Magnetic Resonance Molecular Imaging of the HER-2/neu Receptor

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Abstract

The HER-2/neu receptor is a member of the epidermal growth factor family and is amplified in multiple cancers. It is under intense investigation both as a prognostic marker and for therapy, using monoclonal antibodies targeted against the receptor. We have developed a novel two-component gadolinium-based MR contrast agent to image the HER-2/neu receptor. Positive T1 contrast in MR images was generated by the specific binding of avidin-gadolinium complexes to tumor cells prelabeled with a biotinylated anti-HER-2/neu antibody. Significant intensity enhancement was observed in HER-2/neu-expressing cell lines and in vivo in a breast cancer model. Potential applications of this approach may include determination of the HER-2/neu status for prognosis and for selecting tumors for monoclonal antibody therapy.

Introduction

MRT is a noninvasive technique routinely used in diagnostic imaging. The low sensitivity of MR in comparison with nuclear imaging such as single-photon emission tomography or positron emission tomography is compensated by detection of the abundant water signal from tissues. Image contrast specific to molecular markers or pathways can be detected through the modulation of water T1 or T2 relaxation, using agents typically containing GdDTPA or iron oxide particles. CA can be activated by the chemical cleavage of a protective “cage” restricting water access to the contrast by a specific reporter enzyme, resulting in signal enhancement (1). Cells can also be loaded with CA by internalization using passive endocytosis (2, 3) or by an active transporter system such as a transferrin receptor that shuttles targeted superparamagnetic iron oxide nanoparticles into the cells (4). This technique has been used to track cell migration or detect transgene expression (5). Cells can also be labeled extracellularly with a CA attached to a high-affinity mAb targeted to a specific cell surface receptor. Because only a limited number of functional groups can be conjugated to the mAb to avoid compromising its binding affinity, the CA concentrations achieved by direct labeling of the mAb are low and frequently are not sufficient to generate detectable MR contrast (6). Relaxivity can be increased while maintaining high binding efficiency by attaching mAbs to a larger complex such as dendrimer particles (7) or liposomes, which have multiple sites for CA labeling. The latter approach was successfully used to image αvβ3 integrins expressed in the neovascularity of angiogenic tumors with Gd-labeled polymerized liposomes (8). The large molecular size of these constructs, however, significantly restricts their delivery and diffusion in tissue and poses a serious limitation for the application of such CAs for imaging receptors in solid tissues.

We developed and applied a two-component Gd-based avidin-biotin system for MR molecular imaging of HER-2/neu receptors. The HER-2/neu receptor is an important target for tumor prognosis and therapy (9). It is overexpressed, usually as a result of gene amplification, in approximately 25% of human breast cancers, and HER-2/neu expression correlates with poor prognosis for breast and other forms of human cancer (10). HER-2/neu is also a target for immuno-therapeutic agents, such as the humanized mAb Herceptin, which showed efficacy against HER-2/neu-overexpressing breast cancers (11, 12). In our approach, the extracellular domain of the receptor is prelabeled by a biotinylated mAb. After clearance of the unbound mAb, Gd-labeled avidin is administered and binds, with high affinity, to the biotinylated mAb. Because the CA is delivered as two separate smaller components (molecular weight of 150,000 for mAb and 70,000 for avidin-Gd conjugate), the delivery is more efficient in comparison with a single, large molecular size CA. The method was tested in an experimental mouse model of breast carcinomas derived from HER-2/neu transgenic mice (13) and demonstrated high positive T1 contrast both in vitro and in vivo in experimental animal tumor models.

Materials and Methods

Cell Lines. NT-5 HER-2/neu-expressing cells were derived from spontaneous mammary tumors in female HER-2/neu transgenic mice, which express the nontransforming rat proto-oncogene as described previously (13). NT-5 cells were grown in culture using modified RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Sigma), 300 mg/L L-glutamine, 1% nonessential amino acids, 110 mg/L sodium pyruvate, penicillin/streptomycin, gentamicin, and 0.8 mg/mL insulin and maintained at 37°C, 5% CO2. The mouse carcinoma EMT-6 cell line, which does not express rat HER-2/neu, was maintained in culture using RPMI 1640 with 10% fetal bovine serum and antibiotics at 37°C, 5% CO2.

Flow Cytometry Analysis. NT-5 and EMT-6 cells were analyzed for the expression of HER-2/neu receptors using biotinylated mAb (Ab-9; clone B10; NeoMarkers, Fremont, CA) raised against rat HER-2/neu protein. Primary antibodies were detected with avidin-FITC conjugate (Molecular Probes, Eugene, OR). Non-specific biotinylated antibodies were used in the control studies. To protect surface proteins, cells were harvested from the flasks using enzyme-free cell dissociating buffer (Invitrogen, Carlsbad, CA) for up to 30 min at room temperature. All data were acquired with a FACScan flow cytometer (Becton Dickinson, San Diego, CA). Expression level of the receptor was evaluated using a reference sample consisting of biotinylated microspheres (Molecular Probes) probed with the same avidin-FITC conjugate as the cells.

