Article

Human IgG1 $C_\gamma1$ Domain Is Crucial for the Bioactivity of the Engineered Anti-CD20 Antibodies

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

In this study, we discussed the necessity of human IgG1 $C_\gamma1$ domain for recombinant antibody using computer-aided homology modeling method and experimental studies. The heavy (V_H) and light (V_L) chain variable regions of 1-28, a murine IgM-type anti-CD20 mAb, were ligated by linker peptide $(Gly_4Ser)_3$ to form the single-chain Fv fragment (scFv). Then, the engineered antibody (LH1-3) was generated by fusing scFv with the entire IgG1 heavy constant regions. The 3-D structure of LH1-3 was modeled using computer-aided homology modeling method and the binding activity of LH1-3 was evaluated theoretically. Compared to the 3-D structure of the Fv fragment of the parent antibody, the conformation of the active pocket of LH1-3 was remained because of the rigid support of $C_\gamma1$. Further experimental results of flow cytometry showed that the engineered anti-CD20 antibody possessed specifically binding activity to CD20-expressing target cells. The anti-CD20 antibody fragments could also mediate complement-dependent cytotoxicity (CDC) of human B-lymphoid cell lines. Our study highlights some interests and advantages of a methodology based on the homology modeling and analysis of molecular structural properties. ***Cellular & Molecular Immunology*****. 2007;4(2):121-125.**

Key Words: CD20, engineered antibody, binding activity, molecular modeling

Introduction

Although nonhuman monoclonal antibodies have an excellent target-binding specificity, they are still limited in clinical applications due to the intrinsic properties of antibodies, especially their associated effector functions (1). Many of these problems can be overcome by the use of specific humanized antibodies. Recombinant antibody technologies were developed to reduce the immunogenicity of murine monoclonal antibody (mAb) and to gain effector functions through potential interaction with the human host immune system (2-4). However, the bioactivity of recombinant antibody was usually impaired in specificity and affinity. A general method is needed to direct the design of recombinant antibody so as to overcome such problems and obtain the active antibody as good as possible.

Molecules in the immunoglobulin (Ig) superfamily contain one or more domains known as Ig fold. Each Ig fold contains a disulfide bridge and approximately 100 amino acids (5). The highly conserved folding of the protein family and the quantity of experimentally determined 3-D structure of Ig make this superfamily ideal for modeling using a knowledge-based strategy (6). And so, computer-aided homology modeling method has been a powerful tool to design engineered antibodies with high bioactivity (7, 8).

In our previous study, we obtained a murine IgM-type anti-CD20 antibody, named as 1-28, with neutralized activity (9). Initial efforts to produce a chimeric anti-CD20 antibody were unsuccessful (10), leading us to generate engineered antibody fragment, LH23, V_L-V_H with a $(Gly_4Ser)_3$ linker fused to human IgG1 hinge and Fc regions (V_L-linker-V_H-H_γ-$C_\gamma2$-$C_\gamma3$). However, the hinge of human IgG1 interfered with the CDR pocket, the binding activity of the LH23 was impaired severely (11). And then, further work is required to recover the bioactivity of engineered antibody.

Considered as the hinge of human IgG1 interfered with the CDR pocket and formed intramolecular hydrogen bond with the scFv fragment in LH23, the rigid character may be able to resolve the above question. In the current work, we retained the human $C_\gamma1$ domain and generated an engineered antibody, LH1-3 (V_L-linker-V_H-$C_\gamma1$-H_γ-$C_\gamma2$-$C_\gamma3$). The results

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

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

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

Received January 18, 2007. Accepted March 8, 2007.

of molecular modeling and the experimental studies showed that LH1-3 could bind to $CD20^+$ cell lines. That is to say, its rigid character of the $C_\gamma1$ domain is critical for the bioactivity of the engineered antibodies to CD20.

Materials and Methods

Molecular modeling

3-D structure model of murine anti-CD20 scFv antibody V_L-Linker-V_H was obtained by proceeding along the following steps. First, the variable domains, V_H and V_L were modeled separately based on the homologous structures of antibodies from the Protein Data Bank (PDB) using Homology Module of Insight II (2000) software package (Accelrys Inc, San Diego, 2000). Then, the antibody fragment was docked to obtain a model structure of the V_L-V_H complex considering the surface electrostatic potentials distribution and side chain conformations in the interacting CDR loops. In the third step the linker peptide $(Gly_4Ser)_3$ was selected to form scFv using Ab initio modeling method. Finally, the whole scFv (V_L-Linker-V_H) conformation was manually modified to eliminate clashes and to optimize the interactions.

