Targeted Signal-Amplifying Enzymes Enhance MRI of EGFR Expression in an Orthotopic Model of Human Glioma

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Abstract

Epidermal growth factor receptor (EGFR) imaging in brain tumors is essential to visualize overexpression of EGFRvIII variants as a signature of highly aggressive gliomas and to identify patients that would benefit from anti-EGFR therapy. Seeking imaging improvements, we tested a novel pretargeting approach that relies on initial administration of enzyme-linked anti-EGFR monoclonal antibodies (mAb; EMD72000) followed by administration of a low-molecular-weight paramagnetic molecule (diTyr-GdDTPA) retained at the site of EGFR mAb accumulation. We hypothesized that diTyr-GdDTPA would become enzyme activated and retained on cells due to binding to tissue proteins. In support of this hypothesis, mAb-enzyme conjugates reacted with both membrane-isolated wild-type (wt) EGFR and EGFRvIII, but they bound primarily to EGFRvIII-expressing cells and not to EGFRwt-expressing cells. In vivo analysis of magnetic resonance (MR) tumor signal revealed differences in MR signal decay following diTyr-GdDTPA substrate administration. These differences were significant in that they suggested differences in substrate elimination from the tissue which relied on the specificity of the initial mAb binding: a biexponential signal decay was observed in tumors only upon preinjection with EGFR-targeted conjugates. Endpoint MRI in this setting revealed detailed images of tumors which correlated with immunohistochemical detection of EGFR expression. Together, our findings suggest an improved method to identify EGFRvIII-expressing gliomas in vivo that are best suited for treatment with therapeutic EGFR antibodies.

Introduction

Epidermal growth factor receptor (EGFR) is a member of ErbB receptor family, which is overexpressed predominantly in non–small cell lung cancer, colorectal and squamous carcinomas as well as in glioma cells (1–3). EGFR plays an important role in cancer pathogenesis by readily undergoing ligand-dependent dimerization followed by autophosphorylation of the EGFR dimer resulting in downstream proliferative and antiapoptotic signaling in cancer cells (4). Wild-type EGFRwt overexpression closely correlates with receptor gene amplification and has been previously established as a significant and independent unfavorable predictor of overall survival of glioblastoma patients (5). The truncated EGFRvIII variant of the receptor is constitutively activated and is a hallmark of aggressive gliomas (6, 7). Since EGFR level is low or even undetectable in normal brain cells, this receptor is an appealing molecular target not only for molecular therapies but also as a potential marker molecule for visualization and characterization of gliomas during their response to therapy. Several recombinant monoclonal antibodies (mAb) have been developed during the past decade for achieving specific targeting of the N-terminal extracellular domain III of EGFR with the resultant inhibition of EGF binding and/or receptor dimerization (8–10). Anti-EGFR mAb (11, 12) and EGF ligand (13, 14) have recently been suggested as agents for targeted imaging of EGFR expression using near-IR fluorescence. Unlike in vivo imaging of fluorescence, MRI does not suffer from the drawback of limited depth penetration and scattering of light. However, the sensitivity of MRI to the local molar concentration of contrast agent (CA) is orders of magnitude lower than fluorescence or radionuclide imaging, which limits applicability of MRI for receptor imaging (15–17). Proton MR receptor imaging relies on the ability of CAs associated with the receptor site to shorten relaxation times of nearby water molecules. The number of CA molecules (e.g., the number of chelated paramagnetic cations that can be used for direct labeling of mAbs while still maintaining the appropriate binding affinity of mAbs to the target site) is usually not sufficient for generating adequate MR contrast. Other studies circumvented the problem of insufficient sensitivity by coating iron oxides with mAbs (18–20) or by using gadolinium (Gd)-based targeted micelles (21) and dendrimers (22). MRI sensitivity is thus increased due to either clustering of many Gd cations or, alternatively, due to high
superparamagnetism of iron oxide. However, linking of nanosized CAs to antibodies can result in a decrease in tissue penetration after extravasation in tumors and in an increase of nonspecific MR signal (25, 26).

