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Molecular Magnetic Resonance Imaging With Targeted Contrast Agents

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Abstract Magnetic resonance imaging (MRI) produces high-resolution three-dimensional maps delineating morphological features of the specimen. Differential contrast in soft tissues depends on endogenous differences in water content, relaxation times, and/or diffusion characteristics of the tissue of interest. The specificity of MRI can be further increased by exogenous contrast agents (CA) such as gadolinium chelates, which have been successfully used for imaging of hemodynamic parameters including blood perfusion and vascular permeability. Development of targeted MR CA directed to specific molecular entities could dramatically expand the range of MR applications by combining the noninvasiveness and high spatial resolution of MRI with specific localization of molecular targets. However, due to the intrinsically low sensitivity of MRI (in comparison with nuclear imaging), high local concentrations of the CA at the target site are required to generate detectable MR contrast. To meet these requirements, the MR targeted CA should recognize targeted cells with high affinity and specificity. They should also be characterized by high relaxivity, which for a wide variety of CA depends on the number of contrast-generating groups per single molecule of the agent. We will review different designs and applications of targeted MR CA and will discuss feasibility of these approaches for in vivo MRI. J. Cell. Biochem. 90: 518–524, 2003.

Key words: MRI; targeted contrast agents; receptor imaging; specific binding

The most widely used class of magnetic resonance imaging (MRI) relaxation agents is based on gadolinium (Gd) chelates that are strictly extracellular low molecular weight compounds with high T1 relaxivity such as Magnevist®. Tissue specific contrast agents (CA) that target liver, lymph nodes, and atherosclerotic plaques or accumulate in the cells of the reticuloendothelial system (RES) were also developed recently [Weinmann et al., 2003]. Several groups have reported a selective accumulation of metalloporphyrins in tumors [Vicente, 2001]. Mechanisms describing the selective accumulation of these tissue specific CA may include phagocytosis or endocytosis of CA by RES, liver, or lymphatic cells and by monocytes and macrophages in atherorosclerotic plaques. In the case of tumors the selective accumulation of porphyrin-based CA may be due to the high affinity of these compounds to necrotic tissue [Ni et al., 1996]. Intravascular or blood-pool CA can be considered as a separate group of tissue specific CA and they are either high molecular weight compounds with long blood circulating times or serum albumin-binding Gd chelates [Schmiedl et al., 1987; Bhujwalla et al., 2000]. For a comprehensive review of currently available tissue specific CA, see Weinmann et al. [2003].

While many tissue specific agents demonstrate favorable relaxation properties and some of them are currently in clinical trials in Europe and USA, they were not designed to recognize specific cellular molecular markers and thus enable imaging of a relatively small number of cells expressing these markers. In the current review, we will focus our attention at the recent developments in the design and applications of a distinct group of specific CA targeted to unique cell surface molecular epitopes. Highly specific target recognition and

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binding for these CA is usually provided by mAb or antibody fragments.

CONTRAST AGENT CLASSES

The two major classes of MR contrast agents are paramagnetic CA, usually based on chelates of Gd generating T1 positive signal enhancement, and super-paramagnetic CA that use mono- or polycrystalline iron oxide to generate strong T2 negative contrast in MR images. To improve relaxation properties of Gd-based CA, several macromolecular carriers that can carry large number of Gd groups per molecule have been designed. Protein-based platforms include albumin Gd conjugates, which are a classic prototype for intravascular imaging agents [Schmiedl et al., 1987; Bhujwalla et al., 2000], poly-L-lysine [Bogdanov et al., 1993; Gohr-Rosenthal et al., 1993], avidin [Artemov et al., 2003a], as well as direct mAb Gd conjugates [Matsumura et al., 1994; Shahbazi-Gahrouei et al., 2001]. Polyamidoamine (PAMAM) dendrimers of different generations are used as carriers for multiple Gd groups [Bryant et al., 1999; Kobayashi et al., 2001]. Cross-linked liposomes can be labeled with a large molar concentration of Gd, which provides an extrordinaly high T1 relaxivity of these nanoparticles [Sipkins et al., 1998; Anderson et al., 2000]. Typical molecular weight and size of these polymer Gd chelates varies from about 80 kD for albumin and avidin based agents to several million Daltons for polymerized liposomes with a molecular diameter of 200 nm. The relaxivity of these complexes depends on the B_0 magnetic field used for MRI and on immobilization of the complexes by specific binding to target receptors.

