BiHC, a T-Cell–Engaging Bispecific Recombinant Antibody, Has Potent Cytotoxic Activity Against Her2 Tumor Cells

Abstract
Among different cancer immunotherapy approaches, bispecific antibodies (BsAbs) are of great interest due to their ability to recruit immune cells to kill tumor cells directly. Various BsAbs against Her2 tumor cells have been proposed with potent cytotoxic activities. However, most of these formats require extensive processing to obtain heterodimeric bispecific antibodies. In this study, we describe a bispecific antibody, BiHC (bispecific Her2-CD3 antibody), constructed with a single-domain anti-Her2 and a single-chain Fv (variable fragment) of anti-CD3 in an IgG-like format. In contrast to most IgG-like BsAbs, the two arms in BiHC have different molecular weights, making it easier to separate hetero- or homodimers. BiHC can be expressed in Escherichia coli and purified via Protein A affinity chromatography. The purified BiHC can recruit T cells and induce specific cytotoxicity of Her2-expressing tumor cells in vitro. The BiHC can also efficiently inhibit the tumor growth in vivo. Thus, BiHC is a promising candidate for the treatment of Her2-positive cancers.

Introduction
Human epidermal growth factor receptor 2 (Her2) is an important diagnostic and treatment target for breast cancer. Her2 overexpression occurs in about 25% of breast cancers which are aggressive and highly metastatic with poor clinical outcome.1,2 Besides breast cancer, Her2 overexpression was also discovered in a variety of other cancers, including colorectal, ovarian, pancreatic, and non–small-cell lung cancers.3 Inhibiting Her2 activity with either monoclonal antibodies (mAbs) or small molecular inhibitors has proven effective for treating Her2-positive metastatic breast cancer. Three Her2-specific mAbs, trastuzumab, pertuzumab, and ado-trastuzumab emtansine, have been approved in the clinic by the FDA and resulted into survival benefits in Her2-positive breast cancer patients. However, many of the patients who initially responded to trastuzumab eventually relapsed.4 Furthermore, current anti-HER2 therapeutics still lack efficacy in tumor cells with relatively low to medium levels of Her2 overexpression. Thus, there is an urgent need to develop anti-Her2 agents that can kill cancer cells with a broad range of Her2 overexpression.

Bispecific antibody is one of promising cancer immunotherapies by engaging immune cells to combat tumor cells.5 A variety of bispecific antibody formats have been studied,6 for example, bispecific T cell
engager (BiTE), which combines two single chain Fvs with a short amino acid linker. One BiTE bispecific antibody, blinatumomab, has already been approved by FDA for the treatment of acute B-cell leukemia. Although potent tumor cell clearance was observed in clinic, blinatumomab has an extremely short half-life. Solutions to enhancing the in vivo half-life of bispecific antibody include fusion with Fc or adopting IgG-like format as of catumaxomab. To facilitate the Fc heterodimerization, “Knobs-into-Holes” strategy has been developed. Other IgG-like bispecific formats have also been proposed to facilitate the Fc heterodimerization while reducing homodimerization.

Recently, employing single-domain antibodies for constructing bispecific antibodies have been proposed due to their easy expression and purification from Escherichia coli. In this present study, we combined the advantages of single-domain antibody and Fc, and constructed an IgG-like bispecific antibody HER2/CD3 (BiHC). The BiHC can specifically bind to Her2-expressing tumor cells and is capable of recruiting T cell to induce cytotoxicity of even Her2 low expression cancer cells in vitro. BiHC can also inhibit tumor growth in vivo. Our findings suggested that the BiHC format may provide a format to produce bispecific antibodies more efficiently with potent antitumor activities.

