Dysfunctional Antibodies in the Tumor Microenvironment Associate with Impaired Anticancer Immunity

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Abstract

Purpose: Studies have demonstrated that cancer-associated matrix metalloproteinases (MMP) can generate single peptide bond cleavages in the hinge region of immunoglobulin G1 (IgG1). This study investigated the cleavage of endogenous IgGs by MMPs in the tumor microenvironment and the consequences of the IgG hinge cleavage for humoral immunity.

Experimental Design: We investigated the occurrence of single peptide bond cleaved IgGs (scIgG) in tumor tissues and plasma samples collected from a cohort of breast cancer patients (n = 60). Samples from healthy people (n = 20) were used as the control. Antibody hinge cleavage was detected by multiple assays, including IHC, ELISA, and flow cytometry. A correlation analysis was conducted between scIGG levels and patient clinical parameters.

Results: Levels of scIgGs in tumors were significantly higher than in normal tissues. In addition, scIGG levels in tumors were enriched compared with that in the plasma of the same patients. The appearance of scIGGs in tumor tissues was associated with altered host IgG content and decreased IgG1. Increased tumor scIgGs were found to be positively correlated with adverse clinical factors, such as elevated tumor-associated macrophages, increased expression of MMP9 and other MMPs, and local metastasis to axillary lymph nodes.

Conclusions: The study contributes to mounting evidence for the presence of hinge-cleaved antibodies with reduced Fc immune effector function in the tumor microenvironment. The results highlight a link between tumor scIgGs and poor patient outcomes, and reveal a component of compromised humoral immunity within tumors that could point to new immunotherapeutic strategies to rescue host immunity. Clin Cancer Res; 1–11. ©2015 AACR.

Introduction

IgG antibodies play important roles in the human immune defense system and are one of the most abundant proteins in the peripheral blood. It is generally considered that antibodies gain access to tissues from circulation, participate in immune surveillance, and are recruited to foreign infections or aberrant self-antigens presented in pathologic sites such as tumor tissues in cancer patients.

Autoantibodies against tumor-associated antigens (TAA) have been reported in cancer patients (1, 2). The use of autoantibodies against TAs for early cancer diagnosis has been extensively explored (2). However, a detailed understanding of the roles and efficacy of autoantibodies versus TAs in anticancer immunity has been elusive since their presence in patients’ circulation does not necessarily confer protection (1–3). The suppression and evasion of host immunity have been recently recognized as important cancer hallmarks, and the therapeutic activation of cellular immunity in the tumor microenvironment has gained increased interest for cancer therapy (4, 5). However, the ability of tumors to actively evade antibodies directed against TAs is largely unstudied.

Antibody Fab regions specifically recognize antigens on foreign or abnormal cells, whereas antibody Fc regions directly interact with the Fc receptors on host immune cells, such as natural killer (NK) cells, to trigger immune effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytic (ADCP) killing of cancer cells. The IgG Fc domain also directs the complement system to target cells. Thus, the Fc regions of IgGs play important roles in eliminating target cells, and several cancer therapeutic monoclonal antibodies have been shown to employ Fc-mediated mechanisms. Our recent studies have demonstrated that tumor-associated proteolytic enzymes, such as matrix metalloproteinases (MMPs), can catalyze single peptide bond cleavages in the hinge of anticancer antibodies and lead to markedly reduced antitumor efficacy in in vivo animal model studies (6–10). The impaired IgG1 immune...
Translational Relevance

This study provides the first evidence that dysfunctional IgGs resulting from the proteolytic cleavage of one peptide bond in the hinge region (scIgG) are present in tumor tissues. Elevated scIgG levels contrasted with lower quantities in paired healthy tissues. The elevated scIgGs were inversely correlated with total IgG antibodies, particularly the IgG1 subclass, and were shown to be impaired in their interactions with the Fc gamma receptors. More significantly, the presence of elevated scIgGs was positively correlated with multiple adverse factors for patient outcome, such as tumor-associated macrophages, elevated matrix metalloproteinases (MMP; particularly MMP9), and local metastasis to axillary lymph nodes. Taken together, these findings point to a previously unappreciated mechanism of protease-mediated inactivation of host IgGs in the tumor environment. The implications of the present findings may extend to new immunotherapeutic strategies to overcome tumor defenses against host immunity.

effector functions were associated with decreased antitumor efficacy as evidenced by a reduced or lost engagement of Fc receptors on immune cells or complement factors (6, 8, 11). We have also shown the presence of hinge-cleaved trastuzumab in patients with breast cancer (9, 10). However, little is known about whether host antibodies that infiltrate the cancer tissues in patients also undergo a corresponding hinge cleavage similar to the therapeutic mAbs in the tumor microenvironment (12).

