A novel therapeutic strategy to rescue the immune effector function of proteolytically-inactivated cancer therapeutic antibodies

Xuejun Fan\textsuperscript{a}, Randall J. Brezski\textsuperscript{d,*}, Hui Deng\textsuperscript{a}, Pooja M. Dhupkar\textsuperscript{a}, Yun Shi\textsuperscript{a}, Anneliese Gonzalez\textsuperscript{b}, Songlin Zhang\textsuperscript{c}, Michael Rycyzyn\textsuperscript{d}, William R. Strohl\textsuperscript{d}, Robert E. Jordan\textsuperscript{d}, Ningyan Zhang\textsuperscript{a,1}, and Zhiqiang An\textsuperscript{a,1}

\textsuperscript{a}Texas Therapeutics Institute, Brown Foundation Institute of Molecular Medicine, \textsuperscript{b}Division of Oncology, Department of Internal Medicine, and \textsuperscript{c}Department of Pathology and Laboratory Medicine, the University of Texas Health Science Center at Houston, 1825 Pressler St., Houston, TX 77030

\textsuperscript{d}Biologics Research, Janssen R&D, LLC, 1400 McKean Rd, Spring House, PA 19002

\textsuperscript{1}To whom correspondence should be addressed:

Zhiqiang An, Ph.D. and Ningyan Zhang, Ph.D.

Texas Therapeutics Institute
Brown Foundation Institute of Molecular Medicine
University of Texas Health Science Center at Houston
1825 Pressler St., Suite 532, Houston, TX 77030

Emails: zhiqiang.an@uth.tmc.edu; ningyan.zhang@uth.tmc.edu
Phone: 713-500-3011; Fax: 713-500-2447

\textsuperscript{*}Current Address

Antibody Engineering, Genentech, One DNA Way, South San Francisco, CA 94080
Financial support: This study was partially funded by grants from Janssen R&D, LLC (N. Zhang and Z. An), the Texas Emerging Technology Fund (N. Zhang and Z. An), and the Welch Foundation Grant No.AU0042 (Z. An).

Disclosure of Potential Conflicts of Interest: This study was partially funded by a grant from Janssen R&D, LLC. Several authors (RJR, MR, WRS, and REJ) are employees of Janssen R&D, LLC.

Key words: Antibody therapy, MMPs, antibody hinge cleavage, Fc effector function, Fc engineering

Running title: Tumor evasion of antibody immunity
Abstract

Primary and acquired resistance to anti-cancer antibody immunotherapies presents significant clinical challenges. Here we demonstrate that proteolytic inactivation of cancer-targeting antibodies is an unappreciated contributor to cancer immune evasion and the finding presents novel opportunities for therapeutic intervention. A single peptide bond cleavage in the immunoglobulin G1 (IgG1) hinge impairs cancer cell killing due to structural derangement of the Fc region. Hinge-cleaved trastuzumab gradually accumulated on the surfaces of HER2-expressing cancer cell lines in vitro, and was greatly accelerated when the cells were engineered to express the potent bacterial IgG-degrading proteinase (IdeS). Similar to cancer-related matrix metalloproteinases (MMPs), IdeS exposes a hinge neo-epitope that we have developed an antibody, mAb2095-2, to specifically target the epitope. In in vitro studies, mAb2095-2 restored the lost ADCC functionality of cell-bound single-cleaved trastuzumab (scIgG-T). In vivo, mAb2095-2 rescued the impaired Fc-dependent tumor suppressive activity of scIgG-T in a xenograft tumor model and restored the recruitment of immune effector cells into the tumor microenvironment. More importantly, an Fc engineered, proteinase-resistant version of mAb2095-2 rescued trastuzumab anti-tumor efficacy in a mouse tumor model with human cancer cells secreting IdeS, while trastuzumab alone showed significantly reduced anti-tumor activity in the same model. Consistently, an Fc engineered proteinase-resistant version of trastuzumab also greatly improved anti-tumor efficacy in the xenograft tumor model. Taken together, these findings point to a novel cancer therapeutic strategy to rescue proteolytic damage of antibody effector function by an Fc engineered monoclonal antibody against the hinge neo-epitope and to overcome cancer evasion of antibody immunity.
Introduction

Antibodies are becoming a major drug modality for the treatment of many human diseases including cancer (1-3). However, development of resistance to antibody therapies is widespread and an understanding of the resistance mechanisms is a topic of great interest and clinical urgency (4-7). In the case of breast cancer, it is well established that the humanized IgG1 antibody trastuzumab can inhibit tumor growth by binding via its variable domain to highly-expressed HER2 receptors on cancer cells to inhibit cell signaling (4, 8-10). As an IgG1 antibody, trastuzumab can also mediate ADCC by recruiting immune effector cells to HER2 over-expressing tumor cells (11). Resistance to trastuzumab was associated with substantially increased expression of EGFR and HER3 (9). Up-regulation of cMET expression (12), loss of PTEN or other genetic mutations in the network (8) were also proposed as possible resistance mechanisms against trastuzumab. In addition, antibody resistance mechanisms suggestive of an evasion of Fc-mediated ADCC have also been proposed (11, 13-15). Our group has recently reported that a single proteolytic cleavage in the lower hinge region results in a profound loss of Fc immune effector functions and reduced in vivo efficacy of trastuzumab. Moreover, the results indicated that the lower hinge of trastuzumab can be cleaved by matrix metalloproteinases – a class of proteinases associated with the tumor environment (4).

In this study, we demonstrated that an anti-hinge antibody against the site of proteinase cleavage can rescue the loss of Fc effect functions of the single hinge cleaved trastuzumab (scIgG-T) in vitro and in vivo. By Fc molecular engineering, we showed that trastuzumab itself and the anti-hinge cleavage site antibody mAb2095-2 used in combination with scIgG-T can be rendered resistant to IdeS hinge cleavage by incorporating select mutations in the lower hinge and adjacent CH2 region. The Fc engineered anti-hinge cleavage site antibody restored immune effector functions and the antitumor
efficacy of hinge cleaved trastuzumab in vivo. Thus, the recognition of proteolytic inactivation of IgGs suggests an immune mechanism to restore their functions within the tumor microenvironment.

