Tumor-Associated Hyaluronan Limits Efficacy of Monoclonal Antibody Therapy

Neti C. Singha1, Tara Nekoroski1, Chunmei Zhao1, Rebecca Symons1, Ping Jiang1, Gregory I. Frost2, Zhongdong Huang1, and H. Michael Shepard1

Abstract

Despite tremendous progress in cancer immunotherapy for solid tumors, clinical success of monoclonal antibody (mAb) therapy is often limited by poorly understood mechanisms associated with the tumor microenvironment (TME). Accumulation of hyaluronan (HA), a major component of the TME, occurs in many solid tumor types, and is associated with poor prognosis and treatment resistance in multiple malignancies. In this study, we describe that a physical barrier associated with high levels of HA (HAhigh) in the TME restricts antibody and immune cell access to tumors, suggesting a novel mechanism of in vivo resistance to mAb therapy. We determined that approximately 60% of HER2+ primary breast tumors and approximately 40% of EGFR+ head and neck squamous cell carcinomas are HAhigh, and hypothesized that HAhigh tumors may be refractory to mAb therapy. We found that the pericellular matrix produced by HAhigh tumor cells inhibited both natural killer (NK) immune cell access to tumor cells and antibody-dependent cell-mediated cytotoxicity (ADCC) in vitro. Depletion of HA by PEGPH20, a pegylated recombinant human PH20 hyaluronidase, resulted in increased NK cell access to HAhigh tumor cells, and greatly enhanced trastuzumab- or cetuximab-dependent ADCC in vitro. Furthermore, PEGPH20 treatment enhanced trastuzumab and NK cell access to HAhigh tumors, resulting in enhanced trastuzumab- and NK cell–mediated tumor growth inhibition in vivo. These results suggest that HAhigh matrix in vivo may form a barrier inhibiting access of both mAb and NK cells, and that PEGPH20 treatment in combination with anticancer mAbs may be an effective adjunctive therapy for HAhigh tumors. Mol Cancer Ther; 14(2); 523–32. ©2014 AACR.

Introduction

Hyaluronan (HA) is an extracellular glycosaminoglycan component of many human tissues, and HA accumulation occurs in a variety of human solid tumors (1–5). HA binds noncovalently to the N-terminal globular domains of proteoglycans, such as aggregan or versican, and other hyaladherins or hyalectans forming a complex network in the extracellular matrix (ECM; refs. 4–6). Accumulation of HA in tumors is associated with malignancy and predictive of more aggressive disease (1–5). Elevation of HA levels in tumors, sometimes in combination with collagen, results in blood vessel compression, increased interstitial fluid pressure (IFP), and decreased perfusion (7, 8). HA depletion from solid tumors with a tumor microenvironment (TME) containing high levels of HA (HAhigh) reverses these physiologic effects, resulting in reperfusion of the tumor vasculature, increased chemotherapeutic drug accumulation, and tumor growth inhibition (TGI) in preclinical animal models (8–11). The HAhigh pericellular matrix may also protect malignant cells from immune cell surveillance (12–14). High HA content contributes significantly to the pro-tumorigenic impact of the tumor stromal compartment (15).

Recent advances in targeted monoclonal antibody (mAb)–derived therapeutics have led to remarkable response and survival with reduced toxicity and have established mAbs as part of the anticancer armamentarium (16–18). Trastuzumab (targeting p185HER2) and cetuximab (targeting the epidermal growth factor receptor [EGFR]) are approved for breast cancer, gastric cancer, colon cancer, and other solid tumors (16). Trastuzumab and cetuximab inhibit tumor growth in human cancers characterized by elevated expression of HER2 and EGFR, respectively (16–20). Although trastuzumab and cetuximab have shown success in the clinic, resistance (intrinsic and acquired) to these mAb therapies has been reported in many cases (17, 21). Multiple mechanisms of resistance to these mAbs have been described previously (17, 19–24). Recently, the role of the tumor ECM has been recognized as a potential contributor to therapeutic resistance (7, 25, 26).

One mechanism of efficacy for mAb therapies is antibody-dependent cell-mediated cytotoxicity (ADCC), which involves therapeutic antibody binding to antigen on the tumor cell surface, and subsequent recognition of the antibody Fc region by immune cell surface FcYRIIA (CD16) receptor, followed by target tumor cell killing by immune cells [predominantly natural killer (NK) cells; refs. 16, 17, 20, 22]. ADCC plays a key effector role in mAb-based therapy by direct killing of target tumor cells, possibly leading to tumor antigen presentation and induction of tumor antigen–directed T-cell response (16, 17, 20, 27–30). In this report, we describe experiments suggesting a novel mechanism of resistance to ADCC in vitro and in vivo, mediated by an HAhigh pericellular matrix and its reversal by depletion of HA using a pegylated recombinant human PH20 (PEGPH20).
Materials and Methods