Using the crystal structure of human IgG1 constant region (PDB code: 1h3w), the engineered antibody fragment, LH1-3, was modeled with Builder Module of Insight II (2000) software package. The surface and interior between scFv and human IgG1 constant region were distinguished and the best connection between C-terminal of scFv and N-terminal of human IgG1 constant region was determined with computer graphics technology. To form the correct connection in orientation, a full rotation/translation scan was performed with a translation grid interval of 0.05 Å and an angular interval of 5°. The most acceptable solution was determined and optimized using Discover Module of Insight II (2000) software package under CVFF and Amber forcefield. To remove sterically unfavorable interactions, 3000 steps of steepest descent and 5000 steps of conjugate gradient energy minimizations were performed. At the end of the cycle the rms (root means square) force was at 0.05 kcal/mol $Å^2$, indicating that a local energy minimum had been reached.

Cell culture

Human non-Hodgkin lymphoma cell lines CD20-expressing cells, Daudi, Raji (Burkitt lymphoma) and T-leukemia cell line, Jurkat cells were grown in RPMI 1640 (GIBCO, USA) with 2 mM L-glutamine, supplemented with 10% heat-denatured fetal bovine serum (FBS), and penicillin-streptomycin (100 U/ml and 50 μg/ml, respectively) and maintained at 37°C with 5% CO_2 in humidified chamber and maintained in logarithmic growth phase. CHO cell line DG44 ($dhfr^-$, from ATCC, USA) cells, were grown in pyy003 (TGS Biotech Co., China) supplemented with HT (Sigma, USA).

Genetic construction

Variable region genes were amplified from a murine anti-CD20 mAb, 1-28 (IgM) by RT-PCR and transferred into immunoglobulin expression vectors containing the constant region of the human IgG1 and kappa gene (10). This plasmid was used as a template to clone the variable heavy (V_H) and variable light (V_L) chain domains of anti-CD20 antibody by polymerase chain reaction, meanwhile appropriate restriction enzyme sites and the gene sequences of a 15-amino-acid linker, $(Gly_4Ser)_3$ were introduced. The single-chain Fv fragment (scFv) was cloned into the human IgG1 expression vector pCMV-4 (TGS Biotech Co., China). The constructions of single-chain anti-CD20 antibodies were engineered by fusing single-chain Fv (scFv) with human IgG1 heavy constant regions. The constructions were verified by restricting enzyme digestion and sequencing. Restriction enzymes used for DNA manipulation were obtained from New England Biolabs.

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-kDa-pore size membrane. Concentrated culture supernatants were tested for specific binding activity.

Western blotting analysis

After electrophoresing in SDS-PAGE, LH1-3 proteins were electroblotted onto nitrocellulose membrane. Then, the membranes were blocked with 5% nonfat milk before incubating with anti-human IgG-HRP. The detection was performed with the enhanced chemiluminescences system (ECL) (Amersham, Sweden).

Indirect binding assays by flow cytometry

Antigen binding activity was assessed by indirect immuno-fluorescence on the Daudi, Raji and Jurkat cell lines. Briefly, a total of 2×10^4 cells/tube were washed in ice-cold 2% FBS-PBS, followed by incubation with supernatants from cells expressing engineered antibodies. Antihuman Fc IgG-FITC (Pierce, USA) was added for 30 min on ice, and fluorescence was analyzed using a flow cytometry (Becton Dickson, CA). As a control, a different irrelevant mouse-human antibody orsupernatants from cells transfected with pCMV-c4 vector without inserts was used.