Several studies have looked into alternative uses of mAbs for imaging tumor-associated receptors using contrast-enhanced MRI (27–29). For example, a pretargeting technique has been suggested for enhancing mammmary adenocarcinomas by injecting Gd-labeled avidin (25) or dendrimers (29) as a way of achieving specific association with HER-2/new-bound biotinylated mAb (trastuzumab; ref. 28). We have previously developed a novel pretargeting approach on the basis of an enzyme-mediated amplification of MR signal as a result of accumulation of small molecular weight Gd-labeled substrates at the target site (30). The advantage of this imaging strategy is that small CA molecules accumulate in brain tumors at a faster rate and with less heterogeneity than macromolecular agents (26). The resultant target-to-background contrast ratio can potentially be achieved earlier than in the case of nanoparticle or dendrimer pretargeting due to much faster elimination of the nonreacted CA substrate from the circulation as compared to nonbound Gd-labeled macromolecules or nanoparticles. The other potential advantage is that in achieving higher MR signal due to the Gd relaxation increase that results from the enzyme-activated substrate binding to proteins (31, 32). The increase in relaxivity depends on magnetic field strength and strongly contributes to the MR signal increase at 1.0 to 2.0 Tesla.

The main goals of the current work were (i) to test in vitro the tumor-pretargeted enzyme-mediated amplification system using cells expressing either EGFRwt, or both EGFRwt and EGFRvIII; (ii) to compare in vivo kinetics of MR signal decay after the administration of Gd-labeled peroxidase substrate (diTyr-GdDTPA) with or without pretargeting of the EGF receptor with mAb conjugated to a signal amplification pair of enzymes.

Materials and Methods

Substrate synthesis and bioconjugation Horseradish peroxidase (HRP)-reducing substrate di(tyramido)-DTPA(Gd) and conjugates (Fig. 1) were synthesized as described in the work of Querol and colleagues (32); mAb conjugates were synthesized using bisaromatic hydrazine method, purified on Superdex200 HPLC columns (GE Life Sciences), and analyzed as described in the work of Bogdanov and colleagues (30).

Testing of conjugates in cell culture Gli36ΔEGFR (33) and wild-type Gli36wt cells (34) were propagated on 10% FCS (fetal calf serum) 90% RPMI 1640 in the presence of penicillin/streptomycin and 0.5 μg/mL puromycin (Gli36ΔEGFR). mAb conjugates were cross-titered on 96-well plated live cells using sequential dilutions in the range of 1,000 to 7.5 ng total conjugate (i.e., a mixture of mAb-HRP and mAb-GOx) per well and peroxidase activity associated with the cells was determined as described in the work of Bogdanov and colleagues (30).

Flow cytometry Flow cytometry of Gli36ΔEGFR and Gli36wt cells was performed by using 1 μg/mL Alexa Fluor 488–labeled EMD72000 or cetuximab (humanized mAb C225; ref. 35).

Internalization experiments Cell internalization was studied by using mAb-HRP:mAb-GOX conjugate mixture at 1:2 (w/w ratio). Adherent cells in 6-well plates (4 million cells/well) were incubated with conjugate mixtures either at 4°C or at 37°C. The surface-bound conjugates were eluted with 0.5 mL cold 0.2 mol/L glucose, pH 2.5, for 15 minutes. The eluate was immediately neutralized to pH 8.0. To extract internalized conjugates, 0.5 mL of 1.0% Igepal CA-630 in the presence of protease inhibitors was added to each well and plates incubated for 15 minutes. The amount of bound and internalized conjugates was determined by measuring the initial rates of HRP/GOX-coupled ABTS (2.2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) oxidation by adding of 5 mmol/L ABTS, 5 mmol/L glucose (final concentrations) in sodium citrate, pH 5.5, to the sample aliquots and measuring absorbance at 405 nm over time. The serially diluted conjugate mixture at a constant mAb-HRP:mAb-GOX ratio and known concentrations were used for calibration.

Immunoblotting Membrane proteins were extracted using CNM compartmental protein extraction procedure (BioChain Institute Inc.) following manufacturer’s recommendations with subsequent immunoblotting of protein-normalized lysates on PVDF (polyvinylidene difluoride) membranes using either anti-EGFR mAb (ab3103; AbCam) as primary antibody or EMD72000-HRP conjugates with subsequent digital imaging.

