Construction and Characterization of a Bispecific Anti-CD20 Antibody with Potent Antitumor Activity against B-Cell Lymphoma

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Abstract

To develop more effective anti-CD20 reagents for B-cell lymphoma, we designed and constructed a bispecific tetravalent anti-CD20 antibody, 11B8/2F2(ScFvHL)4-Fc, derived from two fully human monoclonal antibodies (mAb), 2F2 and 11B8. 2F2 is a type I CD20 mAb, which is potent in complement-dependent cytotoxicity (CDC) assays but poor at inducing apoptosis, whereas 11B8 is a type II CD20 mAb, which is effective in induction of apoptosis but ineffective in CDC. Our results showed that 11B8/2F2(ScFvHL)4-Fc possessed apoptosis-inducing activity markedly superior to that of 2F2, and even 11B8, 11B8 plus 2F2, and 2F2(ScFvHL)4-Fc, a 2F2-derived monospecific tetravalent antibody developed previously. Interestingly, 11B8/2F2(ScFvHL)4-Fc displayed a similar ability to mediate CDC as 2F2(ScFvHL)4-Fc, although two of its four antigen-binding arms originated from 11B8. To explore why 11B8/2F2(ScFvHL)4-Fc was so potent in both CDC and apoptotic activity, a bispecific divalent antibody composed of 2F2 and 11B8, denoted as 11B8/2F2-ScFvFc, was constructed and characterized. Our results partially explained the reason for the potent CDC and apoptosis-inducing activity of 11B8/2F2(ScFvHL)4-Fc. Further in vivo therapy studies showed that 11B8/2F2(ScFvHL)4-Fc had a significantly more potent antitumor activity compared with 2F2, 11B8, 2F2 plus 11B8, and 2F2(ScFvHL)4-Fc. These data suggest that 11B8/2F2(ScFvHL)4-Fc may serve as a potential therapeutic agent for B-cell lymphoma. Cancer Res; 70(15); 6293–302. ©2010 AACR.

Introduction

The mouse/human chimeric anti-CD20 antibody rituximab (C2B8) is the first therapeutic monoclonal antibody (mAb) approved for the treatment of relapsed/refractory low-grade or follicular B-cell non-Hodgkin’s lymphomas (NHL; refs. 1–3). Despite the effectiveness of rituximab, only about half of patients respond to the treatment and complete responses are <10% (4, 5). The successful improvement of therapeutic efficacy relies in large part on an understanding of the mechanism by which rituximab functions. Previous studies have suggested that several mechanisms may be involved in providing therapeutic efficacy, including complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), and the induction of apoptosis (6). Anti-CD20 mAbs are often defined as either type I or II based on their ability to redistribute CD20 into lipid rafts (7–9). Type I mAbs (rituximab and most anti-CD20 mAbs) are able to efficiently shift CD20 complexes into rafts, but type II mAbs (B1 and 11B8) are not. The in vitro assays show that type I mAbs are potent in CDC assays and less effective in induction of apoptosis, whereas type II mAbs are effective in inducing apoptosis but ineffective in CDC. In addition to rituximab, the type II mAb B1 has also been approved for treatment of NHL as an 131I conjugate. Interestingly, even as a "naked" mAb, B1 is capable of inducing objective responses in NHL patients, including several complete responses (7, 10).

The multivalent tumor-reactive antibody often shows greater capability to induce apoptosis of tumor cells than its native divalent antibody (11–13). In a previous study, we developed a genetically engineered tetravalent anti-CD20 antibody derived from the type I mAb 2F2 (14). 2F2, a fully human CD20 mAb with exceptionally potent CDC activity (9), was shown to have more potent in vivo antitumor activity than rituximab (14). The tetravalent version of 2F2, 2F2(ScFvHL)4-Fc, was as effective as 2F2 in mediating CDC against B-lymphoma cells, but its apoptosis-inducing activity was markedly superior to that of 2F2 (14). Pharmacokinetic analysis indicated that 2F2(ScFvHL)4-Fc was highly stable...
in vivo, showing a similar serum half-life as the parental divalent IgG1,κ antibody 2F2. Importantly, 2F2(ScFvHL)4-Fc was shown to be far more effective than 2F2 in prolonging the survival of severe combined immunodeficient (SCID) mice bearing systemic human B lymphoma cells (14). These data suggest that the anti-CD20 antibody effective in both mediating CDC and inducing apoptosis may be able to kill CD20-positive lymphoma cells more effectively.