The objective of this study was to investigate the potential cleavage of endogenous IgGs by proteolytic enzymes in the tumor microenvironment and the resulting consequences of the IgG hinge cleavage for humoral immunity. To this end, we surveyed a panel of tumor samples obtained from a cohort of breast cancer patients (n = 60) to assess the presence of cleaved host IgGs. The results supported an association between antibody hinge cleavage in the tumor microenvironment and an impairment of antibody function. The results also pointed to close correlations among antibody hinge cleavage and high levels of tumor-associated macrophages (TAM) and MMP-9 in the tumor microenvironment, suggesting that antibody hinge cleavage may be linked to general immune evasion in cancer.

Materials and Methods

Patient tumor tissues

Surgically removed tumor tissues were collected from breast cancer patients (n = 60) with informed consent under the approved Institutional Review Board protocols # HSC-MS-10-0580 and HSC-MS-11-0559 at the University of Texas Medical School at Houston. All cancer patients enrolled in this study were naïve to any cancer therapies, and surgery was the first-line treatment. Fresh tumor tissues (1–10 mg) were snap frozen in liquid nitrogen in the surgical suite immediately after surgery in the local university hospital and stored at −80°C until analysis. Clinical information of the cancer patients is provided in the Supplementary Tables S1 and S2. Monoclonal antibodies used for the paired study of IgG1 and IgG2 isotypes were produced in HEK293 suspension cells (Invitrogen) using a mammalian expression vector system as previously reported (13). A panel of antihinge rabbit polyclonal antibodies for the detection of antibody hinge cleavage was developed and supplied for the study by Biologics Research, Janssen R&D as was previously described (14). Other assay reagents were purchased from vendors as indicated.

Tumor lysate preparation and IgG extraction from breast tumor tissue

Tumor lysates were prepared using the gentleMACS cell dissociator (Miltenyl Biotec) by adding RIPA buffer containing proteinase inhibitor cocktails into finely sliced frozen tumor pieces (1 mg each into 1 mL buffer). IgGs in tumor lysates were extracted using protein A/G magnetic beads and eluted by 10 mmol/L glycine buffer, pH 3.1, and neutralized with 1 mol/L Tris buffer, at pH 8.0, similar as previously reported (9, 10, 15).

Immunohistochemistry

Detection of scIgGs in patient tumor tissues was optimized using a mixture of three specific antihinge polyclonal antibodies (7, 12) as the primary detection antibody at 0.25 μg/mL of each antibody and stained with secondary anti-rabbit antibody conjugated to horseradish peroxidase (HRP) in an Avidin/Biotin Complex (ABC) system (vector lab). A similar IHC procedure was also used for detection of TAM with a TAM surface marker, CD68 antibody (Abcam). Staining intensities of each slides were scored in a blinded fashion according to the H-scoring system as reported (16), and images were taken under a light microscope (Nikon) using 20 to 40× objective lens.

Measurement of MMP expression by reverse phase protein array kit

A panel of MMPs was profiled using a reverse phase protein array (RPPA; Ray Biotech Inc). Quantitation of each MMP was conducted using a standard curve established for individual MMPs using the protease standard panel supplied in the kit. MMPs were calculated as nanograms (ng) of MMPs per microgram (μg) of tissue lysate protein (10).

Construction of MMP-9–expressing cancer cell

The MMP-9 coding cDNA clone was purchased from GenScript and constructed into a Tet off retroviral expression vector system (Clontech). Stable cancer cell line expressing MMP-9 (BF474-MMP-9) was selected using the method described previously (9).

ELISA detection of hinge cleavage

The three rabbit polyclonal anti-hinge antibodies used for IHC detection were combined to achieve a final concentration of each at 2 μg/mL in PBS and coated on ELISA high-binding plates (Nunc) at 4°C overnight. The scIgGs in tumor lysates were captured on the specific antihinge antibody-coated wells and detected with a goat anti-human Fc (species specific) conjugated with HRP (Jackson ImmunoResearch). TMB (3,3′,5,5′-tetramethylbenzidine) from Sigma Aldrich was used as substrate for detection. Absorbance at 450 nm was read by a Spectramax plate reader ( Molecular Devices).
Detection of IgG hinge cleavage by Western blotting

Tumor cell lysates were separated on SDS-PAGE in denaturing and reducing running conditions, and scIgG-cleaved fragment was detected as described previously (10) and imaged using a FluorChem M system (Protein Simple).