Animal model All procedures were performed as approved by the UMMS Institute Animal Care and Use Committee. An orthotopic human glioma xenograft model was obtained by stereotaxically implanting 1 × 10⁶ Gli36ΔEGFR or Gli36wt cells in 3 μL of 10% Matrigel in serum-free RPMI under aseptic conditions at 37°C (2.5 mm posterior to bregma, 2 mm lateral to midline, and depth of 3.5 mm) in the brain of athymic rats (Harlan, 150–180 g, n = 16) 14 days before the first imaging session. Throughout the imaging procedure, the animals were maintained at 37°C and anesthetized using 1.5% isoflurane in a 30% oxygen/70% nitrogen mixture. A 45-mm diameter, 30-mm long birdcage RF (radio frequency) coil was used. A 26-gauge catheter capped with a needle port was placed in the tail vein for CA administration.

MRI protocol, pulse sequences, and measurements MRI measurements were performed using a Philips Achieva 3.0 T/60-cm bore MRI scanner equipped with 80 mT/m actively shielded gradients. To monitor temporal evolution of signal enhancement following CA delivery, multislice, T₁-WT MR images (TR/TE = 700/8.2 ms) were acquired at various time points following CA infusion. Other imaging parameters were: slice thickness = 1.5 mm; slice separation = 0.15 mm;
field-of-view = 25.6 mm × 25.6 mm; data acquisition matrix = 256 × 128 (zero padded to 256 × 256), 4 NEX. Two weeks after tumor cell implantation, each animal was imaged on 2 occasions: on day 1 (2 weeks after tumor cell implantation), a precontrast image was acquired followed by i.v. injection of 0.1 mmol/kg diTyr-GdDTPA. Twenty $T_1$-WT images were then acquired continuously over a 2-hours period. On day 2 (the day after day 1), the animals were first preinjected with 150 mg/g mAb-HRP/mAb-GOX mixture at 2:1 (w/w ratio, 0.3 mL) in the tail vein. A separate group of Gli36D EGFR tumor animals were preinjected with nonspecific antibody conjugates. Five hours later, a precontrast image was acquired followed by i.v. injection of 0.1 mmol/kg diTyr-GdDTPA. Thirty $T_1$-WT images were then acquired continuously over a 3-hours period. Precontrast $T_2$-WT images were acquired on both days to corroborate the presence of tumor observed in the $T_1$-WT slices. The temporal evolution of the signal decay in the tumor following infusion of diTyr-GdDTPA was evaluated separately for each of the animal groups. Depending on the size of the tumor, one to four slices were selected. Image analysis was performed using region of interest (ROI) for tumor interface and core regions with an aid of ImageJ software (36).

Histology

Following the MRI studies, animals were euthanized and their brains were removed. Under a dissecting microscope, histologic slices were obtained ±2 mm anterior/posterior of the needle track and then embedded in OCT medium. Frozen sections (6 μm) were fixed in acetone, treated by TBS/EDTA, pH 8, at 65°C for inhibiting endogenous phosphatase activity and blocked using 2% serum/TBS. The sections were incubated with anti-EGFR mouse mAb (Abcam) or anti-HRP mAb (Abcam) followed by anti-mouse alkaline phosphatase (AP)-linked antibodies and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Roche). Staining for residual peroxidase activity was achieved by using a DAB kit (Vector Labs). Immunofluorescent staining was performed on frozen sections blocked with 2% serum, 0.05% Tween-20, PBS, pH 7.4. Alexa Fluor 488-labeled EMD72000, Cy3-labeled mouse anti-rat CD31 mAb (clone TLD-3A12; Abcam) were used for visualizing EGFR and endothelial CD31 expression, respectively. For detecting HRP in the tissue sections, digoxigenin-labeled mouse anti-HRP mAb (clone 2H11; Abcam) was used. Digoxigenin-labeled antibody binding was visualized using custom Cy5.5-labeled anti-digoxigenin F(ab')2 (Roche; ref. 37). Detailed methods are included in Supplementary Materials.

