Development and characterization of a fully functional small anti-HER2 antibody

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The penetrating of monoclonal antibodies (mAbs) into solid tumor may be hampered by their large size. The antibody mimetics, composed of two complementarity-determining regions (CDRs) through a cognate framework region (FR), have been demonstrated to have the capacity to penetrate tumors superior to its parental intact IgG. In this study, we used CDR and FR sequences from the humanized anti-HER2 monoclonal antibody trastuzumab to design four antibody mimetics. These antibody mimetics were fused to human IgG Fc to generate mimetics-Fc small antibodies. One of the four mimetics-Fc antibodies binds well to HER2-overexpressing SK-BR3 cells and effectively inhibits the binding of trastuzumab. This mimetics-Fc, denoted as HMTI-Fc, was shown to be effective in mediating antibody-dependent cellular cytotoxicity and exhibit an antiproliferative effect in SK-BR3 cells. To our knowledge, the HMTI-Fc antibody shown here is the smallest fully functional antibody and may have a potential for treatment of cancer. [BMB reports 2009; 42(10): 636-641]

INTRODUCTION

Although over 85% of human cancers are solid tumors, only 25% of the 8 monoclonal antibodies (mAbs) approved for cancer therapy are directed at solid tumor surface antigens (1). This shortfall may be due to barriers to achieving adequate exposure in solid tumors. Solid tumors are characterized by features such as heterogeneous vessel distribution, increasing density of extracellular matrix and tighter collagen organization, which are severe barriers for mAbs diffusion in solid tumors (2). The limited ability of native antibodies to penetrate solid cancers has stimulated the search for smaller alternatives, as a major determinant of speed of mAbs diffusion through tumors is molecular size (3). The rate of diffusion is inversely proportional to the molecular radius, or approximately to the cube root of molecular weight. For example, single-chain antibody (scFv) diffuses approximately 6 times faster than IgG, due to their smaller size and other factors (4).

Recent design variations of engineered antibodies have included reduction in size to Fab', scFv or dissection into minimal binding fragments such as V_{H} domains (5, 6). These antibody fragments have significantly diminished the molecular weight of parent monoclonal antibody. Dramatically, Qiu and colleagues have further whittled bulky IgG antibodies to small antibody mimetics of ~3 kDa, about 1/50 of their normal size (7). These antibody mimetics, which were composed of two complementarity-determining regions (CDRs), V_{H}CDR1 and V_{L}CDR3, through a cognate framework region (V_{H}FR2), retain the antigen recognition of their parent molecules, but have a superior capacity to penetrate tumors. These antibody mimetics are the smallest known antigen-binding fragments of antibodies so far (8).

Antibody has six CDRs residues all of which are more or less involved in antigen recognition. Whereas antibody mimetics composed of CDRs represent a particularly interesting target, the mimetics design remains a big problem (9, 10). Qiu and colleagues proposed four guidelines for mimetic designing (7). First, mimetic should contain at least two antigen-binding sites: one from the V_{H} and the other from the V_{L} domain. Second, the CDR3 loop is an essential component of the mimetic. Third, the CDR3 loop should be complemented by either the CDR1 or CDR2 loop of the other variable domain. Fourth, the C-terminus of the selected CDR1 or CDR2 loop and the N-terminus of the selected CDR3 loop should be joined with a framework region selected from either the VH or the VL domain. Referring to the above guidelines, we used CDR and framework region sequences from trastuzumab, the humanized anti-HER2 monoclonal antibody, to design four small antibody mimetics comprising two interacting V_{H} and V_{L}-derived CDRs. HER2 (erbB2/neu) gene encodes an epidermal growth factor receptor (EGFR)-related ty-
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Fig. 1. Antigen binding assays for mimetics-Fc antibodies. SK-BR3 cells were incubated with a series concentration of the antibodies for 45 min at 4°C. Cells were washed and incubated with FITC labeled goat anti-human IgG for 45 min at 4°C. Cells were then washed and analyzed by FCM. The control is the mean fluorescence intensity of trastuzumab at the highest concentration (6830 pM). All data expressed as % of control and as the mean ± SD (n = 3).