RPMI and McCoy 5A medium, Accutase, and human AB serum were from Mediatech. Aggrecan (Elastin Products), calcein AM and G418 (Sigma-Aldrich), cetuximab (Prodigy), trastuzumab (Prodigy), and recombinant human interleukin (IL)-2 (rhIL2; Peprotek) were purchased from commercial sources. Murine anti-human CD16 and murine immunoglobulin (Ig) G1 isotype control antibodies were from eBioscience. MDA-MB-231, SKOV3, and SKBR3 cell lines were purchased from the American Type Culture Collection. MDA-MB-231/Luc cell line was from Caliper Lifescience Inc., and cell lines MDA-MB-231/Luc/HAS2 (overexpressing luciferase and HAS2 genes), SKOV3/HAS2, and SKBR3/HAS2 were made by genetic engineering (incorporating HAS2 gene using retroviral infection followed by G418 antibiotic selection) as described previously (the cell lines have not been authenticated; ref. 9). HAS2 gene overexpression was determined by qPCR for HAS2 mRNA (Supplementary Fig. S1A). MDA-MB-231 cells were cultured in RPMI with 10% FBS. Peripheral blood mononuclear cells (PBMC) were isolated from fresh healthy donor buffy coats (San Diego Blood Bank) using Histopaque-1077 (Sigma-Aldrich) gradient centrifugation and stored (10 days to 2 months) using Histopaque-1077 at −80°C until use.

Antibody-dependent cell-mediated cytotoxicity

To study HA^{high} pericellular matrix-mediated resistance to ADCC and its reversal by PEGPH20, we first established trastuzumab-dependent NK cell–mediated ADCC with adherent SKBR3 (HER2^{+)} cells in culture. Target cells (10,000–15,000 cells/well) were seeded in 96-well plates and allowed to grow for 2 days to a 70% to 80% confluent adherent monolayer. On the day of assay, monolayer cells were labeled with calcein AM (as described by the manufacturer), followed by treatments with HA-binding proteoglycan aggrecan (1 mg/mL final concentration, 1 hour at 37°C), PEGPH20 (1,000 U/mL, and cetuximab, trastuzumab, or human IgG1 isotype in succession (each for 1 hour at 37°C). The antibody-treated cells were then incubated with extracellular HA^{high} expanded human NK effector cells (at NK to target cell ratio of 15:1) for 5 hours (37°C with 5% CO_{2}). At the end of incubation, the number of live cells was determined by measuring calcein AM fluorescence (excitation: 488 nm; emission: 537 nm) on a SpectraMax M2 plate reader (Molecular Devices). In the ADCC blocking assay, NK cells were pretreated with mouse anti-human CD16 or mouse IgG1 isotype (5 μg/mL) for 30 minutes before being added to the antibody-loaded target cells. The statistical significance of differences in the target cell killing under different conditions was calculated using the Student t test.

Flow cytometry

Cells were harvested and washed with fluorescence-activated cell sorting (FACS) buffer (PBS with 5% FBS) and labeled with fluorescence-labeled antibody for 1 hour at 4°C. Alexa Fluor 488 conjugated to trastuzumab (T-AF488) and cetuximab (C-AF488) was made following the manufacturer’s protocol (Invitrogen) and tested for HER2 or EGFR binding by live cell imaging. Fluorescein isothiocyanate (FITC)–conjugated anti-CD44 and Alexa Fluor 488–conjugated anti-CD24 were from BioLegend. Flow cytometry was performed on a BD FACS scan machine (Becton Dickinson).

Particle exclusion assay and movie

Cancer cells were grown in 96- or 48-well plates (2,000–5,000 cells/well) for 2 days. On the day of the assay, cells were labeled with calcein AM, and then treated with 1 mg/mL aggrecan for 1 hour, followed by incubation with antibody ± PEGPH20 for 1 hour at 37°C. Human NK cells (0.5–1 × 10^6) were added and allowed to settle for 15 to 20 minutes before microscopic photography. One thousand cells were seeded on an eight-chamber glass slide and a movie was generated from images captured every 30 seconds at 32°C. All images were captured using a Nikon Eclipse TE2000i inverted microscope (Nikon) with a SPOT camera (SPOT imaging solutions) with a 40× objective lens.

Immunofluorescence and HA ELISA

Cells were incubated for 1 hour with aggrecan (1 mg/mL) followed by T-AF488 (4 μg/mL), or C-AF488 (10 μg/mL) ± PEGPH20 (1,000 U/mL) for 1 to 2 hours in a humidified incubator with 5% CO_{2} at 37°C in the growth medium, followed by fluorescent microscopic imaging using a 60× objective lens. HA ELISA was performed following the manufacturer’s protocol (Hyaluronan duoSet; R&D Systems).

