Integrated Systems and Technologies

Targeting uPAR with Antagonistic Recombinant Human Antibodies in Aggressive Breast Cancer

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Abstract

Components of the plasminogen activation system, which are overexpressed in aggressive breast cancer subtypes, offer appealing targets for development of new diagnostics and therapeutics. By comparing gene expression data in patient populations and cultured cell lines, we identified elevated levels of the urokinase plasminogen activation receptor (uPAR, PLAUR) in highly aggressive breast cancer subtypes and cell lines. Recombinant human anti-uPAR antagonistic antibodies exhibited potent binding in vitro to the surface of cancer cells expressing uPAR. In vivo these antibodies detected uPAR expression in triple negative breast cancer (TNBC) tumor xenografts using near infrared imaging and 111In single-photon emission computed tomography. Antibody-based uPAR imaging probes accurately detected small disseminated lesions in a tumor metastasis model, complementing the current clinical imaging standard 18F-fluorodeoxyglucose at detecting non-glucose-avid metastatic lesions. A monotherapy study using the antagonistic antibodies resulted in a significant decrease in tumor growth in a TNBC xenograft model. In addition, a radioimmunotherapy study, using the anti-uPAR antibodies conjugated to the therapeutic radioisotope 177Lu, found that they were effective at reducing tumor burden in vivo. Taken together, our results offer a preclinical proof of concept for uPAR targeting as a strategy for breast cancer diagnosis and therapy using this novel human antibody technology. Cancer Res; 1–12. ©2013 AACR.

Introduction

Breast cancer is a remarkably heterogeneous disease composed of multiple subtypes, each representing a distinct biological signature that responds to unique therapeutic regimens (1, 2). Therapeutics that target specific subtypes, such as trastuzumab (Herceptin) in the HER2-positive subtype, have been effective at treating primary and metastatic breast cancer, but ultimately drug resistance and clinical relapse occur in a majority of patients (3, 4). Despite the recent U.S. Food and Drug Administration (FDA) approval of several new drugs for the treatment of breast cancer, therapeutic options for metastatic breast cancer are few (5). Once cells from the primary tumor metastasize to the bone and soft tissue, the primary goal of therapy is palliative (6). Molecular targets of aggressive subtypes are needed for the treatment and evaluation of the disease. Agents directed toward these targets can be used as diagnostic probes or targeted therapeutics. Diagnostic imaging probes would allow for the noninvasive identification of aggressive tumors that are nonresponsive to standard chemotherapeutics, and allow patients to receive alternative therapeutic options sooner. If the target is reflective of tumor viability, metastatic lesions can be identified earlier and response to therapy can be dynamically quantified. Furthermore, therapeutics directed toward molecular targets could reduce patient morbidity associated with nontargeted systemic therapies, and ultimately prolong survival.

The plasminogen activation system (PAS) presents several molecular targets that can be exploited for diagnostic and therapeutic purposes in metastatic breast cancer. The overexpression of the serine protease urokinase plasminogen activator (uPA) and its receptor uPAR have been found to contribute to the aggressive phenotype of a number of cancers (7, 8). In breast cancer, high levels of uPA and its cognate inhibitor plasminogen activator inhibitor-1 (PAI-1) in tumor tissue were found to correlate with poor clinical prognosis and were predictors of tamoxifen resistance (9, 10). Several investigators found that uPAR expression in breast tissue is directly correlated with an aggressive tumor phenotype and low disease-free survival. uPAR expression has been documented in triple-negative breast cancer (TNBC), tamoxifen refractory breast cancers, and metastatic breast cancers. By targeting uPAR, the treatment of breast cancer, therapeutic options for metastatic breast cancer are few (5). Once cells from the primary tumor metastasize to the bone and soft tissue, the primary goal of therapy is palliative (6). Molecular targets of aggressive subtypes are needed for the treatment and evaluation of the disease. Agents directed toward these targets can be used as diagnostic probes or targeted therapeutics. Diagnostic imaging probes would allow for the noninvasive identification of aggressive tumors that are nonresponsive to standard chemotherapeutics, and allow patients to receive alternative therapeutic options sooner. If the target is reflective of tumor viability, metastatic lesions can be identified earlier and response to therapy can be dynamically quantified. Furthermore, therapeutics directed toward molecular targets could reduce patient morbidity associated with nontargeted systemic therapies, and ultimately prolong survival.