**Material and Methods**

**Antibody Design and Purification**

The BiHC antibody was constructed as shown in Figure 1. A signal sequence (pelB) was added at the N-terminal end of two respective polypeptides for periplasmic expression. His6-tag and Flag-tag were added at the c-terminal for purification and detection. BiHC was formed via the heterodimerization of VH-VL (anti-CD3)-CH2CH3 (T366S, L368A, Y407V, Hole mutant) and anti-Her2 VHH-CH2CH3 (T366W, Knob mutant). The VH and VL of anti-CD3 (humanized UCHT1 15), the camel anti-HE2 VHH (GenBank: JX047590.1), and “Knobs-into-Holes” mutants 11,12 were synthesized (Genescript) and then cloned into the pET21a or pET26b plasmids.

To purify BiHC, periplasmic expression and extraction were performed as described previously. BiHC was then purified by Protein A affinity chromatography. Gel filtration was performed on a Superdex-200 10/300 GL column (GE Healthcare, 17-5174-01) using ÄKTA Avant (GE Healthcare). Protein standard (Sigma Aldrich, Cat: MWGF200) was loaded as control for analysis.

**Cell Lines**

Cell lines, including the Her2-positive human breast cancer cell lines MDA-MB-435 and SK-BR-3, human ovarian cancer cell line SKOV-3, Her2-negative Chinese hamster ovary cell line CHO, human ovarian cancer cell line LS174T, human embryonal kidney cell line HEK293T, and T cell line Jurkat, were purchased from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. The cell lines were cultured in DMEM or RPMI-1640 (Thermo, China) with 10% HI fetal bovine serum (Thermo, USA) and 1% penicillin/streptomycin (Hyclone) at 37°C with 5% CO2.

**Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and T Cells**

Human PBMCs were prepared from healthy donors’ blood using Ficoll density centrifugation as described previously. T cells were

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**Figure 1.** BiHC can be expressed and purified from *E. coli* efficiently as a heterodimer. (A) The constructs of BiHC for bacterial expression. Each construct contains a signal sequence, an anti-CD3 ScFv or anti-Her2 VHH, Fc mutants, and a Flag tag or His6 tag at c-termini for detection. (B) Diagram of BiHC structure formed by heterodimerization. (C) Detection of purified BiHC after Protein A affinity chromatography. Top panel, Coomassie blue staining of SDS-PAGE; middle panel, anti-His Western blot; bottom panel, anti-Flag Western blot. (D) Gel filtration chromatography of BiHC; based on protein markers, BiHC run at approximately 95 kDa.
then isolated from the PBMCs using an EasySep Human CD3 Positive Selection Kit (STEMCELL Technologies, Inc., Vancouver, Canada) according to the manufacturer’s instructions. The isolated T cells were cultured in complete RPMI 1640 with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO2 humidified incubator before assays.

**Flow Cytometry Analysis**

Her2 binding properties of BiHC were analyzed using the following flow cytometry method. A total of 1 x 10^6 cells per sample were collected by centrifugation at 1000 rpm for 5 minutes and then washed with 1x phosphate-buffered saline (PBS) containing 0.2% BSA. The cell pellet was resuspended in 100 μl of ice-cold PBS + 0.1% BSA and then incubated with 5 μg BiHC or control primary antibody on ice for 1 hour followed by washing twice with ice-cold PBS + 0.1% BSA. After washing, the cells were incubated with Alexa488-conjugated anti-human IgG1 (Invitrogen, A11013) for another 1 hour on ice. Cells were then washed and resuspended in 500-μl 1x PBS buffer. Flow cytometric analysis was performed on FC500 (Beckman Coulter). Anti-HER2/neu-PE mab (BD cat. 340552) was used as positive control for Her2 binding.

**Immunofluorescence Assay**

To analyze the binding of antibodies to cell surface Her2, immunofluorescence assay was performed. The cells were plated on the class bottom dish (cellvis, cat. D35-10-1-N) and then washed by PBS three times before fixing by 4% paraformaldehyde. After blocking with PBS and 5% BSA for 1 hour at room temperature, the cells were incubated with BiHC and then goat anti-Hu IgG(H + L)-AF488 (Invitrogen, A11013). After washing with PBST, samples were then examined using Zeiss EC Plan-Neofluar 40×/1.30 Oil DIC M27 objective and analyzed with ZEN software.