Results

Synthesis and testing of targeted MR signal amplification system in vitro

The synthesis of peroxidase (HRP) and glucose oxidase (GOX) conjugates of humanized chimera antibody (EMD72000; Merck) that catalyze oligomerization of substrate di(tyramido)-DTPA(Gd) (Fig. 1) was performed using conditions optimized to facilitate enzyme conjugation with minimal antibody binding affinity loss (30). Gel electrophoresis of purified conjugates showed the formation of mAb-GOX.
(290 and 460 kD bands) and mAb-HRP (220 and 400 kD bands; Fig. 2A). Since GOX is a 2-subunit enzyme, the band corresponding to the 69-kD deglycosylated GOX subunit was present in the SDS-treated mAb-GOX conjugates (Fig. 2A, lane 4). The conjugation reaction conditions prevented the formation of cross-linked products as evidenced by the lack of binding specificity to EGFR-positive cells. Immunoblotting experiments showed that optimized conjugates reacted with the same set of receptor variants (i.e., EGFRwt in Gli36wt and EGFRwt/EGFRvIII in Gli36wt cells) as did the control anti-EGFR antibody C225 or by using HRP-EMD72000 conjugate. EGFR variants are identified on the right. C, titration of the mixture of anti-EGFR mAb-HRP and mAb-GOX on Gli36ΔEGFR cells at the optimized complementing ratio (1:2, w/w). D, binding and internalization of conjugate mixture at the optimized ratio (1:2, w/w) in Gli36ΔEGFR (Δ) and Gli36wt (WT) cells; 1, 3, 5, and 7: cell-surface-bound fraction of conjugates; 2, 4, 6, 8: internalized fraction of conjugates.

Figure 2. A, SDS-PAGE (4%–15% gradient) of anti-EGFR mAb (EMD72000) conjugation products or with deglycosylated enzymes: HRP (37 kD, lanes 1 and 2) and with GOX (69 kD subunit, lanes 3 and 4); lanes 1 and 3: before; and 2 and 4: after the purification of conjugates. B, immunoblotting of membrane proteins isolated from Gli36ΔEGFR (Δ) and Gli36wt (WT) cells using mouse monoclonal anti-EGFR antibody C225 or by using HRP-EMD72000 conjugate. EGFR variants are identified on the right. C, titration of the mixture of anti-EGFR mAb-HRP and mAb-GOX on Gli36ΔEGFR cells at the optimized complementing ratio (1:2, w/w). D, binding and internalization of conjugate mixture at the optimized ratio (1:2, w/w) in Gli36ΔEGFR (Δ) and Gli36wt (WT) cells; 1, 3, 5, and 7: cell-surface-bound fraction of conjugates; 2, 4, 6, 8: internalized fraction of conjugates.
that was used for animal experiments. Mixtures of conjugates and paramagnetic substrate diTyr-GdDTPA in the presence of α-glucose always resulted in shorter $T_1$ relaxation times (i.e., higher average longitudinal relaxivity $r_1$, see Supplementary Table S1). The measured difference in molar relaxivity between the substrate alone and reaction mixtures containing HRP and GOX conjugates was clearly greater at lower magnetic field (0.47 T vs. 3 T). The reaction resulted in 2.7 times higher $r_1$ if measured at 0.47 T as opposed to 5% increase of $r_1$ at 3 T. The simple addition of proteins in the solution did not result in large relaxivity gains. However, in the presence of both plasma proteins and mAb conjugates, the relaxivity increase was measurable and higher at 3 T and showed a 20% $r_1$ increase.

