Characterization of a Novel Humanized Anti-CD20 Antibody with Potent Anti-Tumor Activity against Non-Hodgkin’s Lymphoma

Haifeng Zhang\textsuperscript{a,b} Liping Song\textsuperscript{b} Hongtu Ye\textsuperscript{b} Lide Hu\textsuperscript{b} Wenlu Liang\textsuperscript{b} Datao Liu\textsuperscript{b}

\textsuperscript{a}Department of Biochemistry and Molecular Biology, Second Military Medical University, Shanghai, \textsuperscript{b}Central Research Institute, Shanghai Pharmaceuticals Holding Co., Ltd., Shanghai

Key Words

Humanization • Anti-CD20 antibody • Immunogenicity • Non-Hodgkin’s lymphoma

Abstract

Background: Rituximab, a mouse Fab and human Fc chimeric antibody, has been widely used to treat Non-Hodgkin’s lymphoma (NHL). However, only 48% of patients respond to the treatment and complete response rate is below 10%. Also, immunogenicity was reported in 17-20% patients receiving the treatment, making it unsuitable for long term diseases such as autoimmune disorders. It has been a hot research field to “humanize” rituximab toward improved efficacy and reduced immunogenicity. Methods: In this study, an advanced antibody humanization technology was applied to the sequence of the anti-CD20 antibody 2B8, its sequence of which was based on the original murine monoclonal antibody of rituximab in Roche. The complementarity-determining regions (CDRs) of the humanized antibodies were further optimized through computer-aided molecular dock. Results: Five novel humanized anti-CD20 antibodies 1-5(1635, 1534, 3637, 1634 and 1536) were generated and their immunogenicity was significantly decreased when compared to rituximab. The novel humanized anti-CD20 antibodies 1-5 retained the binding activity of their murine counterpart, as demonstrated by the fluorescence-activated cell-sorting analysis (FACS). When compared to rituximab, the humanized antibodies still have the similar properties on both complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). Furthermore, its anti-tumor efficacy in xenograft model is comparable to that of rituximab. Conclusion: The humanized anti-CD20 antibodies 1-5 have lower immunogenicity than rituximab. And at the same time, they still retain the anti-tumor effect both \textit{in vitro} and \textit{vivo}.
Zhang/Song/Ye/Hu/Liang/Liu: Characterization of a Novel Humanized Anti-CD20 Antibody

Introduction

Non-Hodgkin's lymphoma (NHL) is one of the most common cancers in the blood system. The most dominant form of NHL is associated with B cell and refractory to many traditional chemotherapeutics. The mouse/human chimeric anti-CD20 antibody, rituximab, is the first therapeutic monoclonal antibody (mAb) approved for the targeted treatment of relapsed/refractory low-grade or follicular B-cell non-Hodgkin lymphomas [1, 2]. With the growing use of rituximab, the mortality rate of NHL decreased gradually. In the following 10 years, a number of therapeutic regimens were developed based on rituximab, with the aim to enhance efficiency of rituximab and improving clinical outcome. With these agents, the remission rate and complete remission rate of NHL patients further increased with improved prognosis.

The most contributing factor in the success of rituximab is the choice of its target, CD20, which represents an ideal target for monoclonal antibodies (mAbs) [3-5]. The CD20 molecule is a 30- to 35-kDa trans-membrane protein highly expressed by B lymphocytes in early stages of differentiation and by most B-cell lymphomas [6, 7]. The CD20 has four transmembrane domains and both of its N-termini and C-termini are located on the inner side of cytoplasm. It has a large extracellular loop with 43 amino acids to form primary epitope. This epitope is well exposed and easy to be approached by an antibody. Probing with various anti-CD20 antibodies, the physiological functions of CD20 on B cell are inferred to include cell proliferation and, differentiation, signal transduction, and the transmembrane deliver of Ca^{2+}.