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cancer, and in a subset of Her2-positive breast tumors, all of which are classified as aggressive (11–13). In vitro overexpression of uPAR in breast cancer cells was able to induce the epithelial-to-mesenchymal transition (EMT), suggesting that uPAR overexpression can promote an aggressive phenotype (14). Because of its accessibility on the surface of cancer cells, uPAR is of particular interest as a molecular target for breast cancer.

The development of human recombinant anti-uPAR antagonistic antibodies, by panning a fragment–antigen binding (Fab) phage display library against recombinant human uPAR, has been previously reported (15). Two antibodies, 3C6 and 2G10, were characterized for their ability to inhibit uPAR function. Using in vitro methods, 3C6 was found to prevent the association of uPAR with β1 integrin, whereas 2G10 prevented uPA’s association with uPAR. Both antibodies were found to be selective for human uPAR and did not cross-react with murine uPAR. In this report, we document the use of 3C6 and 2G10 as molecular imaging and therapeutic agents in preclinical models of aggressive breast cancer. 3C6 and 2G10 IgGs detected uPAR expression in breast cancer cell–derived orthotopic xenograft tumors, and in disseminated lesions of cardiac dissemination model (CDM) mice by near infrared (NIR) optical imaging and, the clinically relevant nuclear imaging modality, single-photon emission computed tomography (SPECT). The 111In-labeled anti-uPAR IgG SPECT probes complemented the clinical imaging standard 18F-fluorodeoxyglucose (18FDG) positron emission tomography (FDG-PET) by detecting lesions missed by FDG-PET. In a high-dose monotherapy study, both 2G10 IgG and 3C6 IgG resulted in decreased tumor growth with no growth observed in the 2G10 IgG–treated group. A radioimmunotherapy (RIT) study with 177Lu-2G10 IgG resulted in complete tumor regression, suggesting uPAR as a viable therapeutic target for breast cancer. This investigation shows that high uPAR expression is a prominent clinical feature of aggressive breast cancer, corroborating in vitro cell studies, and that our antibodies allow uPAR targeting for diagnostic and therapeutic purposes.

**Materials and Methods**

**Cell culture**

Human breast cancer cell lines MDA-MB-231, MDA-MB-435, MDA-MB-436, MDA-MB-453, MDA-MB-468, BT-549, SK-BR3, and MCF-7 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and were maintained in their respective recommended media, supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C. The drug-resistant cell lines MCF-7 TamR, MCF-DoxR, MDA-MB-231 TaxR, and MDA-MB231 DoxR were a generous gift from Dr. Laura L. Murphy (Southern Illinois University School of Medicine, IL) and were cultured as mentioned earlier. Human mammary epithelial cells (HMEC) were purchased from Lonza and cultured using the MEGM BulletKit. The cell lines were authenticated using short-tandem repeat profiling provided by the vendor.

**uPAR mRNA expression analysis in the NKI dataset**

Using the Netherlands Cancer Institute (NKI) dataset, which reports mRNA levels for 24,498 genes in 295 women with breast cancer, uPAR mRNA levels were assessed and their significance in several breast cancer subtypes was compared (16). The data were stratified according to previously reported methods (17). Patients diagnosed with basal (BLBC), Her2 (ERBB2), Luminal A, Luminal B, or normal-like breast cancer were grouped. A nonparametric Wilcoxon’s t test was conducted to determine which group had significant uPAR mRNA. uPAR mRNA levels in patients falling under the TNBC subtype with all other breast cancer subtypes were compared.

**uPAR gene expression analysis in breast cancer cell lines**

RNA was prepared from each cell line (~2 × 10⁶ cells/cell line) using an RNeasy kit (Qiagen). After RNA isolation, each sample was treated with Turbo DNA-free (Ambion) to remove any residual DNA. RNA was synthesized to cDNA using the high-capacity RNA-to-cDNA kit (Applied Biosystems). For each gene, Taqman qPCR was conducted in quadruplicate using the Taqman Universal PCR Master Mix (Applied Biosystems). The following Taqman Gene Expression Assay probes were used: uPAR-Hs00182181_m1 PLAU, uPA-Hs01547054_m1 PLAU, PAI-1 Hs01126666_m1, and 18s ribosomal 1 (reference gene) Hs03928985_g1 RN18S1. All qPCR was done on an ABI 7300 Real Time PCR system instrument. qPCR raw data (Ct) for each sample was normalized to the reference gene. Data were analyzed using the comparative Ct method (fold change = 2−ΔΔCt) with data normalized to the negative control cell line, MDA-MB-453.