**In vivo imaging experiments and corroboration**

The paramagnetic substrate-mediated enhancement of human glioma xenografts (Gli36ΔEGFR and Gli36wt) was studied using MRI *in vivo*. To account for tumor heterogeneity, the experiments were performed on consecutive days in each animal. On the first day, only the CA substrate was administered (day 1). On the next day—after CA had been completely eliminated—a second experiment was conducted with pre-injection of mAb conjugates 5 hours before injecting the CA (day 2). Figure 3A shows sequential $T_1$-WT rat brain MR images depicting Gli36ΔEGFR xenograft enhancement as a function of time post-i.v. injection of diTyr-GdDTPA. The top row of images shows the temporal washout of diTyr-GdDTPA with no anti-EGFR mAb conjugate pretreatment (day 1). The bottom row of images shows the temporal washout of diTyr-GdDTPA at 5 hours following pretreatment with anti-EGFR mAb conjugates (day 2) in the same slice for the same animal. For both days, the $T_1$-WT images showed strong initial enhancement of the tumor within minutes after i.v. diTyr-GdDTPA injection. However, following i.v. injection of diTyr-GdDTPA with EGFR-targeted conjugate pretreatment (Fig. 3A, day 2: 8 minutes), the initial enhancement was significantly higher than without EGFR-targeted conjugate pretreatment (Fig. 3A, day 1: 9 minutes), especially in the tumor interface region. Moreover, images obtained after the EGFR-targeted conjugate preinjection (Fig. 3A, bottom row) showed more detailed tumor structure with areas of focal enhancement in both the tumor/normal brain interface and core at all time points postinjection of the paramagnetic MR CA. With or without conjugate pretreatment, the tumor interface consistently showed higher $T_1$-WT signal enhancement compared with the core.

Animals preinjected with anti-EGFR mAb conjugates displayed a delayed retention of MR CA compared with animals not receiving EGFR-targeted conjugate pretreatment. Without a preinjection of anti-EGFR conjugates (day 1), most of the diTyr-GdDTPA had washed out of both the tumor/normal brain interface and core regions by 117 minutes after i.v. injection (Fig. 3A, top row). However, after allowing the EGFR-targeted conjugates to accumulate in the tumors (day 2), significant $T_1$-WT hyperintensity persisted in the tumor interface at approximately the same time point (Fig. 3A: day 2 - 116 minutes) post-diTyr-GdDTPA injection.

![Figure 3. A, 3 T MRI of Gli36ΔEGFR human glioma xenografts without and with preinjection of EGFR-targeted conjugates. $T_1$-WT sequential rat brain images depicting enhancement as a function of time postinjection of diTyr-GdDTPA. Top row, temporal washout of diTyr-GdDTPA with no conjugate preinjection (day 1); bottom row, washout of diTyr-GdDTPA following pretreatment with anti-EGFR conjugates (day 2) in the same slice for the same animal. Time intervals (in minutes) after the injection of diTyr-GdDTPA are shown below. B, Gli36ΔEGFR xenografts without and with preinjection of EpCAM-targeted conjugates. The images correspond to the same pattern as shown in A. C, MRI and comparative histology. The images were obtained pre-, immediately post-, and 1 hour post-diTyr-GdDTPA administration. D, left, detection of EGFR overexpression using anti-EGFR antibody digoxigenin/anti-digoxigenin AP system in the tumor shown in C. Right, detection of HRP activity in the same tumor on the parallel section using diaminobenzidine staining. Arrowheads point to tumor location; arrows show presence of tumor expansion as microdeposits in normal brain tissue stained for EGFR expression. Bars in C, D = 1 mm.](cancerres.aacrjournals.org)
signal intensities in the Gli36 tumors. Figure 3A and B. The relative percent were derived from the tumor images of the same representative regions as a function of time post-CA injection. These plots EGFR-targeted imaging. Figure 5 shows the normalized as a function of time was performed to assess the efficacy of signal intensity decay (SID) curves (Fig. 5).

Furthermore, $T_1$-WT enhancement in the tumor interface remained visible for 170 minutes after CA administration (Fig. 3A: day 2 - 170 minutes). In contrast to EGFR-targeted conjugates preinjection, no retention of MR signal was observed when Gli36ΔEGFR-bearing animals were preinjected with anti-EpCAM–targeted conjugates which do not bind to Gli36ΔEGFR cells (Fig. 3B, compare top and bottom rows).

The corroborative histology performed after the final MRI session showed areas of EGFR-positive staining and revealed 2 tumor masses (2.7–3 mm in diameter) with multiple microdeposits around the tumor/normal brain interface (Fig. 3D, arrows). These same features were also easily identifiable on MR images after the injection of anti-EGFR conjugate followed by the CA (Fig. 3C). These areas of tumor/brain were also positively stained for HRP activity using diaminobenzidine (Fig. 3D). By performing immunofluorescent visualization of EGFR and CD31 expression, we observed the presence of multiple blood vessels feeding the expanding tumor (Fig. 4A). Visualization of HRP accumulation by using anti-HRP antibodies (Fig. 4B) verified the results obtained using peroxidase enzymatic activity detection in brain sections (Fig. 3D).

