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Cleavage of the interchain disulfide bonds in rituximab increases its affinity for FcγRIIIA

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ABSTRACT

The Fc region of human IgG1 mediates effector function via binding to Fcγ receptors and complement activation. The H and L chains of IgG1 antibodies are joined by four interchain disulfide bonds. In this study, these bonds within the therapeutic IgG1 rituximab (RTX) were cleaved either by mild reduction followed by alkylation or by mild S-sulfonation; consequently, two modified RTXs – A-RTX (alkylated) and S-RTX (S-sulfonated) – were formed, and both were almost as potent as unmodified RTX when binding CD20 antigen. Unexpectedly, each modified RTX had a higher binding affinity for FcγRIIIA (CD16A) than did unmodified RTX. However, S-RTX and A-RTX were each less potent than RTX in an assay of antibody-dependent cellular cytotoxicity (ADCC). In this ADCC assay, each modified RTX showed decreased secretion of granzyme B, but no change in perforin secretion, from effector cells. These results provide significant information on the structures within IgG1 that are involved in binding FcγRIIIA, and they may be useful in the development of therapeutic antagonists for FcγRIIIA.

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1. Introduction

More than 25 monoclonal antibodies (mAbs) have been developed for treatment of cancer or of immunological and inflammatory diseases [1]. Most of them are chimeric mAbs, humanized mAbs, or human-type mAbs. Antibodies not only bind to antigens, but also exert effector functions – including phagocytosis, complement-dependent cytotoxicity, and antibody-dependent cellular cytotoxicity (ADCC) – via binding to Fcγ receptors (FcγRs) [2,3].

FcγRs play important roles in the activation and suppression of immune responses [4]. FcγRI (CD64) has a high affinity for IgG1, and FcγRII (CD32) and FcγRIII (CD16) each have low affinity for IgG1. IgG1 binds to an FcγR via the hinge and the upper region of the C_H2 domain within the IgG1 [5]. There are 2 disulfide bonds between the two H chains and 1 disulfide bond between each H chain and an L chain; therefore, 4 disulfide bonds join the four chains in the region of the hinge and the upper C_H2 domain. In addition to those interchain disulfide bonds, there are 12 intrachain disulfide bonds, each of which is located in a separate domain of IgG1. The interchain disulfide bonds are much more sensitive to cleavage by S-sulfonation or by reduction followed by alkylation (reduction/alkylation) than are the intrachain bonds [6,7].

The interchain disulfide bonds help maintain the conformation of the hinge region and of the upper C_H2 region of IgG1 antibodies; therefore, we hypothesized that cleavage of all four bonds would change the conformation of IgG1 and thereby result in altered effector function. In fact, reduction of the disulfide bonds reportedly breaks down the open hinge structure and decreases the apparent molecular size of IgG3 [8]; moreover, missense mutations in this region decrease complement-dependent cytotoxic activity [2]. Mutant forms of the middle and/or upper hinge within mAbs decreased or increased binding activity to FcγRIIIA and the suppression or activation, respectively, of ADCC activity [9,10]. Finally, removal of fucose from polysaccharides that are attached to the upper hinge region enhances binding with FcγRIIIA and, therefore, ADCC activity [11].

Rituximab (RTX) is a chimeric IgG1 antibody that specifically recognizes CD20; RTX has been used to treat non-Hodgkin lymphoma [12]. One clinical study of RTX indicated that ADCC was the major mechanism of therapeutic action [13]. ADCC occurs via binding of antibodies to FcγRIIIA on natural killer (NK) cells

Abbreviations: RTX, rituximab; S-RTX, S-sulfonated rituximab; A-RTX, reduced and then alkylated rituximab; mAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; FcγR, Fcγ receptor; KHYG-1/FcγRIIIA, KHYG-1 cells stably expressing FcγRIIIA; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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[3–5]. Here, we mildly S-sulfonated or mildly reduced/alkylated RTX to cleave the interchain disulfide bonds, and the affinity for FcγRIIIA and ADCC activity of each modified RTX were evaluated to assess the importance of the interchain disulfide bonds within IgG1.

2. Materials and methods

2.1. Cell culture and reagents

Human Burkitt's lymphoma Ramos, Raji, or Daudi cells were cultured in RPMI1640 (Wako Pure Chemical Industries Ltd., Osaka, Japan) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/mL streptomycin; KHYG-1 cells were also cultured in this medium with an additional 10 ng/ml IL-2 (Wako Pure Chemical Industries). The KHYG-1 cell lines that stably expressed FcγRIIIA (KHYG-1/FcγRIIIA) were developed by cloning a FcγRIIIA-Val158 cDNA into the pMXs-puro retrovial vector, transfecting this construct into Plat-E packaging cells, and co-culturing the transfected Plat-E cells with KHYG-1 cells (E. Kobayashi et al., unpublished results).

RTX and trastuzumab were purchased from Zenyaku Kogyo Co., Ltd. (Tokyo, Japan) and Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Trastuzumab was used as the non-specific IgG1 control.