**Fab and IgG production**

2G10 (λ light chain) and 3C6 (κ light chain) Fabs and IgGs were produced as previously described (15) and the IgGs were purified on a Protein A FF column (GE Life Sciences), and then on an S75 HiLoad Prep column. A11, isotype matched control IgG, in this study, was expressed and purified as originally described (18).

**Flow cytometry**

MDA-MB-231, MCF-7, and HMEC cells were washed with DPBS and harvested with TrypLE (Gibco). A total of 1 × 10⁶ cells were incubated with 10 nmol/L 2G10 or 3C6 IgG for 20 minutes at 4°C, followed by FITC-labeled antihuman IgG antibody (BD) for another 20 minutes at 4°C. Stained samples and controls were assayed on a BD FacsCalibur. In the experiments for determining apparent dissociation constants for MDA-MB-231 cells, cells were harvested as described, and separated into 1 × 10⁶ aliquots. The antibody constructs, 2G10 and 3C6 (Fab and IgG) and A11 isotype control IgG, were incubated at 4°C with the cells for 90 minutes. For apparent Kᵦ calculations, all cells were incubated with their defined antibody concentration for 12 hours in DMEM-H21 at 4°C to account for the attenuated Kᵦ of IgG-treated samples affecting calculated apparent Kᵦ. Fab samples were also incubated for 4 hours to ensure that data for samples incubated for longer periods were not skewed by dead cells (98% cell viability after 1 hour, vs. 95% viability after 12 hours, data not shown).
Samples and controls were probed with a phycoerythrin-labeled anti-human Fc, and assayed on an LSRII flow cytometer (BD). All concentrations were done in triplicate.

**Surface plasmon resonance**
Soluble uPAR was immobilized on a CM5 chip via EDC/NHS chemistry, where exposed lysines on suPAR’s surface were covalently linked to the dextran surface. 2G10 and 3C6 Fab samples were made at 1, 4, 16, 64, and 256 nmol/L for analysis. 2G10 and 3C6 IgG samples were made at 0.0390625 nmol/L, 0.15625, 0.625, 2.5, 10, 40, and 160 nmol/L for analysis. Surface plasmon resonance (SPR) experiments were conducted in a Biacore T100 apparatus. All samples were flown over the chip surface at 30 ìls/min for 30 seconds, followed by a 120-second dissociation phase, and removed with Glycine pH 2.5 for 30 seconds. Response curves were evaluated in the BiaEvaluation software. For determination of the dissociation constants, a 1:1 Langumurian best-fit binding model was employed for both Fab and IgG binding curves.

**Animal models for imaging**
The animal work was conducted in accordance with an UCSF Institutional Animal Care and Use Committee protocol and was done by the Preclinical Therapeutics Core at UCSF. Six- to 7-week-old nu/nu mice were purchased from Taconic Farms. Nude mouse xenografts were generated by subcutaneous injection of cell line (1 x 10^6 cells/mL; 100 ìL per site/mouse) in the mammary fat pad of the mouse. MCF-7 mice were subcutaneously implanted with a slow-release estrogen pellet (0.8 mg of 17β-estradiol) in the contralateral flank. The xenograft study was analyzed using qPCR and reverse transcription polymerase chain reaction (RT-qPCR) as well as immunohistochemistry (IHC) with a clone 2G10 labeled 2G10 IgG. Gene expression of the clone used for transfection was done with a lentiviral particle according to the manufacturer’s protocol. After puromycin treatment, clones were selected using flow cytometry with AlexaFluor 488–labeled 2G10 IgG. Gene expression of the clone used for the xenograft study was analyzed using qPCR and flow cytometry.

**Biodistribution study**
Mice (n = 4/time point) bearing MDA-MB-231 xenografts between 120 and 250 mm^3 in volume were injected with 25 ìCi (2.5 ìg) of 111In-2G10 and 111In-3C6. At 24, 48, and 72 hours, the mice were euthanized for analysis in accordance with UCSF Animal Care and Use Committee guidelines. Mice (n = 3/xenograft) bearing MDA-MD-231(uPAR-), MCF-7, MCF-7 TamR, and MDA-MB-435 xenografts between 120 and 250 mm^3 in volume were injected with 25 ìCi (2.5 ìg) of 111In-2G10 and 111In-3C6 were euthanized for analysis at 72 hours. Blood was collected by cardiac puncture. The tumor, heart, lung, liver, spleen, kidneys, and muscle were harvested, weighed, and counted in an automated γ-counter (Wizard2; Perkin Elmer). The percentage injected dose per gram (% ID/g) of tissue was calculated by comparison with standards of known radioactivity. The uPAR knockout cell line was generated using uPAR shRNA Plasmid (h) sc-36781-SH from Santa Cruz. Transfection was done with a lentiviral particle according to the manufacturer’s protocol. After puromycin treatment, clones were selected using flow cytometry with AlexaFluor 488–labeled 2G10 IgG. Gene expression of the clone used for the xenograft study was analyzed using qPCR and flow cytometry.
In vitro and in vivo therapeutic studies

The matrigel invasion and clonogenic survival assays were conducted as previously described (15, 20).