Fig. 2. Binding of FITC-trastuzumab to human SK-BR3 cells in the presence of increasing concentrations of the antibodies. SK-BR3 cells were incubated with 1 μg/ml FITC-trastuzumab and increasing concentrations of competing antibodies for 45 min at 4°C. The cells were then washed and analyzed by FCM. Maximal fluorescence means the mean fluorescence intensity obtained in the absence of competitor antibodies. All data were expressed as the mean of triplicate samples.
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Fig. 3. Cell growth inhibition assays. SK-BR3 (A) and MCF-7 cells (B) at a density of $1.5 \times 10^4$ per well were seeded in 96-well plates overnight. A series concentration of antibodies was added to the cells and incubated for 24 h. Then cell viability was determined by Cell Titer 96 non-radioactive cell proliferation assay kit. All data were expressed as the mean of triplicate samples.

Fig. 4. ADCC tests. ADCC activities of antibodies were measured by lactate dehydrogenase (LDH)-releasing assay using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit. Percentage of specific lysis was calculated according to the following formula: % lysis = (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100. *P < 0.05. All data were expressed as the mean ± SD (n = 3).

cell lysis was not induced by HMTI-Fc and considered as nearly background. No effect was detectable in parallel assays carried out with HER2-negative MCF-7 cells (data not shown). These results demonstrate the specificity of the HMTI-Fc-dependent cell-mediated cytolytic activity, clearly based on both binding abilities of the mimetics-Fc antibody: (I) to the cognate receptor with its antigen binding sites; (II) to natural killer cells with its Fc effector domain.

DISCUSSION

Monoclonal antibodies have become the protein therapeutics of choice for targeting tumor (1). Currently, eight mAbs have been approved for cancer therapy. However, only trastuzumab, cetuximab and bevacizumab are approved to treat solid tumors and actually bevacizumab is directed at a soluble ligand (vascular endothelial growth factor), not at a surface protein within solid tumors. Full-length antibodies, especially the high-affinity binders, do not generally penetrate far into solid tumors (15, 16). Such a dilemma reflects that there is an urgent demand in improving the penetration of mAbs in solid tumors (3).

Reducing the size of mAbs represents a promising way to improve the penetration of mAbs (7, 8). Recent design variations of engineered antibodies have included reduction in size to single-chain antibody, dissection into minimal binding fragments such as VH domains, and rebuilding of scFvs into multivalent high avidity oligomeric scFvs (5). These recombinant antibody fragments, such as scFvs, whose activities depend on the correct folding of the proteins, are not very convenient in expression and purification (15). Antibodies have six CDRs (three in each light and heavy chain), which determine their binding affinity and specificity. On each chain, the CDRs are interspersed with four framework regions (FRs) that maintain the CDRs in their proper orientations. Small antibody mimetics composed of various CDRs combination with or without FRs exhibit weak binding affinity but retain antigen-specificity (7, 10). It has been proposed that two CDRs alone (one from the heavy chain and one from the light chain) might retain antigen specificity if separated by an FR that allows them to assume a conformation similar to that of the parental antibody after antigen binding (8).

In the present study, we designed four small antibody mimetics of anti-HER2 humanized antibody trastuzumab and fused them to the human IgG Fc, yielding fully functional antibodies (mimetics-Fc). One of the four mimetics-Fc antibodies, HMTI-Fc, was shown to bind well to HER2-positive cells, though its affinity was reduced compared with the full size parental antibody. The three dimensional structure of HMT-I was built by homology modeling and shown in ribbon representation (Supplementary Fig. 2). VLFR2, which links VLCDRl and VHCDR3 in the native Fab, plays a determinant role to connect the VHCDR1 and VLCDR3 and keep them in a “quasi-physiological” binding-site surface (Supplementary Fig. 2), which is in consistent with the results reported by Qiu et al (7). Previous stud-
ies found that the lowest-affinity scFv had the most uniform distribution throughout the tumor, whereas the highest affinity scFv was found mainly in the perivascular region of the tumor (16). To avoid such a "binding-site barrier," we have retained weakened affinity instead of enhanced affinity of parental antibody against targets.

