Multiparametric Monitoring of Tumor Response to Chemotherapy by Noninvasive Imaging

Zdravka Medarova, Leonid Rashkovetsky, Pamela Pantazopoulos, and Anna Moore

Molecular Imaging Laboratory, MGH/HST Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital/Harvard Medical School, Boston, Massachusetts

Abstract

With the emerging concept of individualized cancer therapy, it becomes crucial to develop methods for the noninvasive assessment of treatment outcome. With this in mind, we designed a novel approach for the comprehensive evaluation of response to chemotherapy with the established agent doxorubicin in a preclinical breast cancer model. This approach delivers information not only about change in tumor size but also about target antigen expression. Our strategy relies on a tumor-specific contrast agent (MN-EPPT) targeting the underglycosylated MUC-1 (uMUC-1) tumor antigen, found on more than 90% of breast cancers and predictive of chemotherapeutic response. MN-EPPT consists of superparamagnetic iron oxide nanoparticles (MN) for magnetic resonance imaging (MRI) modified with Cy5.5 dye (for optical near-IR fluorescence (NIRF) imaging) and conjugated to peptides (EPPT), specifically recognizing uMUC-1. In vivo, treatment of mice bearing orthotopic human breast carcinomas with doxorubicin led to a reduction in tumor mass and resulted in down-regulation of uMUC-1. The tumor-specific accumulation of MN-EPPT allowed the assessment of change in tumor volume by noninvasive imaging. Furthermore, in mice injected with MN-EPPT, tumor delta-T2 was significantly reduced after treatment with doxorubicin, indicating a lower accumulation of MN-EPPT and reflecting the reduced expression of uMUC-1. With these studies, we have shown the utility of magnetic resonance imaging for the multiparametric characterization of breast tumor response to chemotherapy. This approach has the potential of significantly advancing our ability to better direct the development of molecularly targeted individualized therapy protocols because it permits the monitoring of therapy on a molecular scale. [Cancer Res 2009;69(3):OF1–8]

Introduction

The emergence of molecularly targeted cancer therapies mandates the development of methods to directly determine their efficacy. Noninvasive imaging techniques are currently available for visualizing different pathologic conditions. However, their use for cancer monitoring is limited due to the lack of tumor-specific imaging probes. We have previously developed a multimodal imaging probe (MN-EPPT) targeting the underglycosylated mucin-1 tumor antigen (uMUC-1), which is overexpressed and underglycosylated on more than 90% of breast tumors and whose abundance seems to be linked to tumor progression and response to chemotherapy (1). Our tumor-specific multimodal imaging probe consists of superparamagnetic iron oxide nanoparticles (MN) for magnetic resonance imaging (MRI) modified with Cy5.5 dye [for optical near-IR fluorescence (NIRF) imaging] and has peptides (EPPT) specifically recognizing uMUC-1 attached to its dextran coat (1). The dual-modality approach combines the high-resolution, tomographic capability, and unlimited tissue penetration of MRI with the high sensitivity and low cost of in vivo optical imaging as a validation tool (2).

In the past, we have done extensive studies validating the utility of MUC-1 as a candidate antigen and the specificity of MN-EPPT as a multimodal targeted probe in various cancer models (1, 3). In our new studies described here, we used this imaging probe for the simultaneous quantitative assessment of tumor volume and target antigen availability in breast tumors before and after chemotherapy. The importance of these studies is underscored by the fact that breast cancer is the second leading cause of cancer-related deaths in women of the western world. In the United States alone, more than 180,000 new cases are diagnosed each year. Of these patients, ~25% will succumb to their disease despite early diagnosis and aggressive therapeutic intervention, possibly due to the inability to optimally tailor-fit treatments on an individualized basis.

As of today, the most reliable predictor of overall breast cancer survival and therapeutic outcome in breast cancer patients is the stage at which the tumor is diagnosed. At the same time, early therapeutic intervention holds promise because of its life-saving potential. Therefore, it is critical to devise ways in which to explore the molecular complexity of breast cancer for early diagnosis and monitoring of treatment progress. There is a wealth of imaging modalities capable of evaluating the tumor and the tumoral response to therapy, including mammography and sonography (4–7), MRI (8–11), and scintigraphy or positron emission tomography (12–14). These modalities, however, primarily evaluate tumor volume and metabolism but do not provide dynamic information about the molecular profile of the lesion, which would be a much earlier and more reliable predictor of tumor outcome (4).

