Molecular Magnetic Resonance Imaging Approaches Used to Aid in the Understanding of Angiogenesis *In Vivo*: Implications for Tissue Engineering

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In tissue engineering it is often necessary to assess angiogenesis associated with engineered tissue grafts. The levels of vascular endothelial growth factor receptor 2 (VEGF-R2) is elevated during angiogenesis. The goal of this study was to develop and assess a novel magnetic resonance imaging (MRI) molecular probe for the *in vivo* detection of VEGF-R2 in an experimental rodent model of disease. The possible use of the probe in tissue engineering applications is discussed. The molecular targeting agent we used in our study incorporated a magnetite-based dextran-coated nanoparticle backbone covalently bound to an anti-VEGF-R2 antibody. We used molecular MRI with an anti-VEGF-R2 probe to detect *in vivo* VEGF-R2 levels as a molecular marker for gliomas (primary brain tumors). Tumor regions were compared with normal tissue. Nonimmune nonspecific normal rat immunoglobulin G coupled to the dextran-coated nanoparticles was used as a control. Prussian blue staining for iron-based nanoprobe was used to confirm the specificity of the probe for VEGF-R2 in glioma tissue. VEGF-R2 levels in tumor tissues were also confirmed in western blots and via immunohistochemistry. Based on our results, *in vivo* evaluation of tissue angiogenesis using molecular MRI is possible in tissue engineering applications.

Introduction

IN TISSUE ENGINEERING it is often necessary to assess angiogenesis associated with engineered tissue grafts to determine if successful tissue and/or cell growth is occurring. The levels of vascular endothelial growth factor receptor 2 (VEGF-R2) is elevated during angiogenesis. In a study on subcutaneously implanted precellularized scaffold for tissue engineering of intestinal mucosa, it was found that at 4 weeks postorganoid unit implantation, there was recognizable mucosa and submucosa present on the luminal surface of the scaffold with an associated increase in VEGF-R2 positive cells.¹ In another study a biopolymeric construct (polyglycolic acid-poly-L-lactic acid scaffold) seeded with just vascular progenitor cells was found to develop no microvessels; however, capillary-like structures were formed when endothelial progenitor cells were seeded with human smooth muscle cells, as determined by an increase in VEGF-R2.² In a recent study, baboon endothelial progenitor cells were used for tissue-engineered vascular grafts, and it was found that the

successful grafts developed an increase in VEGF-R2-positive cells.³ Monitoring of successful angiogenesis in tissue grafts with the use of *in vivo* imaging would be quite beneficial.

Molecular magnetic resonance imaging (mMRI) is an implementation of MRI that allows noninvasive visualization of *in vivo* biological processes at cellular and molecular levels. mMRI utilizes a signaling component, such as superparamagnetic iron oxide (SPIO)-based particles to generate a negative signal contrast (T_2 relaxation contrast) that occur as a result of the binding of an affinity component to molecular markers. The probes enhance proton magnetization relaxation rates and thus decrease relaxation times at their sites of accumulation, making them ideal for diagnostic purposes.⁴

The molecular targeting agents we used in our study incorporated a dextran-coated magnetite-based nanoparticle backbone covalently bound to an anti-VEGF-R2 antibody (Ab). The objective of this project was to develop and assess novel MRI molecular probes for the *in vivo* detection of VEGF-R2 in an experimental rodent model for gliomas. VEGF-R2 is a potential marker that can also be used to

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evaluate tissue-engineered grafts or transplants to monitor tissue angiogenesis.

Methods

Rat glioma models

C6 gliomas. Three-month-old male Fischer 344 rats (250–350 g; $n = 4$) were anesthetized (3% isoflurane at 2.5 L/min oxygen) and placed on a stereotaxic device (Stoelting, Wood Dale, IL). The heads of anesthetized rats were immobilized, and using aseptic techniques, a 1 mm burr hole was drilled in the skull 2 mm anterior and 2 mm lateral to the bregma on the right-hand side of the skull. C6 cells (10 μ L of a 10^6 /mL cell suspension) in an ultra-low gelling temperature agarose were injected into the cortex to a 3 mm depth at a rate of 2 μ L/min.^{5,6} A waiting time of 2 min was implemented following injection and bone wax was put in the burr-hole to prevent any reflux. The wound was sutured and covered with surgical glue. The surgery was performed in sterile conditions. The syringes containing the C6 cells were kept at 37°C until the moment of use. Rats were maintained on a choline-deficient diet throughout the studies, as the tumor cells have been shown to be tumorigenic in choline-deficient Fisher rats. C6 implanted cells (glial cell strain cloned from a rat glial tumor induced by *N*-nitrosomethylurea) yield intracerebral growth with minimal distant metastasis.^{7,8}

Synthesis of the anti-VEGF-R2 contrast agent

Synthesis of nanoparticles involves coprecipitation of ferrous and ferric salts in an alkaline medium, and surface complexing agents, such as dextran, to provide colloid stability and biocompatibility.^{9–11} A dextran-based crosslinked iron oxide (CLIO) molecular-targeted IO nanoparticle-based anti-VEGF-R2 probe was synthesized. To recognize VEGF-R2 a mouse monoclonal anti-VEGF-R2 Ab against amino acids 1158–1345 mapping at the C-terminus of Flk-1 (designated as VEGF-R2; Flk-1 [A-3; Santa Cruz Biotech., Santa Cruz, CA]) was used.

