kD-pore size membrane. Concentrate culture supernatants were tested for specific binding activity.

Western blotting

After electrophoresing in SDS-PAGE, LH23 and HL23 proteins were electroblotted onto nitrocellulose membrane. Then, the membranes were blocked with 5% nonfat skim milk before incubating with goat anti-human IgG conjugated to HRP (1/1,000). The detection was performed with the

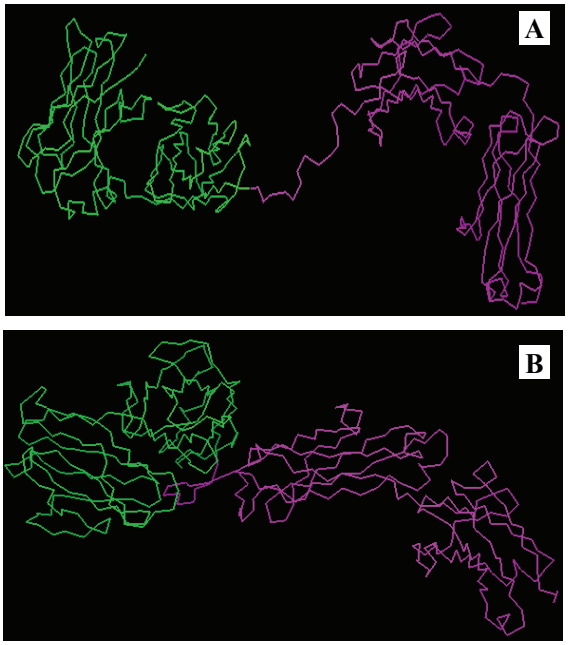

Figure 2. The optimized 3-D structure of HL23 (A) and LH23 (B). The green denoted the α-carbon atom orientation of scFv and the pink denoted the α-carbon atom orientation of human IgG1 constant region.

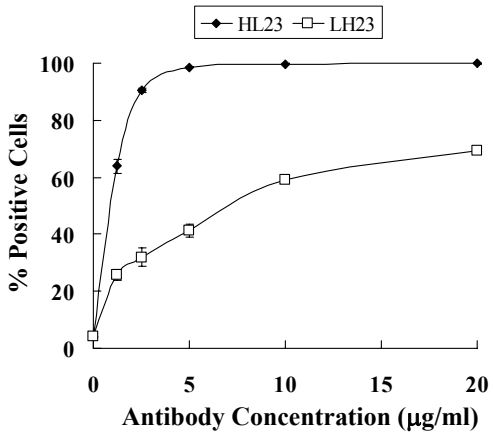

Figure 3. Binding activity of the two engineered anti-CD20 antibodies demonstrated by flow cytometry. A total of 3×10^5 Raji cells were incubated with various concentrations of HL23 or LH23 in 100 μl PBS containing 2% FBS for 45 min on ice. The cells were washed and incubated with FITC-labeled GAH IgG for 30 min on ice, and then were analyzed by flow cytometry.

enhanced chemiluminescences system (ECL) (Amersham, Sweden).

Flow cytometric detection

Indirect immuno-fluoroscence was used to assess the binding activity of engineered antibodies to antigen on the Raji and Jurkat cell lines. Briefly, after washed, cells were incubated with supernatants containing engineered antibodies for 30 min on ice and then washed. FITC conjugated GAH (Pierce, USA) was added to detect the antibodies binding to the cell surface. After the cells were washed with ice-cold 2% FBS-PBS, fluorescence was analyzed using a flow cytometry (Becton Dickson, CA).

Fluorescence microscopy

After flow cytometric analysis, slide smears were prepared from Raji cells, and analyzed under fluorescent light. Fluorescence images were obtained with fluorescence microscopy (Nikon, Japan) and manipulated with Adobe PhotoShop on a Macintosh computer immediately.

Results

The difference of 3-D structure between LH23 and HL23 predicted their binding difference

Based on the 3-D structure of anti-CD20 antibody 1-28 Fv fragments, considering the orientation of N-terminal of V_H and C-terminal of V_L, the 3-D structure of the other form of anti-CD20 1-28 scFv was constructed. The final optimized structures of the two forms were shown in Figure 1. Apparently, the conformation between V_H-Linker-V_L and V_L-Linker-V_H was very different. Due to the distance between N-terminal of V_H and C-terminal of V_L and the existence of arginine residue (Arg^{L107}), the linker peptides in V_H-Linker-V_L interfered the intra-molecular interaction of CDRs.

The original structures of scFv unit, human IgG1 hinge region, CH_2 and CH_3 domains that were used to model the antibody LH23 and HL23 were manipulated as rigid bodies. These models assumed to be in tail-to-head fashion compatibility. The distance between C-terminal of scFv (i.e., main chain carbon atom C_α) and N-terminal of human IgG1 hinge (main chain carbon atom C_α) was determined as common distance of C_α-C_α, and the orientation between COO^- of scFv and NH_3^+ of IgG1 hinge was chosen. The optimized structures of LH23 and HL23 were shown in Figure 2. The orientations of human IgG1 hinge in LH23 and HL23 were different because of the difference of property in C-terminal between V_H and V_L. Compared to the 3-D structure of 1-28 Fv fragment, the 3-D structure of the pocket formed by CDRs in HL23 was kept while that in LH23 was changed. So, it was predicted that the binding activity of HL23 would be remained just like its parental antibody while that of LH23 might be impaired severely.