Immunohistochemistry and HA staining in human tumors

Four human breast cancer tissue arrays, including 58 invasive ductal carcinoma, four intraductal carcinoma, three invasive lobular carcinoma, and four of other tumor type with HER2 expression status, were purchased from US BioMax. The arrays were stained for HA using biotinylated HA-binding protein followed by horseradish peroxidase and 3,3’-diaminobenzidine (DAB) detection. The array slides were scanned with a ScanScope CS (Aperio Technologies, Inc.) using bright field imaging at ×20 magnification, and analyzed for HA distribution using pixel count algorithm of the Spectrum program (Aperio Technologies). HA staining was scored as high (HA^{high}), medium (HA^{medium}), and low (HA^{low}) corresponding to strong positive pixel area of 25%, 10% to 25%, and <10%, respectively (9). The intensity threshold was established using HA staining of positive control tumors, where the strong positivity is consistent from batch to batch.

Ex vivo imaging of tumors

Athymic nude mice (Taconic) were maintained in the animal facility of Halozyme Therapeutics, Inc. The animal use protocol was reviewed and approved by Halozyme’s Institutional Animal Care and Use Committee. Female, 5- to 7-week-old nude mice (NCr-nu/nu) were inoculated intramuscularly into pretibial space with 5 × 10^6 SKOV3/HAS2 cells. When tumor sizes reached 300 mm^3, mice (5/group) were treated intravenously twice weekly with trastuzumab, or human IgG1 isotype in succession (each for 1 hour at 37°C). The antibody-treated cells were then incubated with extracellular HA^{high} expanded human NK effector cells (at NK to target cell ratio of 15:1) for 5 hours (37°C with 5% CO_{2}). At the end of incubation, the number of live cells was determined by measuring calcein AM fluorescence (excitation: 488 nm; emission: 537 nm) on a SpectraMax M2 plate reader (Molecular Devices). In the ADCC blocking assay, NK cells were pretreated with mouse anti-human CD16 or mouse IgG1 isotype (5 μg/mL) for 30 minutes before being added to the antibody-loaded target cells. The statistical significance of differences in the target cell killing under different conditions was calculated using the Student t test.

Flow cytometry

Cells were harvested and washed with fluorescence-activated cell sorting (FACS) buffer (PBS with 5% FBS) and labeled with fluorescent-labeled antibody for 1 hour at 4°C. Alexa Fluor 488 conjugated to trastuzumab (T-AF488) and cetuximab (C-AF488) was made following the manufacturer’s protocol (Invitrogen) and tested for HER2 or EGFR binding by live cell imaging. Fluorescein isothiocyanate (FITC)–conjugated anti-CD44 and Alexa Fluor 488–conjugated anti-CD24 were from BioLegend. Flow cytometry was performed on a BD FACS scan machine (Becton Dickinson).

In vivo TGI

The mice were inoculated intramuscularly with 5 × 10^6 SKOV3/HAS2 cells. When tumor sizes reached 200 mm^3, mice (6/group) were dosed intravenously twice weekly with ex vivo–expanded human NK cells (8 × 10^6 cells/mouse) and trastuzumab (0.2 mg/kg) with and without PEGPH20 (40 μg/kg). Tumor
size was measured weekly using ultrasound imaging (Vevo 2100 Imaging System; VisualSonics). Statistical analysis of difference in tumor volume between the control and treatment groups 1 day after the last treatment dose was performed using one-way ANOVA, with a P value less than or equal to 0.05 (P ≤ 0.05) considered statistically significant (32).

Results

Simultaneous occurrence of high HA level and EGFR or HER2 expression in human tumors

Extracellular HA accumulation occurs in a wide variety of cancers [e.g., breast, prostate, colorectal, gastric, and head and neck squamous cell carcinoma (HNSCC)], and high levels of HA surrounding tumor and/or stromal cells are associated with epithelial–mesenchymal transition, host–tumor interactions, and other protumorigenic processes (1–5). We investigated the frequency of occurrence of accumulation of high HA levels in primary HER2+/ breast adenocarcinoma tumors and EGFR+ HNSCC tumors using biotinylated HA-binding protein. Twenty-four of 63 EGFR+ HNSCC samples (38%) and 14 of 24 HER2+ breast tumor samples (58%) were found to be HAhigh (Table 1). This finding indicates that a substantial fraction of HER2+/ or EGFR+ tumors exhibit an HAhigh phenotype, and provides a rationale for exploring the role of pericellular matrix-associated HA accumulation in resistance to anticancer therapies such as trastuzumab or cetuximab.