Analysis of complement-mediated cytotoxicity (CDC)

The CDC activity of engineered anti-CD20 antibodies on malignant cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT), which was used to measure mitochondrial function. Briefly, 3×10^4 cells of Daudi or Raji cells were treated with engineered anti-CD20 antibodies (0 to 10 μg/ml) at 37°C for 20 min. Rabbit complement (diluted 1/20) was added as a source of complement and the cells incubated for additional 30 min at 37°C. All samples were done in triplicate. The MTT (5 mg/ml) was added to each well. After 4 h incubation at 37°C, the cells were then lysed with 100 μl of 10% SDS/0.01 M HCl. The absorbance was measured with a microplated

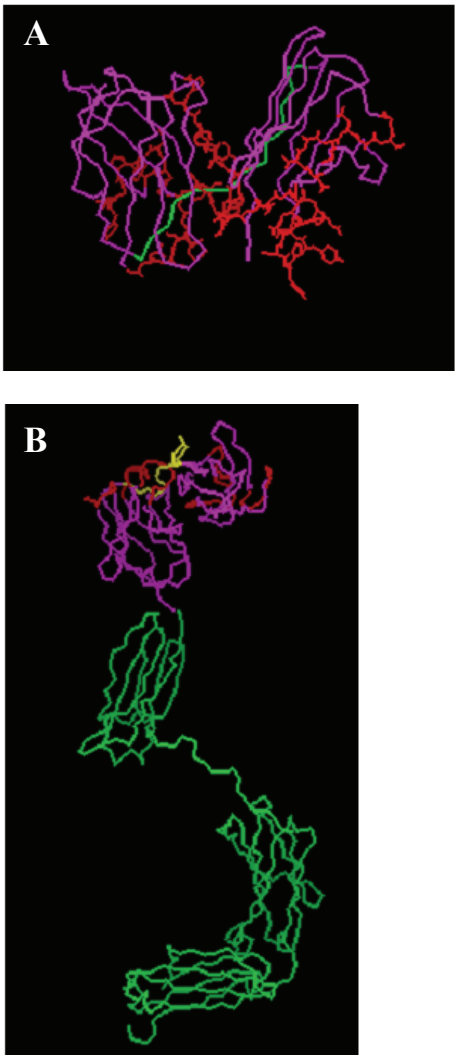

Figure 1. The 3-D modeling structure of the antibody fragment. (A) The structural model of scFv (V_L-Linker-V_H). FRs were indicated with pink, CDRs were indicated with red and the linker peptide was indicated with green. (B) The structural model of LH1-3. FRs was indicated with pink, CDRs were indicated with red, the linker peptide was indicated with yellow and the constant region was indicated with green.

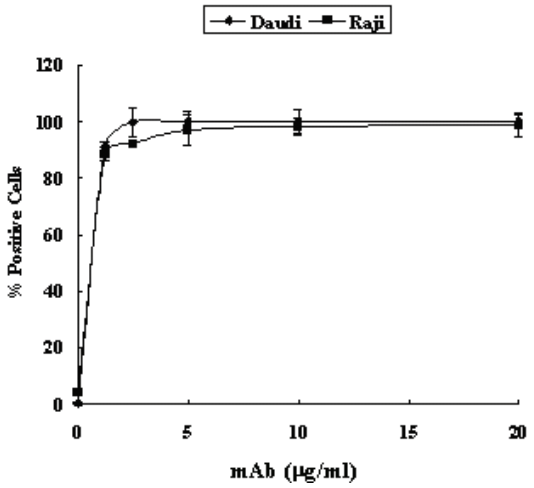

Figure 2. Binding activity of the engineered anti-CD20 antibodies was demonstrated by flow cytometry. A total of 3 × 10^5 cells Daudi or Raji cells were incubated with various concentrations of LH1-3 in 100 μl PBS containing 2% FBS for 30 min on ice. Then, cells were washed and incubated with anti-human Fc IgG-FITC for 30 min on ice. Cells were analyzed by flow cytometry.

reader (PerkinElmer, USA) at 570 nm. Wells without cells were used as blanks and were subtracted as background from each sample. Results were shown as a percentage of control.

Results

3-D modeling structures of LH1-3

Using the sequence alignment based on Blastp (http://www.ncbi.nlm.nih.gov/) program, a structure model of the anti-CD20 antibody 1-28 Fv fragment was generated by homology modeling method. Analysis results of the structure using the Procheck program showed that 98% of the non-glycine and non-proline residues adopted either the most favorable or additional allowed regions on a Ramachandran plot. Considering measurements of bad non-bonded interactions and the planarity of peptide bond, the model met the requirements for a valid structure indicated by Profile-3D.

Based on the modeling structure of the scFv and the crystal structure of human IgG1 constant region, the structural models of the engineering antibody fragment LH1-3 were obtained and optimized. The 3-D structures LH1-3 were shown in Figure 1B. As comparison, the 3-D structure of the scFv (V_L-Linker-V_H) was shown in Figure 1A. Due to the existence of the human IgG1 $C_\gamma1$ and its rigid character, the human IgG1 constant region did not interfere with the conformation of the scFv fragment.