For the MTT assay, 2,000 cells were plated in 96-well format. After 24 hours, the cells were incubated with 2.5 μg of unlabeled antibodies or 2.5 μg of antibodies labeled with 177Lu at activities of 50 and 25 μCi. The 177Lu-antibodies were prepared under the same conditions as the 111In-antibodies for imaging. The cells were incubated for 96 hours with the radiolabeled antibodies and then the cell viability was determined using an MTT assay kit (Promega) according to the manufacturer’s protocol. For the in vivo studies, the animal models were generated by the Preclinical Therapeutics Core at UCSF. Briefly, suspended tumor cells in a buffered solution of 1.0 × 10^6 cells/100 µL were injected into the subcutis of the mammary fat pad of 6 to 7-week-old nude female mice. When the mean tumor volume of mice with established palpable tumors were measured to be >100 mm³, the mice were randomized into different treatment groups (n = 10/arm). When the study began at Day 0, tumor volumes of the animals were between 75 and 200 mm³. The mice were treated with 30 mg/kg of 2G10, 3C6, A11, and saline at days 3, 10, 17, and 24. For the RIT study, randomized MDA-MB-231 xenograft mice (n = 10/arm), with tumor volumes > 100 mm³, were treated with 111In-2G10 (75 μCi, 2.5 μg/dose), 177Lu-EDTA (75 μCi), saline, and 2G10 (4 mg/kg) at days 7 and 21. Mice were weighed weekly and the tumor volumes were calculated using the formula: volume = 0.5236 × L × W × H. Animals were removed from the study and sacrificed when tumor volumes were >1,000 mm³, in accordance with our animal protocol.

Statistical analysis

On box plots, the highest horizontal line represents the highest value in a group, the topmost section represents the second highest quartile, the lowermost quartile represents the lowest quartile, the part of the white box represents the second highest value in a group, and the lowermost section represents the median. The correlation of TN status with uPAR mRNA levels was evaluated. A nonparametric Wilcoxon test was used to analyze uPAR levels between BLBC and other subtypes, or TNBC and other subtypes (21–23). Although the correlation between uPAR mRNA levels and BLBC was strong (P = 6 × 10^-3, Fig. 1A), the correlation between uPAR mRNA levels and TNBC was stronger (P = 1.2 × 10^-10, Fig. 1B). Longitudinally, uPAR mRNA levels were further correlated with poor clinical prognosis and an earlier recurrence of cancer progression.

Results

uPAR expression is associated with aggressive breast cancer in vivo and in vitro

Using the NKI dataset, uPAR mRNA levels were compared between patients of different breast cancer subtypes (16, 17). As shown in Fig. 1A, the basal-like breast cancer (BLBC) subtype was found to have the highest uPAR mRNA levels among the subtypes. Knowing that BLBCs often exhibit a high level of uPAR expression is associated with aggressive breast cancer (15). For SPECT imaging, the IgGs were labeled with a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaetic acid (DOTA) chelate derivative. The DOTA-labeled IgGs were chelated with the long-lived isotope 111In and administered intravenously to MDA-MB-231 and MCF-7 xenograft mice. In the reconstructed SPECT/CT data, 111In-2G10 and 111In-3C6 showed pronounced tumor localization and retention in the MDA-MB-231 xenograft 72 hours postinjection (Fig. 3A). No localization was

2G10 and 3C6 preferentially accumulate in MDA-MB-231 orthotopic xenograft tumors in vivo

NIR optical was initially used to investigate the specific localization of the antibodies to uPAR expressing xenografts and to acquire pharmacokinetics leading to the informed selection of the appropriate isotope for nuclear imaging. AlexaFluor 680-labeled antibodies were injected into bilateral orthotopic MDA-MB-231 and MCF-7 xenograft tumor-bearing mice and serially imaged. 2G10 and 3C6 exhibited strong localization to the uPAR expressing MDA-MB-231 xenograft (Fig. 2). Specificity was evident 48 hours postinjection, and persisted well out to 96 hours. NIR probe localization was neither observed in the MCF-7 xenografts nor in MDA-MB-231 xenograft mice injected with scrambled isotype control AlexaFluor 680-labeled IgGs (Supplementary Fig. S2).