HAhigh pericellular matrix inhibits NK cell access to tumor cells in vitro

Parental HER2+ SKBR3 and SKOV3, and EGFR+ MDA-MB-231 breast cancer cells with low levels of HA (HAlow) were transfected with a retrovirus carrying human HA synthase 2 (HAS2) cDNA to generate HAS2-overexpressing SKBR3/HAS2, SKOV3/HAS2, and MDA-MB-231/Luc/HAS2 cell lines. These HAS2-overexpressing cell lines secreted higher levels of HA compared with parental cells in culture (Supplementary Fig. S1B). Humann HAS2 gene overexpression did not alter HER2, EGFR, CD44, or CD24 levels on cell surface (Supplementary Fig. S2).

In the presence of aggrecan, an HA-binding proteoglycan, SKBR3/HAS2 (HAhigh) cells formed enlarged pericellular matrices in vitro (Fig. 1A) compared with parental SKBR3 cells (Fig. 1B), which, in turn, restricted the access of human NK cells to tumor cells in culture. The pericellular matrix surrounding SKBR3/HAS2 cells was diminished by PEGPH20 treatment, allowing increased proximity of NK cells to tumor cells as shown in Fig. 1C, where the NK cells can be seen closely surrounding the tumor cells. This finding was further extended to HAhigh MDA-MB-231/Luc/HAS2 cells. Compared with parental MDA-MB-231 cells, MDA-MB-231/Luc/HAS2 cells formed enlarged pericellular matrix coats, which inhibited access of human NK cells to tumor cells (compare Fig. 1D with Fig. 1E; Supplementary Movie S1). PEGPH20 treatment removed the HA-rich pericellular matrix, allowing NK cells to access the target tumor cells (Fig. 1F and Supplementary Movie S1). We also observed NK cell synapse with tumor cells following PEGPH20 treatment, resulting in morphologic changes in the tumor cells, indicating initiation of NK cell–mediated killing of the target tumor cells (Fig. 1G). Overall, HAS2 overexpression led to formation of an HAhigh pericellular matrix coat capable of preventing access of NK cells to tumor cells in cell culture, and this physical restriction was removed by HA depletion following PEGPH20 treatment.

HAhigh pericellular matrix inhibits ADCC in vitro

We conducted ADCC studies to test the functional potential of HA coats in preventing NK cell–mediated tumor cell lysis. During ADCC, antibody-dependent synapse between NK cells and target tumor cells is essential for tumor cell killing (16, 20, 22). Monolayer-adherent SKBR3 parental and SKBR3/HAS2 cells were incubated in the presence of aggrecan to study the effect of an HAhigh pericellular matrix coat on ADCC. In the presence of NK cells, trastuzumab-dependent targeted killing of SKBR3/HAS2 cells was consistently lower than that of parental SKBR3 cells (Fig. 2A), suggesting that an HA coat was mediating resistance to antibody-dependent NK cell-mediated cytotoxicity of HAhigh tumor cells. The difference in ADCC between SKBR3/HAS2 and SKBR3 cells appeared to be trastuzumab concentration-dependent, with the biggest difference observed at 40 ng/mL trastuzumab with 46% cytotoxicity in HAS2-transfected cells versus 72% in untransfected cells (Fig. 2A). Smaller differences between SKBR3/HAS2 and SKBR3 were observed at 0.8 and 2,000 ng/mL trastuzumab (Fig. 2A). A similar trend in resistance to antibody-dependent NK cell–mediated cytotoxicity was observed with MDA-MB-231/Luc/HAS2 breast tumor cells in the presence of cetuximab, with reduced killing of MDA-MB-231/Luc/HAS2 cells compared with parental MDA/MB/231 cells (Fig. 2B). This is not related to the differences in HER2 or EGFR surface expression between transfected and nontransfected cell populations (Supplementary Fig. S2) or HA–aggrecan complex interference in trastuzumab or cetuximab binding to HER2 or EGFR on SKBR3/HAS2 or MDA-MB-231/Luc/HAS2 cells, respectively (Fig. 2C). In addition to resistance to ADCC, the HA-rich pericellular matrix also showed a basal level (non-mab-mediated) resistance to NK cell–mediated cytotoxicity (to a lesser extent than resistance to ADCC) in SKBR3/HAS2 and MDA-MB-231/Luc/HAS2 tumor cells (Fig. 2A and B, control). Pretreatment of NK cells with mouse anti-CD16 antibody blocked its ADCC activity on SKBR3 cells (Supplementary Fig. S3), suggesting that the ADCC observed on adherent SKBR3 cells is CD16 dependent. The observed resistance of HAS2-overexpressing tumor cells to ADCC and basal NK cell–mediated cytotoxicity supports the observation that an HAhigh pericellular matrix on the tumor cell surface interferes with functional access of NK cells to target tumor cells, and partially protects the tumor cells from NK cell–mediated cytotoxicity.