Here we have addressed the need to develop new tumor antigen–specific contrast agents in combination with high-resolution in vivo imaging methods for the monitoring of response to therapy. We believe that this study is significant because it explores a noninvasive imaging approach to monitor change in tumor size and the relative availability of a tumor antigen (uMUC-1). uMUC-1 provides a unique advantage in this case because it shows predictable changes in phenotype directly linked to breast cancer progression as well as to therapeutic outcome (15). Considering that related iron oxides are already in clinical use (16), if successful, our studies would establish the feasibility of a...
multiparameter method for monitoring breast cancer response to chemotherapy, based on the expression of molecular markers implicated in disease progression.

Materials and Methods

Probe synthesis and characterization. The uMUC-1–targeted MN-EPPT probe and the scrambled control probe MN-SCR were synthesized and characterized as described (1, 3). Peptide sequences were as follows: EPPT, C-AAA-A-G-E-P-P-T-T-F-A-Y-W-G-K (FITC); SCR, C-AAA-A-G-E-P-P-T-T-F-A-Y-W-G-K (FITC). Synthesis resulted in a triple-labeled nanoparticle, consisting of FITC on EPPT peptide (for fluorescence microscopy), superparamagnetic iron oxide (MN, a magnetic label for MRI), and Cy5.5 dye (Amersham Biosciences) attached to the MN (for NIRF optical imaging). Iron concentration and peptide/FITC and Cy5.5 payloads were determined as described in ref. 1. The resultant probe had an average iron concentration of 7.3 ± 0.16 mg/mL, 2.1 Cy5.5 molecules per nanoparticle, and 4.43 peptides per nanoparticle.

In vitro cell treatment. To validate the observation that treatment with doxorubicin down-regulates uMUC-1 in BT-20 breast adenocarcinoma cells, cells were incubated with 0.4 μmol/L of doxorubicin-HCl (Sigma) for 48 h, as previously described (17). PBS-treated control cells served as controls. Following incubation, the cells were analyzed for uMUC-1 expression by quantitative reverse transcription-PCR (RT-PCR) analysis and Western blot, as described below. Alternatively, to assess the relative accumulation of MN-EPPT in these cells, following treatment with doxorubicin, the cells were incubated at 37°C overnight with MN-EPPT or the scrambled control probe (MN-SCR); washed, fixed in 2% paraformaldehyde, suspended in 0.5-mL PCR tubes, and imaged by MRI as described below. Following the MRI session, the cells were transferred to fluorescence-activated cell sorting tubes and analyzed by flow cytometry.

Real-time quantitative RT-PCR. Total RNA was extracted from breast adenocarcinoma cells and tissue using the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen, Inc.). Relative levels of uMUC-1 mRNA were determined by real-time quantitative RT-PCR (TaqMan protocol). TaqMan analysis was done using an ABI Prism 7700 sequence detection system (PE Applied Biosystems). The PCR primers and TaqMan probe specific for MUC-1 mRNA were designed using Primer express software 1.5. Primer and probe sequences are as follows: forward primer, 5’-ACAGGTTCGCTATCTGCAA-3’ (nucleotides 64–84 in the 5’ nonrepetitive region); reverse primer, 5’-CTCACAGGCATTCTCAGTA-3’ (nucleotides 139–164 in the 5’ nonrepetitive region); and TaqMan probe, 5’-FAM-TGGGAAAGGAGACTTGCTACCCAGA-TAMRA-3’ (nucleotides 96–124 in the 5’ nonrepetitive region). For Western blot. For Western blot, total cell extracts were prepared by solubilization of BT-20 breast adenocarcinoma cells with radioimmunoprecipitation assay lysis and extraction buffer (Pierce) with added Halt Protease Inhibitor Cocktail (Pierce). Protein concentration in the extracts was determined using the BCA Protein Assay Kit (Pierce). Samples were incubated with 1% SDS and 3% 2-mercaptoethanol at 100°C for 5 min and centrifuged. Approximately 100 μg of total protein were separated on a 12% polyacrylamide gel (SDS-PAGE). Precision Plus Protein Kaleidoscope Standards (Bio-Rad) were used as molecular weight standards. Separated proteins were transferred onto a polyvinylidene difluoride membrane and developed with One-Step Western Advanced Kit for mouse primary antibody (GenScript Corp.). Primary anti-Muc-1 mouse monoclonal antibodies specific for the backbone peptide APDTRPA (VU4H5 clone, Santa Cruz Biototechnology) and anti-tubulin mouse monoclonal antibodies as an internal standard (V10178, Biomed) were used at 200- and 10,000-fold dilutions, respectively.