2.2. S-sulfonation and reduction/alkylation of RTX

S-sulfonated RTX (S-RTX) and reduced/alkylated RTX (A-RTX) were prepared as described previously [14]. Briefly, 0.5 mg/mL of RTX was allowed to react with 270 mM sodium sulfite and 70 mM sodium tetrathionate at 37 °C for 4 h to generate S-RTX; the solution was then dialyzed against phosphate-buffered saline (PBS). To generate A-RTX, 0.5 mg/mL of RTX was reduced with 10 mM dithiothreitol at 37 °C for 1 h and then treated with 50 mM iodoacetamide at 37 °C for 30 min; this solution was then dialyzed against PBS.

2.3. SDS–PAGE and gel filtration HPLC

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and gel filtration HPLC were performed as described previously [14,15]. SDS–PAGE was performed under non-reducing conditions on 12% acrylamide gel, and the protein bands were stained with Coomassie. The contamination of polymeric and dimeric RTX was assessed by gel filtration HPLC on a Protein Pak 300SW column (Waters Corporation, Milford, MA).

2.4. CD20-binding assay by flow cytometry

Flow cytometry for CD20-binding assay was described previously [15]. Ramos, Raji, and Daudi cells were allowed to react with RTX, S-RTX, or A-RTX and then stained with FITC-conjugated goat anti-human κ chain (Millipore, Billerica, MA). The mean fluorescence intensities were measured using a FACS LSR flow cytometer equipped with CellQuest software (Becton Dickinson, Franklin Lakes, NJ).

2.5. FcRIIIA-binding assay

Enzyme-linked immunosorbent assays (ELISAs) were performed with the extracellular fragments of either of two FcγRIIIA variants, FcγRIIIA Val158 or FcγRIIIA Phe158 [15]. Each variant was used to coat wells of separate 96-well plates, and RTX samples were allowed to react with separately with each variant. The bound RTXs were detected with horseradish peroxidase (HRP)-conjugated goat $F(ab')_2$ anti-human IgG (Chemicon International Inc., Temecula, CA). KHYG-1/FcγRIIIA cells were suspended at 1.0×10^6 cells/mL in FACS buffer (PBS containing 0.1% bovine serum albumin) and then allowed to stand on ice for 30 min. After being washed with FACS buffer, the cells were allowed to react with 670 nM RTX, S-RTX or A-RTX at 4 °C for 30 min. After another wash, cells were stained with FITC-labeled goat anti-human κ chain antibodies on ice for 30 min. After yet another wash, the cells were suspended in 0.5 mL FACS buffer and passed through filters with 59-μm meshes. Fluorescence intensities of cells were measured using a FACS LSR flow cytometer equipped with CellQuest software.

2.6. ADCC assay

ADCC activity was measured using human NK cell line KHYG-1/FcγRIIIA as the effector cells, three types of target cells (Ramos, Raji, or Daudi), and assay methods described previously [15]. Briefly, target cells were stained with 25 μM Calcein-AM (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) at 37 °C for 30 min. KHYG-1/FcγRIIIA cells and the target cells were mixed at an effector/target (E/T) ratio of 25/1 and incubated at 37 °C for 4 h. The fluorescence intensity of calcein released from the target cells was measured with an excitation wavelength of 485 nm and emission wavelength of 538 nm.

2.7. Assays of granzyme B and perforin

Ramos cells were suspended in RPMI1640 containing 2 mM L-glutamine, 1% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 2×10^5 cells/mL and allowed to react separately with each one of five concentrations of modified or unmodified RTX at room temperature with gentle rotation for 1 h. Ramos cells (0.1 mL of 1×10^5 cells/mL) were then mixed with KHYG-1/FcγRIIIA cells (0.1 mL of 25×10^5 cells/mL). Each cell suspension was incubated at 37 °C in 5% CO_2 for 4 h.

ELISA kits for granzyme B and perforin were purchased from Mabtech AB (Nacka Strand, Sweden); granzyme B and perforin were measured according to the manufacturer's instructions. Briefly, an anti-granzyme B mAb (GB10) was used to coat 96-well plates, and bound granzyme B was measured with biotinylated mAb (GB11) and HRP-streptavidin. Similarly, an anti-perforin mAb (Pf-80/164) was used to coat 96-well plates, and bound perforin was detected with a biotinylated anti-perforin mAb (Pf-344).

3. Results

3.1. Cleavage of the interchain disulfide bonds and effect on RTX-CD20 binding

The interchain disulfide bonds within mature RTX were cleaved by one of two methods – mild S-sulfonation or mild reduction/alkylation. SDS–PAGE with non-reducing conditions was then used to analyze RTX and two RTX variants, S-RTX and A-RTX, which were generated by S-sulfonation and reduction/alkylation, respectively (Fig. 1A). S-RTX and A-RTX preparations each comprised mostly H and L chain and only small amounts of H_2, whereas RTX preparations comprised H_2L_2; these findings indicated that either S-sulfonation or reduction/alkylation cleaved the interchain disulfide bonds; more than 90% of interchain disulfide bonds were cleaved in the S-RTX and in the A-RTX preparations. Aggregation of IgG generally increases binding affinity to FcγRs [14]; therefore, the molecular weights of proteins within the RTX, S-RTX, or A-RTX preparations were examined by gel filtration HPLC. Only

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