Table 1. HA distribution in EGFR-positive HNSCC and HER2+ breast adenocarcinoma tissues

<table>
<thead>
<tr>
<th>HA score</th>
<th>HNSCC (n = 63)</th>
<th>Breast (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>10/63 (16%)</td>
<td>1/24 (4%)</td>
</tr>
<tr>
<td>Medium</td>
<td>28/63 (44%)</td>
<td>9/24 (38%)</td>
</tr>
<tr>
<td>High</td>
<td>24/63 (38%)</td>
<td>14/24 (58%)</td>
</tr>
</tbody>
</table>

NOTE: HA staining was scored as high, medium, and low corresponding to strong positive pixel area of 25%, 10–25%, and <10%, respectively (9).
cetuximab then incubated with NK cells to induce ADCC. HA depletion following PEGPH20 treatment resulted in a 40% increase in trastuzumab-dependent NK cell–mediated killing of SKBR3/HAS2 cells (Fig. 3A and Supplementary Table S1). Conversely, a much smaller increase of trastuzumab-mediated ADCC of parental HAlow SKBR3 cells resulted following PEGPH20 treatment (Fig. 3B and Supplementary Table S1). Pretreatment of NK cells with PEGPH20 showed no effect on NK cell–mediated ADCC, suggesting that the observed increase in ADCC on SKBR3/HAS2 following PEGPH20 treatment is not due to altered NK effector function caused by PEGPH20 (Supplementary Fig. S4A).

In HAlow MDA-MB-231/Luc/HAS2 cells, PEGPH20 treatment also resulted in a significant increase in cetuximab-dependent ADCC (Fig. 3C and Supplementary Table S1), further supporting the observation that an HAhigh pericellular matrix plays a role in ADCC inhibition. As expected, PEGPH20 only caused a small increase in cetuximab-mediated ADCC of HAlow parental MDA-MB-231 cells (Fig. 3D and Supplementary Table S1). The increase in ADCC on SKBR3/HAS2 (HAhigh) and MDA-MB-231/Luc/HAS2 (HAhigh) cells following PEGPH20 treatment appeared to be antibody concentration dependent, with the increase in ADCC diminished at a higher antibody concentration (Fig. 3A and C). This is probably due to a greater number of antibody-bound receptors on target cells at higher antibody concentration, which results in effective cell killing despite fewer encounters with NK cells. The effect of limited NK cell accessibility, mediated by an HAhigh pericellular matrix, is likely to be most sensitive at intermediate antibody concentration. The observed increase in ADCC following PEGPH20 treatment in HAhigh tumor cells is supported by a moderate increase in basal NK cell–mediated cytotoxicity by...
PEGPH20 (Fig. 3A and C, control), suggesting NK access inhibition in HA\textsuperscript{high} tumor cells.

HA depletion increased antibody and NK cell accessibility to HA\textsuperscript{high} xenograft tumors

Sensitization of drug-resistant cancer cells to chemotherapeutic agents by hyaluronidase treatment suggests higher drug exposure to tumor cells following HA depletion (33). Similarly, HER2 receptor in HA\textsuperscript{high} tumors can be masked by pericellular/stromal HA (34, 35); however, in our \textit{in vitro} experiments, HA–aggrecan complex did not interfere with trastuzumab or cetuximab binding to HER2 on SKBR3/HAS2 or EGFR on MDA-MB-231/Luc/HAS2, respectively, in the presence of aggrecan (left) and aggrecan and PEGPH20 (right). Cells were incubated with aggrecan to generate HA–aggrecan complex followed by incubation with Alexa Fluor 488-conjugated trastuzumab (T-AF488) or cetuximab (C-AF488) antibody with or without PEGPH20. Antibody binding was detected using fluorescent live cell imaging.

PEGPH20 treatment was determined by immunohistochemistry (IHC) and ELISA to confirm PEGPH20 hyaluronidase activity in vivo. PEGPH20 caused approximately 60% HA removal in SKOV3/HAS2 tumors (Supplementary Fig. S5A and S5B). In contrast to the \textit{in vitro} experiment (Supplementary Fig. S4B), PEGPH20 treatment significantly increased tumor-associated trastuzumab levels in animals dosed with T-AF488 (Supplementary Fig. S5C and S5D). Similarly, HA depletion by PEGPH20 treatment resulted in increased trastuzumab levels in tumors of animals concomitantly dosed with T-AF488 and NK-PKH26 (Fig. 4A; top middle vs. right). Quantitation of the fluorescence signal intensity showed an approximately 2-fold increase in trastuzumab levels (Fig. 4B). To our surprise, PEGPH20 treatment showed no statistically significant increase in tumor-associated NK levels in animals dosed with NK-PKH26 in the absence of antibody (Supplementary Fig. S5E and S5F). However, PEGPH20 treatment increased access of NK cells to tumors in the animals dosed with T-AF488 plus NK-PKH26 (Fig. 4A; bottom middle vs. right). NK cell infusion in the PEGPH20/NK cell/trastuzumab–treated tumors increased by 3-fold compared with the trastuzumab/NK cell–treated tumors without PEGPH20 (Fig. 4C). Trastuzumab or NK cells were not detected in kidney, muscle, or heart of the mice treated with PEGPH20/NK cell/trastuzumab.