Avidin-Gd Conjugate. To prepare the conjugate, purified avidin (Proryme, San Leandro, CA) was conjugated with DTPA (Sigma, St. Louis, MO) and labeled with Gd ions using a modified protocol of Huotawich et al. (14). Briefly, protein was allowed to react with a 20-fold molar excess of cyclic anhydride of DTPA for 24 h at 4°C, and after separating low molecular weight reagents, the conjugates were reacted with Gd(NTA)3 for 24 h at 4°C. To separate the conjugate from low molecular weight compounds, the reaction mixture was filtered through a centrifugal filter device (Ultrafree-15; 10,000 cutoff value; Millipore, Bedford, MA). The labeling efficiency was estimated from T1 relaxation time of avidin-GdDTPA using T1 of albumin-GdDTPA as a reference [T1 relaxivity of Albumin(Gd)10 per protein concentration at 4.7 T is 128 mM−1 s−1 (15)].
**In Vitro MRI.** For in vitro MR studies, cells were prelabeled with biotinylated anti-HER-2/neu mAb (Ab-9; clone B10; NeoMarkers) at a 1:50 dilution in PBS with 0.5 mg/ml BSA for 30 min at room temperature. After extensive washing, cells were incubated with avidin-GdDTPA conjugate (1 mg/ml) for 5 min at room temperature. Control cells were incubated with a nonspecific antibody and/or avidin-GdDTPA. After washing, cells were fixed with 2% paraformaldehyde in PBS, and 10^7 cells were embedded in a layer of soft agarose gel (total volume of 50 μl) in a 5-mm NMR tube. T1 (repetition time, TR = 1 s; spin echo time, TE = 8 ms) and T2 (TR = 3 s, TE = 35 ms) weighted images of the sample were obtained with an Omega-400 spectrometer (Omega, GE/Bruker, Billerica, MA) equipped with microimaging system, using two-dimensional spin echo imaging and a 5-mm proton MRI probe (GE/Bruker).

**MRI Animal Studies.** NT-5 tumors and control EMT-6 murine mammary carcinomas were inoculated s.c. in female severe combined immunodeficient (SCID) mice (average body weight, 20 g). Solid tumors of approximately 200 mm^3 [measured by a caliper as (a x b x c), where a, b, and c are the principal axes of the tumor] were used for the studies. All treated animals received biotinylated anti-HER-2/neu mAb (Ab-9; Clone B10; NeoMarkers; 0.2 ml, i.v.), followed 12 h later by 0.2 ml of 60 mg/ml avidin-GdDTPA conjugate administered i.v. in saline. Control NT-5 tumor-bearing animals received 0.2 ml of 1 mg/ml BSA in saline i.v. followed by avidin-GdDTPA. For the imaging experiments, animals were anesthetized with an i.p. injection of a ketamine/acepromazine mixture (25 and 2.5 mg/kg, respectively, in saline). Anesthetized animals were positioned in a home-built 20-mm volume RF coil and multislice T1-weighted images (repetition time, TR = 300 ms; echo time, TE = 15 ms; flip angle of 90°; field of view of 32 mm; slice thickness ST = 2 mm) were acquired with a 4.7 T animal MR scanner (Omega; GE/Bruker). Sixteen slices were acquired within an experimental time of 15 min. Animal body temperature was maintained at 37°C by heat generated from a pad circulating with warm water. All animal experiments were performed in accordance with institutional guidelines.

**Western Blot Analysis.** Western blot analysis of HER-2/neu expression level in cultured NT-5 and EMT-6 cells as well as in experimental tumors and livers of the carrier mice was performed according to standard techniques. Briefly, equal amounts of proteins from cell lysates (10 μg) were separated on a 10% polyacrylamide gel. Protein bands were transferred to nitrocellulose membranes. Immunoblotting was performed with primary rabbit polyclonal anti-HER-2/neu antibodies [Neu(C-18); Santa Cruz Biotechnology, Santa Cruz, CA] and secondary antirabbit peroxidase-conjugated antibodies. The bands were detected with a chemiluminescence detection system (ECL kit; Amersham Biosciences, Piscataway, NJ), and densitometry analysis of the films was performed with NIH Image software.

**Immunohistochemistry.** Five-μm, paraffin-embedded, NT-5 tumor sections were immunohistochemically stained for HER-2/neu expression with Neu(C-18) antibodies and ABC staining system (Santa Cruz Biotechnology). Sections were counterstained with hematoxylin and digitized with a microscope equipped with a digital imaging system (Eclipse TS100; Nikon USA, Melville, NY).