Materials and Methods

Cell lines and reagents

MCF7, SKOV-3, SKBR-3, and BT474 cancer cell lines were obtained from ATCC in 2010 (Manassas, VA). All cancer cells were maintained as previously described (4). Trastuzumab (IgG-T) was purchased from a specialty pharmacy (IDIS) (4). Single hinge cleaved trastuzumab (scIgG-T) was prepared by enzymatic digestion with the IgG-degrading enzyme S (IdeS) as previously described (4, 16). Anti-hinge cleavage site antibody (mAb2095-2), proteinase-resistant antibodies, rabbit anti-human cleavage antibodies, and control antibodies were prepared as previously described (4, 17, 18). Recombinant streptococcal immunoglobulin-degrading enzyme (IdeS) was expressed in Escherichia coli and purchased from Genovis AB. Antibodies for pHER2 (Y1248) and pAKT (S473) were purchased from Epitomics (Burlingame).

Detection of MMP expression in cancer cells and antibody hinge cleavage by cancer cells

MMP expression in cancer cells was detected as described previously (4). To determine the effect of trastuzumab concentration on single hinge cleavage, cancer cells were treated with varying concentrations of trastuzumab (0.1, 1.0 and 10 µg/ml) for 24 hrs. To determine the cleavage kinetics over time, cells were treated with trastuzumab (10 µg/ml) over the period of 0, 4, and 24 hrs. Trastuzumab concentration in the cell lysates was quantified with ELISA using the HER2 ECD protein (Sinobiologicals). Same amount of antibody was used for enrichment based on the ELISA results from each cancer cell lysates. Enrichment of single hinge cleaved trastuzumab (scIgG-T) was performed using Protein A dynabeads followed by SDS-PAGE electrophoresis under denaturing and
reducing conditions and detected by Western blotting as described previously (4). Detection of antibody hinge cleavage by flow cytometry was carried out by anti-poly-cleavage antibodies cocktails followed by anti-rabbit-FITC antibody (Jackson ImmunoResearch Laboratories). The mean florescence intensity (MFI) was measured using a Guava easyCyte HT instrument (Millipore).

**Construction of BT474-IdeS and SKOV-3-IdeS stable cell lines**

BT474-IdeS and SKOV-3-IdeS stable cell lines were constructed based on protocols described previously (19) using the RevTet-off system (Clontech Laboratories). The design strategy is summarized in Fig. S1. For the detection of IdeS expression, BT474-IdeS, SKOV-3-IdeS, and vehicle control cells were cultured in RPMI 1640 media and cell culture supernatants were collected, and concentrated by centrifugation at 2000 rpm for 30 minutes at 4°C in Amicon Ultra tubes (Millipore). Anti-flag M2 beads (Sigma) was used to pull down the IdeS protein and subjected to SDS-PAGE separation under denature and non-reducing conditions, and the expression of IdeS was detected by Western blotting.

**Quantitation of trastuzumab level in mouse sera and tumor tissues by ELISA**

HER2 ECD was coated on 96-well plates at 2 µg/ml in PBS buffer and incubated at 4°C overnight. The plates were then washed with 300 µl/well with PBS containing 0.05% Tween-20 (PBS-T) and blocked with 5% non-fat milk (Bio-Rad Laboratories) for 1 hour at RT. The trastuzumab concentration was measured in the mouse sera and tumor tissues. Goat-anti-human Fc horseradish peroxidase (HRP) (Sigma) conjugate (1:5K dilution) was added and incubated for another 1 hour at RT. The plates were developed using the 3,3’,5,5’ – Tetramethylbenzidine (TMB) substrate system (Sigma Life Science) and stopped by the addition of 2M H2SO4. The plates were read at 450 nm using Spectra Max M4 (Molecular Devices).

**Binding of the anti-hinge cleavage site antibody mAb2095-2 to sIgG-T on cancer cells and antibody dependent cellular cytotoxicity (ADCC) assay**
Specific binding of the anti-hinge cleavage site antibody mAb2095-2 to scIgG-T on SKOV-3 and BT474 cancer cells was measured using a Guava easyCyte HT instrument based on manufacturer’s instructions and as described previously (4). R-PE-conjugated streptavidin was used as a negative control. ADCC activities were assayed as described previously (20) using the xCELLigence instrument (ACEA Biosciences). scIgG-T was added in 3-fold titration starting from 30 nM as reported previously (4) in the presence or absence of mAb-2095-2 at 60 nM concentration. For the detection of antibody concentration in mouse sera and tumor tissues, blood samples and tumor tissues from athymic nude mice (n=4-5) were collected one day after the last antibody administration. The antibody concentrations were determined by ELISA as described previously (4). ADCC activities using SKOV-3-IdeS target cells were examined by a single (at 0 hour) or multiple addition (0, 24, 48 and 72 hours) of trastuzumab, mAb2095-2, cleavage-resist version of trastuzumab (PR- trastuzumab) or PR-2095-2 as indicated.

**Mouse xenograft tumor models**

Immunodeficient homogeneous athymic background nude mice (Charles River Laboratories) were used for mouse xenograft tumor studies. These studies were carried out in accordance with the animal care and use guidelines and the protocol was approved by the Animal Welfare Committee (AWC) of the University of Texas Medical School at Houston. BT474 and BT474-IdeS breast cancer cells were implanted subcutaneous (s.c.) in mice of 6-8 weeks old as previously reported (20). When the tumor size reached at 50-100 mm$^3$, mice were randomized into treatment groups with 5-6 mice per group. Antibody was administered twice weekly intraperitoneally (i.p.) at 5 mg/kg for 5 weeks. Tumor size was measured and recorded two times every week using a Vernier scale caliper. Xenograft tumor tissues were collected and stored at -80°C until further ex vivo analysis. Mouse spleens were collected in 10 ml of RPMI media until flow cytometry analysis.
Detection of immune cell infiltration in xenograft tumor tissues and antibody single hinge cleavage in breast tumor tissues from high HER2 expressing breast cancer patients treated with trastuzumab by immunohistochemistry (IHC)

The tumor tissues were embedded in Optimal Cutting temperature (O.C.T.) (Sakura Finetek). Serial 4-μm thick sections were made for IHC staining. The tumor tissue slides were blocked with 5% normal serum in PBS. A drop from the Biotin blocking kit (Vector Laboratories) and a drop from the Avidin blocking kit (Vector Laboratories) were added and incubated for 30 minutes at RT. αM/CD11b and F4/80 (R&D Systems) were applied as primary antibodies and positive staining was visualized using a three-step staining procedure with an Elite ABC kit (Vector Laboratories) and counterstained with Haematoxylin (Vector Laboratories). Three tumor tissue sections (n=3) were prepared from each treatment group and the entire tumor sections were scanned under 200x (magnification = 20x10) to identify all positive stained cells using a Carl ZEISS microscopy equipped with the Cameras AxioCamMRC5 software (Monument). Positive stained immune cells were counted from eight views of each tumor tissue slide. Average number of immune cell infiltration per tumor tissue slide under 200x magnifications was plotted for each treatment group and standard error was calculated from the 3 tumor tissue slides in 24 images.