MRI of cell phantoms. To show that the specificity of MN-EPPT for uMUC-1 can be used to probe for down-regulation of uMUC-1 by doxorubicin, we performed MRI of cell phantoms prepared as described in the previous section. Imaging was done using a 9.4T Bruker horizontal bore scanner equipped with ParaVision 3.0 software. The imaging protocol consisted of coronal T2-weighted spin echo (SE) pulse sequences with the following parameters: SE TR/TE = 3.00/0[18, 16, 24, 32, 40, 48, 56, 64]; field of view, 40 × 40 mm; matrix size, 128 × 128 pixels; slice thickness, 0.5 mm; in-plane resolution, 312 × 312 μm. Image reconstruction and analysis were done using Marvisi 3.5 software (Institute for Biodiagnostics, National Research Council, Canada). T2 maps were constructed according to established protocol by fitting the T2 values for each of the eight echo times (TE) to a standard exponential decay curve. T2 relaxation times were calculated by manually segmenting out the cell pellet on magnetic resonance images.

Flow cytometry. Flow cytometry was done to confirm if the MN-EPPT specificity for uMUC-1 translates in reduced probe accumulation in breast adenocarcinoma cells, following uMUC-1 down-regulation by doxorubicin. Flow cytometry was done on cells treated as described in “In vitro cell treatment,” using a FACSCalibur (Becton Dickinson) equipped with the Cell Quest software package (Becton Dickinson).

Tumor model. To establish a preclinical orthotopic tumor model of human breast cancer, 5- to 6-wk-old female nu/nu mice (n = 10; Massachusetts General Hospital Radiation Oncology breeding facilities) were inoculated in the right mammary fat pad with the uMUC-1–positive human breast adenocarcinoma cell line BT-20 (American Type Culture Collection), as previously described (18).

All animal experiments were done in compliance with institutional guidelines and the animal protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital.

Doxorubicin treatment. To establish a clinically relevant treatment model, we used the standard chemotherapeutic agent doxorubicin. The treatment protocol involved i.v. injection of 7 mg/kg doxorubicin in saline solution once weekly for 2 consecutive weeks beginning 8 d after tumor implantation once tumors had reached a diameter of 0.5 cm, as previously suggested (17). Saline solution–injected animals served as nontreated controls. As previously described, to evaluate tumor response to chemotherapy, in vivo imaging was done 1 d before the beginning of treatment and 1 d after the completion of treatment (3).

In vivo MRI. MRI was done before and 24 h after i.v. injection of MN-EPPT or the scrambled control probe, MN-SCR (10 mg Fe/kg), using a 9.4T Bruker horizontal bore scanner equipped with Varivison 3.0 software using sequences as for in vitro MRI. Image reconstruction and analysis were done as described for in vitro MRI.

The tumor volume and tumor T2 relaxation times were calculated by manually segmenting on one mouse tumor on magnetic resonance images. Quantitative evaluation of differential tumor growth by MRI was based on multislice T2-weighted images. The volume was estimated according to the formula for the volume of an ellipsoid: V = 4/3πabc, where a and b are the equatorial radii (along the x and y axes) and c is the polar radius (along the z axis). For T2 map analysis of relaxation times, the terminal slices were not included in the analysis to avoid interference from partial volume effects. Relative MN-EPPT accumulation in the tumors was estimated based on the following formula: T2 before injection – T2 after injection (delta-T2, in milliseconds).