Iron-oxide-based CA usually consist of a monocrystalline (MION) or polycrystalline (SPIO) magnetic core with a diameter of 5 to 30 nm embedded within a polymer coating (such as dextran or other polysaccharide) for a total particle diameter of 17–50 nm, respectively. The major types of these high molecular weight imaging platforms suitable for targeted MRI are summarized in Table I. Generally, the relaxation properties of these CA depend on the molecular size of the carrier and larger molecules have higher relaxivity per molecule, respectively. Therefore to improve the sensitivity of detection, it is feasible to use large, highly efficient CA in situations where there is a limited number of target binding sites per cell. On the other hand, the large molecular size can prevent effective extravasation of the CA molecules from the vasculature, and reduce diffusion of the CA through the interstitium. This problem is not relevant for imaging of vascular endothelium [Sipkins et al., 1998; Anderson et al., 2000] and to a certain extent can be less important in tumors with highly permeable vasculature [Brigger et al., 2002]. Efficiency of the CA delivery to cellular targets generally depends on the rate of vascular extravasation and pharmacokinetics of the CA in the plasma. CA with longer circulation time could show better kinetics at the target site. Depending on the molecular size and surface characteristics, different mechanisms of CA clearance are active in vivo [Brigger et al., 2002]. Several strategies to prolong the plasma life time have been proposed. Long circulating iron oxide particles (LCDIO) were produced using dextran coating of the iron core [Moore et al., 2000]; coating the nanoparticles with hydrophilic polymers such as polyethyleneglycol (PEG) generally helps to reduce binding of plasma proteins and subsequent clearance by macrophages [Oussoren and Storm, 1997]. Anionic modification of dendrimer surface groups reduces clearance and

TABLE I. Typical Properties of the Platforms for Targeted MR Contrast Agents (CA)

Class	Representative compound	Number of metal atoms	Molecular diameter (nm)	Molecular weight (Da)
Low molecular weight	GdDTPA Magnevist®	1 [Gd^{3+}]	—	743
Protein carriers	Albumin	30 [Gd^{3+}]	8	~80,000
	Poly-L-Lysin	65 [Gd^{3+}]	—	~52,000
Dendrimers	PAMAM generation 4	64 [Gd^{3+}]	6–8	~60,000
	Gadomer-17	24 [Gd^{3+}]	5–6	17,500 (35,000 apparent size)
Iron oxide nanoparticles	MION-46	2,064 [Fe] 4.6 nm iron core	34	~775,000
	SPIO Feridex®	~6.5 nm Fe_3O_4 core	70–140	Megadalton range
Liposomes	Gd-perfluorocarbon nanoparticles	~50,000 [Gd^{3+}]	200	Megadalton range

cytotoxicity of dendrimers in vivo [Malik et al., 2000]. Currently it seems that the delivery of high molecular weight CA to the targeted site is a critical problem for targeted molecular MRI. A careful consideration of CA delivery aspects is a key to successful in vivo applications. Another serious concern is biodegradation of the Gd complexes that can result in a release of toxic Gd [Runge et al., 2002]. Diethylene triaminepentaacetic acid cyclic anhydride traditionally used for protein modification may not provide sufficient stability of chelates if the CA is eventually internalized by the target cell. More complex chemical conjugation strategies using derivatives of DTPA and/or 1,4,7,10- tetraazacyclododecane–N,N′,N″,N‴–tertraacetic acid can be required for development of stable polymer Gd conjugates that can survive internalization and cell processing of the CA by the target cells [Liu and Edwards, 2001].