Quantitation of IgG subtypes and total IgG antibodies in tumor tissues by ELISA

Quantitation of 4 human IgG subclasses (IgG1, 2, 3, and 4) was conducted using an ELISA kit (Invitrogen). Extracted IgG samples were compared with standard curves of each IgG subclass using the standard IgGs provided in the assay kit. Triplicates were conducted for all experiments. Total IgG antibodies in tumor samples were measured by coating protein A/G at 1 mg/mL in high-binding (Nunc) 96-well plates and capturing IgG antibodies on the plate. Total IgGs were measured using an HRP-conjugated goat F(ab')2 fragment against human F(ab')2 (Jackson ImmunoResearch Laboratories). Purified human serum IgG mixtures were used to develop standard curve for determination of total IgG levels in tumor samples.

Fc gamma receptor binding by ELISA

Interactions of antibody IgGs with activating Fc gamma receptors were measured by an ELISA method as described previously (10).

Fc immune cell engagement by flow cytometry

THP-1 human monocytic cells (ATCC) were used for measuring antibody interactions with Fc gamma receptors expressed on the immune cells. THP-1 cells were cultured in a humidified CO2 (5%) incubator as suggested in the ATCC manual to 1 x 10⁶ cells/mL. Cells were collected and washed with PBS containing 1% BSA before addition of antibody IgG-containing tumor lysates at 37°C incubator for 2 hours. Detection of IgGs bound to Fc gamma receptors on the THP-1 cell surface was measured using a flow cytometer (Guava EasyCyte, EMD Millipore), after staining with a goat F(ab')2 fragment specific for human F(ab')2, and conjugated with R-Phycoerythrin (PE).

ADCC measurement by xCELLigence

Antibody-mediated cytotoxic killing of cancer cells (ADCC) by peripheral blood mononuclear cells (PBMC) was measured using live monitoring cell index changes by an xCELLigence instrument (ACEA Biosciences, Inc.). ADCC activity at a given time point was calculated using the formula % of cancer cell killing = 100 x (1 – (cell index of treated cells/cell index of control cells)) as reported previously (17).

Mouse xenograft tumor

The animal protocol was approved by the institutional animal care committee of the University of Texas Medical School at Houston. BT474 and IdeS-expressing BT474 cancer cells were prepared and implanted s.c. in fat pad of athymic nude mice (6–8 weeks age) as described (10). Tumors were harvested upon reaching 500 to 700 mm³, snap frozen in liquid nitrogen, and stored at −80°C until analysis.

Statistical analysis

Statistical analysis was conducted using GraphPad Prism software. Non-pairwise t test was used to compare differences of two groups and calculate P values. Correlation of two parameters was analyzed with the Pearson correlation constant, R.

Results

Detection of elevated levels of IgGs with hinge cleavage (scIgGs) in tumors of breast cancer patients

As reported previously (7), we developed specific antiIgG antibodies by immunization of rabbits with a panel of peptide analogues of the cleaved human IgG hinge. A schematic model of single-strand hinge cleavage of an IgG1 and the molecular weights of the different fragments are shown in Fig. 1A. Antibody specificity for scIgG neoepitopes in the lower hinge was demonstrated in ELISA, and there was little background binding detected to intact IgGs (Fig. 1B). We then used the same antiIgG antibodies for detection of IgG hinge cleavage in breast tumor tissues by IHC and ELISA. For IHC detection of cleaved IgG, FFPE breast tumor tissues were used, and staining intensities were semiquantitated by H-scores based on the method reported previously (16). There was clear detection of cleaved IgGs in breast tumor tissues by IHC staining (Fig. 1C). Based on the semiquantitative H scores on IHC staining, we categorized the staining intensity into three groups: high, medium, and low (Fig. 1C). The percentages of patients in each category are shown in Fig. 1D. Among the breast cancer patients tested in the cohort, about half (20 of 43) of the patients showed high levels of IgG cleavage, about 23% (10 of 43) had medium levels, and 30% (13 of 43) of patients showed low levels (Fig. 1D).