Previous studies have suggested that several mechanisms could be working through the therapeutic efficacies of anti-CD20 antibodies, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) [8-10] and the induction of apoptosis [11, 12].

Although rituximab has been widely used in the treatment of lymphoma, only 48% of patients respond to the treatment, and complete responses are less than 10% [13-15]. Rituximab belongs to one of the human-mouse chimeric antibodies with 30% of its sequence from the parental murine origin. During the clinical application, immunogenicity was reported in the 17 -20% of patients. Hence, it is restricted to treatment of long time disorders such as autoimmune disease. Humanization of rituximab for high efficacy and lower immunogenicity is urgently required. In this study, we described the in vitro and in vivo antitumor activities of five humanized anti-CD20 antibodies 1-5 (1635, 1534, 3637, 1634 and 1536), as well as their comparisons to rituximab.

Materials and Methods

Cell lines, antibodies and animals

The Chinese hamster ovary cell lines CHO-S were purchased from Invitrogen (Shanghai, China). The T lymphocytic leukemic cell line Jurkat and two human Burkitt lymphoma cell lines, Raji and Ramos, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Rituximab was purchased from Roche Ltd (Basel, Switzerland). Eight-week-old nude BALB/c mice were purchased from China Academy of Science (Shanghai, China). All mice were housed in individual cages in a temperature-controlled room with a 12h light/dark cycle. Food and water were provided ad libitum. All the experiments were approved by the Second Military Medical University, China in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (publication No. 96-01).

Humanization of anti-CD20 mAb 2B8

Two separate BLASTP searches were performed for light chain variable region (VL) and heavy chain variable region (VH) of 2B8, the original murine monoclonal antibody of rituximab in Roche. The complementarity-determining regions (CDRs) with human germline antibody frame sequence and high
sequence identity to that of 2B8 were chosen and analyzed. The selected VH and VL genes of humanized versions of 2B8 were synthesized by overlapping PCR.

**Computer-guided molecular modeling**

The online modeling server Swiss-PdbViewer (http://spdbv.vital-it.ch/) was utilized to analyse the three-dimensional structure of 2B8 using the framework sequences of the variable regions of 2B8. Considering the distribution of surface electrostatic potential and structural characteristics, the 3D complex structure of 1–5 fragment was constructed using Molecular Docking method. Using homology modeling method, the 3D structure of humanized 1–5 variable regions was constructed and optimized.

**Construction and expression of humanized antibodies**

The light and heavy chain expression vectors for humanized antibodies were constructed using the same method as described in previous report [16]. The resulting light and heavy chain expression vectors (pcDNA3.1-Fc for heavy chain and pcDNA3.1-κ for light chain, respectively) were co-transfected into CHO-S cells using Lipofectamine 2000 reagent. After 72-hour incubation, the transfected cells were seeded at the density of 2.0×10^5 cells /10cm dish and were selected with 500μg/ml G418 (GIBCO) and 12.5μg/ml puromycin (Sigma) for resistant clones.

**Specificity assay of humanized antibodies**

Fluorescence-activated cell-sorting analysis (FACS) was performed to determine the specific binding of recombinant mAbs to Ramos cells (CD20^+) with Jurkat cells (CD20^-) as negative control. Briefly, Ramos cells and Jurkat cells at 1.0×10^6 cells/mL were incubated with different dilutions of the humanized antibodies for 2 h at 4°C. The cells were washed and incubated with FITC-labeled goat anti-human IgG (H + L) for 1 h at room temperature. And then the cells were washed and analyzed by FACS.