VISUALIZATION OF MOLECULAR TARGETS WITH MR

Several successful attempts of in vivo and/or cellular MRI of various molecular targets with specific MR agents were reported. The most direct approach that is related to standard nuclear imaging protocols uses labeling of monoclonal antibodies with GdDTPA. One of the early studies using this approach was by Matsumura et al. with MAb against 9L glioma cells [Matsumura et al., 1994]. Enhanced contrast in the tumor was detected, although no control experiments with protein-cojugated GdDTPA were performed. Shahbazi-Gahrouei et al. have reported postmortem relaxometry of human MM-138 melanoma xenografts in nude mice probed in vivo with anti-melanoma Mab GdDTPA conjugate [Shahbazi-Gahrouei et al., 2001]. Selective retention of the CA in tumors was detected 24-h post administration. In this study the reported ratio of Gd to MAb was about 37 and at this ratio a significant decrease in immunoreactivity of MAb is very likely. A different strategy was proposed for MRI of the $\alpha_v\beta_3$ integrin that is a molecular marker of the angiogenic endothelium. High accessibility of this marker enables the use of large blood-pool nano-complexes such as paramagnetic polymerized liposomes [Sipkins et al., 1998] or Gd-perfluorocarbon nanoparticles [Anderson et al., 2000] that can contain several thousands Gd ions per particle. These nanoparticles are targeted to the receptor by MAb attached via biotin-avidin linkers. The high concentration of Gd provides high T_1 positive contrast in vivo, however the large molecular size of this CA may prevent its use for imaging cell-surface receptors expressed on tumor cells in solid tumors because of inefficient extravasation and very slow diffusion in the interstitial compartment. Conjugation of MAb against tumor cell-surface markers with macromolecular Gd carriers was reported for imaging of mucin-like protein expressed in many types of gastrointestinal carcinomas [Gohr-Rosenthal et al., 1993]. MAb were conjugated to poly-L-Lysine-GdDTPA with a labeling ratio of 65 Gd ions per molecule. The molecular weight of the conjugate was about 200 kD. Konda et al. reported the development of a folate-conjugated dendrimer complexed with GdDTPA [Konda et al., 2000]. A fourth-generation PAMAM was used as the imaging platform for targeted MRI and initial results were obtained for human folate receptor expressing ovarian tumor xenografts grown in nude mice [Konda et al., 2000]. Breast cancers expressing HER-2/neu receptors were imaged with a two-step labeling protocol using biotinylated Herceptin MAb and avidin-GdDTPA conjugates [Artemov et al., 2003a]. The maximum estimated number of Gd atoms per receptor is a product of the number of Gd per avidin molecule (12.5) and the number of biotins per MAb.

The conjugation of superparamagnetic iron-oxide particles with MAb or MAb fragments is another approach for imaging receptors expressed on the cell surface of target cells. This approach was used for imaging leukocytes with a MAb against the leukocyte common antigen conjugated to SPIO particles [Bulte et al., 1992]. E-selectin expressed in human endothelial cells was imaged using the $F(ab)_2$ fragment of anti-human E-selectin MAb conjugated to CLIO nanoparticles [Kang et al., 2002]. A similar method was used for imaging the HER-2/neu receptors on the surface of malignant breast cancer cells using Herceptin MAb and SPIO complexes [Artemov et al., 2003b]. A significant advantage of the iron-oxide based CA is that their high T2 relaxivity produces strong negative T2 contrast at nanomolar concentrations of the CA. However, most of these studies were performed with immobilized cells. In vivo applications are problematic because of the large molecular size of the conjugates, which may prevent effective delivery to the target site. Two

examples of successful in vivo applications of iron-oxide targeted CA include imaging of inflammation sites with human polyclonal IgG attached to MION particles [Weissleder et al., 2000], and imaging of apoptosis in EL4 solid tumor models exposed to a chemotherapeutic agent [Zhao et al., 2001]. In the latter study, SPIO particles were conjugated to the C2 domain of the protein synaptotagmin that binds to phosphatidylserine present on the outer leaflet of the plasma membrane in apoptotic cells. Efficient delivery of the CA to these sites can be explained by significantly increased vascular permeability in the sites of inflammation or in treated tumors.