Binding activity detection of the engineered anti-CD20 antibody

The relative binding activities to CD20 of the two fusion proteins were compared on CD20-expressing Raji cells using flow cytometric assay. In the assay, the binding activity was tested by adding various concentrations (0-20 μg/ml) of the two anti-CD20 engineered antibodies to cells followed by staining with FITC-conjugated GAH IgG. The results of flow cytometry indicated that the positive proportion of $CD20^+$

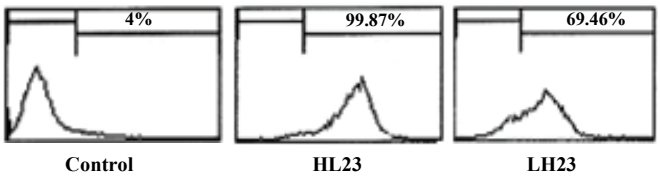

Figure 4. Detection fluorescence intensity by flow cytometry. Raji cells were incubated with supernatants from cells transfected with pCMV-c4 vector without inserts, with 20 μg/ml the HL23 or LH23 and then with FITC-labelled GAH. Cells were analyzed by flow cytometry. Data are representative of three concordant experiments.

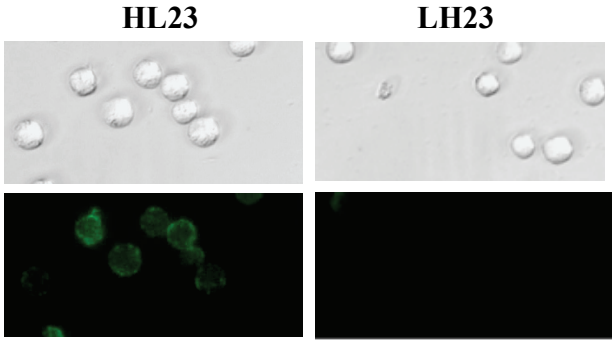

Figure 5. Photographs under fluorescence microscopy. Raji cells were incubated with 20 μg/ml HL23 or LH23, and then with FITC-labelled GAH. Cells were analyzed by fluorescence microscopy. A light microscopic picture of samples was taken from the same area with the fluorescence picture. Magnification, 400×.

cells incubated with HL23 were obviously superior to those of the same concentrations of LH23 (Figure 3). Moreover, as shown in Figure 4, a significant shift in fluorescence intensity was observed for HL23.

In addition, the binding activities to CD20 of HL23 and LH23 were examined using fluorescence microscopy. The results showed that fluorescence was not found on the Jurkat cells (data not shown) but was seen on the Raji cells. Moreover, the fluorescence was much more intense for HL23 than for LH23 (Figure 5).

Discussion

The Fv fragment, consisting of the variable domains of the heavy and light chains, is the smallest antibody fragment with the entire antigen-binding site. However, the absence of covalent bonds makes it unstable, so the Fv fragment tends to dissociate at low protein concentrations (9). One of the approaches to improve the stability of the Fv domain association involves construction of scFvs composed of two variable domains linked *via* a short flexible peptide (10). The most frequently used linker is the 15 residues $(Gly_4Ser)_3$, introduced by Huston et al. (11). This linker spans the 35Å distant between the carboxyl terminus of one variable domain and the amino terminus of the other without distorting the conformation of the antigen-binding site (9, 11). However, the scFv still retains a number of limitations. The lack of binding activity may be due to monovalent binding that might limit the effectiveness and sensitivity of scFv in many immunochemical applications such as FACS and ELISA (12). The lack of effector functions may be due to the absence of human immunoglobulin heavy constant domains that limit the potential interaction with the human host immune system. Given above two points, it would be desirable to connect scFv with Fc portion (hinge, CH2 and CH3 domain) of human IgG antibody. To do so, the scFv-Fc fusion protein possesses the constant domains of human IgG antibody. This will be helpful for scFv-Fc fusion protein to obtain ideal property. It is also in accordance with the theory that this combines the affinity and specificity of the bivalency and effector functions of a complete immunoglobulin (13-15).

However, there are two selections in the construction of scFv-Fc fusion protein, V_H-Linker-V_L-Fc or V_L-Linker-V_H-Fc. In the previous studies about scFv proteins, either V_H-Linker-V_L or V_L-Linker-V_H is better depending on different antibody. However, it is well known that the function of a protein is correlated tightly with its structure. The development of crystal structure and the emergence of molecular modeling make it possible to get the structure of an interesting protein. So, we believe that it will be helpful for the construction of scFv-Fc fusion protein, as well as other engineered antibody, to predict its activity firstly by computer-aided molecular modeling.

In this study, we constructed the models of two engineered anti-CD20 antibodies using computer-aided homology modeling method, V_H-Linker-V_L-H_γ-$C_\gamma2$-$C_\gamma3$ (HL23) and V_L-Linker-V_H-H_γ-$C_\gamma2$-$C_\gamma3$ (LH23). Based on the theoretical prediction, because the 3-D structure of the pocket formed by CDRs in HL23 was kept while that in LH23 was changed, the binding activity of HL23 would be remained while that of LH23 might be impaired severely. More important, the experiment result was consistent with the prediction. The binding activity depends on the order of the variable regions and the domain order should be V_H followed by V_L. So, it was demonstrated that the computer-aided homology modeling method used in our study was feasible and the modeling results were believable. That is to say, molecular modeling could be used to instruct our operation to obtain the more satisfactory results.

In conclusion, we reported the design of two engineered antibodies using computer-aided methods. Base on the result of modeling, the binding activities of the two antibodies were predicted. Biological experimental results demonstrated that the methods of molecular modeling could be used to instruct operation. The study highlights the potential relationship between the antibody binding activity and their 3-D conformation, which appears to be worthwhile in providing direction for future antibody design and construction of IgM antibody. Of course, further studies should be done to test the computer-aided modeling method.

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