To confirm the presence of single-cleaved IgGs, an ELISA was configured to specifically detect this version (scIgG) and to provide a more quantitative estimates of scIgGs in tumor and normal breast tissues. Protein lysates were made from the snap-frozen tumor tissues from cancer patients and normal breast tissues from healthy people. Levels of scIgGs in the lysates were determined by ELISA using scIgG capture with the same anti–hinge-specific antibodies followed by detection with an anti-Fc reagent. This was the first application of an assay that specifically detects single-cleaved IgGs after extraction from complex biologic samples. The ELISA results indicated that the scIgG levels in breast tumor tissues were significantly higher than those in normal breast tissues (Fig. 1E). To further compare scIgG levels in tumor tissues and peripheral circulation from the same cohort, scIgGs were determined in the paired patient plasma samples, and, interestingly, tumor tissues showed much higher scIgGs than in plasma (Fig. 1F) when normalized to total IgG (scIgG signal/µg of antibody IgGs). These results provided the first evidence for a localized elevation of scIgGs in breast tumor tissues.

The levels of scIgGs showed an inverse correlation with total IgGs in tumor tissues

Although IgGs are among the most abundant proteins circulating in human peripheral blood, less is known about IgG concentrations in the interstitial fluid in tissues such as tumors. Nevertheless, it is well established that cancer therapeutic antibodies can successfully penetrate solid tumor tissues by targeting tumor antigens (18, 19) as demonstrated by the anticancer efficacy of tumor-targeting antibodies such as trastuzumab (IgG1), which binds to HER2-expressing solid tumors. To investigate the relationship between scIgG levels and the concentrations of total IgGs in tumor tissues, we determined the concentrations of total IgG levels in breast tumor tissues using an

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ELISA-based method. There were significantly reduced total IgG levels (ng IgGs/µg of lysate protein) in patient tumor tissues in comparison with that in normal breast tissue (Fig. 2A). The amounts of scIgG levels in tumor tissues showed an inverse correlation with the total IgG levels among the tested cohort samples (Fig. 2B). As scIgGs are resultant from hinge cleavage of intact IgGs, the inverse relationship presumably reflects increased proteolytic cleavage of targeting IgGs on tumor cell surfaces and/or depletion of total soluble intact IgGs in the tumor environment. To investigate possible mechanisms underlying the apparent inverse relationship, we constructed a stable cancer cell line (BT474-IdeS) that expresses the particularly potent IgG hinge cleavage protease, immunoglobulin-degrading enzyme from Streptococcus pyogenes (IdeS; refs. 20, 21). The BT474-IdeS cell line was compared with wild-type BT474 with regard to within-tumor IgG cleavage in a mouse xenograft tumor model. Consistent with...
the findings shown for tumors from cancer patients, a lower concentration of IgGs in BT474-IdeS tumors was found compared with wild-type tumors (Fig. 2C), which also contrasted with the increased levels of scIgGs in the tumor lysates of BT474-IdeS (Fig. 2D). The accumulation of scIgGs in tumors that occurs in parallel with a decline in the total IgGs may reflect a number of complex factors within the tumor microenvironment such as differential proteolysis of cell-targeted versus circulating IgGs; isotype discrepancies; angiogenesis-related blood volume disparities related to tumor stages; and rates of in vivo clearance of scIgG versus intact IgG. Notwithstanding the biologic complexities involved, the findings of the present study clearly highlighted a localized, protease-mediated processing of host IgGs within the tumor microenvironment.

The increased levels of scIgGs were associated with reduced levels of the IgG1 subclass in tumor tissues

IgG1 and IgG2 constitute more than 85% of total human IgG antibodies in peripheral circulation, with IgG1 being the most abundant (22, 23). Previous in vitro studies showed that IgG1 antibodies were the most sensitive to hinge cleavage by numerous human and pathologic proteases, whereas IgG2 antibodies were the most resistant subclass (11, 24, 25). In order to determine whether the generation of scIgGs in tumor tissues affects the relative proportions of IgG subclasses, we investigated the amounts and subclass ratios of tumor-infiltrated IgGs by ELISA based on the standard curves of respective subclasses (Supplementary Fig. S1). Breast tumor tissues were found to contain a significantly lower IgG1 subclass, a higher proportion of IgG2 subclass, and a significantly reduced G1–G2 ratio in comparison with that in the peripheral plasma from the same group of patients (Fig. 3A–C). The IgG3 and IgG4 subclasses contributed a lower percentage (10%–15%) of total IgGs, and percentages were similar between tumor tissues and patient plasma samples (Supplementary Fig. S2). To examine the consequences of the observed subclass shift from IgG1 to IgG2, we compared the binding of trastuzumab engineered as an IgG1 or IgG2 to Fc gamma receptors as well as antibody-dependent immune effector cell killing of cancer cells. As expected, IgG2 showed much reduced binding to all Fc gamma receptors (Supplementary Fig. S3). More significantly, IgG2 triggered much weaker antibody-dependent killing of cancer cells than did the IgG1 counterpart in a cell culture study (Fig. 3D). Collectively, these results show a reduction of both total IgGs and the IgG1 subclass in tumor tissues and support the notion that the generation of scIgGs together with a
profile shift of antibody IgG subclasses may weaken local IgG humoral immunity against cancer.