An important concept for molecular imaging is amplification of the label. This can be performed in the extracellular compartment using, for example, avidin-biotin system [Goldenberg et al., 2003] or by loading cells with the CA using active transport through a specific promoter. This concept was successfully demonstrated in a study by Weissleder et al., where 9L glioma cells expressing the engineered transferring receptor (ETR, with distrupted negative feedback regulation) were imaged in vivo using MION nanoparticles conjugated to human holotransferrin [Weissleder et al., 2000]. ETR expressing cells internalized the targeted CA and produced negative T2 contrast, which was detected in vivo by T2*-weighted gradient echo MRI. The hydrodynamic radius of the MION particles used in the study was around 17 nm, which corresponds in size to a protein with a mass of 775 kDa. Effective contrast uptake to the tumor was most likely a result of the long circulation time of the agent, and efficient amplification of the label by cell internalization.

Another group of "smart" CA includes agents that can be activated or deactivated by specific biological transformations. Processes activated by β-galactosidase, proteases, and DNA cleavage or hybridization can be studied [Bremer and Weissleder, 2001; Perez et al., 2002]. Although these CA can be "targeted" to specific chemical reactions, their design properties and applications are not discussed in the current review.

METHODOLOGY

As mentioned earlier, the delivery of the CA to the target site is one of the most important issues in the development of MRI molecular markers. Although several studies suggest an efficient delivery of relatively large CA particles (with diameters close to 20 nm) to tumors, in general, extravasation and diffusion barriers for these nanocomplexes can significantly reduce their usefulness in viable solid tumors following systemic administration [Brigger et al., 2002]. Indeed, extensive studies have demonstrated that tumors are generally characterized by permeable vasculature. The vascularization pattern of solid tumors, however, is heterogeneous. Regions of high vascular permeability are often spatially different from regions of high vascular volume [Bhujwalla et al., 2000]. Tumors typically have areas with reduced vascularization and blood perfusion and, consequently, the delivery of imaging and/or therapeutic high molecular weight agents to these regions may be significantly reduced. One way to circumvent limitations imposed by delivery is to use multistep labeling or pretargeting labeling concept sketched in Figure 1 [Goldenberg et al., 2003]. Prelabeling is used in nuclear imaging to increase the target/background ratio and match the pharmacokinetics of radionuclides to their half lifetime [Paganelli et al., 1999]. A traditional pretargeting method includes the avidin/streptavidin–biotin system, which combines a high binding affinity and signal multiplication by binding four biotins per protein molecule. However, avidin and streptavidin are foreign proteins and may induce host immune response. This significantly limits successful applications of the technique in vivo especially when repetitive imaging is required. For targeted MRI, prelabeling can provide an additional advantage by splitting a large CA into smaller components with improved delivery properties. This approach was used for in vivo MRI of HER-2/neu receptors [Artemov et al., 2003a] where two-step labeling with biotinylated MAb and GdDTPA-labeled avidin produced measurable contrast in breast cancer tumor xenografts expressing the HER-2/neu receptor. The molecular weight of the largest component of the CA, MAb,was 160 kDa which provided effective delivery of the CA to the tumor. To further improve existing methods, several key problems need to be addressed. Firstly, highly efficient bispecific probes are required. These probes, which can be MAb fragments, minibodies, or diabodies, should be of a relatively low molecular weight and size and provide high affinity of binding both to the