In an attempt to develop more effective anti-CD20 reagents to further improve the efficacy of antibody therapy for B-cell lymphoma, in this study, we generated a bispecific tetravalent anti-CD20 antibody with two antigen-binding arms derived from 2F2 and the other two from 11B8. 11B8 is a type II fully human CD20 mAb potent at inducing apoptosis (8). This bispecific tetravalent antibody, denoted as 11B8/2F2(ScFvHL)4-Fc, has the same structure as the monospecific tetravalent antibody 2F2(ScFvHL)4-Fc (Fig. 1A). Our data indicated that 11B8/2F2(ScFvHL)4-Fc had significantly stronger apoptosis-inducing activity than 2F2, and even 2F2 (ScFvHL)4-Fc, 11B8, and 2F2 plus 11B8. Intriguingly, 11B8/2F2(ScFvHL)4-Fc displayed a similar ability as 2F2(ScFvHL)4-Fc to mediate CDC, although two of its binding sites originated from 11B8. Immunotherapeutic studies further revealed that 11B8/2F2(ScFvHL)4-Fc showed a significantly more potent antitumor activity than did 11B8, 2F2, 11B8 plus 2F2, and 2F2(ScFvHL)4-Fc. Finally, a bispecific divalent antibody, which is composed of 2F2 and 11B8, has been constructed to explore why 11B8/2F2(ScFvHL)4-Fc is so potent in both CDC and apoptotic activity.

Materials and Methods

Cell lines, antibodies, and animals
Two human Burkitt lymphoma cell lines (Raji and Daudi) and Chinese hamster ovary (CHO-K1) cell line were obtained from the American Type Culture Collection. These cell lines were obtained within 2 years before being used in this study. All the cell lines were authenticated twice by morphologic and isoenzyme analyses during the study period. Cell lines were routinely checked for contamination by Mycoplasma using Hoechst staining and consistently found to be negative. The anti-HER2 humanized antibody trastuzumab (anti-HER2) was purchased from Roche Ltd. The 11B8, 2F2, and 2F2(ScFvHL)4-Fc antibodies were expressed and purified as described previously (14). Eight-week-old female BALB/c SCID mice and 6-week-old female athymic nude mice were housed in pathogen-free conditions and treated in accordance with guideline of the Committee on Animals of the Second Military Medical University.
**Construction of expression vectors for the anti-CD20 bispecific tetravalent antibody**

The 11B8 heavy chain variable region gene was fused to the 5’ end of its light chain variable region gene via a linker sequence (Gly4Ser)3 to generate the 11B8 single-chain antibody gene 11B8(ScFvHL). The single-chain antibody gene 2F2(ScFvHL) was generated using the same method. To construct the expression vector for the anti-CD20 bispecific tetravalent antibody 11B8/2F2(ScFvHL)4-Fc (Fig. 1A), the 2F2(ScFvHL) gene was linked to the 11B8(ScFvHL) gene via a short sequence coding for a flexible peptide linker (ASTGS, refs. 15, 16) to generate the 2F2(ScFvHL)/11B8(ScFvHL) gene. Then, the 2F2(ScFvHL)/11B8(ScFvHL) gene was genetically fused in frame to the 5’ terminus of the human IgG1Fc (hinge, CH2 and CH3 domains) gene, and the resulting 2F2(ScFvHL)/11B8(ScFvHL)-hinge-CH2-CH3 gene was cloned into the pcDNA3.1(+). For the expression vector for 11B8/2F2(ScFvHL)4-Fc, the two expression vectors were cotransfected into CHO-K1 cells using Lipofectamine 2000 reagent (Invitrogen), yielding the expression vector for 11B8/2F2(ScFvHL)4-Fc.

**Expression and purification of 11B8/2F2(ScFvHL)4-Fc**

The expression vector for 11B8/2F2(ScFvHL)4-Fc was transfected into CHO-K1 cells using Lipofectamine 2000 reagent (Invitrogen). After transfection, the stable transfectants were isolated by limiting dilution in the presence of 450 μg/mL G418. The cell clones producing the highest amount of antibody were selected and grown in serum-free medium. Finally, the bispecific tetravalent antibody 11B8/2F2(ScFvHL)4-Fc was purified by affinity chromatography on protein A-Sepharose (Amersham Biosciences) from the serum-free culture supernatants. Purified proteins were analyzed by SDS-PAGE and Western blot. A detailed description of SDS-PAGE and Western blot methods is provided as Supplementary Data.