**Cytotoxicity Assays**

Cytotoxicity assays were performed as described previously with minor modifications. Briefly, human T cells or PBMCs were used as effector cells. Tumor cell lines were used as target cells. Target cells (2.5–5 x 10^5 cells/well) were plated into 96-well flat-bottomed plates. After 12-hour culture, effector cells (2.5–5 x 10^5 cells/well) were added with indicated amount of BiHC. After 72-hour incubation, live cells were measured using Cell Counting Kit-8 reagent (cck8, Dojindo). Survival rate was calculated as: (OD450 BiHC + Effector – OD450 medium)/ (OD450 Effector – OD450 medium) x 100%.

**In vivo Efficacy Study**

In vivo tumor growth inhibition assays were performed as described previously. Briefly, fresh cultured SKOV-3 cells (1 x 10^5) were mixed with fresh isolated human PBMCs (5 x 10^6) in 200-μl volume and subcutaneously co-implanted into the right flank of 5-week-old male NOD/SCID mice. Two hours after tumor cell injection, mice were treated with BiHC (5 mg/kg) or PBS alone every 3 days by intraperitoneal injection for four doses. Mice were weighed and tumor growth was measured twice a week using calipers. Tumor volume was calculated as 1/2(length x width^2). NOD/SCID mice (Vital River Co. Ltd., Beijing) were maintained in the Animal Experiment Center of Sun Yat-Sen University under standardized environmental conditions.

**Results**

**Expression and purification of BiHC from E.coli**

BiHC, a bispecific antibody with Fc, was obtained by heterodimerization of two polypeptides with anti-Her2 and anti-CD3 modalities, respectively (Figure 1). To facilitate the periplasmic expression in E. coli, a signal sequence (PelB) was added to the N-terminal of both polypeptides. The “Knobs-into-Holes” mutations, which consist of a T366W (Knob) mutation in one Fc and T366S, L368A, and Y407V (Hole) mutations in the other Fc, were added individually to the anti-CD3 domain (Figure 1, A and B), were linked with each individual polypeptide. To engage T cells, the ScFv of anti-CD3 clone UCHT1 was used. To recognize Her2-positive tumor cells, the single-domain anti-Her2 VHH (GenBank: JX047590.1) was used. The two constructs were cloned into the E. coli expression vectors pET21a or pET26b for protein expression.

After induction in E. coli, BiHC can be purified by Protein A affinity chromatography with a final yield of 0.2 mg/l (Figure 1C). The presence of both polypeptides can be identified by their distinct C-terminal tags, anti-His for the anti-Her2 VHH-Fc around 40 kDa and anti-Flag for the anti-CD3 ScFv-Fc around 55 kDa (Figure 1C). Unlike bispecific antibodies with both arms using conventional ScFv, the different molecular weights of two polypeptides, ~40 kDa for anti-Her2 VHH and ~55 kDa for anti-CD3, can be visualized individually by Coomassie blue staining (Figure 1C). The Coomassie blue staining also revealed the two polypeptides with similar molar ratio, suggesting heterodimerization. Gel filtration analysis also showed that BiHC protein ran as a single peak with a molecular size close to 95 kDa, which was the expected size of a single heterodimeric BiHC molecule, suggesting that BiHC is in the form of heterodimeric monomer (Figure 1D).

**BiHC recognizing Her2 antigen**

To confirm whether BiHC can bind Her2 tumor cells, flow cytometry analysis was performed (Figure 2). Both LS174T cells (Her2 medium expression) and MDA-MB-435 cells (Her2 low expression) cells can be bound by positive control antibody, PE-conjugated anti-Her2/neu (Figure 2, A and B), with LS174T cells showing higher binding intensity and MDA-MB-435 cells showing lower binding intensity. The cells were also analyzed with BiHC and then anti-human IgG Alexa488 as secondary antibody. Similar pattern of binding of BiHC on the two cell lines, higher on LS174T cells and lower on MDA-MB-435 cells, was observed for BiHC (Figure 3, C and D), suggesting that BiHC is able to bind Her2 molecules on the surface of Her2-positive tumor cells and the binding correlates with Her2 expression levels.