**Figure 2.**

HA\textsuperscript{high} pericellular matrix inhibits ADCC on tumor cells and HA–aggrecan complex does not interfere with mAb binding to HER2 or EGFR surface receptors. A, dose-dependent trastuzumab-mediated ADCC of monolayer SKBR3 (HA\textsuperscript{low}) and SKBR3/HAS2 (HA\textsuperscript{high}) cells. B, cetuximab-mediated ADCC of monolayer MDA-MB-231 (HA\textsuperscript{low}) and MDA-MB-231/Luc/HAS2 (HA\textsuperscript{high}) cells. t tests were performed to compare responses of parental and HAS2-overexpressing cells. **, P < 0.05; ***, P < 0.005; bars, SEM; n = 5. C, trastuzumab or cetuximab binding to HER2 on SKBR3/HAS2 or EGFR on MDA-MB-231/Luc/HAS2, respectively, in the presence of aggrecan (left) and aggrecan and PEGPH20 (right). Cells were incubated with aggrecan to generate HA–aggrecan complex followed by incubation with Alexa Fluor 488-conjugated trastuzumab (T-AF488) or cetuximab (C-AF488) antibody with or without PEGPH20. Antibody binding was detected using fluorescent live cell imaging.
with T-AF488, NK-PKH26, or T-AF488 plus NK-PKH26 with or without PEGPH20 (Fig. 4D and Supplementary Fig. S6) despite the fact that both muscle and heart have substantial HA content (36). Taken together, these data show that HA depletion by PEGPH20 increased trastuzumab and NK cell accessibility to HAHigh xenograft tumors in the SKOV3/HAS2 tumor model, probably as a result of tumor vasculature decompression (3, 8–11).

HA depletion from HAHigh tumors following PEGPH20 treatment increased TGI by increasing ADCC

Higher antibody and NK cell accessibility to HAHigh tumors following HA depletion and stromal remodeling by PEGPH20 treatment suggests that PEGPH20 may enhance ADCC in vivo, leading to improved TGI. We tested this possibility in the SKOV3/HAS2 tumor model. In this tumor model, NK cell exposure to tumor peaked at day 2 and NK cells were detectable within tumor up to day 5 after dosing with NK-PKH26 (Fig. 5A). SKOV3/HAS2 tumor-bearing mice were treated twice weekly with trastuzumab (0.2 mg/kg), human NK cells (8 × 10⁶ cells/mouse), and PEGPH20 (40 mg/kg) individually or in combinations to study the effect of PEGPH20 on antibody-dependent NK cell–mediated TGI. The trastuzumab/NK combination treatment group showed higher TGI (41%) compared with the trastuzumab (22%) or NK treatment groups (24%; Fig. 5B), suggesting the requirement of a human NK supplement for optimal tumor sensitivity. Treatment with PEGPH20 alone showed 46% TGI (Fig. 5B). The triple combination of PEGPH20/NK cell/trastuzumab treatment resulted in 68% TGI (Fig. 5B). These in vivo data demonstrate that HA depletion following PEGPH20 treatment enhanced trastuzumab and NK cell–induced TGI, which is likely a consequence of increasing trastuzumab and NK cell exposure to tumor cells in SKOV3/HAS2 tumors (Fig. 5).

Discussion

The majority of HA is localized in tumor ECM, where it binds with multiple hyaladherins or hyalectans (5, 6, 37, 38). Versican is predominantly expressed in HAHigh tumors (5, 37, 38) and has limited commercial availability. Aggrecan is a proteoglycan with structure composition similar to versican and binds to HA efficiently and was used in the in vitro studies reported here. High levels of HA accumulation in primary human tumors were found in a substantial percentage of HER2⁺ breast tumor tissues and EGFR⁺ HNSCC tissues, and these findings are consistent with a previous report that high levels of HA is associated with HER2⁺ breast cancer tissues (1). HAHigh glioma cells and mouse sarcoma cells have been previously shown to exhibit pericellular matrix–dependent resistance to CTL-mediated cytotoxicity in vitro (12–14). Thus, we hypothesized that the pericellular matrix of HAHigh tumor cells may form a physical barrier capable of interfering with access of antibody and immune cells to target tumor cells. In this study, we investigated PEGPH20-sensitive pericellular matrix–mediated inhibition of trastuzumab- or cetuximab-dependent ADCC targeting HAHigh solid tumors.