**Binding of CD20 mAbs to Raji cells**

Flow cytometry analysis (FCM) was performed to determine the binding of CD20 mAbs to Raji cells using a FACScan flow cytometer (Becton Dickinson). Briefly, Raji cells (1 × 10^6/mL) were incubated with different concentrations of FITC-labeled CD20 antibodies for 1 hour at 4°C. Then, the cells were washed and analyzed by FCM.

**Dissociation rate of CD20 mAbs**

To determine the dissociation rate of CD20 mAbs from Raji cells, the cells were incubated with saturating FITC-labeled CD20 mAbs (10 μg/mL) at 37°C for 45 minutes, washed twice, and resuspended in complete medium. After different time intervals, the samples were taken, washed, and analyzed by FCM to determine the percentage of the remaining cells that were still stained.

**Cytotoxicity assays**

CDC and ADCC activities of CD20 mAbs were measured by lactate dehydrogenase (LDH) releasing assay using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega). A detailed description of cytotoxicity assays is provided as Supplementary Data.

**Apoptosis assay**

The cells were incubated with different concentrations of CD20 mAbs at 37°C for 16 hours. After washing, cells were treated with Annexin V-FITC (BD Biosciences Pharmingen), washed again, and analyzed by FCM.

**Construction of the anti-CD20 bispecific divalent antibody**

The single-chain antibody genes 11B8(ScFvHL) and 2F2(ScFvHL) were genetically fused in frame to the human IgG1Fc (hinge, CH2 and CH3 domains) gene. The resulting 11B8(ScFvHL)-hinge-CH2-CH3 and 2F2(ScFvHL)-hinge-CH2-CH3 fusion genes were fused to a COOH-terminal His6 tag and a COOH-terminal FLAG tag, respectively. Then, the 11B8(ScFvHL)-hinge-CH2-CH3-His6 and 2F2(ScFvHL)-hinge-CH2-CH3-FLAG genes were respectively cloned into the pcDNA3.1 and pcDNA3.1Zeo vectors (Invitrogen), yielding the pcDNA3.1-11B8(ScFvHL)-hinge-CH2-CH3-His6 and pcDNA3.1Zeo-2F2(ScFvHL)-hinge-CH2-CH3-FLAG expression vectors. For the expression of 11B8(ScFvFc) and 2F2(ScFvFc), these two expression vectors were respectively transfected into CHO-K1 cells using Lipofectamine 2000 reagent. After transfection, the stable transfectants were isolated by limiting dilution in the presence of 450 μg/mL G418 or 250 μg/mL zeocin. To express the anti-CD20 bispecific divalent antibody 11B8/2F2-ScFvFc, the two expression vectors were cotransfected into CHO-K1 cells and the stable transfectants were isolated by limiting dilution in the presence of 450 μg/mL G418 and 250 μg/mL zeocin. The cell clones producing the highest amount of recombinant mAbs were selected and grown in serum-free medium. Finally, 11B8(ScFvFc) and 2F2(ScFvFc) were purified by affinity chromatography on protein A–Sepharose. The bispecific divalent antibody 11B8/2F2-ScFvFc was purified by affinity chromatography on protein A–Sepharose. Purified antibodies were identified by SDS-PAGE, Western blot, and ELISA assays. A detailed description of SDS-PAGE, Western blot, and ELISA assays is provided as Supplementary Data.

**Assessment of raft-associated CD20**

As a rapid assessment of the presence of CD20 in raft microdomains, we used a flow cytometry method as described previously (9). Briefly, cells were incubated with FITC-conjugated mAbs (10 μg/mL) for 15 minutes at 37°C. After washing, one half of the sample was maintained on ice to allow calculation of 100% surface antigen levels; the other was treated with 0.5% Triton X-100 for 15 minutes on ice to determine the proportion of antigens remaining in the insoluble raft fraction. Cells were maintained at 4°C throughout the assay, washed once, and assessed by FCM.