The nuclear imaging modality SPECT was used to assess the extent of 3D probe localization and pharmacokinetics in vivo. For SPECT imaging, the IgGs were labeled with a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaetic acid (DOTA) chelate derivative. The DOTA-labeled IgGs were chelated with the long-lived isotope 111In and administered intravenously to MDA-MB-231 and MCF-7 xenograft mice. In the reconstructed SPECT/CT data, 111In-2G10 and 111In-3C6 showed pronounced tumor localization and retention in the MDA-MB-231 xenograft 72 hours postinjection (Fig. 3A). No localization was
observed in the MCF-7 xenografts injected with $^{111}$In-2G10 (Fig. 3B). The time activity curves for $^{111}$In-2G10 and $^{111}$In-3C6 found tumor uptake at its zenith 72 hours postinjection (Supplementary Fig. S3). A biodistribution study showed at 24, 48, and 72 hours, confirmed high tumor uptake for both probes (Fig. 3C). At 72 hours, the %ID/g values were 53.2% and 31.8% for $^{111}$In-2G10 and $^{111}$In-3C6, respectively (Fig. 3C). $^{111}$In-2G10 had more favorable tumor-to-blood (T/B) and tumor-to-muscle (T/M) ratios compared to $^{111}$In-3C6 with values of 12 and 114 for $^{111}$In-2G10 and 4, and 36 for $^{111}$In-3C6. MDA-MB-231 (uPAR-) xenografts, with uPAR expression knocked-out using shRNA, did not show significant tumor retention of the probes at 72 hours postinjection nor did the uPAR negative cell lines MCF-7 and MDA-MB-435 (Fig. 3D). MCF-7 TamR, with high uPAR mRNA expression, had tumor uptake values for the probes nearly identical to the MDA-MB-231 xenografts at 72 hours (Fig. 3D). At 120 hours postinjection, $^{111}$In-2G10 cleared from all secondary tissues and a scintigraphic signal
was only observed in the tumors (Supplementary Movie M1). A subsequent pharmacokinetics study found the in vivo half-lives of $^{111}$In-2G10 and $^{111}$In-3C6 to be 9.1 and 5.8 days (Supplementary Fig. S4).

2G10 and 3C6 identify MDA-MB-231 CDM model lesions in vivo

Further evaluation of 2G10 and 3C6 was conducted in MDA-MB-231 CDM models to test probe localization in smaller, dispersed lesions. Intracardiac injection of MDA-MB-231 cells in mice generated tumors analogous to breast cancer metastases in humans (19). Because the MDA-MB-231 cells were engineered to stably express luciferase, the formation of the micrometastases was followed with BLI. 3C6 and 2G10 were potent imaging agents in the CDM model, able to detect lesions millimeters in size (Fig. 4 and Supplementary Fig. S5). The SPECT signal from 3C6 coregistered with the observed BLI signal, resolving a 15.9 mm$^3$ osteolytic lumbar lesion (Fig. 4A). 2G10 identified lesions in the ilium (53 mm$^3$) and knee (14.3 mm$^3$) that coregistered with BLI (Fig. 4B). 2G10 detected a number of osseous and soft-tissue lesions, including a 63 mm$^3$ lesion at the base of the skull (Fig. 4C). The lesions depicted in Fig. 4 were found to be non-FDG avid. Interestingly, the tumor shown in the reconstructed data of Fig. 4C showed FDG uptake in areas surrounding the tumor, but not in the tumor itself. Fifteen mice, representing 36 BLI detectable lesions, were imaged with the uPAR probes and FDG. Non-FDG-avid tumors imaged by the uPAR probes represented 34 of 36 lesions (94%), while the remaining 2 showed exclusive FDG uptake and were not detectable by the uPAR probes (Supplementary Fig S8).