Detailed kinetic analysis of in vivo MR signal enhancement as a function of time was performed to assess the efficacy of EGFR-targeted imaging. Figure 5 shows the normalized $T_1$-WT signal intensities in the Gli36ΔEGFR tumor interface and core regions as a function of time post-CA injection. These plots were derived from the tumor images of the same representative animals shown in Figure 3A and B. The relative percent change in $T_1$-WT signal intensity of the interface and core regions was significantly higher with EGFR-targeted conjugate administration (day 2, Fig. 5B), as compared with without pretreatment (day 1, Fig. 5A), at the initial time points. With EpCAM-targeted conjugate administration (day 2), there was no significant change in initial $T_1$-WT signal intensities of the interface and core regions between days 1 and 2; both days showed similar washout behavior of the interface and core signal intensity decay (SID) curves (Fig. 5).

Interestingly, the SID curves for tumor interface and core regions in animals pretreated with EGFR-targeted conjugates (day 2; Fig. 5B) depict 2 separate decay components (long $T_1$ and short $T_2$), whereas the washout curves for the corresponding regions without EGFR-targeted conjugate injection (day 1; Fig. 5A) show only a single decay component ($T_0$). On the basis of the $\chi^2$ analysis using an $F$ test (Supplementary Material), a biexponential function was found to best model the SID curves for the tumor interface and core regions after injection of EGFR-targeted conjugates (day 2). Without EGFR-targeted conjugate preinjection (day 1), a monoexponential function was found to best model both the regions. For treatment with and without EpCAM-targeted conjugates and Gli36wt tumors (treated with and without EGFR-targeted conjugates), a monoexponential elimination function was the best model for all SID curves [see Table 1 for all decay time constant (DTC) values].

Discussion

Imaging of receptor expression in cancer with high spatial resolution usually requires MRI assisted with imaging probes (38, 39). Due to the inherent nonspecific uptake of nanosized probes in nontarget cells and slow elimination of nonbound CA from blood, the specific imaging signal is frequently obscured. With a goal of imaging EGF receptor expression in gliomas, we investigated a novel strategy on the basis of specific local retention of paramagnetic products of di(tyramido)-DTPA(Gd) (diTyr-GdDTPA; Fig. 1), that is, a substrate of peroxidase (30, 40). We applied the strategy in models of orthotopic human gliomas that either expressed the wild-type EGFR or both the wild-type receptor and EGFRvIII (33). The anti-EGFR mAb (EMD72000) was covalently linked with HRP and GOX, that is, to the enzymes that function as a self-complementing enzymatic signal amplification system (30). In cell culture experiments, these conjugates showed a remarkable preference for truncated EGF/RvIII mutant form expressed in Gli36ΔEGFR cells but not in Gli36wt counterpart. This effect can be explained by the steric constraints in accessibility of
epitope (Ser460-Gly461) on EGFR domain III recognized by EMD72000 antibody (41). Since even after prolonged incubation in cell culture 25% of mAb conjugates were resistant to internalization, we anticipated that our strategy enables reliable MR imaging of EGFRvIII with high specificity due to the retention of CA at the sites of mAb conjugate colocalization.