Detection of scIgG-T in breast tumor tissues were carried out in accordance with the IRB approved clinical protocol (UTHSC-MS-11-0559 - Protocol No. T-11-102). The patients tumor tissues were excised freshly, fixed in 4% paraformaldehyde and embedded in paraffin. Serial 4-mm thick sections were prepared for IHC staining. Rabbit anti-human cleavage antibody cocktail (anti-APE, anti-ELLG and anti-PAP) were applied as primary antibody, and positive staining was visualized using a three-step staining procedure with the Elite ABC kit (Vector Laboratories) as described above.

Cell lysate preparation from tumor tissues
Mouse xenograft tumor tissues were homogenized using gentle MACS (MACS Miltenyi Biotec) according to manufacturer’s procedure in a cell lysis buffer in the presence of proteinase inhibitor cocktail (Calbiochem). Protein concentration was measured by Bio-Rad DC Protein Assay (BIO-RAD), and protein was detected by Western blotting on SDS-PAGE.

**Detection of immune cells in dissociated splenocytes by flow cytometry**

The mice splenocytes (n=3) were minced to single cells with a pair of tweezers and filtered through cell strainer (70µm) (BD Falcon). Harvested cells were centrifuged at 2000 rpm for 8 minutes at 4°C. The supernatant was discarded and 1ml ACK lysis buffer (Gibco Life Technologies) was added to each sample for 5 min at room temperature. The splenocytes were washed with PBS (pH 7.4) and cells (1x10⁶/tube) were stained with four labeled antibodies: Fluorescein isothiocyanate (FITC) conjugated anti-Mouse CD11b (BD Pharmingen), Phycoerythrin (PE) conjugated anti-Mouse CD49b (BD Pharmingen), Peridinin-chlorophyll proteins (PerCP) conjugated anti-Mouse F4/80 (BioLegend), and Allophycocyanin (APC) conjugated anti-Mouse Ly-6G (BD Pharmingen) for 30 min. The stained cells were washed with 1% BSA-PBS. Cells were then suspended in 2% BSA-PBS buffer and positive stained cells were analyzed using a Guava easyCyte HT instrument according to manufacturer’s manual.

**Statistical analysis**

Statistical analysis was performed using paired Student’s t-test. A p-value less than 0.05 between treatment groups is considered significantly different.

**Results**

**Trastuzumab hinge cleavage occurs upon antibody binding on high HER2-expressing cancer cells**

Cancer cells produce many types of MMPs and their expression can be detected in both culture media and cell lysates (Fig. S2). To test if MMPs expressed by cancer cells can mediate antibody single hinge cleavage, we examined the cleavage of the trastuzumab hinge using a
panel of cancer cell lines with low or high HER2 expression. Cancer cells were cultured in the presence
of trastuzumab for 24 hours; trastuzumab was enriched by Protein A bead adsorption from the culture
media and cell lysate, respectively; and single hinge cleavage of trastuzumab was measured by Western
blotting. Molecular weights of the different antibody types and fragments are shown in Fig. 1A.
Measurable quantities of single hinge cleavage were detected when trastuzumab was enriched from cell
lysate of the three high HER2 expressing cancer cell lines (SKOV-3, SKBR-3, and BT474), but no
single hinge cleavage was detected in trastuzumab enriched from cell lysates of the low HER2
expressing MCF7 cancer cell line (Fig. 1B), suggesting that single hinge cleavage takes place when
trastuzumab is bound to the cell surface. In support of this conjecture, no single hinge cleavage was
detected in the culture media collected from high HER2 expression cancer cells (Fig. S3). For the high
HER2 expressing cell lines, trastuzumab cleavage was barely detectable in the cell lysate after four
hours of incubation, but cleavage was readily detectable after 24 hours of incubation (Figs. 1C-E).
Collectively, these results suggest that proteolytic hinge cleavage requires the antibody binding to the
cell surface and the cleavage process is accumulative and time-dependent.

To investigate if single hinge cleavage of trastuzumab occurs in human breast tumors, we stained
the surgical breast tumor tissues from two patients with HER2-overexpressing breast cancer. The two
patient surgical breast tumor tissues were sampled after six months of adjuvant treatment with
trastuzumab and chemotherapy. Compared with the negative IHC staining control (Fig. 1F), strong singe
hinge cleaved antibodies were detected to be associated with tumor tissues from the two patients (Figs.
1G-H). These results demonstrate that the cleavage of trastuzumab and generation of scIgG-T occur in
the tumor microenvironment.

**Overexpression of IdeS in cancer cells mediates strong single hinge cleavage of trastuzumab in
vitro and in vivo**
As high HER2 expressing cancer cells such as BT474 showed trastuzumab single hinge cleavage upon antibody binding on the cell surface (Figs. 1B&E), the antibody cleavage site(s) and the proteinase(s) which mediated the cleavage have yet to be established. Moreover, the enzymatic cleavage kinetics mediated by the natural cancer cells is relatively slow (Fig. 1C-E). To generate a robust model of the proteinase mediated antibody hinge cleavage in vitro and in vivo, we constructed BT474 and SKOV-3 cell lines that stably expressed the IdeS proteinase (Fig. S1). IdeS cleaves the hinge of IgG antibody with high specificity at the –LLG\(^{236}\)\(\text{▼}G^{237}\)PS-site and has shown strong single cleavage of the antibodies in our previous studies (4, 16). The expressed protein was readily detected in the IdeS-expressing cancer cells using an anti-flag antibody, but not in the vehicle transfected control cells (Fig. 2A). Overexpression of IdeS in both BT474 and SKOV-3 cancer cells showed rapid antibody single hinge cleavage when trastuzumab was added in the cultures (Fig. 2B). In addition, overexpression of IdeS showed no impact on proliferation of cancer cells (Fig. 2C). To further confirm that the single hinge cleaved trastuzumab (scIgG-T) were bound to cancer cell surfaces; we detected cell surface bound scIgG-T using flow cytometry after incubation of trastuzumab with BT474-IdeS cells for 24 hours. A large number of scIgG-T molecules were detected on the surface of BT474-IdeS cells (MFI=1228); whereas scIgG-T molecules were minimally detectable on vehicle control cells (MFI = 25) compared to the baseline signal (MFI = 6) (Fig. 2D).