Table 1. Calculated $K_D$ values of 2G10 and 3C6 Fab and IgG using SPR and MDA-MB-231 cells using flow cytometry

<table>
<thead>
<tr>
<th>Antibody Construct</th>
<th>$K_D$ (SPR) (mol/L)</th>
<th>$K_D$ (MDA-MB-231 cells) (mol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2G10 Fab</td>
<td>$10 \times 10^{-9}$</td>
<td>$50 \pm 5 \times 10^{-9}$</td>
</tr>
<tr>
<td>3C6 Fab</td>
<td>$50 \times 10^{-9}$</td>
<td>$800 \pm 115 \times 10^{-9}$</td>
</tr>
<tr>
<td>2G10 IgG</td>
<td>$2 \times 10^{-12}$</td>
<td>$2 \pm 0.5 \times 10^{-9}$</td>
</tr>
<tr>
<td>3C6 IgG</td>
<td>$230 \times 10^{-12}$</td>
<td>$5 \pm 0.5 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

Anti-uPAR antibodies affect tumor growth in vivo

Encouraged by the imaging data, the therapeutic benefit of the antibodies was next investigated in vitro and in vivo. Any
Targeting uPAR in Breast Cancer

putative antitumor effects of 2G10 or 3C6 on cells were initially investigated using the antibodies to inhibit migration of MDA-MB-231 cells in a Matrigel invasion assay. Both antibodies were potent inhibitors of invasion (Fig. 5A). At 10 nmol/L, 2G10 inhibited about 30% of invasion of MDA-MB-231 cells, and 3C6 inhibited about 14% of invasion. At twenty times less antibody, 2G10 inhibited about 13% of invasion of MDA-MB-231 cells, and 3C6 inhibited about 30% of invasion of MDA-MB-231 cells, and 3C6 also had a pronounced effect on the clonogenic survival of treated cells, but the level was similar to MCF-7 suggesting that it was because of the inherent toxicity of the isotope and antibody diffusion, rather than targeted localization of the radionuclide. 2G10 was investigated as a radioimmunotherapeutic because of its long in vivo half-life and favorable tumor retention properties compared to 3C6. RIT, as with antibody imaging, requires a small amount of material compared to monotherapy. A monotherapy dose at 30 mg/kg for 1 animal would require 500 μCi of 2G10 whereas an RIT agent would only require 2 μg of material. 2G10 was radiolabeled with the therapeutic β particle-emitting radionuclide 177Lu for RIT. A radioactive MTT assay with 177Lu-2G10 and 177Lu-A11 showed that a dose of 50 μCi of 177Lu-2G10 resulted in the preferential killing of MDA-MB-231 cells, but not MCF-7 cells (Fig. 5D). Unlabeled 2G10, at a mass used for RIT, was not toxic to either cell line. 177Lu-A11 did show some toxicity in MDA-MB-231 cells, but the level was similar to MCF-7 suggesting that it was because of the inherent toxicity of the isotope and antibody diffusion, rather than targeted localization of the radionuclide. 177Lu-2G10, and a low monotherapy dose of 2G10 (4 mg/kg), were evaluated in MDA-MB-231 xenografts. A 150 μCi dose of 177Lu-2G10, fractionated into 2 equivalent doses of 75 μCi

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(2.5 μg/dose) 2 weeks apart, was used for the study. In addition to less myelotoxicity, fractionated dosing allows for a greater total dose to be administered, thus compensating for in vivo IgG clearance, and the radionuclide decay. At days 7 and 21, the mice were injected with saline control, 75 μCi of 177Lu-2G10, 75 μCi of 177Lu chelated to EDTA—to represent nontargeted 177Lu- or 2G10 (4 mg/kg; Fig. 5E). Mice treated with 177Lu-2G10 had marked tumor regression starting after the first dose and the emission of γ photon by 177Lu decay allowed them to be imaged during the course of treatment (Supplementary Fig. S6). After 35 days, the 177Lu-2G10–treated mice had tumor mean volumes of 50 mm³ whereas the tumor volumes of the control arms (saline and 177Lu-EDTA) increased to greater than 1,000 mm³. The tumors of the 177Lu-2G10 group were undetectable at day 49 and none of the tumors of that group (10/10) had recurred by day 84. Despite a much smaller injected mass (4 mg/kg vs. 30 mg/kg) and decreased dosing frequency, the low-dose 2G10 monotherapy was effective at mitigating tumor growth with only a 3-fold tumor volume increase by day 49.