To test this assumption, we designed experiments that directly compared in vivo elimination kinetics of diTyr-GdDTPA in the same tumor-bearing animals. These experiments were performed initially in the absence of mAb conjugates and then after the preinjection of a mixture of conjugates at the optimized HRP:GOX ratio. As a result, we were able to compare kinetics of the measured MR signal decrease due to the washout of diTyr-GdDTPA and its reactive products from the total tumor volume, as well as from volumes corresponding to highly vascularized tumor "interface" (Fig. 4 and Supplementary Fig. 2S) and the remaining "core" volumes. From the quantitative kinetic analysis of the MR signal intensities, we concluded that the initial MR signal intensity enhancement for both interface and core regions was significantly higher for Gli36ΔEGFR tumors that were pretreated with EGFR-targeted mAb conjugates (day 2) as compared with the same tumors before the injection of conjugates (day 1) or Gli36wt tumors. When nontargeted mAb conjugates (control) were preinjected into Gli36ΔEGFR tumors, no significant signal change was observed compared with day 1. Binding of paramagnetic products of enzymatic reaction are more efficient CAs and their binding to the EGFRvIII-positive tumors resulted in a long-term retention/enhancement (Fig. 3A), whereas oligomerization or binding to proteins in the extracellular compartment resulted in a short-term tumor enhancement. The effects caused by paramagnetic products binding were previously observed and characterized in several similar enzyme-mediated reactions (32, 42).

The retention of MR signal enhancement in tumors that were pretreated with specific targeted conjugates relative to those that were not, resulted in a slower CA elimination consistent with the binding of the CA to the target cells in the presence of targeted enzyme amplification pair. As expected, signal enhancement of the tumor interface was initially higher than that of the rest of the tumor volume ("core"), independent of whether the animals were preinjected with conjugates or not. This was caused by high vascular density of the tumor interface (Fig. 3), which corresponds to an area of enhanced transvascular permeability and higher functional blood volume (43). The higher local permeability translates into higher concentrations of targeted conjugates as well as the substrate compared with less vascularized core. The results of direct staining for HRP activity or detection of HRP in tumors by using immunofluorescence suggest this difference (Figs. 3 and 4).

The inevitable variations in tumor size and tumor heterogeneity dictated the need in a parameter that would allow comparing MRI results obtained in different animals and tumors. We determined that kinetic analysis of temporal decay of the normalized MR signal intensities can be used for this purpose (Table 1). The calculated DTC values can be correlated to tumor perfusion, vascular permeability, and the volume of the extravascular extracellular space (EES; see Supplementary Material).
Table 1. DTC for diTyr-GdDTPA or its products in the tumor interface and core regions with (day 2) or without conjugate preinjection (day 1)

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<th>Tumor core DTC, min</th>
<th>Tumor interface DTC, min</th>
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<th>Tumor core DTC, min</th>
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<tr>
<td><strong>Gli36(\Delta)EGFR tumors, EMD72000</strong> (anti-EGFR) conjugates (experiment)</td>
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<td>No preinjection of conjugates (day 1)</td>
<td>(t_0), min(^a)</td>
<td>(t_0), min(^b)</td>
<td>(t_0), min(^b)</td>
<td>(t_0), min(^h)</td>
<td>(t_0), min(^b)</td>
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<tr>
<td>With preinjection of conjugates (day 2)</td>
<td>(t_1), min(^c)</td>
<td>36 ± 10(^a)</td>
<td>54 ± 20(^a)</td>
<td>54 ± 28</td>
<td>92 ± 46</td>
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<td>P values</td>
<td>(P &lt; 0.005)</td>
<td>(P &lt; 0.0002)</td>
<td>(P &lt; 0.004)</td>
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| **Gli36\(\Delta\)EGFR tumors, anti-EpCAM conjugates (control) |                     |                          |                     |                          |                     |
| Tumor core DTC, min | \(t_0\), min\(^c\) | \(t_0\), min\(^d\) | \(t_0\), min\(^a\) | \(t_0\), min\(^a\) | \(t_0\), min\(^a\) |
| Tumor interface DTC, min | \(t_1\), min\(^d\) | \(t_1\), min\(^e\) | \(t_1\), min\(^f\) | \(t_1\), min\(^f\) | \(t_1\), min\(^f\) |
| P values | \(P < 0.01\) | \(P < 0.03\) | \(P < 0.004\) | \(P < 0.01\) | \(P < 0.01\) |

| **Gli36-WT tumors, EMD72000 conjugates (control) |                     |                          |                     |                          |                     |
| Tumor core DTC, min | \(t_0\), min\(^c\) | \(t_0\), min\(^a\) | \(t_0\), min\(^a\) | \(t_0\), min\(^a\) | \(t_0\), min\(^a\) |
| Tumor interface DTC, min | \(t_1\), min\(^d\) | \(t_1\), min\(^f\) | \(t_1\), min\(^f\) | \(t_1\), min\(^f\) | \(t_1\), min\(^f\) |
| P values | \(P < 0.01\) | \(P < 0.03\) | \(P < 0.004\) | \(P < 0.01\) | \(P < 0.01\) |

\(^a\)ROI placement is explained in Supplementary Figure 2S.