**Immunotherapy**

Groups of 10 female 8-week-old SCID mice were injected via the tail vein with 1 × 10^7 Daudi or Raji cells on day 0, followed 6 days later by the i.v. injection of 10 or 20 μg of CD20 mAbs. Animals were observed daily and euthanized at the onset of hind leg paralysis.
Six-week-old female athymic nude mice were irradiated with 400 rads to suppress innate natural killer activity and, 2 days later, injected s.c. with $1 \times 10^7$ Daudi cells in the right flank. When tumors reached $\sim 60$ mm$^3$ in size, the mice were randomized into groups of 8. Then, the mice were injected i.v. with 20 μg of CD20 mAbs twice weekly for 3 consecutive weeks and tumor volumes were monitored by caliper measurement. Tumor volume was calculated using the following formula: tumor volume (mm$^3$) = length × (width)$^2$/2.

**Tumor penetration studies**

Anti-HER2 or different CD20 mAbs were labeled with Cy5 using Cy5 Maleimide Mono-Reactive Dye kit (Amersham Biosciences). Six-week-old female athymic nude mice were irradiated with 400 rads and, 2 days later, injected s.c. with $1 \times 10^7$ Daudi cells in the right flank. When tumors reached $\sim 300$ mm$^3$ in size, the mice were randomized into groups of three and administered with 100 μg of different Cy5-conjugated mAbs by i.v. tail vein injection at time 0. At 24 hours after injection, tumors were harvested and embedded into OCT mounting medium at $-80^\circ$C. Frozen tumor sections (10 μm) were cut using a Leica cryostat and stained with 4′,6-diamidino-2-phenylindole (Sigma) to visualize nuclei. Then, the tumor sections were examined with Confocal LSM510 microscope (Carl Zeiss Microimaging, Inc.).

**Statistical analysis**

Statistical analysis was performed by Student’s unpaired t-test to identify significant differences unless otherwise indicated. Differences were considered significant at $P < 0.05$.

**Results**

**Construction and characterization of 11B8/2F2 (ScFvHL)$_4$Fc**

SDS-PAGE analysis under nonreducing conditions showed a single band of $\sim 186$ kDa for the bispecific tetravalent antibody 11B8/2F2(ScFvHL)$_4$-Fc (Fig. 1B). Under reducing conditions, 11B8/2F2(ScFvHL)$_4$-Fc migrated as a single band of $\sim 93$ kDa, corresponding approximately to the calculated molecular mass of the monomeric polypeptides. The monospecific tetravalent antibody 2F2(ScFvHL)$_4$-Fc displayed a similar pattern of protein bands to that observed by SDS-PAGE analysis of 11B8/2F2(ScFvHL)$_4$-Fc. The identity of the purified mAbs was further confirmed by Western blot using polyclonal antibodies against human IgG (Fig. 1C).

The binding of 11B8/2F2(ScFvHL)$_4$-Fc to Raji cells was assessed in antigen-binding assays. As shown in Fig. 2A, 11B8/2F2(ScFvHL)$_4$-Fc and 2F2(ScFvHL)$_4$-Fc gave similar binding curves, suggesting that 11B8/2F2(ScFvHL)$_4$-Fc bound to Raji cells with the antigen-binding activity similar to that of 2F2 (ScFvHL)$_4$-Fc. Dissociation experiments were further performed by means of a direct immunofluorescence assay in which Raji cells were incubated with FITC-labeled anti-CD20 mAbs, and the stained cells were quantified after different incubation times. As shown in Fig. 2B, 11B8/2F2(ScFvHL)$_4$-Fc dissociated much more slowly than both 2F2 and 11B8. It exhibited a similar off-rate to that of 2F2(ScFvHL)$_4$-Fc, indicating that both of these two tetravalent constructs engaged more than two of its binding sites and were able to persist for longer time on the cell surface.

**CDC activity of 11B8/2F2(ScFvHL)$_4$-Fc**

The anti-CD20 mAbs were compared for their ability to lyse CD20-positive lymphoma cells in the presence of complement. As shown in Fig. 3A, 2F2 and 2F2(ScFvHL)$_4$-Fc were equally potent in mediating CDC against Daudi cells, which was in agreement with that reported previously (14). As expected,
the type II CD20 mAb 11B8 was ineffective at mediating CDC. So it is not surprising that 2F2 plus 11B8 was only as potent as 2F2 in CDC activity. Interestingly, 11B8/2F2(ScFvHL)₄-Fc lysed Daudi cells as effectively as 2F2(ScFvHL)₄-Fc, although two of its binding sites were derived from the 11B8 antibody. The similar results (Fig. 3A) were obtained with Raji cells, which are more resistant to CDC than Daudi cells.