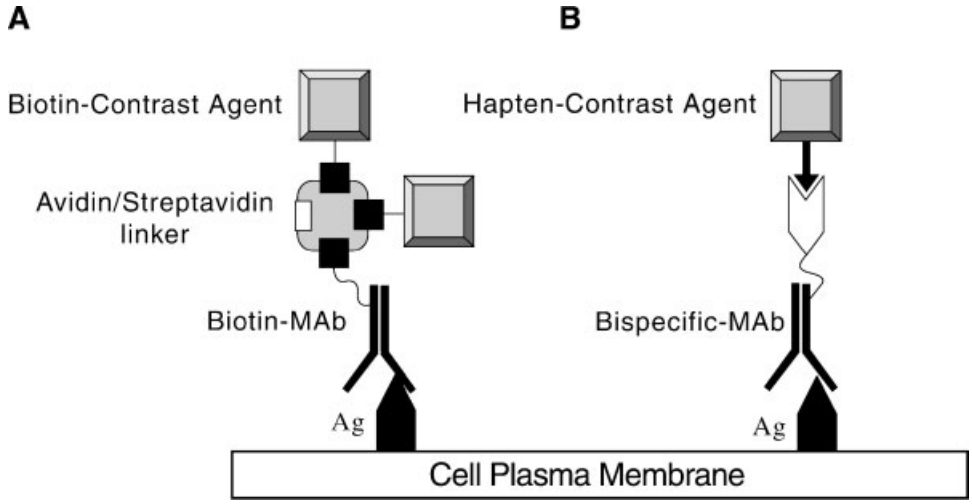

Fig. 1. Design of the specific labeling schemes with pretargeting. **A**: Three-step labeling with biotinylated antibody, streptavidin/avidin linker, and biotinylated probe(s). **B**: Two-step labeling with bispecific antibody and a hapten/probe conjugate.

molecular target and to the contrast-generating component of the CA. Extensive experiments with albumin-GdDTPA contrast agent demonstrate that this blood-pool contrast agent (molecular weight close to 80 kDa) can efficiently extravasate into the tumor interstitium in several human tumor models [Bhujwalla et al., 2000]. Based on these considerations, the molecular weight for the imaging component of the optimized CA should be in the range of 50–100 kDa. This is well below the size of iron-oxide based CA and requires the use of Gd based paramagnetic complexes. Detectable concentrations of Gd in vivo are just below the millimolar range and a large number of Gd ions should be directed to the molecular target to generate detectable MR contrast. The most promising platforms for Gd CA are spherical dendrimers and linear polymers such as poly-Lysine [Bogdanov et al., 1993]. The ligands used for complexation of Gd should provide high thermodynamic and kinetic stability and enable straightforward modification of the polymer carrier using available surface groups [Liu and Edwards, 2001]. Good examples are the 1B4M derivative of GdDTPA [Kobayashi et al., 2001] and of DOTA [Bryant et al., 1999] that use the linker attached to the carbon backbone of the molecule, which does not affect the stability of the complex. Another consequence of the low sensitivity of MRI is that majority of the available molecular targets should be occupied by the CA to provide detectable contrast. If the targets are functional cell-surface receptors then blocking them with the CA molecules can interfere with cell signaling and result in significant physiological changes. For instance using Herceptin as a targeting agent for HER-2/neu imaging may have a therapeutic effect for HER-2/neu expressing tumors.

To enable effective MRI, optimized MR protocols are also required. A significant advantage of Gd-based CA is the positive T_1 signal enhancement that, generally, is preferable in vivo over the signal reduction generated by iron-oxide based T_2 CA. Most current studies use T_1 weighted imaging, and signal enhancement in the target site (such as tumor) is measured relative to the signal of control tissue (such as muscle). While this approach is experimentally simple, it does not necessarily produce optimum sensitivity. Indeed if we expect a modest decrease in T_1 relaxation time in the target site then the optimal repetition delay of the T_1-weighted imaging experiment should be close to T_1. It is difficult to optimize this parameter without previous knowledge of the relaxation properties of the CA. Also the labeling protocol is usually quite long (can take up to 24 h) and the subject has to be removed from the magnet between the pre- and post-contrast examination. This makes coregistration of pre- and post-contrast images difficult. Quantitative T_1 imaging offers an opportunity to simplify the registration problem and to allow more objective assessment of the efficiency of contrast enhancement. MRI experiments with targeted CA do not require high temporal resolution, so

the increased acquisition time of the quantitative T_1 imaging in comparison with T_1-weighted protocols is not essential. Also modern fast-imaging MR techniques enable fast acquisition of quantitative T1 maps within a relatively short experimental time [Nekolla et al., 1992; Bhujwalla et al., 2000].

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