In alignment with our hypothesis, exogenous HAS2-overexpressing tumor cells formed an enlarged pericellular matrix that limited access of NK cells to tumor cells, and depletion of HA following PEGPH20 treatment allowed NK cell access to tumor cells. These results suggest that an HAHigh pericellular matrix may contribute to the innate resistance of tumors to host immune...
surveillance and promote tumor cell survival. This conclusion is further supported by (i) the observed resistance of HAS2-overexpressing tumor cells to ADCC, and (ii) a dramatic increase of ADCC by PEGPH20 treatment in HA<sup>high</sup> tumor cells in vitro. Although HA<sup>high</sup> tumor cells were reported to mask HER2 from binding to trastuzumab (34–35), we have not detected HA/aggrecan–mediated HER2 or EGFR receptor masking in HAS2-overexpressing cells in vitro. Limited access of NK cells to tumor cells by the HA<sup>high</sup> pericellular physical barrier likely contributed to the observed resistance of HAS2-overexpressing tumor cells to ADCC in vitro. Restricted therapeutic antibody access to tumor as well as masking of receptors by HA may also contribute to resistance to ADCC in other HA<sup>high</sup> tumor cells.

HA depletion from HA<sup>high</sup> tumors has been shown to be effective in increasing intratumoral exposure of chemotherapeutic agents, suggesting an HA-dependent limitation of drug access in HA<sup>high</sup> tumors mediated by compression of blood vesicles by intratumoral pressure (3, 8–11). Here, we demonstrate that HA depletion by PEGPH20 increased large-size therapeutics (both trastuzumab and NK cell) access to HA<sup>high</sup> tumors. Furthermore, neither exogenously administered trastuzumab nor NK cells were present at detectable levels in normal tissues even in the presence of PEGPH20, suggesting that the effect of PEGPH20 on trastuzumab and NK cells is predominantly tumor-specific and HA<sup>high</sup> tumor-specific activity of PEGPH20 would likely contribute to an improved therapeutic index as a result of pharmaceutical activity on the HA<sup>high</sup> (high-IFP) tumors. These results demonstrate an HA-dependent TME-mediated inhibition of antibody and NK cell access to HA<sup>high</sup> tumors, which suggests that these tumors may be sensitized to ADCC following PEGPH20 treatment. Indeed, HA depletion by PEGPH20 resulted in higher TGI in the triple-combination group (PEGPH20/NK cells/trastuzumab) compared with the double-combination group (trastuzumab/NK cells) in the SKOV3/HAS2 tumor model. This is consistent with previous reports that PEGPH20 can enhance TGI of HA<sup>high</sup> tumors when combined with chemotherapy in several preclinical animal models (8–11). The observed statistically significant increase in TGI in the PEGPH20/NK cells/trastuzumab group compared with NK
cells/trastuzumab is rather modest in our tumor model. This is likely caused by tumor heterogeneity and a low NK cell to target cell number ratio in vivo and unlikely to be caused by limited PEGPH20 availability into the tumor, because PEGPH20 has a serum half-life of 10.3 hours, which is dramatically higher than rhuPH20 (t½ < 5 minutes). And HA removal from tumors by PEGPH20 is sustained for up to 72 hours (3,8). Both stromal cells and tumor cells may provide HA to the TME, and our results demonstrate that an HA<sup>high</sup>-TME contributes to limiting the access of NK cells and therapeutic antibody into the tumor. Although, while we have not observed reduced antibody binding to HA<sup>high</sup> tumor cells in vitro, receptor masking in HA<sup>high</sup> tumors in vivo may contribute to resistance to mAb therapy (34,35). Thus, enhanced tumor access of NK cells and antibody, as well as increased antibody binding to exposed target receptors, can be achieved by depletion of stromal HA with PEGPH20 treatment.

Growing evidence indicates that HA provides a protumorigenic environment and exogenous hyaluronidase administration showed significant antitumor activity in HA-overexpressing tumors (3,7–11,15). Conversely, increased hyaluronidase activity has been detected in tumor tissues and/or serum from several primary and metastatic cancers (3–5,39,40). Although hyaluronidase expression (predominantly associated with hyaluronidase 1, which has very little enzymatic activity at physiologic activity) in the tumor has been shown to potentially increase metastasis in some preclinical animal models (3,9,40), PEGPH20 treatment did not increase metastasis in an HA<sup>high</sup> tumor xenograft model and PEGPH20 in combination with gemcitabine reduced metastasis burden in a KPC mouse model (3,11). Although, the possibility of increasing metastasis following HA depletion by PEGPH20 cannot be ruled out by the current studies, available literature indicates that an increase in metastatic potential by PEGPH20 treatment is unlikely (3,11). Additional investigation is required to understand the protumorigenic and/or prometastatic role of hyaluronidase and it is likely tumor type dependent.