**Complement dependent cytotoxicity assay**

Raji cells were used to examine the complement dependent cytotoxicity (CDC) activity of humanized anti-CD20 antibodies 1-5 (1635, 1534, 3637, 1634 and 1536). Briefly, cells were washed and suspended in serum-free RPMI-1640 at a concentration of 1.0×10^6 cells/mL. Eighty microliters of the cell suspension was added to each of a 96-well, flat-bottom plate. Twenty five microliters of serially diluted antibodies 1-5 was added to each well in triplicate and incubated for 10 mins. Then, 50μL normal human serum (Bioreclamation) was added. The plate was incubated at room temperature for 10 minutes and then was incubated at 37°C for 1 h. After the incubation, the plate was centrifuged at 200g for 5mins, and 50 μl supernatant of each well was transferred to a fresh 96-well plate. The CCK-8 kit was applied to detect the fluorescence intensity at 450 nm. Controls include background control (cells only, used as negative control) and rituximab (used as positive control).

**Antibody-dependent cell-mediated cytotoxicity assay**

Antibody-dependent cell-mediated cytotoxicity (ADCC) activities of anti-CD20 mAbs were measured by lactate dehydrogenase (LDH)-releasing assay using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI). Briefly, the Ramos cells (CD20^+) were incubated with CD20 mAbs for 1 h in phenol red-free DMEM culture medium in a 5% CO₂ incubator at 37°C, followed by the addition of human peripheral blood mononuclear cells (PBMC) as effector cells (effector to target, 40:1 for ADCC assay). After an additional incubation for 5 h at 37°C, the cell lysis was determined by measuring the amount of released LDH, which is positively correlated with the OD at 490nm. Controls include human IgG (used as negative control) and rituximab (used as positive control).

**The immunogenicity assay of humanized antibody**

The blood from normal volunteers (n = 20) was drawn into a heparinized syringe, mixed with same volume of HBSS without Ca²⁺/Mg²⁺, gently loaded onto lymphocytes gradient separating medium, and centrifuged at 800g for 30 minutes. The peripheral blood mononuclear cells (PBMC) were collected from the boundary surface, washed with HEPES-buffered saline, and re-suspended in RPMI 1640 medium. The PBMC cells were then cultured in 50ml serum-free AIM V medium with 1:100 dilution of beta-mercaptoethanol for 2 hrs. Cells were added with 800 units/ml of GM-CSF and 500 units/ml of IL-4 and further cultured for...
5 days. Five days later, 0.2 units/ml TNF-α and 50 units/ml IL-1α were added and cells were cultured for 2 more days. On day 7, 50 mg/ml of mitomycin C was added to the culture and incubated for 1 hour to stop the differentiation of dendritic cells (DC). The mature dendritic cells were harvested by centrifuging at 600 g and seeded at 2×10^4 cells/well (100 μl) in a round-bottomed 96-well plate. The CD4+ Cellect Kit (Biotex) was applied to collect human CD4+ cells. The CD4+ cells were harvested at 2×10^6/ml in AIMV medium containing 2% human serum. Rituximab and humanized anti-CD20 antibodies1-5 (1635, 1534, 3637, 1634 and 1536) were added at 5mM to a 96-well plate containing 100 μl dendritic cells, followed by addition of 100 μl CD4+ cells. The proliferation of activated CD4+ cells was determined with Alamarblue reagent and the results were converted to a T-cell Stimulation Index [17]. Rituximab was used as control.

**Immunotherapy**

The BALB/C nude mice were injected with Ramos cells (1×10^7 cells/0.1 ml/per mouse) subcutaneously at day 0. Tumor size was measured with caliper and calculated using the formula of length×width^2/2. When the average tumor size achieved 100mm^3 (80-160mm^3), the tumor-bearing mice were randomly assigned into seven groups (n=8). On every Monday, Wednesday and Friday in the following two weeks, mice were intraperitoneally administered with 5mg/kg of humanized anti-CD20 antibody 1534,1634,1535,1635,3637, PBS (as a negative control) or rituximab (as a positive control) for a total of 7 times. The volume of the tumors was measured every Monday, Wednesday and Friday. PBS was used as control.