Rescuing ADCC activity by an anti-hinge cleavage site antibody mAb2095-2

We have previously reported that single hinge cleaved trastuzumab (scIgG-T) lost binding to Fc\(\gamma\)Rs on immune effector cells and had minimal ADCC activity when compared with the intact trastuzumab (IgG-T) (4). To test if the loss of Fc-effector function in scIgG-T can be rescued by an intact Fc provided by another antibody, we used the anti-hinge cleavage site antibody mAb2095-2 in in vitro ADCC studies. As recently reported by our group, mAb2095-2
specifically recognizes the IdeS hinge cleavage site G^{236} ▼ G^{237}, but not the intact hinge (17). As indicated in the Figs. 3A&B histograms, mAb2095-2 bound to SKOV-3 and BT474 high HER2-expressing cells when scIgG-T was opsonized on the cancer cells, but not when the intact trastuzumab was bound to the cells. To determine whether mAb2095-2 can rescue ADCC activity of scIgG-T, SKOV-3 cells were used as target cells and human PBMC as effector cells in an in vitro ADCC assay. While the scIgG-T alone was not able to mediate ADCC, mAb2095-2 rescued the ADCC activity of scIgG-T in a dose-dependent manner (Fig. 3C) and reached 56% cell lysis at 30 nM at 72 hours (Fig. 3D). We then compared the ability of mAb2095-2 to reconstitute the ADCC activity of scIgG-T with that of intact trastuzumab. In comparison with scIgG-T alone (24%), the intact trastuzumab (IgG-T) mediated high ADCC activity (78%); remarkably, mAb2095-2 restored scIgG-T’s ability to induce ADCC (56%) close to the intact antibody at the same concentration of 30 nM (Figs. 3C-D). Also as a control, mAb2095-2 alone did not induce ADCC mediated cell lysis because of the lacking of direct binding on cancer cells (Fig. 3D).

**Restoring the tumor inhibiting efficacy of scIgG-T in vivo by the anti-hinge cleavage site antibody mAb2095-2**

In order to determine if the IdeS proteinase cleaves antibody hinge in vivo, we intraperitoneally (IP) dosed purified IdeS at 25 µg per mouse in tumor (BT474 breast cancer xenograft) bearing mice weekly 30 minutes after trastuzumab treatment. Administration of IdeS abolished the antitumor efficacy of trastuzumab (Fig 4A). Administration of IdeS also resulted in a significant loss of serum trastuzumab (0.3 µg/ml and 118 µg/ml in the presence or absence of IdeS, respectively) due to the hinge cleavage (Fig. 4B). Similarly, administration of IdeS resulted in a 10-fold reduction of antibody infiltration in tumor tissues (6.7 and 68 ng trastuzumab/mg tumor lysate protein in the IdeS treated and control mice, respectively) (Fig. 4C). As detected by Western blotting, large quantities of scIgG-T was detected in tumor tissues treated with IgG-T in the absence of IdeS, but only small amount IgG-T was detected in
tumor tissues treated with IgG-T in the presence of IdeS (Fig. 4D). Of the small quantity of trastuzumab detected in tumor tissues treated with IgG-T in the presence of IdeS, a significant portion was single hinge cleaved (Fig. 4D). These results suggest that IdeS converted IgG-T to the rapidly-cleared F(ab')\(_2\) derivative prior to the antibody gaining access to the tumor site. To investigate if the anti-hinge cleavage site antibody mAb2095-2 could rescue the loss of efficacy of scIgG-T resulting from the compromised Fc effector function as we reported previously (4), we co-administrated scIgG-T and mAb2095-2 in mice bearing BT474 xenograft tumors. As shown in Fig. 4E, the intact trastuzumab effectively inhibited tumor growth, while scIgG-T exhibited partial tumor inhibition. However, co-administration of the anti-hinge cleavage site antibody mAb2095-2 fully restored the tumor inhibiting efficacy of scIgG-T (Fig. 4E). As expected, an isotype control antibody and the anti-hinge cleavage site antibody mAb2095-2 possessed no tumor inhibiting efficacy on their own. The ex vivo tumor studies showed that trastuzumab (IgG-T), scIgG-T, and mAb2095-2 in combination with scIgG-T treated tumor extracts had similar levels of pHER2 and pAKT inhibition when compared with tumor extracts treated with the isotype control or mAb2095-2 alone (Fig. 4F). Therefore, the rescue effect of the anti-hinge cleavage site antibody mAb2095-2 on antitumor efficacy was the result of restoring the compromised Fc effector function of scIgG-T.

We have reported previously that scIgG-T showed both compromised Fc effector functions and reduced recruitment of immune cells in the tumor microenvironment (4). Here, co-administration of the anti-hinge cleavage site antibody mAb2095-2 with the scIgG-T showed similar levels (shown in the right graph of each panel) of CD11b\(^+\) (a pan myeloid marker) and F4/80\(^+\) (a marker for macrophages) immune cell infiltration as the intact trastuzumab treatment in the tumor tissues; and the anti-hinge cleavage site antibody mAb2095-2 alone did not trigger significant tumor infiltration of immune cells (Figs. 5A&B). As expected, the number of CD11b\(^+\)
and F4/80+ immune cells infiltrated in the scIgG-T treated xenograft tumors were at similar levels compared with the tumors treated with the isotype control IgG1 (Figs. 5A&B), confirming that the comprised Fc effect function in scIgG-T was associated with the reduced immune cell infiltration in the tumor microenvironment.