As reported previously (8, 10), monoclonal scIgGs have reduced binding affinities to Fc human gamma receptors compared with intact IgG counterparts in ELISA (Supplementary Fig. S4A). In this study, we further investigated if the increased host scIgGs in tumor tissues have compromised interactions with Fc gamma receptors. Comparative tests of FcγRII and FcγRIII binding by antibodies extracted from tumor tissues and normal breast tissues were conducted using both ELISA and flow cytometry.

Antibodies from tumor lysates showed a trend toward reduced ELISA binding to Fc gamma receptors compared with antibodies prepared similarly from normal breast tissues with P values of 0.2688 and 0.1712 for FcγRII and FcγRIII, respectively (Supplementary Fig. S4B). The reduced engagement with Fc gamma receptors by IgG antibodies in tumor tissues was also demonstrated using flow cytometric measurements of antibody binding to human monocyte cells (THP-1), and a representative flow histogram shows the overlay of the binding on THP-1 immune cells by IgGs from either tumor (T-IgGs) or normal breast tissues (N-IgGs; Supplementary Fig. S4C and S4D). The average of the mean fluorescence intensity (MFI) for the antibodies from tumor tissues (114 ± 32, T-IgGs) was lower than that from normal breast tissues (241 ± 145, N-IgGs) with a P value of 0.0936 (Supplementary Fig. S4C and S4D). These results are consistent with both IgG hinge proteolysis contributing to compromised Fc interaction with Fc gamma receptors, and also with a decreased IgG1/IgG2 ratio in tumor IgGs.

Increased scIgGs were associated with high macrophage infiltration in tumor tissues

Several studies have indicated that TAMs promote tumor progression and are correlated with poor outcomes in cancer patients (26, 27). TAMs are often attributed with the production of multiple cytokines and MMPs in the tumor microenvironment. Because several MMPs are capable of generating scIgGs in vitro (8, 24), we investigated the relationships between scIgGs and TAMs in tumor tissues. The levels of TAMs in breast tumor tissues were assessed by IHC using the CD68 surface marker for identification (Fig. 4). Based on the CD68 IHC staining intensity for TAMs, we grouped patient tumor tissues into high (H), medium (M), and low (L) as shown in Fig. 4A. The patient group with high TAM content made up a large proportion (>40%) of the total population (Fig. 4B). Of particular interest, the patient tumor
tissues in the highest TAM group were correlated with a high content of scIgGs (horizontal bar graph), whereas the tumors with low TAM content had a higher percentage of tumors with a lower level of scIgGs (>40%; Fig. 4C). The average of scIgG levels in the high TAM tumor group was significantly higher than that in those with low to medium TAM staining tumor tissues (Fig. 4D).

Association of MMP9 with increased scIgGs in tumor tissues
MMPs are often produced by TAMs and promote tumor progression and metastasis. We have reported that several MMPs can generate scIgGs in vitro (6, 11). To investigate if MMPs are associated with the elevated scIgGs in tumor tissues, we quantitatively assessed a number of MMPs in tumor lysates using an RPPA. Consistent with previous studies (26, 28), the sum of a group of seven MMPs comprising of MMP1, 2, 3, 8, 9, 10, and 13 was significantly higher in tumor tissues than in normal breast tissues with MMP9 being the predominant MMP in the majority of the tested tumors (13 of 14; Supplementary Fig. S5A and S5B). In contrast with normal breast tissues, the mean level of MMP9 in breast tumors was significantly higher (Fig. 5A). Of interest, a correlation analysis between MMP9 expression and scIgG levels showed a positive relationship among the tumor extracts from this group of patients (Fig. 5B). To verify hinge cleavage by MMP9, we constructed MMP9 overexpressing cancer cells (Supplementary Fig. S5C) and compared the hinge cleavage of trastuzumab by the MMP9-expressing cancer cells with the wild-type high HER2 cancer cells. After a 24-hour incubation of the antibody with the cancer cells in culture, there were increased scIgG levels in the MMP9-expressing cells compared with wild-type cells as detected by Western blotting (Fig. 5C and D), supporting the notion that elevated MMP9 expression contributes to the increased IgG hinge cleavage in breast tumor tissues.