**ADCC activity of 11B8/2F2(ScFvHL)₄-Fc**

To explore the capacity of CD20 mAbs to mediate ADCC, a standard LDH assay was performed. Purified human peripheral blood mononuclear cells (PBMC) from healthy donors were used as effector cells and CD20⁺ lymphoma cells were used as target. The results summarized in Fig. 3B clearly indicated that 2F2, 11B8, 2F2 plus 11B8, 2F2(ScFvHL)₄-Fc, and 11B8/2F2(ScFvHL)₄-Fc were equally effective in inducing ADCC against Daudi or Raji cells in a dose-dependent manner.

**Induction of apoptosis in CD20⁺ cell lines by 11B8/2F2(ScFvHL)₄-Fc**

Induction of apoptosis was assessed by FITC-Annexin V assays in Daudi and Raji cells. As shown in Fig. 3C, apoptosis induced by 2F2(ScFvHL)₄-Fc was substantially higher than that induced by 2F2 [P < 0.05 for 2F2(ScFvHL)₄-Fc compared with 2F2 at concentrations of 0.4, 2, and 10 μg/mL], which was consistent with the results in our previous report (14). As expected, given its type II status, 11B8 was much more potent in triggering apoptosis than the type I mAb 2F2, and it was able to cause the same level of apoptosis as 2F2(ScFvHL)₄-Fc. 2F2 plus 11B8 seemed to be as effective as 11B8 in triggering apoptosis. It is particularly noteworthy that 11B8/2F2(ScFvHL)₄-Fc was shown to be the strongest inducer of apoptosis. Compared with 2F2 (ScFvHL)₄-Fc, 11B8/2F2(ScFvHL)₄-Fc induced a significantly higher amount of apoptotic cells in Daudi cells at concentrations of 0.4 μg/mL and higher (P < 0.05), although these two mAbs had the same structure. The markedly higher level of...
apoptosis of Raji cells was also observed with 11B8/2F2 (ScFvHL)4-Fc than with 2F2(ScFvHL)4-Fc (Fig. 3C).

Characterization of 11B8/2F2-ScFvFc

In an attempt to explore why the bispecific tetravalent antibody 11B8/2F2(ScFvHL)4-Fc was so potent in both CDC and apoptotic activity, a bispecific divalent antibody, 11B8/2F2-ScFvFc (Fig. 4A, I), and two monospecific divalent antibodies, 2F2ScFvFc and 11B8ScFvFc (Fig. 4A, I), were constructed and characterized. First, the purity and the molecular weight of three purified divalent ScFvFc mAbs were determined by SDS-PAGE. Under nonreducing conditions, all of the three mAbs—11B8ScFvFc, 2F2ScFvFc, and 11B8/2F2-ScFvFc—yielded one protein band with molecular mass of ~100 kDa (Fig. 4A, II). SDS-PAGE analysis under reducing conditions showed a single band of ~50 kDa for 11B8ScFvFc, 2F2ScFvFc, or 11B8/2F2-ScFvFc (Fig. 4A, II). These results indicated that all of the three mAbs were disulfide-linked dimers. The identity of 11B8ScFvFc, 2F2ScFvFc, and 11B8/2F2-ScFvFc was also confirmed by Western blot assay.