To further confirm that BiHC can bind Her2-positive cells, immunofluorescence analysis was also performed. Using Her2-positive cells, LS174T, and Her2-negative cells, CHO, BiHC showed strong binding to LS174T but not CHO (Figure 3E), confirming that BiHC can specifically bind to Her2-positive cells.

**Potent T cell-dependent cytotoxicity of BiHC against tumor cells**

To evaluate whether BiHC can mediate killing of tumor cells, cytotoxic assays were performed for cells with or without Her2 expression. Freshly isolated nonstimulated human T cells were used as effector cells. In the absence of effector cells, BiHC has no cytotoxicity against HER2-negative cells CHO and Her2-positive cells SKBR-3,
SKOV3, LS174T, or MDA-MB-435 cells (Figure 3, A-E). However, in the presence of effector cells, increased cytotoxicity was observed for all the Her2-positive cells but not Her2-negative cells (CHO cell) (Figure 3, A-E). The target-specific cytotoxicity against HER2-positive cells also exhibited a dose-dependent manner. BiHC also showed higher cytotoxic activities against Her2 higher-expression tumor cells (SKOV3 and SKBR3) than the Her2 medium-expression cell line LS174T or low-expression cell line MDA-MB-435, consistent with previous

Figure 2. Flow cytometry analysis of BiHC binding on tumor cells. (A and B) Tumor cells LS174T (A, left panel) and MDA-MB-435 (B, right panel) using control antibody anti-Her2 (neu)-PE. Gray dot line: cells with PBS staining; black solid line: anti-Her2 (neu)-PE staining. (C and D) Tumor cells LS174T (C, left panel) and MDA-MB-435 (D, right panel) using BiHC and secondary antibody Alex488-conjugated anti-human IgG1 (Invitrogen, A11013). Gray dot line: cells with PBS staining; black solid line: BiHC, then Alex488-conjugated anti-human IgG; black dash line: Alex488-conjugated anti-human IgG1 only. (E) The immunofluorescence staining of CHO (top panel) and LS174T (bottom panel) using DAPI to staining nucleus (left), BiHC as primary and then Alex488 conjugated anti-human IgG1 (Invitrogen, A11013) (middle). The merged images are on the right.

Figure 3. BiHC mediates potent cytotoxic activities against Her2 tumor cells in the presence of T cells. Freshly isolated human T cells were used as effector cells. Different Her2-positive or -negative cell lines were used as target cells. Different cells: (A) CHO, (B) SKOV3, (C) SKBR3, (D) LS174T cells, and (E) MDA-MB-435 were used as target cells (E/T 10:1) in the presence of different concentrations of BiHC. The dotted lines: target cells with BiHC only without effector cells. Solid lines: target cells with BiHC in the presence of effector cells. The figures were plotted using Excel. (F) SKOV3 and (G) LS174T cells were treated with different concentrations of BiHC (triangle) or trastuzumab (square) in the presence of PBMCs. The viable cells were measured after culture for 3 days. Survival rates were calculated as described in the Methods and Materials. The figures were plotted using Prism6. All data are the mean of triplicates with error bars representing the standard deviation.
results using Herceptin-based bispecific antibodies. Thus, BiHC mediates potent T-cell–dependent cytotoxicity against Her2-positive tumor cells.

Anti-Her2 monoclonal antibody trastuzumab is currently the standard therapy for Her2-overexpression breast cancer patients. To test the cytotoxic activity of BiHC, SKOV3 and LS174T cells were treated with trastuzumab and BiHC along with PBMCs. PBMCs were used as BiHC uses T cells as effector cells and trastuzumab uses natural killer or macrophage as effector cells to perform antibody-dependent cell-mediated cytotoxicity. Trastuzumab with fresh isolated human PBMCs showed minimal cytotoxic activities against SKOV3 and LS174T cells (Figure 3, F and E). However, the same amount of BiHC showed much higher cytotoxic activities (Figure 3, F and E), suggesting more potent cytotoxic activities of BiHC against Her2-positive cancer cells.