Table 1. The intramolecular hydrogen bond formed by scFv fragment and human IgG1 constant region in LH23

Donor	Acceptor	Distance (Å)	Angle (°)
scFv: Ser^{66}(L):HG	CH: Thr^{236}:OG1	1.95	136.46
CH: Glu^{226}:HE2	scFv: Val^{12}(H):O	1.70	153.04
CH: Ser^{229}:HN	scFv: Lys^{13}(H):NZ	2.45	147.58
CH: Ser^{229}:HG	scFv: Lys^{13}(H):NZ	2.02	168.49
scFv: Lys^{13}(H):HZ2	CH: Pro^{227}:O	1.85	172.28
CH: Lys^{228}:HZ2	scFv: Ser^{15}(H):OG	2.07	160.61
scFv: Lys^{75}(H):HZ1	CH: Cys^{242}:O	1.90	166.96
scFv: Asn^{76}(H):HD21	CH: Lys^{345}:NZ	2.28	173.19
scFv: Gln^{81}(H):HE21	CH: His^{237}:O	1.89	170.77
scFv: Ser^{115}(H):HG	CH: Glu^{226}:OE2	1.79	157.66

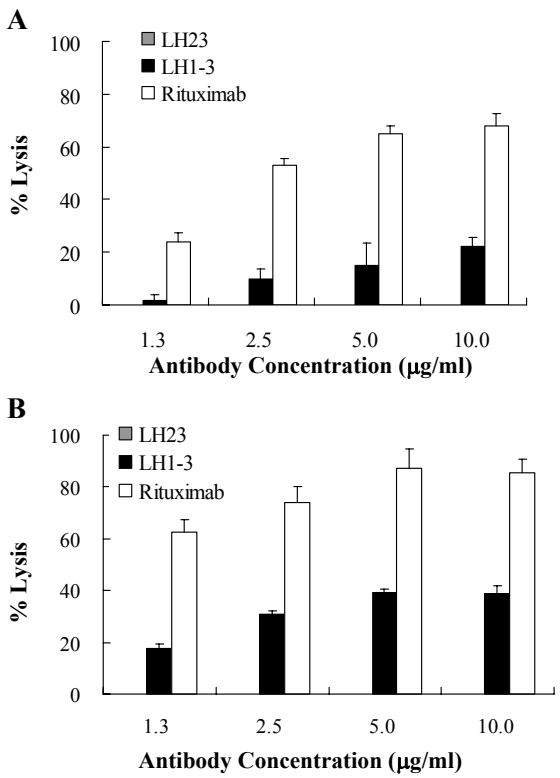

Figure 3. CDC assay of the two engineering anti-CD20 antibodies. Daudi (A) or Raji (B) cells (3×10^4) were incubated with anti-CD20 antibodies for 20 min and rabbit complement were added as a source of complement and the cells incubated at 37°C for 30 min. Cell lyses rate was assessed.

Binding activity of LH1-3

The binding activities to CD20 of the LH1-3 were conducted on $CD20^+$ cell lines, Daudi, Raji cells using flow cytometric assay. The results of flow cytometry indicated that LH1-3 could bind specifically to CD20 expressed on Daudi and Raji cells (Figure 2).

CDC of LH1-3

The biological activity of the engineered anti-CD20 antibody fragments was assessed in CDC with Rituximab as a control. We have tested the capacity of the different lymphoma lines to be killed by CDC. As shown in Figure 3, the LH1-3, anti-CD20 antibody fragments were potent in mediating lyses positive a range of concentrations. However, The LH23 had no any effects on complement-mediated lysis.

Discussion

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 fusion protein to predict its activity firstly by computer-aided molecular modeling.

In previous study, we constructed a scFv-Fc anti-CD20 engineered antibody, LH23 (11). The results of molecular modeling showed that the hinge of human IgG1 interfered with the CDR pocket and formed intramolecular hydrogen bond with the scFv fragment in the LH23 (as listed in Table 1). In the current work, we retained the human $C_\gamma 1$ domain and generated an engineered antibody fragment, LH1-3. Firstly, the 3-D structures of the LH1-3 engineered antibody fragment were modeled using computer-aided homology modeling method. The results showed that the human IgG1 constant region did not interfere with the conformation of the scFv fragment because of its rigid character of the human IgG1 Cγ1 (Figure 1). The results of flow cytometry also showed that LH1-3 could bind specifically to CD20 expressed on Daudi and Raji cells (Figure 2).