\(^b\)Monoexponential fitting model was used.

\(^c\)Biexponential fitting of SID curves. A monoeponential function was used for the tumor regions in animals with

\(^d\)Gli36\(\Delta\)EGFR group preinjected with EMD72000 conjugates (day 2) and

\(^e\)Gli36-WT group preinjected with anti-EGFR (EMD72000) conjugates (day 2). P values correspond to comparisons within

\(^f\)Gli36\(\Delta\)EGFR group (preinjected with EMD72000 conjugates) between:

\(^g\)\(t_0\) and \(t_1\) in the tumor core;

\(^h\)\(t_0\) and \(t_2\) in the tumor core;

\(^i\)\(t_0\) and \(t_1\) in the tumor interface;

\(^j\)\(t_0\) and \(t_2\) in the tumor interface;

\(^k\)\(t_0\) and \(t_1\) in the tumor core;

\(^l\)\(t_0\) and \(t_2\) in the tumor core;

\(^m\)\(P < 0.01\) for \(t_0\) values in the tumor core and interface;

\(^n\)\(P < 0.03\) for \(t_0\) values in the tumor core and interface;

\(^o\)\(P = 0.0005\) for \(t_0\) values in the tumor core and interface;

\(^p\)No significant differences in DTCs were observed in the tumor regions between days 1 and 2 in animals with Gli36-WT tumors (preinjected with EMD72000 conjugates);

By performing SID analysis, we found that in the absence of conjugate preinjection, the washout of diTyr-GdDTPA was monoeponential throughout the tumor volume generating a single DTC (\(t_0\)) in both tumor models (Fig. 5A, Table 1). In each case, \(t_0\) was attributed to the washout of the free CA since the substrate does not bind to plasma proteins. This assignment is justified because diTyr-GdDTPA is not expected to have any affinity for tumor cells in the absence of receptor-targeted conjugates. The control EpCAM-targeted conjugates in Gli36\(\Delta\)EGFR tumors (day 2) as well as EGFR-targeted conjugates in Gli36WT cells (day 2) similarly showed monoeponential decay (Table 1) since control anti-EpCAM conjugates did not bind to glioma cells of our study and Gli36WT cells were binding very low amounts of EMD72000 conjugates.

Following the preinjection of Gli36\(\Delta\)EGFR-bearing animals with EGFR-targeted conjugates on day 2, the diTyr-GdDTPA elimination changed from monoeponential to biexponential for both the tumor interface and core regions (Fig. 5, Table 1). The elimination of free (and/or oligomerized) substrate was responsible for short DTC (\(t_2\)), whereas the long DTC (\(t_1\)) component was attributed to paramagnetic reaction products retained by tumor cells. Importantly, the comparison of \(t_1\) and \(t_2\) DTC values suggest that in addition to dynamic acquisition delayed MR imaging can also be useful when imaging receptors: the retention of tumor-bound paramagnetic product resulting from a specific enzymatic reaction can be detectable in the tumor interface for several hours postadministration of the substrate while the free substrate is eliminated within an hour.

In conclusion, we performed quantitative analysis of MR dynamic signal enhancement in human glioma xenografted animals that were (i) imaged first by using “nonspecific” paramagnetic CA and (ii) imaged with the same CA after preinjecting of anti-EGFR antibody conjugates. The enzyme linked to EGFR-targeted antibodies converted the CA into reactive products thereby profoundly changing the tissue elimination kinetics. Therefore, in addition to imaging of the MR signal enhancement associated with tumor tissues in vivo, the amplification strategy results in a kinetic signature. The analysis of images acquired dynamically or by using endpoint imaging can establish the presence of cell surface marker and quickly delineate the areas where this marker molecule can be targeted for therapeutic purposes.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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