**In Vivo Biodistribution Studies.** Delivery of mAbs and avidin to tumor, liver, and muscle was detected in fresh tissue slices using fluorescence-labeled antibodies and avidin. Animals received i.v. injection of FITC-labeled non-specific antirabbit mAb (1 mg/kg; NeoMarkers) or FITC-avidin conjugate (5 mg/kg; Molecular Probes). After sacrificing the animal, tissue slices (thickness of 0.5 mm) were prepared and mounted on microscope coverslips. Images were obtained with an Eclipse TS100 inverted fluorescence microscope using 1× and 2× objectives and digitized with a charge-coupled device camera (Coolpix; Nikon USA) attached to the microscope.

**Results**

**HER-2/neu Receptor Expression in Model Cell Systems and in Mouse Tumor Models.** Expression of the HER-2/neu receptor in NT-5 and EMT-6 cells growing in culture at 70% confluence was detected with fluorescence-activated cell-sorting analysis. Data shown in Fig. 1A demonstrate a significant shift of fluorescence intensity for NT-5 cells probed with a specific antibody. Expression of the rat isoform of the receptor that is recognized by the mAb was not detected in EMT-6 cells. Expression levels of HER-2/neu were quantified using fluorescence of standard calibrated microspheres. The expression level of the receptor was estimated at 7 x 10^5 receptors/cell. Low background, produced by nonspecific binding of avidin to the cell surface, demonstrates the high specificity of receptor recog-

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**Fig. 1.** (A) Fluorescence-activated cell-sorting analysis of HER-2/neu expression on the membrane of breast cancer cells. NT-5 cells have constitutively high levels of the receptor, which is detected as a significant shift in fluorescence in cells probed with the specific antibody (solid line). Expression levels of HER-2/neu in NT-5 and EMT-6 cells were determined using fluorescence-activated cell-sorting analysis. Data shown in Fig. 1A demonstrate a significant shift of fluorescence intensity for NT-5 cells probed with a specific antibody. Expression of the rat isoform of the receptor that is recognized by the mAb was not detected in EMT-6 cells. Expression levels of HER-2/neu were quantified using fluorescence of standard calibrated microspheres. The expression level of the receptor was estimated at 7 x 10^5 receptors/cell. Low background, produced by nonspecific binding of avidin to the cell surface, demonstrates the high specificity of receptor recog-

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**Cruz, CA** and secondary antirabbit peroxidase-conjugated antibodies. The bands were detected with a chemiluminescence detection system (ECL kit; Amersham Biosciences, Piscataway, NJ), and densitometry analysis of the films was performed with NIH Image software.
Results of Western blot analysis presented in Fig. 1B also demonstrate high levels of HER-2/neu protein in NT-5 cells. Expression of the protein was not detected in EMT-6 cells. High HER-2/neu expression levels were also detected in experimental NT-5 tumors. Endogenous receptors were not detected in liver samples (Fig. 1B). Immunohistochemical analysis of NT-5 and EMT-6 tumor slices also demonstrated an intensive staining of HER-2/neu receptors in NT-5 tumors only, as shown in Fig. 1C. As anticipated, in NT-5 tumors, the brown staining was localized to the cell membrane.

**MRI Detection of the HER-2/neu Receptor in Cell Samples.** A photograph of the cell sample prepared in a 5-mm NMR tube and a T1 weighted MR image obtained from the sample are shown in Fig. 2. NT-5 and EMT-6 cells were labeled with anti-HER-2/neu mAbs and probed with avidin-GdDTPA complexes as described in “Materials and Methods.” A schematic of the antibody-CA complex bound to the cell surface receptors is also shown in the figure. On average, we had 12.5 GdDTPA groups attached to an avidin molecule. Positive T1 contrast was detected in the layer of HER-2/neu-expressing NT-5 cells, whereas EMT-6 cells produced a weak background signal. Although the cell density in this in vitro sample was about 20% of the tissue cell density (10^6 cells/mm^3), the CA generated detectable positive contrast in the MR images of HER-2/neu-expressing NT-5 cells.