**Statistical analysis**

Data are expressed as means±SD for each experiment. The number of samples is indicated in the description of each experiment. We used an analysis of variance (ANOVA) followed by a Student-Newman-Keuls post hoc test for statistical analysis. We performed experiments for quantification in a blinded fashion.
Results

Molecular modeling of anti-CD20 monoclonal antibody 1-5

The nucleotide sequences of VH and VL of antibodies 1-5 were determined and the deduced amino acid sequences of VH and VL of antibodies 1-5 are shown in Fig. 1.

The model of Swiss-PdbViewer was applied to analyse the three-dimensional structure of 2B8. Considering the surface electrostatic potential distribution and structural character, the 3D complex structure of the humanized antibodies fragment was constructed using Molecular Docking method. The molecular model of the variable regions of the humanized antibodies was obtained (Fig. 2).

Humanization of 2B8 antibody

The CDRs of heavy chain and light chain of the murine monoclonal antibody 2B8 were analyzed based on the FR sequence of the variable region. Two separate BLASTP searches
were performed to find human germline antibody FR sequence which is similar to the FR sequence of 2B8. The sequences were then analyzed by the “in silicon” method to find out which has lower affinity with MHCII (HLA-DR), and to confirm the candidates of the FR sequences. Finally, five humanized antibodies 1-5 (1635, 1534, 3637, 1634 and 1536) were selected.

Specific binding assay
The humanized antibodies 1-5 were expressed in CHO-S cells and then the CHO-S cell serum-free culture supernatant was purified with Protein A affinity chromatography. In the flow cytometry assay, the humanized antibodies 1-5 were found to bind to the CD20-positive Ramos cell specifically since they did not bind to the CD20-negative T lymphocyte leukemia Jurkat cell. Among them, antibody 3637 has relatively higher binding activity with CD20-positive Ramos cell (Fig. 3).

CDC activity of humanized antibodies 1-5
The capacity of humanized antibodies 1-5 to mediate CDC was assessed in CD20-positive human lymphoma cell lines-Raji. The results (Fig. 4) showed that humanized antibodies 1-5 were as effective as rituximab in lysing Raji cells in the presence of human complement.

ADCC activity of humanized antibodies 1-5
The ability of humanized antibodies 1-5 to lyse CD20⁺ lymphoma cells in the presence of human PBMCs was investigated and compared with rituximab. Our data (Fig. 5) clearly indicated that the humanized antibodies 1-5 and rituximab were equally effective in mediating ADCC against Ramos cells in a dose-dependent manner.
Immunogenicity of humanized antibodies 1-5

The immunogenicity of humanized antibodies 1-5 was assessed by the standard DC-T cell activation assay. First of all, the dendritic cells (DC) were produced from PBMCs and induced to mature cells in the presence of GM-CSF, IL-4, TNF-α and IL-1α. And then, the CD4\(^+\) cells were collected by the CD4\(^+\) collect kit. The results summarized in Fig. 6 show humanized antibodies 1-5, are equally effective in activating CD4\(^+\) cells. Encouragingly, the T-cell Stimulation Index of the humanized antibodies 1-5 were significantly less than rituximab, indicating that the immunogenicity of humanized antibodies 1-5 were lower than that of rituximab.

The in vivo anti-tumor activity of humanized antibody

In vivo therapy studies were performed in BALB/c nude mice bearing Ramos tumors. The volume of the tumors were measured. The results (Fig. 7) showed that all of the five humanized anti-CD20 antibodies (1534, 1634, 1535, 1635 and 3637), PBS and Rituximab. Mice were monitored daily and the volume of the tumors were measured when administration. Data are expressed as means ± SD (n = 8). P<0.05 vs. control.

Discussion

In this study we described the humanization of a marketed anti-CD20 monoclonal antibody, rituximab. The aim of the study is to make the immunogenicity of rituximab lower through humanization while still retaining its anti-tumor efficacy.