Potent biological activity was demonstrated in CDC assays. The results showed that LH23 was inactive due to the absence of Cγ1 domain, while the LH1-3 was potent in mediating lysis. It is required for at least 2-headed binding to engage and activate C1q that anti-CD20 antibodies were effective in recruiting complement in CDC assays (12). The ligation of CD20 by complement-lytic mAbs could induce the Ab:Ag complexes to redistribute into lipid rafts and make them have a sharp rise in a comparatively small area of the plasma membrane (12). Concentrating engineered antibody fragments, LH1-3, could provide an ideal density of juxtaposed Fc regions for engaging the C1q heads, therefore triggering the classical complement path way. The binding activity of the LH23 was impaired severely so that LH23 could not show any bioactivity in CDC assays. At the same concentration, the activity of LH1-3 was much weaker than Rituximab. The possible explanation is the differences of two antibodies in the recognizing epitope and the antibody isotype of parental antibodies (13).

In conclusion, the rigid structure between the hinge region and scFv is necessary for the binding activity of engineered antibodies of IgG isotype that is from IgM-type antibody. Our study shows that a knowledge-based molecular modeling approach can lead to important insights into structure-function and structure-origin relationships of antibody.

Acknowledgements

This study was supported by National Natural Sciences Foundation (No.30490240) and "973" Foundation (No. 001CB510005, 2003CB515508) of China.

References

1. Kortt AA, Dolezal O, Power BE, Hudson PJ. Dimeric and trimeic antibodies: high avidity scFvs for cancer targeting. Biomol Eng. 2001;18:95-108.
2. Cooper JC, Morgan G, Harding S, et al. Alefacept selectively promotes NK cellular deletion of $CD45RO^+$ human T cells. Eur J Immunol. 2003;33:666-675.
3. Heuser C, Ganser M, Hombach A, et al. An anti-MUC1-antibody-interleukin-2 fusion protein that activates resting NK cells to lyses of MUC1-positive tumour cells. Br J Cancer. 2003;

89:1130-1139.
4. Lorenzo CD, Tedesco A, Terrazzano G, et al. A human, compact, fully functional anti-ErbB2 antibody as a novel antitumour agent. Br J Cancer. 2004;91:1200-1204.
5. Alzari PM, Lascombe MB, Poljak RJ. Three-dimensional structure of antibodies. Annu Rev Immunol. 1988;6:555-580.
6. Petit S, Brard F, Coquerel G, Perez G, Tron F. Structural models of antibody variable fragments: a method for investigating binding mechanisms. J Comput Aided Mol Des. 1998;12:147-163.
7. Willuda J, Honegger A, Waibel R, et al. High thermal stability is essential for tumor targeting of antibody fragments: engineering of a humanized anti-epithelial glycoprotein-2 (epithelial cell adhesion molecule) single-chain Fv fragment. Cancer Res. 1999;59:5758-5767.
8. Hoedemaeker FJ, Signorelli T, Johns K, Kuntz DA, Rose DR. A single chain Fv fragment of P-glycoprotein-specific monoclonal antibody C219. J Biol Chem. 1997;272:29784-29789.
9. Li A, Sun Y, Hong H, et al. Preparation and function assay of monoclonal antibodies against human CD20 antigen. Zhonghua Wei Sheng Wu Xue He Mian Yi Xue Za Zhi. 2002;22:385-388.
10. Wang YG, Huang Y, Gu X, et al. Construction and expression of chimeric anti-human CD20 monoclonal antibody. Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi. 2006;22:363-367.
11. Geng SS, Feng JN, Li Y, et al. Binding activity difference of anti-CD20 scFv-Fc fusion protein being derived from variable domain exchange. Cell Mol Immunol. 2006;3:439-443.
12. Cragg MS, Morgan SM, Chan HTC, et al. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. Blood. 2003;101:1045-1052.
13. Michaelsen TE, Ihle o, Beckstrom KJ, et al. Construction and functional activities of chimeric mouse-human immunoglobulin G and immunoglobulin M antibodies against the Neisseria meningitides PorA P1.7 and P1.16 epitopes. Infect Immun. 2003;71:5714-5723.