Figure 4. Characterization of bispecific divalent antibody 11B8/2F2-ScFvFc. A, structure and size comparison of bispecific divalent antibodies and nonspecific divalent antibodies. I, schematic representation of the structures of 2F2ScFvFc, 11B8ScFvFc, and 11B8/2F2-ScFvFc. II, SDS-PAGE analysis of purified anti-CD20 mAbs under nonreducing and reducing conditions. Lane 1, 2F2; lane 2, 2F2ScFvFc; lane 3, 11B8ScFvFc; lane 4, 11B8/2F2-ScFvFc. III, Western blot analysis of purified anti-CD20 mAbs separated by SDS-PAGE. Lane 1, 2F2; lane 2, 2F2ScFvFc; lane 3, 11B8ScFvFc; lane 4, 11B8/2F2-ScFvFc. B, ELISA assay. Different concentrations of 2F2ScFvFc, 11B8ScFvFc, or 11B8/2F2-ScFvFc were added to ELISA plates coated with anti-FLAG antibody and incubated for 45 min. After washing, horseradish peroxidase-conjugated goat anti-His antibody was added and the plates were further incubated for 45 min. Finally, 3,3',5,5'-tetramethylbenzidine was added as a substrate and the absorbance was measured at 450 nm. Points, mean (n = 3); bars, SD. C, antigen-binding activity of anti-CD20 antibodies. Raji cells were incubated with different concentrations of 2F2ScFvFc, 11B8ScFvFc, or 11B8/2F2-ScFvFc for 1 h at 4°C. The cells were washed and analyzed by FCM. D, translocation of CD20 into Triton X-100-insoluble membrane fraction. Raji cells were incubated with FITC-labeled anti-CD20 mAbs (10 μg/mL) for 15 min at 37°C. After washing, one half of the sample was maintained on ice to allow calculation of 100% surface antigen levels; the other was treated with 0.5% Triton X-100 for 15 min on ice to determine the proportion of antigens remaining in the insoluble raft fraction. All of samples were washed and analyzed by FCM to assess bound FITC-CD20 mAbs. Columns, mean (n = 3); bars, SD.
The in vitro antitumor activity of 11B8/2F2-ScFvFc

To investigate the capacity of 11B8ScFvFc, 2F2ScFvFc, and 11B8/2F2-ScFvFc to mediate CDC, Daudi and Raji cells were used for this experiment. As shown in Fig. 5A, 11B8/2F2-ScFvFc lysed CD20⁺ human B-lymphoma cells slightly less effectively than did 2F2ScFvFc, whereas 11B8ScFvFc exhibited undetectable CDC activity. In the ADCC assay, the data indicated that all of the three antibodies showed similar cytotoxic activity against both Daudi and Raji cells (Fig. 5B). Subsequently, we assessed the ability of 11B8/2F2-ScFvFc to evoke apoptosis. As seen in Fig. 5C, 11B8/2F2-ScFvFc was as effective as 11B8ScFvFc in inducing apoptosis in Daudi cells and the apoptosis induced by 11B8/2F2-ScFvFc was significantly higher than that induced by 2F2ScFvFc. This greater level of apoptosis with 11B8/2F2-ScFvFc than with 2F2ScFvFc was also confirmed using Raji cells (Fig. 5C). These data indicated that 11B8/2F2-ScFvFc was potent in both CDC and apoptotic activity.

11B8/2F2(ScFvHL)₄-Fc effectively prolongs the survival of SCID mice bearing systemic Daudi or Raji tumors

The in vivo therapy studies were performed in SCID mice bearing systemic Daudi or Raji tumors. The survival curves were plotted according to Kaplan-Meier method and compared using the log-rank test. As shown in Fig. 6, all of the anti-CD20 mAbs—2F2, 11B8, 2F2(ScFvHL)₄-Fc, and 11B8/2F2(ScFvHL)₄-Fc—were able to significantly prolong the survival of SCID mice bearing disseminated Daudi tumors (SCID/Daudi; *P* < 0.01 for each compared with anti-HER2). The type I mAb 2F2 showed a markedly improved antitumor activity compared with the type II mAb 11B8 (*P* < 0.01). In an agreement with the results of our previous study (14), the tetravalent version of 2F2, 2F2(ScFvHL)₄-Fc, seemed to be more effective than 2F2 in prolonging the survival of SCID/Daudi mice (*P* < 0.01). Importantly, a pronounced difference in survival was noticed between 2F2(ScFvHL)₄-Fc and 11B8/2F2(ScFvHL)₄-Fc treatment groups (*P* < 0.01), and 11B8/2F2(ScFvHL)₄-Fc showed significantly more potent antitumor activity. Similar results were obtained using Raji cell-bearing SCID mice (SCID/Raji), which were treated with the same dose of 2F2, 11B8, 2F2(ScFvHL)₄-Fc, and 11B8/2F2(ScFvHL)₄-Fc (Fig. 6). Further studies indicated that 11B8/2F2(ScFvHL)₄-Fc also had a markedly improved antitumor activity compared with a combination of 2F2 and 11B8 in SCID/Daudi (*P* < 0.01) and SCID/Raji mice (*P* < 0.01; Supplementary Fig. S1).