Association of high scIgGs with lymph node positivity and triple-negative breast cancer tumor tissues
The patient profiles (Supplementary Tables S1 and S2) in this study encompassed triple negative (21.7%), HER2 high expression (16.6%), and estrogen receptor positive (ER+)/progesterone receptor positive (PR+; 61.6%) patient populations. To investigate potential associations between scIgG levels and cancer characteristics of patients, a correlation analysis of scIgG levels in tumor tissues was conducted among groups with different cancer subtypes, lymph node involvement, cancer grades, and stages. Notably, a higher mean scIgG level was found in the triple-negative group of cancer patients (Fig. 6A) in comparison with those in patient subgroups with HER2+ and/or ER+ and PR+.

Figure 4.
High scIgGs in tumor tissues were closely associated with high levels of TAMs. A, tumor tissues from breast cancer patients (n = 45) showed differential intensity of macrophage (CD68+) infiltration. A representative image is shown for each group, and brown color indicates positive IHC staining, H for high TAM, M for medium TAM, and L for low TAM. B, grouping of tumor tissues based on IHC staining scores of TAM, the vertical bar graph indicates the percentage of breast tumors with high, medium, and low TAM staining. C, high TAM (H) staining tumors have higher population with high scIgG levels (scIgG-H with H scores of 100 to 300), whereas groups with medium (M) to low (L) levels of TAM are associated with more patient tumors with medium (scIgG-M) to low scIgG (scIgG-L) levels with H score < 100. D, levels of scIgGs in high TAM group (TAM-H) are significantly higher than those in the combined low and medium TAM groups (TAM-M/L). Means of scIgG staining scores for each group (Y-axis) are indicated by the horizontal lines in the scatter dot graph. ***, P < 0.01.
There was no significant difference between the HER2+ and ER+/PR+ patient groups (Fig. 6A). Neither tumor grades nor their stages of progression showed a clear correlation with scIgG levels in tumor tissues (Supplementary Fig. S6). It was striking that those patients with lymph node positivity showed significantly higher scIgGs levels than those without lymph node involvement (Fig. 6B). These data support the potential link between the elevated scIgGs and the development of local metastasis in breast cancer.

Discussion

The presence of elevated scIgGs in tumor tissues from breast cancer patients is an indicator of immune evasion by cancer.

Cancer evasion and suppression of host immunity in the tumor microenvironment have been well documented, but the concept was established mainly based on evidence from studies on cellular immunity such as high infiltration of myeloid-derived suppressor cells and regulatory T cells (4, 29). To our knowledge, the present investigation provides the first evidence for a potential contribution to cancer immune evasion through hinge cleavage of host IgGs. Studies have reported the existence of autoantibodies to many tumor-associated antigens in cancer patients, such as HER2, but the anticancer function of those autoantibodies has not been established (2). Elevated scIgGs in tumor tissues provide a novel mechanism to explain the confounding ability of some cancers to evade host antibody immunity (autoantibodies against cancer antigens) or to resist immunotherapeutic mAb treatment. IgG antibodies constitute one of the most important host adaptive immune mechanisms and bridge the interactions between innate immunity such as mediated by NK and phagocytic macrophages with adaptive cellular immunity mediated by T cells (18, 19, 22). It is well documented that the Fc regions of IgGs play key roles in eliminating target cells, and Fc-mediated effector functions have been shown to contribute to the efficacy of several cancer therapeautic monoclonal antibodies (30–32). Our recent studies demonstrated that single-strand hinge cleavage of the anti-HER2 IgG1 antibody trastuzumab impaired its antitumor efficacy due to the loss of Fc-mediated effector functions (6, 10). The present study has demonstrated that breast tumor tissues exhibit hinge cleavage of endogenous autoantibodies to a markedly higher degree than tissue samples obtained from healthy individuals, indicating that the breast tumor microenvironment may present a compromised humoral immunity due to the generation of dysfunctional scIgG antibodies against cancer.