Tumor growth inhibition by BiHC in vivo

The antitumor activity of BiHC was further evaluated in vivo using a SKOV-3 tumor xenograft model. Her2-positive SKOV-3 cells were engrafted with or without human PBMCs subcutaneously on NOD/SCID mice and then treated with either PBS or BiHC (Figure 4). For mice transplanted with SKOV3 cells, rapid tumor growth was observed. In the presence of human PBMCs, tumor growth was modestly inhibited. When the mice transplanted with PBMCs were treated with BiHC, significant tumor growth inhibition was observed (Figure 4). In the mice that were treated with BiHC, tumor developed only in one of the five mice. No tumor growth was observed in the other four mice treated with BiHC. No apparent toxicity was observed in all mice. These results demonstrated that BiHC can inhibit tumor growth in xenograft mouse models.

Discussion

As one of promising cancer immunotherapy approaches, bispecific antibody has been generating great interests in cancer therapy. Currently, over 60 different bispecific antibody formats have been investigated. Each of these different BsAb formats brings different properties in binding valency, geometry of antigen-binding sites, pharmacokinetics, or effector functions. The Fc-based IgG-like format is attractive because such antibodies are close to natural IgG architecture and possesses desirable biophysical properties, such as high stability, large-scale manufacturing capability, low immunogenicity, and a long serum half-life.

However, formats, especially those based on IgG or Fab structures, are difficult to express in E. coli and have the pairing problems of either interheavy chains or light to heavy chains.

Recently, employing single-domain antibodies for constructing bispecific antibodies has been proposed due to the ease of expression and purification of these proteins from E. coli. The single-domain antibodies, or VHHs from the dromedary or llama, have a number of advantages compared to the Fab, or scFv. For example, only one domain is necessary to generate an antigen-binding fragment. Antibodies using VHH linked to Fc offers an attractive alternative to obtain antigen binding site with smaller molecular weight, full function of antibody, and less immunogenicity.

In this article, we describe a potent Her2/CD3 bispecific antibody, BiHC, based on the heterodimerization of Fc using “Knobs-into-Holes” mutants. Single-domain antibody anti-Her2 VHH was used to target Her2-positive tumor cells. The anti-CD3 clone UTC1 ScFv was used to recruit T cells. Different from bispecific antibodies with VHH on both arms, or both arms with Sc-Fv, or regular Fab fragment, the size difference of VHH and ScFv makes it easy to determine the efficiency of heterodimerization of BiHC (Figure 2). The size difference will also make it easy to obtain heterodimeric bispecific antibodies. Although we have not tried the homodimeric anti-CD3 ScFv-Fc or anti–Her2-VHH-Fc, those formats are expected to have different molecular weight based on size exclusion chromatography.

Due to the properties of anti-CD3 clone UTC1 ScFv and the anti-Her2 VHH, BiHC can be expressed and purified in an active form from periplasm of E. coli even with Fc fusion. With the anti-Her2 and anti-CD3 moieties, BiHC can kill Her2-positive cancer cells with high potency by recruitment of T cells. The cytotoxic activity of BiHC is likely affected by the expression level of Her2 in cancer cells as the Her2 high-expressing cell lines, SKBR3 and SKOV3, are more sensitive to BiHC than the Her2 low-expression cell line MDA-MB-435 (Figure 3), consistent with previous Her2 targeting bispecific antibodies. However, more data are needed to validate the correlation. Those studies confirmed that BiHC is a potent bispecific antibody against Her2-positive tumor cells. With its additional advantages in purification and heterodimer characterization, BiHC is a promising immunotherapeutic candidate for Her2-positive tumors.

Conflicts of Interest

The authors declare no conflicts of interest.

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