In vivo and ex vivo optical imaging. In vivo NIFR optical imaging was done immediately after each MRI session. Animals were placed into a whole-mouse imaging system (Imaging Station IS2000MM, Eastman Kodak Company) and imaged in the Cy5.5 channel. At the end point of each experiment following the last imaging session, mice were sacrificed; tumors were excised, placed in the optical imaging system, and imaged ex vivo. Image analysis was done using the Kodak 1D 3.6 Network software. The actual volumes of excised tumors were determined by measuring tumor dimensions ex vivo using calipers.

Immunohistochemistry and in situ apoptosis detection. To detect the accumulation of MN-EPPT in tumors at the microscopic level, we performed confocal immunohistochemistry. Tumors were embedded in Tissue-Tek optimum cutting temperature compound (Sakura Fineteck) and imaged in the Cys5.5 channel. At the end point of each experiment following the last imaging session, mice were sacrificed; tumors were excised, placed in the optical imaging system, and imaged ex vivo. Image analysis was done using the Kodak 1D 3.6 Network software. The actual volumes of excised tumors were determined by measuring tumor dimensions ex vivo using calipers.
fluorescence microscopy of frozen tumor sections. Note the good colocalization of FITC (EPPT) and Cy5.5 (MN) fluorescence. Bar, 20 μm.

Results

MN-EPPT accumulation in an orthotopic model of human breast adenocarcinoma can be detected by MRI and NIRF imaging. In this study, we used a preclinical orthotopic model of human breast cancer to obtain information about tumor response to the standard chemotherapeutic agent doxorubicin. As a first step toward this goal, we evaluated whether MN-EPPT accumulates in the orthotropic breast tumor model and whether this accumulation is sufficient to generate detectable contrast on T2-weighted magnetic resonance and optical images. Eight days after tumor implantation once tumors had reached a diameter of 0.5 cm, female nu/nu mice were injected with the uMUC-1–targeted MN-EPPT probe. Twenty-four hours after probe injection, mice were subjected to MRI and fluorescence optical imaging. Representative T2-weighted images and their corresponding T2 maps before and 24 hours after MN-EPPT injection are shown in Fig. 1. Before injection of the contrast agents, breast tumors appeared with characteristically long T2s (T2 = 46.8 ± 2.0 ms). After administration of MN-EPPT, there was a significant decrease in tumor T2 relaxation time (T2 = 30.9 ± 1.2 ms) compared with the pre-contrast value (P < 0.0011, n = 9; Fig. 1). In vivo NIRF optical imaging was done immediately after the post-contrast MRI session. In mice injected with MN-EPPT, there was a high-intensity fluorescence signal coming from the implanted tumor (4,388.8 ± 420.4 relative fluorescence units; Fig. 2A). Ex vivo imaging of excised tumors (Fig. 2B) revealed bright fluorescence associated with the tumor compared with muscle tissue, which was used to define background fluorescence. Furthermore, ex vivo fluorescence microscopy on frozen tumor sections revealed very extensive accumulation of the probe in tumor cells (Fig. 2C). The good

Statistical analysis. All data were represented as mean ± SE. Statistical analysis was done using two-tailed Student’s t test and linear regression where indicated. P ≤ 0.05 was considered statistically significant.
colocalization of fluorescence in the NIR channel (Cy5.5, MN) and in the green channel (FITC, EPPT peptide) indicated integrity of the probe after persistence in the circulation (Fig. 2C).

With these experiments, we established that MN-EPPT accumulated in orthotopic breast tumor models and that this accumulation can be detected by noninvasive MRI and NIRF optical imaging. These findings set the stage for our next studies in which we assessed the utility of the described imaging approach for the monitoring of tumor response to chemotherapy. Because the affinity of MN-EPPT for tumors is a function of its uMUC-1-targeted nature (1, 3), we speculated that the uptake of this probe by tumors would permit not only tumor delineation for the calculation of tumor volume but also measurement of target antigen expression as reflected by tumor T2 relaxation times.