11B8/2F2(ScFvHL)₄-Fc potently suppresses tumor growth

The athymic nude mice s.c. inoculated with Daudi cells developed tumors of ~60 mm³ in size by day 10. Then, the tumor-bearing mice were treated with 20 µg of anti-HER2, 2F2, 11B8, 2F2 plus 11B8, 2F2(ScFvHL)₄-Fc, or 11B8/2F2(ScFvHL)₄-Fc twice weekly for 3 consecutive weeks. As illustrated in Fig. 7, all of the CD20 mAbs—2F2, 11B8, 2F2 plus 11B8, 2F2(ScFvHL)₄-Fc, and 11B8/2F2(ScFvHL)₄-Fc—were shown to significantly arrest tumor growth from day 20 onwards (*P* < 0.05 for each compared with control anti-HER2, Mann-Whitney test; Fig. 7). 2F2(ScFvHL)₄-Fc and 2F2 plus 11B8 seemed to be equally potent in inhibiting the growth of tumor, and both of them were shown to be more effective than either 2F2 or 11B8 (Fig. 7). It is particularly noteworthy that the bispecific tetravalent antibody 11B8/2F2(ScFvHL)₄-Fc showed a significantly more potent antitumor activity than all of other CD20 antibodies (*P* < 0.05 for 11B8/2F2(ScFvHL)₄-Fc in comparison with 2F2, 11B8, 2F2(ScFvHL)₄-Fc, and 2F2 plus 11B8 from day 30 onwards, Mann-Whitney test; Fig. 7).

Tumor penetration studies

Because the bispecific tetravalent antibody 11B8/2F2(ScFvHL)₄-Fc is ~25 kDa higher than that of a normal IgG antibody, we evaluated its ability to penetrate CD20-positive tumors. One hundred micrograms of Cy5-conjugated anti-HER2, 2F2, and 11B8/2F2(ScFvHL)₄-Fc were injected i.v. into nude mice bearing Daudi tumors of ~300 mm³ in size (three mice per group). At 24 hours after injection, tumors were harvested, sectioned, and examined for the presence of Cy5-conjugated antibody molecules. As shown in Supplementary Fig. S2, 11B8/2F2(ScFvHL)₄-Fc had a similar ability as the normal IgG antibody, 2F2, to penetrate Daudi tumors, with about half of the tumor cells displaying a robust staining. Our data also showed that the negative control, anti-HER2, did not stain Daudi tumors, showing that targeting by 11B8/2F2(ScFvHL)₄-Fc and 2F2 was CD20 antigen dependent.

Discussion

The primary mechanism of action of rituximab in vivo remains unresolved, with continuing debate over the relative importance of various potential mechanisms, including ADCC, CDC, and direct induction of apoptosis. However, it is becoming clear that the multiple potential mechanisms are not independent and mutually exclusive but are likely to be interactive and potentially cooperative components of rituximab-mediated therapy (6). In this study, we first developed a bispecific tetravalent anti-CD20 antibody, 11B8/2F2...
(ScFvHL)_4-Fc, derived from the type I mAb 2F2 and type II mAb 11B8. 11B8/2F2(ScFvHL)_4-Fc consists of two 2F2-ScFv, two 11B8-ScFv, and a human IgG1 Fc domain, thus possessing four antigen-binding sites. Due to its slower off-rate than the parental mAbs 2F2 and 11B8, 11B8/2F2(ScFvHL)_4-Fc clearly used at least three of its binding sites to bind to the CD20 molecule. Results from apoptosis assays indicated that 11B8/2F2(ScFvHL)_4-Fc promoted much more apoptosis of CD20^+ lymphoma cells than did 2F2 plus 11B8 and the monospecific tetravalent antibody 2F2(ScFvHL)_4-Fc. In the in vitro therapy studies, 11B8/2F2(ScFvHL)_4-Fc was further shown to be significantly more effective in prolonging the survival of tumor-bearing SCID mice than 2F2 plus 11B8 and 2F2(ScFvHL)_4-Fc. Because 11B8/2F2(ScFvHL)_4-Fc displayed a similar ability to mediate CDC and ADCC against B-lymphoma cells compared with 2F2 plus 11B8 and 2F2 (ScFvHL)_4-Fc, it could be speculated that the enhanced apoptosis-inducing activity of 11B8/2F2(ScFvHL)_4-Fc might contribute to its improved antitumor therapeutic efficacy.