In our mouse model, if some tumor cells are liberated from HA<sup>high</sup> tumor by PEGPH20, they will likely be sensitized to effector immune cell–mediated elimination.

Despite the breakthroughs brought to cancer treatment by mAbs, innate tumor resistance to drug remains a major problem (17,19,20,23,24). Targeted mAb therapy for human solid tumors relies on binding of the mAb to tumor cell surface target receptors followed by intracellular signaling attenuation to sensitize tumor cells, direct tumor cell killing by FcγRI (CD16)–expressing immune cells, and mAb-mediated host immune response activation. Success of mAb therapies in the clinic is limited by several underlying mechanisms including underappreciated TME-mediated host immune response suppression, such as reduced malignant cell killing by CD8<sup>+</sup> TILs and/or NK cells, and properties of some immune cells harboring protumor activities (25).

In addition, tumor cells have been shown to use multiple immunosuppressive mechanisms to avoid host immune attack, including tumor-induced impairment of antigen presentation (41,42), secretion of immunosuppressive and growth factors (e.g., TGFβ and IL10) in the TME (43,44), amplification of receptor tyrosine kinases (45), upregulation/expression of negative costimulatory signaling molecules (CTLA-4 binding CD80 and/or CD86, PD-L1 and/or PD-L2) by tumor cells (46,47), inhibition of dendritic cell differentiation and maturation (44,46), and increased infiltration of regulatory T-cells (48,49).

Here, we demonstrate that an HA<sup>high</sup>-tumor ECM enables formation of a protective pericellular matrix coat capable of inhibiting access of NK cells to tumor cells in vivo as well as antibody and NK cell access in vitro, suggesting a novel mechanism for cancer cells to escape detection and clearance by antibody- and NK cell-mediated ADCC.

The role of the TME in tumor progression and metastasis supports the approach of targeting one or more TME components for cancer therapeutics (7,15,50). HA in the TME is a promising target for cancer therapeutics due to its role in creating high intratumoral pressure resulting in blood vessel compression and development of hypoxia, epithelial–mesenchymal transition, tumor progression and metastasis, multidrug resistance, and
escape from immune system surveillance. Increasing tumor exposure of therapeutic mAbs and immune cells by depleting HA from the TME is a therapeutic strategy with great potential. Our data show that an HAhigh-TME contributes to inhibition of ADCC in vitro and in vivo that can be reversed by HA depletion following PEGPH20 treatment, and demonstrates the potential beneficial effect of PEGPH20 treatment together with mAb-based therapies for HAhigh solid tumors.

Disclosure of Potential Conflicts of Interest

G.I. Frost has ownership interest in and is a consultant for Halozyme Therapeutics, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: T. Nekoroski, P. Jiang, G.I. Frost, Z. Huang, H.M. Shepard


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.C. Singh, T. Nekoroski, C. Zhao

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N.C. Singh, T. Nekoroski, C. Zhao, P. Jiang, Z. Huang

Stromal HA Elevation Limits mAb-Mediated Cancer Therapy

Writing, review, and/or revision of the manuscript: N.C. Singh, T. Nekoroski, P. Jiang, G.I. Frost, Z. Huang, H.M. Shepard

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Nekoroski

Study supervision: P. Jiang, Z. Huang

Other (histology processing and IHC): R. Symons

Acknowledgments

The authors thank Susan Zimmerman, Gina Wei, and Qiping Zhao for their contributions in cell lines, retroviral vector, and retrovirus generation. Grace Lee for in vivo experiments; Ryan Osgood, Robert Connor, and Calvin Yu for assistance with in vivo studies; Curtis Thompson for critical suggestions in article preparation; and Daniel C. Maneval for article review. The authors acknowledge the pioneering work of Robert Stern, Markku Tammi, and Robert Kebel on role of HA biology in cancer.

Grant Support

All work in this study was funded by Halozyme Therapeutics, Inc.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received July 21, 2014; revised November 25, 2014; accepted December 1, 2014; published OnlineFirst December 15, 2014.

References


Molecular Cancer Therapeutics

Tumor-Associated Hyaluronan Limits Efficacy of Monoclonal Antibody Therapy

Netai C. Singha, Tara Nekoroski, Chunmei Zhao, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-14-0580

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2014/12/16/1535-7163.MCT-14-0580.DC1

Cited articles
This article cites 50 articles, 22 of which you can access for free at:
http://mct.aacrjournals.org/content/14/2/523.full.html#ref-list-1

Citing articles
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
/content/14/2/523.full.html#related-urls

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.