Tracking change in tumor size following chemotherapy

To determine if MN-EPPT can serve as a tumor-targeted agent for the monitoring of tumor response to chemotherapy, we treated tumor-bearing animals with doxorubicin and compared changes in tumor size as measured by MRI and NIRF optical imaging between experimental mice and saline-treated controls. As seen in Fig. 3, whereas in animals treated with doxorubicin there was an actual reduction in tumor volume following the course of therapy, in saline-treated controls tumor size increased dramatically (Fig. 3B). In terms of relative measurements of change in tumor size, the two modalities were highly correlated ($R^2 = 0.89$; Fig. 3C) and accurately defined true differences between experimental and control tumors, as seen ex vivo (Fig. 3D).

Tracking change in tumor antigen expression following chemotherapy

There is evidence in the literature that treatment of breast tumor cells with doxorubicin leads to down-regulation of the uMUC-1 antigen (19). With this knowledge, we speculated that, because the accumulation of MN-EPPT in tumors is predicated on its specificity for the uMUC-1 tumor-specific antigen (1, 3) and the change in T2 relaxation times post-contrast versus pre-contrast (delta-T2) is a function of local contrast agent distribution and abundance (20), by measuring delta-T2 relaxation times we would...
be able to indirectly extract information about the relative availability of uMUC-1 as a result of chemotherapeutic treatment. Obtaining such information would be very valuable from a clinical point of view because changes in uMUC-1 expression directly relate to tumor progression, response to therapy, metastatic potential, and survival (21–24).

We first validated the observation that treatment with doxorubicin down-regulated uMUC-1 in BT-20 breast adenocarcinoma cells. Cells were incubated with doxorubicin for 48 hours as previously described (17). Following incubation, the cells were analyzed for uMUC-1 expression by quantitative RT-PCR analysis and Western blot. Both methods confirmed substantial down-regulation of MUC-1 in BT-20 cells following treatment with doxorubicin (Fig. 4A and B). Quantitative RT-PCR revealed a significant 60 ± 0.7% down-regulation of the gene (P = 0.028; Fig. 4A). This down-regulation effect was accompanied by the induction of considerable levels of apoptosis in the doxorubicin-treated cells as assessed using TUNEL assay (Fig. 4C). Consistent with the known mechanisms of cytotoxicity of doxorubicin, there was abundant nuclear as well as extranuclear TUNEL staining, indicating the formation of multiple micronuclei and loss of nuclear membrane integrity. These events accompany mitotic catastrophe and reflect advanced stages of doxorubicin-induced apoptosis (25) and successful mediation of cytotoxicity by the drug (Fig. 4C). The down-regulation effect was uMUC-1 specific because housekeeping genes [18S (quantitative RT-PCR) and tubulin (Western blot)] were not down-regulated (Fig. 4A and B).

Next, we wanted to see if we could use the uMUC-1 selectivity of MN-EPPT to probe for these changes. Populations of cells treated with doxorubicin or PBS as described above were incubated with PBS, MN-EPPT, or a scrambled control probe, MN-SCR (3), which is identical to MN-EPPT except that the targeting EPPT peptide is replaced with a scrambled peptide. Following incubation, cell phantoms were imaged by MRI. As seen in Fig. 5A and B, cells incubated with MN-EPPT displayed significantly lower T2 relaxation times than cells incubated with MN-SCR, further confirming the specificity of MN-EPPT for uMUC-1 (P < 0.0001, n = 6; Fig. 5A and B). Treatment with doxorubicin resulted in significantly higher T2 relaxation times of MN-EPPT–incubated cells (P = 0.0003, n = 6; Fig. 5A and B), indicating a lower accumulation of the probe and closely mirroring the reduced expression of the antigen in these cells as shown by quantitative RT-PCR and Western blot. Unlike in cells incubated with MN-EPPT, in cells incubated with the MN-SCR probe, treatment with doxorubicin did not change the T2 relaxation times of the cells (Fig. 5A and B).

The findings by MRI were also confirmed using flow cytometry in the FL4 channel (Cy5.5), done immediately after the MRI session (Fig. 5C and D). The relative fluorescence of cells incubated with MN-EPPT was significantly higher than that of cells incubated with MN-SCR (P = 0.007; Fig. 5C and D), reflective of the specificity of the probe for uMUC-1. In addition, relative fluorescence was lower in cells treated with doxorubicin and further incubated with MN-EPPT than in control-treated cells (P = 0.007; Fig. 5C and D).
reflecting the reduced availability of the antigen in the presence of doxorubicin. By contrast, when MN-SCR was used as a contrast agent, relative fluorescence levels in doxorubicin-treated cells were not different than in controls (Fig. 5C and D). These studies confirmed that MN-EPPT specific accumulation in breast adenocarcinoma cells is a function of the relative availability of the target antigen.