**Discussion**

Several groups have shown that uPAR expression in breast tumor tissue is highly correlated with aggressive phenotypes (12, 25). Aberrant uPAR expression is typically seen concomitantly with that of uPA and PAI-1. In vitro studies of the PAS use particularly aggressive TNBC cell lines as model systems, and several groups have found that simultaneous uPAR and Her2 overexpression effected a higher degree of tumor aggressiveness (13, 26). Interestingly, when nonaggressive breast cancer cells were cultured under hypoxic conditions, uPAR overexpression and a subsequent aggressive phenotype were observed (14). Furthermore, there is an inverse relationship between uPAR expression in breast cancer and the efficacy of tamoxifen in treatment (11). This is corroborated by our finding that tamoxifen-resistant breast cancer cells have a
marked increase in PAS component expression. These studies highlight the potential importance of uPAR in cancer cell proliferation and invasion. Internal and external stresses on the tumor may activate pathways leading to uPAR overexpression and subsequent phenotypic changes that help cancer cells escape unfavorable milieus.

Here, the ability of 2G10 and 3C6, 2 human recombinant anti-uPAR antagonistic antibodies, to bind to uPAR overexpressing breast cancer cells in vitro, and target these cells in vivo, is described. Previous agents directed toward uPAR have met with mixed results as imaging agents. Antagonistic peptides of uPAR have been used for PET imaging, but poor affinity and limited bioavailability have limited their further development and clinical translation (27). One antibody-based strategy targeting rat uPAR with a 125I-labeled probe showed little tumor accumulation that was likely because of the enhanced permeation and retention (EPR) effect and not selective targeting (28). Our data suggest that 2G10 and 3C6 localization in the tumor models was the direct result of uPAR binding and not simply because of the EPR effect or other hemodynamic

Figure 5. The therapeutic efficacy of 3C6 and 2G10 in vitro and in vivo. A, the effect of 3C6 and 2G10 on the Matrigel-coated transwell migration of MDA-MB-231 cells. Media containing 5% FBS was added to the bottom chamber, and the migration of cells was monitored over 24 hours in a fluorescence plate reader. B, the effect of the 3 antibodies 2G10, 3C6, and A11 on the clonogenic survival of uPAR-expressing MDA-MB-231 cells and nonexpressing MDA-MB-435 and MCF-7 cells. C, tumor growth of MDA-MB-231 xenograft mice treated with a high dose (30 mg/kg) of 2G10-, 3C6-, and A11-matched isotype control IgG and saline control. Mice (n = 10/group) were dosed 4 times over the course of the study as denoted by (X). Asterisk denotes indicated P < 0.05 via a Student t test. D, the targeted cytotoxicity of 177Lu-2G10 on MDA-MB-231 cells compared to MCF-7 cells as measured by MTT assay. MDA-MB-231 and MCF-7 cells in a 96-well plate were treated for 96 hours with unlabeled 2G10 (2.5 µg), 50 and 25 µCi of 177Lu-2G10 (2.5 µg), or A11 (2.5 µg). E, the therapeutic effect of 177Lu-2G10 was tested in SCID mice bearing MDA-MB-231 tumors. Mice (n = 10/group) were treated with 177Lu-2G10 (75 µCi–2.5 µg), 177Lu-EDTA (75 µCi), saline, and 2G10 (4 mg/kg) at the indicated time points; “X” denotes dosing. Asterisk denotes indicated P < 0.05 via a Student t test.
forces. Three-dimensional SPECT/CT imaging confirmed that 2G10 and 3C6 labeled with $^{111}$In permeated the MDA-MB-231 tumors (i.e., they did not pool in the vascularized periphery of the tumor) and that this signal was as robust and long-lived as other antibody probes in clinical development, although $^{111}$In-2G10 had more favorable pharmacokinetic properties than $^{111}$In-3C6 (29, 30). Enhanced $^{111}$In-3C6 clearance was observed as evident by increased hepatic uptake and a decreased T/B ratio. The decreased uptake by 3C6 could be explained by its lower affinity for uPAR or epitone occlusion. The epitone occlusion argument is plausible—intriguen is more abundant on the cell surface compared to uPAR and uPA binding is believed to cause a conformational change in the integrin binding region (31).