**In Vivo Detection of HER-2/neu-expressing Tumors.** Mice bearing NT-5 or EMT-6 tumors were imaged using a 4.7 T MR scanner. All animals received avidin-GdDTPA CA i.v., and tumors were prelabeled with specific biotinylated anti-HER-2/neu antibodies. In control studies, three NT-5 tumor-bearing animals were imaged without prelabeling of the receptors with antibodies. Typical results obtained for NT-5 and EMT-6 tumors at different time points, after administration of the CA (avidin-GdDTPA conjugate) and at 1, 8, 24, and 48 h after contrast. Arrows show enhanced signal from the tumor at the 8 and 24 h time points for the HER-2/neu-expressing NT-5 tumor. B. signal enhancement in the tumor relative to the muscle tissue at 24 h after contrast. The presented results are means for five NT-5 tumors treated with the primary biotinylated antibody, three control NT-5 tumors treated with BSA instead of antibody, and three EMT-6 tumors treated with anti-HER-2/neu biotinylated antibody. Error bars represent the SE. The signal enhancement was significant (P < 0.05) for prelabeled NT-5 tumors.
receptor in the tested cells, the estimated MR detection limit is in the range of 10^5 receptors/cell. This number is within the clinically relevant range of HER-2/neu receptor expression, which may exceed 4 million receptors/cell in breast tumors with HER-2/neu histological score of “3+” (16). The two main advantages of this in vivo molecular MRI approach are the relatively small molecular weight of both components of the avidin-GdDTPA-based targeted CA (150,000 for mAb and 70,000 for avidin-GdDTPA), which resulted in efficient delivery to the tumor interstitium compared with magnetic nanoparticles (7), and the easily identifiable positive signal enhancement due to T1 contrast. Another strategy may be to use Gd-labeled streptavidin, to reduce nonspecific binding compared with avidin. However, a disadvantage is the longer plasma half-life of streptavidin compared with avidin (17). The shorter half-life of avidin in plasma, estimated using fluorescein-labeled avidin to be less than 60 min (data not shown), results in rapid clearance with minimal background in normal tissue. Whereas the use of avidin-biotin is well documented in nuclear imaging, this is the first report of the use of such a system for MRI of cell receptors. To enable potential application of this technique in clinic, several important issues have to be addressed. Whereas both components of the targeted MR CA have successfully been used clinically to deliver immunotherapy and radioimmunotherapy with mAbs and a radiolabeled avidin-biotin system (18–21), the concentrations used were much lower than those required for MR detection. The therapeutic humanized mAb Herceptin can be given at high doses [250-mg single dose (18)] without significant adverse effects and without invoking host immune response. Avidin-biotin or streptavidin-biotin systems, on the other hand, can induce a profound immune response (19–21). This can limit multiple applications of the targeted CA for repetitive imaging and may also impair the efficacy of targeted therapy using the avidin-biotin system (19). Another important problem is the stability of Gd(III) chelating complexes in vivo (22), especially because of the accumulation of high molecular weight CAs in the liver and kidneys (23). Free Gd(III) ions are cytotoxic (24, 25), and more stable Gd chelates such as those described by Kobayashi et al. (23) may be preferable because of increased stability in vivo. The potential translation of the targeted MR CAs to the clinic depends upon successfully resolving these issues. Once solved, the technique may be of significant importance to screen cancers for receptor status and to follow receptor-blocking therapy by detecting diminished accumulation of the CA in tumors responding to therapy.

Determination of Efficiency of Delivery of the CA in Vivo.
Fluorescent images of tumor, liver, and muscle slices of an experimental animal prepared 2 h after i.v. injection of FITC-labeled avidin are shown in Fig. 4. Intensive fluorescence of the tumor sections demonstrates an efficient and uniform delivery of the protein to the tumor interstitium. Fluorescence detected in the tumor section is comparable or higher than the fluorescence detected in the liver. No uptake of the high molecular weight fluorescent marker was detected in the muscle.

Discussion
We have developed a novel method for noninvasive imaging of HER-2/neu receptor-expressing cells using a two-component, targeted, Gd-based MR CA. The relatively low molecular size of the CA provides efficient delivery to the tumor interstitium. MRI detection of HER-2/neu-expressing cells was demonstrated in isolated cells and in preclinical tumor models. Effective delivery of both components of the CA to the tumor interstitium was confirmed with fluorescent analogues of the agents. Based on the expression efficiency of the contrast, are shown in Fig. 3A. These images correspond to a slice obtained through the center of the tumor. Because animals had to be removed from the magnet between consecutive scans, there was a small variation in the positions of the imaging slices visible in the figure. The contrast is retained in NT-5 tumors at 8–24 h time points as shown in the images. Contrast enhancement decreased to baseline levels in the EMT-6 tumor, after the enhancement observed at early time points. NT-5 tumors not pretreated with biotinylated antibody also did not retain contrast (data not shown). By 48 h, contrast was cleared from all of the experimental tumors studied. MR signal intensity changes at 24 h after contrast time point, averaged over the tumor, for a group of animals are shown in Fig. 3B. For analysis of MR signal intensities, the region of interest was chosen to include the whole tumor within all imaging slices through the tumor position. Statistically significant signal enhancement was observed at the 24 h time point for the NT-5 tumors (P < 0.05, unpaired t test).

References


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