These experiments established an in vitro framework for our subsequent in vivo studies aiming to quantitatively assess relative uMUC-1 availability in preclinical breast cancer tumors. To accomplish this goal, we analyzed tumor T2 relaxation times of animals treated with doxorubicin or saline as described in the previous section and calculated tumor delta-T2 values before the beginning and after the completion of treatment. In animals treated with doxorubicin, there was a 25% decrease in delta-T2 after treatment with doxorubicin ($P = 0.04, n = 5$; Table 1A). In control mice treated with saline, there was no significant difference between pretreatment and posttreatment delta-T2 (Table 1A). The observed drop in delta-T2 after treatment with doxorubicin was indicative of reduced relative MN-EPPT accumulation in the tumors and was accompanied by a corresponding down-regulation of uMUC-1 as seen by quantitative RT-PCR ($P = 0.03$; Table 1B). Unlike in mice injected with the targeted MN-EPPT probe, in control mice injected with MN-SCR as a contrast agent, there were no significant differences in delta-T2 between mice treated with doxorubicin and saline (results not shown). These studies corroborated our in vitro findings and further confirmed that MN-EPPT can be used to noninvasively assess relative uMUC-1 availability in breast tumors.

Discussion

The recent past has witnessed considerable progress in the way human tumors are characterized with a particular gain in knowledge of cancer at the molecular level. This has resulted in a shift toward using molecularly targeted therapies for cancer, necessitating the development of corresponding molecular tools to determine which patients are most likely to benefit from particular therapies (26). Consequently, the capacity to noninvasively assess tumor anatomy and physiology and at the same time registering aberrations in the molecular phenotype of cells is essential for early diagnosis and effective treatment. With these studies, we have shown the utility of MRI for multiparametric characterization of tumor response to chemotherapy in a preclinical breast cancer model. We have confirmed that MRI and NIRF can be combined to obtain complementary information about anatomic changes in tumor size following treatment using the breast cancer model. In addition, we have established that our contrast agent, by virtue of being target specific for a tumor antigen, can provide molecular information about target antigen expression when combined with MRI as a modality with a high quantitative sensitivity to local contrast agent abundance. This approach represents an example of noninvasive semiquantitative assessment of therapy-induced changes in tumor molecular expression levels by MRI.

An important point relevant to our studies is the potential for effects other than probe uptake by the tumor, such as vascularity and the presence of necrosis, which can affect the measurements by MRI. Areas of active angiogenesis as well as edema can result in elevated T2 relaxation times, whereas areas of local hemorrhage or necrosis can appear with short T2. We have attempted to eliminate
or minimize the influence of these factors by focusing on the delta-T2 parameter, which represents an estimate of the change in T2 after probe injection relative to preinjection. Assuming a minimal change in anatomic/physiologic tumor characteristics during the time between the preinjection and postinjection magnetic resonance measurements, the delta-T2 value reflects relative probe accumulation. Consequently, the development of similar targeted probes with a faster clearance rate (e.g., Gd chelates) and optimal target binding kinetics will permit a further shortening of the timespan between pre-contrast and post-contrast imaging and will even more accurately reflect probe uptake by the tumor.

In summary, this study describes a method for antigen-specific noninvasive breast tumor imaging by MRI. Our strategy can be used in vivo to monitor tumor response to therapy based not only on changes in tumor size, which is a late and often unreliable marker of response to therapy, but also on the expression of molecular biomarkers. Considering that many newly established and experimental breast cancer therapies are molecularly targeted, similar probes can be developed for the monitoring of the availability of antigens such as Her2/neu, epidermal growth factor receptor, estrogen receptor, and somatostatin receptor, to name a few. Progress in that direction has already been made (27–31). The ultimate implications of the approach include optimization of existing cancer treatment regimens, testing of novel therapeutic paradigms, and development of personalized medicine protocols.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### References


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