The Fc region of the antibody directly participates in recruiting immune cells in ADCC (8). We fused Fc region to the small mimetics antibody HMT-I to produce HMTI-Fc, aiming to trigger its ADCC function. Our results showed that HMTI-Fc effectively mediated ADCC against HER2-positive breast cancer cells. The in vitro antitumor activity of HMTI-Fc was also demonstrated by the growth inhibition assay.

In summary, the mimetics-Fc antibody HMTI-Fc, which has moderate binding affinity and antitumor activity, is the smallest fully functional antibody so far. The data shown here suggest that this small antibody may have the potential as a novel antitumor agent in solid tumor.

MATERIALS AND METHODS

Materials

Two human breast cancer cell lines, SK-BR3 and MCF-7, and the Chinese hamster ovary (CHO)-K1 cell line were obtained from the American Type Culture Collection (ATCC). The anti-HER2 humanized antibody (trastuzumab) was purchased from Roche Ltd. Trastuzumab was labeled with FITC to produce FITC-trastuzumab. FITC-goat anti-human IgG and horse-radish peroxidase (HRP)-conjugated goat anti-human IgG were purchased from Zymed (San Francisco, CA).

Rational design of the mimetics-Fc antibody

Referring to the above guidelines proposed by Qiu et al. (7), we used CDR and framework region sequences from trastuzumab to design four small antibody mimetics comprising two interacting V_{H} and V_{L}-derived CDRs, denoted as HMT-I, HMT-II, HMT-III and HMT-IV. The heavy and light chain variable region genes of trastuzumab (17) were synthesized by Shanghai Sangon Biological Engineering Technology Company (Shanghai, China). The following primers were used to generate the small antibody mimetics using trastuzumab variable gene as template: for HMT-I (LCDR1-LFR2-LCDR3), the primers were 5'-AACGGT CACCATGGGATGAGGACTTCTCTCTCTTTGGT-3' and 5'-GCTAGCGTGGGAGGAGTAGTATAATGTTGCTGTGCAACCCATTCCAGT GCCATG-3'; for HMT-II (HC
dr1-HCDR2-LCDR3), the primers were 5'-AACGGT CACCATGGGATGAGGACTTCTCTCTCTTTGGT-3' and 5'-GCTAGCGTGGGAGGAGTAGTATAATGTTGCTGTGCAACCCATTCCAGT GCCATG-3'; for HMT-III (HCDR1-LCDR2-LFR3-HCDR3), the primers were 5'-AACGGT CACCATGGGATGAGGACTTCTCTCTCTTTGGT-3' and 5'-GCTAGCGTGGGAGGAGTAGTATAATGTTGCTGTGCAACCCATTCCAGT GCCATG-3'; for HMT-IV (LCDR2-HFR2-LCDR3), the primers were 5'-AACGGT CACCATGGGATGAGGACTTCTCTCTCTTTGGT-3' and 5'-GCTAGCGTGGGAGGAGTAGTATAATGTTGCTGTGCAACCCATTCCAGT GCCATG-3'. The restriction endonuclease recognition sites (HindIII and NheI) were underlined. The resultant antibody mimetics genes were genetically in frame fused to the human IgG Fc gene. Then the mimetics-Fc fusion genes were cloned into the pcDNA3.1 (+)-vector (Invitrogen, Carlsbad, CA) to yield the expression vectors.