In order to understand whether the restored immune cell infiltration in the tumor tissues treated by co-administration of the anti-hinge cleavage site antibody mAb2095-2 with scIgG-T was a reflection of the overall immune response of the treated mice, we investigated peripheral immune cell populations isolated from spleen tissues of the treated mice. CD49b (natural killer cells, NK) positive cells in the spleens from mice treated with the intact trastuzumab were significantly higher than those treated with the scIgG-T or the isotype control antibody. Co-administration of the anti-hinge cleavage site antibody mAb2095-2 with the scIgG-T also enhanced the population of CD49b positive immune cells in the peripheral circulation (Fig. 5C). Similarly, combination of the anti-hinge cleavage site antibody mAb2095-2 with scIgG-T showed increased populations of F4/80 positive immune cells in spleen tissues (Fig. 5D). No significant difference in the number of CD11b and Ly6G positive cells (neutrophils) was observed among the antibody treated groups (Figs. S4A&B), suggesting that the neutrophil population was not significantly affected by trastuzumab treatment. The total numbers of splenocytes dissociated from spleen tissues were similar among the different antibody treatment groups (Fig. S4C). Furthermore, we determined the robustness of HER2+ cancer cell killing by the immune cells isolated from mouse spleens in the presence of trastuzumab. The splenocytes from both the intact trastuzumab and the mAb2095-2 plus scIgG-T groups resulted in enhanced ADCC activities (Fig. 5E), and the results support the notion that anti-hinge cleavage site antibody mAb2095-2 restored antitumor efficacy by rescuing immune effector function in vivo. It is also noted that, even though not statistically significant, both immune cell populations and ADCC mediated by the splenocytes were higher in the
anti-hinge cleavage site antibody mAb2095-2 plus scIgG-T group than that in the intact trastuzumab treatment group (Fig. 5C-E).

**A proteinase-resistant variant of trastuzumab exhibits full tumor inhibiting activity in the proteinase rich tumor microenvironment**

We have demonstrated that anti-hinge cleavage site antibody mAb2095-2 can rescue the immune effector function of scIgG-T *in vivo* in the BT474 wild type cancer cell xenograft tumor model, where cancer cells had limited hinge cleavage activities. In order to study the rescue effect of the anti-hinge cleavage site antibody mAb2095-2 in a tumor microenvironment with high proteolytic activity, we made a variant of the antibody with a proteinase-resistant (PR) Fc hinge (PR-2095-2). Our group has recently reported such engineered IgG1 hinge/Fc variants that were resistant to IdeS cleavage (18). We thus constructed a proteinase-resistant variant of mAb2095-2 (PR-2095-2) and, for comparative purposes, a proteinase-resistant variant of trastuzumab (PR-IgG-T). Whereas trastuzumab and mAb2095-2 were readily cleaved at the hinge region, PR-IgG-T and PR-2095-2 were completely resistant to cleavage when the antibodies were incubated in BT474-IdeS cell culture (Fig. 6A). In an *in vitro* ADCC assay using SKOV-3-IdeS as target cells and human PBMC as effector cells, trastuzumab was not able to trigger ADCC-mediated cell lysis, whereas PR-IgG-T showed over 90% cancer cell killing at 72 hours (Fig. 6B). Next, the ability of PR-2095-2 to rescue the ADCC activity of IgG-T was assessed. The anti-hinge cleavage site antibody mAb2095 without proteinase-resistant Fc engineering was not able to rescue the Fc-mediated ADCC activities of trastuzumab when SKOV-3-IdeS was used as the target cells, but the PR-2095-2 was effective in rescuing the Fc-effector function of trastuzumab, reaching approximately 78% cell killing at 96 hours (Fig. 6C). To study the effect of multiple exposures of trastuzumab on ADCC rescue by mAb2095-2, and PR-2095-2, trastuzumab (5μg/ml) was added in the assay mixture at 0, 24, 48, and 72 hours (Fig. 6D). Consistent with the results from the single exposure of trastuzumab ADCC experiments (Fig. 6C), multiple addition of trastuzumab and mAb2095-2 to the
cell culture conditions did not rescue the loss of ADCC-mediated cell lysis by trastuzumab (Fig. 6D). These results suggest that the proteinase-rich cancer cells are effective in trastuzumab hinge cleavage once the antibodies bind to the cell surface; and the hinge cleaved trastuzumab remains cell-bound and prevent intact trastuzumab from binding to cancer cells. We further tested the tumor inhibition and restorative efficacy of the proteinase-resistant variant of trastuzumab (PR-IgG-T) and mAb2095-2 (PR-2095-2), respectively, in the proteinase-enriched BT474-IdeS xenograft tumor model. The isotype control and the PR-2095-2 alone had no effect on tumor growth since they do not directly engage cancer cells (Fig. 6E). Trastuzumab exhibited partial efficacy as explained by the loss of Fc-mediated ADCC cell killing due to hinge cleavage by the IdeS proteinase. The mAb2095-2 was ineffective at rescuing the loss of Fc function of trastuzumab, because it was also cleaved by IdeS secreted by the BT474-IdeS cancer cells, and as a result, was incapable of providing a functional Fc. In contrast, the proteolytic resistant variant of trastuzumab (PR-IgG-T) completely inhibited tumor growth. Remarkably, the proteinase-resistant variant of mAb2095-2 (PR-2095-2) was able to fully rescue the tumor inhibition efficacy of trastuzumab, suggesting PR-2095-2 provided an intact Fc to bridge the hinge cleaved trastuzumab.

**Discussion**

It is increasingly recognized that antibodies of the IgG1 isotype are susceptible to specific cleavage within hinge region by physiologically-relevant proteinases such as human MMPs and the bacterial proteinase IdeS from *Streptococcus pyogenes* (4, 16, 21-24). The cleavage of the IgG hinge by proteinases secreted by bacterial pathogens has been proposed to contribute to microbial virulence by facilitating evasion of host immune responses (23, 25-27). The potential for a related mechanism in cancer was made evident by many reports of the upregulation of MMPs within the tumor environment (28-31). We recently reported that single hinge cleaved trastuzumab (scIgG-T) showed significantly
reduced immune effector functions such as ADCC and reduced efficacy in a high HER2 expressing human breast cancer xenograft model. Further, the single hinge cleavage of trastuzumab was detected in human tumor tissue obtained from a breast cancer patient who was treated neoadjuvantly with trastuzumab (4). These results suggest that a proteolytic single hinge cleavage of trastuzumab in the tumor microenvironment could result in reduced anti-tumor efficacy by uncoupling the antibody’s ability to engage immune effector functions such as ADCC.