Previous studies indicated that treatment of SCID mice with cobra venom factor to inactivate complement markedly reduced the therapeutic activity of rituximab (17). Support for the role of complement in rituximab therapy also comes from the demonstration that complement is consumed during rituximab treatment (18) and that the resistance of different lymphoma cells to rituximab in vivo may be related to their sensitivity to CDC in vitro (19). 2F2 is significantly more potent in CDC than rituximab (9). The in vivo antitumor effect of 2F2 has been shown to be significantly greater than that of rituximab, further supporting an important role for complement in anti-CD20 mAb immunotherapy (14). In this study, it is intriguing to note that 11B8/2F2(ScFvHL)_4-Fc has a similar ability to mediate CDC as 2F2(ScFvHL)_4-Fc, although two of its four antigen-binding arms are derived from the type II mAb 11B8. To elucidate this reason, the bispecific divalent antibody 11B8/2F2-ScFvFc was constructed and assessed for the CDC activity. First, we investigated the ability of 11B8/2F2-ScFvFc to translocate CD20 into
detergent-insoluble fractions of the plasma membrane. Previous studies have shown that the translocation of CD20 into microdomains seems important for the ability of CD20 mAb to activate complement, probably because the process concentrates the antibody Fc regions in such a way that they very efficiently engage multiple globular heads of the C1q molecules, thereby initiating the classic complement pathway (17, 20, 21). Here, the data showed that 11B8/2F2-ScFvFc was able to translocate CD20 into lipid rafts, whereas the monospecific divalent antibody 11B8ScFvFc was not. In an agreement with this, our results further showed that 11B8/2F2-ScFvFc but not 11B8ScFvFc effectively lysed Daudi and Raji cells in the presence of complement. More importantly, 11B8/2F2-ScFvFc exhibited CDC activity only slightly lower than that observed for the monospecific antibody 2F2ScFvFc. Therefore, we speculate that a divalent anti-CD20 antibody has the potential to translocate CD20 into rafts and mediate CDC if only it contains one arm originating from the type I CD20 mAb. These results also partially explain why 11B8/2F2(ScFvHL)₄-Fc, the bispecific tetravalent antibody with two antigen-binding arms derived from 2F2 and the other two arms from 11B8, can mediate CDC as effectively as 2F2(ScFvHL)₄-Fc, which contains four arms from 2F2.

The CD20 mAbs, such as rituximab, LT20, and 2F2, which are poor at inducing apoptosis, are potent at redistributing CD20 into membrane rafts, whereas others, such as B1 and 11B8, which are potent in apoptosis assays, are ineffective at causing CD20 redistribution. However, a CD20 mAb, AT80, was found to be active in both assays. So movement of CD20 into Triton X-100–insoluble rafts seems independent of apoptosis (8). In this study, 11B8/2F2-ScFvFc and 11B8ScFvFc were shown to be equally effective in triggering apoptosis, but only 11B8/2F2-ScFvFc showed the ability to translocate CD20 into rafts. Thus, the data further support that redistribution of CD20 to the Triton X-100–insoluble compartment may be independent of apoptotic response.

Furthermore, because one arm of 11B8/2F2-ScFvFc is from 11B8 and the other one is from 2F2, we speculate that the potent apoptotic activity of 11B8/2F2-ScFvFc should be attributable to its 11B8 ScFv portion. Based on these data, it can be concluded that the 11B8-derived antigen-binding arm of the bispecific tetravalent antibody 11B8/2F2(ScFvHL)₄-Fc may be responsible for its greater apoptosis-inducing activity than 2F2(ScFvHL)₄-Fc.

In summary, the data shown here indicate that a bispecific anti-CD20 antibody, which is composed of type I and type II mAbs, has the potential to be potent in both CDC and apoptotic activity. The tetravalent antibody 11B8/2F2(ScFvHL)₄-Fc, containing two arms from the type I CD20 mAb 2F2 and the other two arms from the type II CD20 mAb 11B8, has been shown to be a potent antitumor agent in tumor-bearing SCID mice, suggesting that it may be a promising therapeutic agent for B-cell lymphoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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