Expression and purification of mimetics-Fc antibodies

The expression vectors for mimetics-Fc antibodies were transfected into Chinese hamster ovary (CHO)-K1 cells according to the manufacturer's protocols. In brief, cells grown in RPMI containing 10% FCS at 80-90% confluency were transfected with 0.8 μg of expression vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stable transfectants producing the highest amount of recombinant antibodies were selected in the presence of G418 (Sigma, St. Louis, MO) and grown in serum-free medium. Recombinant antibodies were purified by Protein A affinity chromatography as described previously (18).

SDS-PAGE and Western blot

The purified mimetics-Fc antibodies were analyzed by SDS-PAGE and quantified by MicroBCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's instructions as described previously (19). For Western blot analysis, purified mimetics-Fc antibodies were electrophoresed on a 10% SDS-PAGE under non-reducing conditions and then electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). After blockade of its free protein-binding sites, the PVDF membranes were treated with HRP-conjugated goat anti-human IgG. Finally, the bands were detected by enhanced chemiluminescence reagents (Amersham Biosciences).

Flow cytometry

To examine the binding activity of mimetics-Fc antibodies, 1 × 10^6 SK-BR3 cells were incubated with increasing concentrations of purified mimetics-Fc antibodies for 45 min at 4°C. After washing twice with PBS, the cells were incubated with FITC-goat anti-human IgG (Zymed). To examine the specificity of mimetics-Fc antibodies, competitive binding assay was performed as follows. 1 × 10^6 SK-BR3 cells were incubated with 1 μg/ml FITC-trastuzumab and increasing concentrations of purified competing antibodies for 45 min at 4°C. Thereafter, the cells were washed and analyzed by FACScan flow cytometer (Becton-Dickinson, San Jose, CA). The IC_{50} values of competitors were calculated using a four parameter algorithm.

Cell growth inhibition assays

Breast cancer cells (SK-BR3 and MCF-7 cells) at a density of 1.5 × 10^5 per well were seeded in 96-well plates in a 5% CO_2 in-

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incubator at 37°C. After an overnight incubation, different concentrations of mimetics-Fc antibodies were added and incubated for another 24 h. Then cell viability was determined by Cell Titer 96 non-radioactive cell proliferation assay kit according to the manufacturer’s protocol (Promega, Madison, WI) as described previously (20). Briefly, 20 μl MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt)/phenazine methosulfate solution was added into each well. After incubation for 2 h at 37°C, the absorbance was measured at 490 nm using BIO-TEK ELx800 Universal Microplate Reader (BioTek Instruments Inc., Winooski, VT). The IC_{50} values of competitors were calculated using a four parameter algorithm.

**ADCC test**

ADCC activities of mimetics-Fc antibodies were measured by lactate dehydrogenase (LDH)-releasing assay using the CytoTox 96 non-Radioactive Cytotoxicity Assay kit (Promega, Madison, WI) according to the manufacturer’s instructions as described previously (21). Briefly, the cells were incubated with the small antibody mimetics for 1 h in phenol red-free DMEM culture medium in a 5% CO\(_2\) incubator at 37°C, followed by the addition of human peripheral blood mononuclear cells (PBMC) as effector cells (effector to target, 5 : 1; 10 : 1; 25 : 1; 50 : 1; 100 : 1 for ADCC assay). Controls included target cells incubated in the absence of effector. After an additional incubation for 4 h at 37°C, the cell lysis was determined by measuring the amount of LDH released into the culture supernatant. Maximum LDH release was determined by lysis in 0.2% TritonX-100. Percentage of specific lysis was calculated according to the following formula: % lysis = (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100.

**Molecular modeling**

The three-dimensional structure of HMT-I were built by homology modeling based on the crystal structure of trastuzumab using Insight II/Homology Software Package (Accelrys Inc., San Diego, CA). In order to minimize steric clashes and ensure correct bond lengths and angles after modeling, the side chains of the model structure were subjected to 5, 000 steps of energy minimization using steepest descent method and 10, 000 steps of energy minimization using conjugate gradient method, while the α carbon atoms of the main chain were held fixed in position (the convergence criterion is 0.5 and 0.01 KJ/mol respectively).

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