Using the MDA-MB-231 CDM model, $^{111}$In-labeled 2G10 and 3C6 detected soft-tissue and osseous metastatic lesions. Although this is the first such time that a uPAR-targeted antibody has been used to detect lesions like these, the imaging results highlight the potential sensitivity of uPAR SPECT probes in detecting metastases. Furthermore, with this model, it was possible to compare our biomarker-targeted imaging approach with the standard of care metabolic imaging approach for breast cancer, FDG-PET. We found that 2G10 and 3C6 detected more MDA-MB-231 lesions than FDG-PET. Not all tumor cells take up $^{18}$FDG, and FDG-PET is known to produce false positive signals—especially in inflamed tissue around the tumor (32). Given the caveats of FDG-PET, we hypothesized that this $^{18}$FDG uptake shown in Fig. 4C arose from inflamed tissue surrounding the lesion. In Fig. 4C, the $^{18}$FDG-avid cells did not bioluminesce, and subsequent studies showed that $^{111}$In-2G10 did not localize to sites of inflammation (Supplementary Fig. S7). In this study, however, FDG-PET detected 2 lesions that $^{111}$In-2G10 did not (Supplementary Fig. S8). The fact the uPAR probes and $^{18}$FDG identified different tumors derived from the same cell line highlights the impact of microenvironment on tumor heterogeneity, and how it can influence the ability to comprehensively diagnose and treat breast cancer. While further investigation is needed to understand the basis for this heterogeneity, these results suggest that a combined diagnostic approach, using both biomarkers and metabolic targets, can identify a wider array of lesions than by using any singular imaging agent.

A corollary to anti-uPAR IgG localization in tumors is the potential therapeutic benefit resulting from antibody targeting of uPAR. Attempts previously to target uPAR for therapeutic benefit have again met with mixed success. An antibody purported to disrupt the uPAR/uPA system was used to elicit a therapeutic effect in a syngeneic model of rat breast cancer; however, its specificity to uPAR, as well as antagonistic characteristics, were not confirmed (28). Another group showed that concomitant treatment of xenograft tumor-bearing mice with an anti-uPAR antibody and the nucleoside analog gemcitabine effected a stronger therapeutic outcome that treatment with either agent alone (33). Using radiotherapy, uPAR has been targeted with peptides and recombinant proteins. The antagonistic peptide used to image uPAR previously, showed little therapeutic effect and low tumor uptake in a colon cancer model when labeled with $^{177}$Lu (34). In a TNBC model, Barry and colleagues used a recombinant version of PAI-2, an uPA inhibitor expressed during pregnancy, labeled with $\alpha$-emitting isotope $^{212}$Bi to target the uPA/uPAR system (35). This approach worked well against small preangiogenic clusters of cells and reduced tumor growth in vivo. In vitro studies found that our uPAR antibodies affected cell invasion and clonogenic survival, suggesting potential therapeutic benefit in vivo. As a monotherapy at a high dose, 2G10 exhibited a pronounced cytostatic effect and was a more potent therapeutic than 3C6. This difference in therapeutic efficacy is supported by the higher tumor uptake of 2G10 in the imaging studies compared to 3C6 and the longer half-life of 2G10 in vivo. Localization of the antibodies to tumors supported the delivery of a therapeutic payload to the tumor site. Here, we found that 2G10 labeled with the therapeutic radionuclide $^{177}$Lu showed selective cytotoxicity for uPAR expressing cells in vitro and, in an animal study, complete tumor regression. Comparing our in vivo work to the studies on $^{211}$Bi-PAI-2 found that $^{177}$Lu-2G10 was more effective treating established tumors in a TNBC model (35). Currently, RIT is used in the treatment of hematological malignancies with Bexxar and Zevalin as the only 2 FDA approved agents; however, recent studies have shown that RIT is effective in treating solid tumors such as prostate and colon cancer (36, 37). Our preclinical RIT data suggest that uPAR-targeted RIT agents could be viable options for treating aggressive breast cancers when other therapies have failed. Furthermore, although Bauer and colleagues indicate that anti-uPAR therapeutics may play a role in potentiating cytotoxic drug responses, our data with 2G10 suggest that disruption of the uPAR/uPA interaction might be a pivotal axis to target in cancers that show uPAR overexpression. This idea has recently been supported by work where murine antibodies directed toward murine uPAR significantly disrupted tumor growth and progression in vivo (38). The uPAR antibodies described here are fully human, thus allowing for repeated administration of the antibodies as imaging probes, therapeutics and RIT agents with minimal immunologic side effects.

As with other cancer targets, uPAR expression is, indeed, found on other noncancerous cells. uPAR’s role in effecting cellular migration and proliferation lends it to be important for the function of activated leukocytes, but is not highly expressed in otherwise normal states (39, 40). In addition, uPAR plays a role in angiogenesis and wound healing (41, 42). These complications, however, are in the same vein as those for other antibody-based targeted imaging agents and therapeutics. Given the abundance of uPAR on aggressive tumor cells (especially on tumor-associated macrophages and fibroblasts) relative to resting leukocytes and other cells that express uPAR, the data presented here on 2G10 and 3C6 underscore uPAR’s potential as a diagnostic and therapeutic target.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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