Members of the MMP family play important roles in malignant processes. Their functions have been associated primarily with degradation of extracellular matrix components in invasion and metastasis (29, 32, 33). Until relatively recently, little attention was given to endogenous human IgG or specific anti-tumor mAb therapeutics as substrates whose function could be impaired by proteinases in the tumor environment (34). Recent studies from our group have demonstrated that trastuzumab can readily be cleaved by recombinant forms of MMP3, 7, 12, and 13 in vitro (4). We showed in this study that MMPs are expressed by the tested cancer cells and demonstrated for the first time that cancer cells possess the ability to cleave targeting antitumor antibodies and cause impairment of Fc-mediated immune function.

As we reported previously, single hinge cleaved trastuzumab (scIgG-T) by IdeS abolishes the antibody’s ability to induce ADCC-mediated cell lysis in vitro and in vivo (4, 16). The loss of ADCC activity of scIgG-T was a result of the compromised Fc effector function due to reduced interaction with FcγRs (4, 16). Therefore, IdeS expressing cancer cells provided a unique tool for examining the ability of the anti-hinge cleavage site antibody to overcome the effects of antibody hinge cleavage in vitro and in vivo. Pertinent to the concept of in vivo proteolysis of IgGs was the finding that a co-administration of IdeS proteinase with trastuzumab totally abolished anti-tumor efficacy in the BT474 mouse xenograft
model. This finding verified the potent IgG cleavage activity of IdeS and the in vivo consequences of IgG functional impairment by this proteinase and potentially others with relevance to cancer (35).

Proteolytic cleavage of the IgG hinge exposes cryptic epitopes for antibody recognition. The predominant epitopes are those with a free carboxyl terminus; for example –C-P-A-P-COO- resulting from MMP3 cleavage (23, 36). To enable the use of the IdeS proteinase as a model system, we first generated mAb2095-2 against a peptide analog of the neoepitope of the IdeS hinge cleavage site, C-P-A-P-E-L-L-G-COO-. MAb2095-2 binds to this epitope in cleaved IgG1 in a highly specific manner and does not interact with intact IgG1 antibody (17). Therefore, the anti-hinge cleavage site antibody mAb2095-2 was a key tool for investigating if an anti-hinge cleavage site antibody could restore therapeutic efficacy to proteolytically damaged antibody such as trastuzumab.

Indeed, the combination of mAb2095-2 with the inactive scIgG-T fully restored Fc-mediated cell-killing functions in ADCC assays in vitro. The rescue effect is best explained by the complex formed between mAb2095-2 and scIgG-T on the cancer cell surface providing a surrogate intact hinge and functional Fc for engagement of immune effector cells. Since scIgG-T retains its full capacity of binding affinity for the HER2, the binding of mAb2095-2 on the hinge cleavage site of scIgG-T provides the link between cancer cells and effective engagement of immune cells. The IdeS overexpressing BT474 cancer cell tumor model brought about rapid proteolytic hinge cleavage of trastuzumab that greatly impaired the antitumor efficacy of the mAb. These data support previous studies with in vitro generated scIgG-T that also showed reduced antitumor activity in vivo (4).

In the context of low proteolytic activities against antibody hinge such as wildtype BT474 cancer cells, co-administration of mAb2095-2 (IgG1) fully restored scIgG-T to the efficacy level of intact trastuzumab. This indicates that an administered anti-hinge cleavage site antibody, in this case mAb2095-2, can reconstitute immune effector function by the contribution of an intact Fc domain.
Our findings that anti-hinge cleavage site antibody mAb2095-2 restored immune cell populations in tumor tissues and showed modulation of peripheral immune system in mice support the notion that anti hinge antibodies could sufficiently reconstitute host immunity against cancer cells by providing intact Fc engagement of effector cells.

We further propose that these results, obtained in a model system of trastuzumab hinge cleavage by IdeS, could guide the design of new Fc platforms for antibody therapeutics targeting various cancer cells where endogenous proteinases are prevalent. Since anti-hinge antibodies could recognize hinge cleaved antibodies whenever and wherever they are associated with cancer cells, the anti-hinge cleavage site antibody would restore immune effector functions without the need of knowing the targets of the primary (damaged) antibodies. The present results point to an alternative and previously untested strategy to supplement host immunity in cancer.

It is of obvious concern that a therapeutic anti-hinge antibody with the IgG1 hinge would be prone to an analogous hinge cleavage in proteinase-rich physiological conditions. As reported recently by our group, an Fc engineered construct of an anti-CD20 antibody showed full immune effector function and proteinase-resistance (18). In this study, a proteinase-resistant variant of trastuzumab showed its ability to resist IdeS-cleavage \textit{in vivo} and possessed full antitumor efficacy, whereas unmodified trastuzumab that was cleaved by IdeS resulted in the loss of antitumor efficacy in BT474-IdeS mouse tumor model. More interestingly, we used an Fc engineered anti-hinge cleavage site antibody resistant to IdeS hinge cleavage that restored the antitumor efficacy of trastuzumab when it was impaired by IdeS in the BT474-IdeS tumor model. This successful rescue of trastuzumab efficacy by the Fc engineered anti-hinge cleavage site antibody signals the potential use of such a therapeutic approach to overcome the cleavage of antibodies in tumor tissue and to restore the impaired Fc immune effector function.
Disclosure of Potential Conflicts of Interest

This study was partially funded by a grant from Janssen R&D, LLC. Several authors (RJR, MR, WRS, and REJ) are employees of Janssen R&D, LLC.

Acknowledgments

We thank Bin Yuan for constructing the IdeS expression vector and Zhao Huang for assistance on Western blotting. We also want to thank Dr. Michelle Kinder and Dr. Katharine Grugan for critical discussions throughout the studies. This study was partially funded by grants from Janssen R&D, LLC (N. Zhang and Z. An), the Texas Emerging Technology Fund (N. Zhang and Z. An), and the Welch Foundation Grant No.AU0042 (Z. An).