IgGs are produced by subpopulation of B cells, such as plasma cells, and are the most abundant immunoglobulin proteins in circulation. It is likely that tumor-associated antibodies are recruited from the circulation, and/or locally produced by...
Elevated scIgG levels were associated with breast tumor type and lymph node involvement. A, significant higher levels of scIgGs (Y-axis) were detected in tumor tissues from triple negative (TN) cancer patients in comparison with that in HER2+ or ER+/PR+ groups. ScIgG levels were determined in tumor lysates using ELISA, and values are the average of three experimental replications. ***, \( P < 0.01 \) and ns indicates no statistically significant differences. B, high scIgG levels were found in tumor tissues from breast cancer patients with lymph node involvement (+, \( n = 17 \)) in comparison with that from node-negative patients (−, \( n = 13 \)). Graph shows whisker 10–90 percentiles. **, \( P < 0.05 \).

Elevated scIgGs parallel increased levels of MMPs in breast tumor tissues

MMPs are a family of highly conserved zinc endopeptidases comprising more than 25 members in humans (38). In cancer, most attention regarding the function of MMPs has been directed at the degradation of matrix components such as collagen (39) and the promotion of cancer invasion and metastasis (38, 40). The expression of MMP9 and its association with tumor promotion and metastasis has been reported (40, 41). As shown in the present investigation, elevated scIgGs in the tumor tissues from breast cancer patients paralleled higher levels of MMP9 expression. This study showed that overexpression of MMP9 in BT474 cancer cells resulted in an increased single strand hinge cleavage of the IgG1 monoclonal antibody trastuzumab. The results indicate that MMP9 may exert an additional proteolytic function in cleaving the hinge region of human IgG1 antibodies in the tumor microenvironment. In addition to MMP9, several other hinge cleaving MMPs, including MMP2, −3, and −13 (6, 11, 24), were also detected at higher levels in breast tumors compared with normal tissue. The regulation of MMP activation is complex, and...
an involvement of other MMPs in IgG hinge cleavage is likely as was previously shown in vitro (6, 24). Nevertheless, in this study, MMP9 is considered to be a principal contributor to human IgG1 cleavage because its expression in tumor tissues was the highest among the panel of MMPs in the assay array.

Elevated scIGs in breast tumor tissues were associated with adverse factors such as elevated TAM levels and local node metastasis

TAMs produce a number of growth factors and cytokines as well as MMPs and are involved in promoting tumor growth and metastasis. A number of studies have reported that high TAM levels within tumors were linked with local inflammation, angiogenesis, and poor prognosis for cancer patients (27, 42–44). The present study showed that high levels of scIGs in breast tumors were also associated with high TAM levels. The association of scIGs and TAMs suggests a possible mechanistic interconnection among different components of local immune dysfunction, and increased scIGs may represent another important node in the complex network of interactions among humoral and cellular immunity and cancer cells in the tumor microenvironment. It has been well documented that TAMs produce high levels of MMPs (28, 45), and the link shown in this study among scIGs, MMPs, and TAMs suggests an intricate relationships among them in the tumor microenvironment. More importantly, the results of elevated scIGs in tumor tissues from triple-negative subgroup and node-positive breast cancer patients are consistent with the reported association among cancer metastasis and high MMPs and TAM (15, 26–28, 40, 42, 46).

In summary, this study has established the presence of IgG hinge cleavage in tumor tissues from a cohort of breast cancer patients and has demonstrated a potentially important and overlooked proteolytic mechanism of cancer evasion of host humoral immunity. The close associations of antibody single hinge cleavage (scIGs), MMPs, and tumor-associated macrophages further suggest a role for proteolysis within the complex immune interrelationships within the tumor microenvironment. The positive correlations of scIG levels with multiple adverse factors for patient poor outcome, such as high TAMs, high MMPs (particularly MMP9), and lymph node involvement, point to unexpected dysfunctional actions of scIGs in breast tumors. The present findings draw together well-recognized, but disconnected, observations that suggest an additional direction of study to better understand how cancers evade host defenses.

Disclosure of Potential Conflicts of Interest

W.R. Strohl has ownership interests (including patents) in any reports receiving commercial research grants from Janssen R&D, and is a consultant/advisory board member for Covagen Board and MRSAb Scientific Advisory Board. No potential conflicts of interest were disclosed by the other authors.

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