To reduce the immunogenicity of murine CD20 antibody, a method called "CDR grafting" [18] was pioneered by PDL company and it became the primary technology for antibody
humanization. This technology transfers the CDR regions of a murine antibody to the frameworks of a human antibody, and thereby reduces the immunogenicity induced by the framework regions of parental murine antibody. So far, the humanized anti-CD20 antibodies obtained using this technology include 2H7 monoclonal antibody (CN101418045A) from Health Tyco biotechnology company, A20 monoclonal antibody (CN1662557A) from the Immune Medical company; H1286/L373 from Vaccinex company, and 1K1791 monoclonal antibody (WO2009031230A1) from the Osaka University. However, all these products are composed of the humanized framework with murine CDR amino acids and may still be recognized by human T helper cell for subsequent immune response.

The recognition of T helper cell to a heterologous protein relies on the binding of its polypeptide with MHC class II molecules on the surface of the antigen-presenting cell (such as dendritic cell), presenting the peptide-MHC complex to the T-cell receptor, and subsequent activation of the lymphocytes and induction of immune response. The unique antibody humanization technology was applied to the sequences of the murine monoclonal antibody 2B8. We designed and selected human germline antibody frame sequences that have less potential binding sites to the MHC II (HLA-DR). Indeed, we found that the immunogenicity of humanized antibodies 1-5 was significantly reduced as assessed by the DC-T cell activation assay. All five humanized antibodies have an equal effect on the activation of CD4+ cells. Furthermore, the T-cell Stimulation Index of the humanized antibodies 1-5 was significantly decreased compared with rituximab.

A number of novel immunotherapeutic agents have emerged over the past decade as effort to improve clinical outcomes of immunotherapy in non-Hodgkin lymphoma (NHL). Several different strategies have been utilized in the engineering of novel anti-CD20 mAbs in order to amplify their efficacy, including the development of humanized or fully human anti-CD20 mAbs with lower immunogenicity and enhancing CDC and/or ADCC via increased Fc binding affinity to FcγRIIIa receptor (CD16) on immune effector cells [19-21]. However, the higher CDC and ADCC activity can induce serious side effects. For example, ofatumumab exhibits higher CDC induction and greater potency in B cell lysis than rituximab, due to its 2-3 fold tighter affinity to C1q on the cell surface. The preclinical data demonstrated the high efficacy of ofatumumab in rituximab-resistant CLL models, and further a Phase I study in patients with relapsed/refractory CLL was undertaken [22, 23]. A total of 33 patients received four weekly ofatumumab infusions at doses ranging from 100 to 2000 mg. About 51% of patients developed infectious complications with the majority being grade 1/2. Furthermore, ofatumumab was evaluated in a Phase III clinical trial in patients with poor prognosis CLL [24]. On the other hand, Ocrelizumab also demonstrates enhanced ADCC compared to rituximab [25]. However, further clinical development for its use in systemic lupus erythematosus and rheumatoid arthritis was suspended by Roche following excess mortality related to opportunistic infections in the patients receiving Ocrelizumab. In this study, we found that the ability of the humanized anti-CD20 antibodies 1-5 to mediate CDC and ADCC was similar to that of rituximab.

In the xenograft model, the humanized anti-CD20 antibodies 1-5 also exhibited equal antitumor activity compared with rituximab. Therefore, it could be concluded that the humanized anti-CD20 antibodies 1-5 have lower immunogenicity than rituximab. And at the same time, they still retain the anti-tumor effect both in vitro and vivo. In our most recent studies, we selected one of the five humanized anti-CD20 antibodies, 3637, which has lower immunogenicity and higher affinity to CD20 antigen for monkey PK and PD studies. Our preliminary data suggests that continuous administration of 3637 showed a more durable tumor suppressing effect when used at the same dose as rituximab (data not shown), suggesting that it could be a good therapeutic candidate for the treatment of Non-Hodgkin's lymphoma.
Conflict of interest

No potential conflicts of interest were disclosed.

References

Zhang/Song/Ye/Hu/Liang/Liu: Characterization of a Novel Humanized Anti-CD20 Antibody