References


Figure Legends

Figure 1. **Proteolytic cleavage of trastuzumab by high HER2 expressing cancer cells and in human breast cancer tumor tissues.** (A) A diagram of intact (IgG1) and hinge cleaved (scIgG1) antibody and sizes of the different antibody components. scHC, single hinge cleaved heavy chain; Fc(m), Fc monomer; HC, heavy chain; and LC, light chain. (B) Single hinge cleaved trastuzumab (scIgG-T) was detected in the cell lysates of high HER2 expressing cancer cell lines SKOV-3, SKBR-3, and BT474, but not lysate from the low HER2 expressing cancer cell line MCF7. Cancer cells were treated with trastuzumab at 10 µg/ml for 24 hours. scIgG-T was detected by Western blotting with an anti-human Fc antibody under reducing and denaturing conditions. scIgG-T was only detected in high HER2 expressing cancer cell lysates (C-E), but not in the culture media (Figure S1). Single hinge cleavage of trastuzumab by the three high HER2 expressing cancer cell lines (SKOV-3, SKBR-3, and BT474) was detectable after 24 hours of treatment, but barely detectable after 4 hours of treatment. In B-E, scIgG-T = single hinge cleaved trastuzumab loading control; IgG-T = intact trastuzumab loading control; medium = intact trastuzumab incubated in culture medium without cancer cells. IgG-T concentration in the cell lysis was measured by ELISA and equal amount of IgG-T was loaded in each lane. (F-H) Antibody single hinge cleavage in breast tumor tissues as detected by immunohistochemistry (IHC). Images G and H are IHC staining of breast cancer tissues from two high HER2 expressing breast cancer patients treated with trastuzumab. Rabbit anti-human cleavage antibody cocktail (anti-APE, anti-ELLG and anti-PAP) were applied as primary antibody, and positive staining was visualized under a microscope. Arrows in images G and H indicate tumor and stroma tissues. Image F is the negative staining control in which no primary antibodies were added.
Figure 2. **Cleavage of trastuzumab by the IdeS expressing BT474-IdeS and SKOV-3-IdeS cells.** (A) Expression of IdeS by BT474-IdeS cells was confirmed by Western blotting (WB) using rabbit anti-flag antibody followed by anti-rabbit antibody-HRP. (B) Trastuzumab hinge cleavage time course in the BT474-IdeS and SKOV-3-IdeS cell culture supernatants as monitored by WB. (C) Cell growth curve of BT474-IdeS was measured by xCELLigence as compared with BT474-vehicle control and BT474-wild type cells. (D) Trastuzumab hinge cleavage on BT474-IdeS cell surface as measured by flow cytometry using the mAb2095-2 antibody conjugated with Phycoerythrin (PE).

Figure 3. **Binding of mAb2095-2 to scIgG-T on cancer cells and the rescue of scIgG-T mediated ADCC function in vitro by mAb2095-2.** Isotype control, scIgG-T, or IgG-T was incubated with SKOV-3 (A) or BT474 (B) cancer cells. Binding of mAb2095-2 to the cells from isotype control, scIgG-T, or IgG-T was measured by flow cytometry. (C) Rescue of ADCC activity of scIgG-T by mAb2095-2 in a dose dependent manner. (D) ADCC activity mediated by IgG-T, scIgG-T, mAb2095-2 and combination of scIgG-T/mAb2095-2 at 30 nM for 72 hours. ADCC activity mediated by individual antibodies and antibody combinations using the high HER2 expressing SKOV-3 cells as target cells and human PBMCs as the effector cells (n=3) was detected as cell index using the xCELLigence instrument. Percentage of cell lysis was calculated as: (cell index of control group - cell index of treatment group)/cell index of control group x 100. SKOV-3 cells and PBMCs were used as baseline levels of cell growth. Error bars represent the standard deviation (SD) of triplicate measurements.

Figure 4. **Rescuing tumor inhibiting efficacy of scIgG-T by mAb2095-2 in mouse xenograft tumor models.** (A) Administration of the IdeS enzyme completely abolished trastuzumab efficacy in a mouse xenograft model. The nude mice (n=5) were subcutaneously inoculated with 1 x 10^7 BT474 human breast cancer cells. Mice were then treated with trastuzumab at 1 mg/kg twice weekly for a total of 9
doses when tumors reached an average size of 100 mm$^3$. Mice in the trastuzumab in the presence of IdeS arm were injected with the purified IdeS enzyme at 25 µg per mouse after 30 minutes of trastuzumab administration. Tumor sizes were calculated and compared among the treatment groups (*, p<0.05). Administration of the IdeS enzyme depleted trastuzumab in mouse sera (B) and mouse tumor tissues (C). Both sera and tumor samples were collected one day after the last antibody injection. Antibody concentrations were measured by ELISA. (D) Large quantities of scIgG-T was detected by Western blotting in tumor tissues treated with IgG-T in the absence of IdeS, but only small amount of IgG-T was detected in tumor tissues treated with IgG-T in the presence of IdeS. (E) Rescuing scIgG-T tumor inhibition efficacy by mAb2095-2. The Nude mice (n=5) were subcutaneously inoculated with 5 x 10$^6$ BT474 human breast cancer cells. The mice were treated with the antibodies at 5 mg/kg weekly for a total of 5 doses when tumors reached an average size of 50 mm$^3$. The mice with the combination of scIgG-T and mAb2095-2 arm were treated with the mAb2095-2 at 5mg/kg with a 3 days delayed administration of scIgG-T. Tumor sizes were measured and compared among the treatment groups (**, p<0.005). (F) Effect of various antibody treatment on pHER2 (Y1289) and pAKT (S473) levels in BT474 tumor lysates as determined by Western blotting. Tumor lysates were prepared from the frozen xenograft mouse tumor tissues.

Figure 5. **Rescue of anti-tumor immunity by mAb2095-2.** Immune cell infiltration in xenograft tumors treated with trastuzumab, single hinge cleaved trastuzumab (scIgG-T), scIgG-T in the presence of mAb2095-2, mAb2095-2, and the isotype IgG1 control were detected by IHC. Three tumor tissue slides were prepared from each antibody treatment group. Monocytes/macrophages were stained using the anti-mouse integrin αM/CD11b and anti-mouse F4/80 antibodies. (A) Representative images (X200) of CD11b antibody stained tumor tissues. CD11b positive immune cells were stained as brown color. The graph shows the average number of CD11b positive cells from 24 images (3 tumor tissue slides and 8
images per slide per mouse). Student t-test was used to compare the statistical difference between treatment groups and **, p<0.005. (B) Representative images (X200) of F4/80 antibody stained on tumor tissues. F4/80 positive cells were stained with brown color. The graph shows the average number of positively stained cells detected in a total of 24 images from three mice as described in A. (C), CD49b positive splenocytes from mice (n=3) treated with different antibodies. A total of 1x10^6 splenocytes were stained with the PE rat anti-mouse CD49b and positive counts from 10,000 events were measured by flow cytometry. (D). F4/80 positive splenocytes from mice (n=3) treated with different antibodies. A total of 1x10^6 splenocytes were stained with the PerCP anti-mouse F4/80 and positive counts from 10,000 events were measured by flow cytometry. (E). In vitro ADCC activity mediated by the splenocytes from mice (n=3) treated with different antibodies. SKOV-3 cells were used as target cells (T) and splenocytes from each antibody treated group were used as the effector cells (E) at E:T ratio of 100:1. Percentage of cell lysis was calculated as (cell index of control group - cell index of treatment group)/cell index of control group x 100. SKOV-3 cells and splenocytes were used as baseline levels of cell growth for each group. Error bars represent SD of triplicate measurements. Pair wise t test used to compare differences between treatment groups. * p < 0.05, **, p<0.01.

Figure 6. The Fc engineered proteinase resistant PR-mAb2905-2 and variant of trastuzumab (PR-trastuzumab) is resistant to IdeS hinge cleavage in vitro and in vivo. (A) Anti-hinge PR-2095-2 and PR-trastuzumab showed resistant to hinge cleavage by IdeS expressing BT474-IdeS cells. Antibodies were treated with at 10 µg/ ml for 24 hours. The hinge cleaved Fc(m) was detected under reducing and denaturing conditions by Western blotting. (B) PR-trastuzumab is fully efficacious in mediating ADCC in vitro. (C & D) PR-2095-2 but not mAb2095-2 is effective in rescuing ADCC activity of scIgG-T after single dose or multiple exposures to IgG-T. SKOV-3-IdeS cells with high activity of hinge cleavage were used as the target cells and human PBMCs were used as the effector cells. Trastuzumab was added
either as single dose (C) or multiple doses (D), n=4. (E) Cleavage resistant variant of trastuzumab (PR-IgG-T) and PR-2095-2 showed normal tumor inhibition and rescuing efficacy in the high proteolytic tumor microenvironment, BT474-IdeS xenograft tumor model. Nude mice (n=5) were subcutaneously inoculated with 5 x 10^6 BT474-IdeS cancer cells. The mice were treated with the antibodies at 5 mg/kg weekly for a total of 5 doses when tumors reached an average size of 50 mm^3. The combination arms with either mAb2095-2 or PR-2095-2 were treated 3 days after administration of IgG-T. Tumor sizes show average of each treatment group with error bars indicating the standard deviations, (*, p<0.05; **, p<0.01).
Figure 1

A: Diagrams of IgG1 and sclG1 isoforms.

B: Western blot images showing IgG HC and Fc (m) in various cell lines.

C, D: Bar charts comparing IgG-T and IgG-HC levels in SKOV-3 and SKBR-3 cells.

E: Western blot images showing IgG HC and Fc (m) in BT474 cell line.

F: Immunohistochemistry images of tumor and stroma.

G: Close-up images of tumor and stroma regions.

H: Further close-up images of tumor and stroma regions.
Figure 2
Figure 3

A. Isotype control
Binding of 2095-2 to IgG-T
Binding of 2095-2 to scIgG-T

B. Isotype control
Binding of 2095-2 to IgG-T
Binding of 2095-2 to scIgG-T

C. scIgG-T+2095-2
scIgG-T

D. Cell lysis (%)

scIgG-T concentration (nM)

Cell lysis (%)

IgG-T
scIgG-T+2095-2
scIgG-T
2095-2
Figure 4

Tumor volume (mm$^3$) over Days post xenografting.

Antibody concentration (μg/ml) for Isotype IgG1, IgG-T, and IgG-T+Ides.

IgG-T concentration (ng/mg protein) for Isotype IgG1, IgG-T, and IgG-T+Ides.

Tumor volume (mm$^3$) for Isotype IgG1, Trastuzumab, sclG-T, sclG-T+2095-2, and 2095-2.

pHER2 (Y1221/1222) and pAkt (S473) as well as β-Actin expression levels for Isotype IgG1, IgG-T, sc-IgG-T, 2095-2, and sclG-T+2095-2.
Figure 6

A

- sIgG-T
- IgG-T
- 2095-2
- PR-IgG-T
- PR-2095-2

B

- SKOV-3-IdeS+IgG-T
- SKOV-3-IdeS+PR-IgG-T

C

- SKOV-3-IdeS+IgG-T
- SKOV-3-IdeS+2095-2
- SKOV-3-IdeS+PR2095-2

D

- SKOV-3-IdeS+IgG-T
- SKOV-3-IdeS+2095-2
- SKOV-3-IdeS+PR2095-2

E

- PR-IgG-T
- IgG-T+PR-2095-2
- IgG-T+2095-2
- PR-2095-2
- IgG-T
- Isotype IgG1

Tumor volume (mm$^3$)

Days post xenografting

Cell lysis (%)

24 hr 48 hr 72 hr 96 hr

Single antibody exposure at 0 hr

Multiple antibody exposures at 0, 24, 48, 72 hr

Downloaded from mct.aacrjournals.org on June 25, 2017. © 2014 American Association for Cancer Research.
Molecular Cancer Therapeutics

A novel therapeutic strategy to rescue the immune effector function of proteolytically-inactivated cancer therapeutic antibodies


Mol Cancer Ther Published OnlineFirst December 31, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0715

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/12/31/1535-7163.MCT-14-0715.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.