Amine-terminated CLIO (CLIO-NH₂) was synthesized as described by Josephson *et al.*¹² A monodispersed SPIO col-

loid monocrystalline iron oxide nanocompound (MION) was synthesized and crosslinked with epichlorohydrin (Sigma-Aldrich, St. Louis, MO) to prepare CLIO-NH₂.¹³ Briefly, amination is achieved by the addition of concentrated ammonia, followed by heating at 37°C overnight.¹² Low-molecular-weight materials are removed by dialysis against water using dialysis tubing (12–14 K cutoff; Spectra-Por, Laguna Hills, CA).^{12,13} Air was then bubbled through the colloid for 24 h at 37°C.¹² The colloid was subjected to pressure dialysis with the addition of 10 volumes of 5 mM sodium citrate (pH 8).^{12,13} These steps fully oxidize any ferrous iron and remove traces of low-molecular-weight materials.¹² The nanoparticles were characterized by transmission electron microscopy (TEM) and light scattering (Nanotrak particle size analysis).

Preparation of the Ab-SPIO conjugates was done via disulfide exchange reactions in the following steps (as depicted in Fig. 1): (1) Synthesis of amine-terminated CLIO: CLIO-NH₂, (2) *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) activation of CLIO-NH₂, (3) *N*-succinimidyl-S-acetylthioacetate (SATA) activation of immunoglobulin G (IgG), and (4) synthesis of CLIO-IgG, as described by Hermanson.¹⁴ Heterobifunctional crosslinking agents containing an amine-reactive group at one end and a disulfide bond with a good leaving group on the other end are used for making conjugates.¹⁴ The leaving group on the disulfide portion of the crosslinker permits efficient interchange with a free sulfhydryl on the Ab.¹⁴ SPDP is used for conjugation.¹⁴ The activated *N*-hydroxysuccinimide (NHS) ester end of the SPDP reacts with amine groups in SPIO to form an amide linkage.¹⁴ The 2-pyridyldithiol group at the other end reacts with sulfhydryl groups in the Ab to form a disulfide linkage.¹⁴ Ab IgG is activated with SATA, a thiolation reagent.¹⁴ The compound reacts with primary amines via its NHS ester end to form a stable amide linkage.¹⁴ The acetylated sulfhydryl group is stable until deacetylated with hydroxylamine.¹⁴ Thus, Ab molecules may be thiolated with SATA to create the sulfhydryl target functional groups necessary to couple the SPDP-activated CLIO. The molar ratio of Fe to Ab is 1:26. These agents can be widely distributed via the vascular system and maintain long circulation times.^{15,16}

FIG. 1. Steps for the formation of CLIO-based IgG nanoprobe conjugates. The steps include (1) the synthesis of amine-terminated CLIO: CLIO-NH₂, (2) SPDP activation of CLIO-NH₂, (3) SATA activation of IgG, and (4) synthesis of CLIO-IgG. The diagrams were obtained and modified from Josephson *et al.*¹² and Hermanson.¹⁴ CLIO, crosslinked iron oxide; IgG, immunoglobulin G; SPDP, *N*-succinimidyl 3-(2-pyridyldithio) propionate; SATA, *N*-succinimidyl-S-acetylthioacetate.

FIG. 2. T_2 -weighted MRI of the head of a C6 glioma-bearing rat, 21 days after C6 cell implantation, with regions outlined where histological sections were obtained. Hematoxylin and eosin staining of C6 glioma (cells implanted 19 days before): (A) area of normal tissue ($\times 40$), (B) glioma ($\times 40$), (C) tumor infiltration ($\times 10$), and (D) necrosis ($\times 40$). (E) Western blot levels of VEGF-R2 indicate that there are higher levels in C6 glioma tissue compared with normal tissue. MRI, magnetic resonance imaging; VEGF-R2, vascular endothelial growth factor receptor 2. Color images available online at www.liebertonline.com/ten.

FIG. 3. Immunohistochemistry staining (magnification, $\times 40$) for VEGF-R2 levels in normal brain tissue (A), and C6 glioma tissue (B) tumor boundary and (C) tumor periphery, obtained in regions depicted in a T_2 -weighted MRI of the head of a C6 glioma-bearing rat 24 days after C6 cell implantation. Color images available online at www.liebertonline.com/ten.

MRI experiments

In vivo MRI experiments were carried out under general anesthesia (1–2% isoflurane, oxygen 0.8–1.0 L/min). MRI equipment used was a Bruker Biospec 7.0 T/30 cm horizontal-bore imaging spectrometer (Bruker BioSpin MRI, Ettlingen, Germany). Animals were imaged at 7–10 days after the cells were injected and then every 2–3 days until the desired volume of the tumor (75–150 mm³) was reached. Anesthetized (2% isoflurane) restrained rats/mice were placed in a MRI probe, and their brains localized by MRI. The images were obtained using a Bruker S116 gradient coil (2.0 mT/m/A), a 72 mm quadrature multirung radio frequency (RF) coil for RF transmission, and a rat head coil for receiving RF signal. MRI was performed for the purpose of determining the incidence, number, growth rate, and volume of each tumor for the C6 gliomas. Multiple ¹H MRI slices were taken in the transverse plane using a spin echo multislice (repetition time [TR] 0.8 s, echo time [TE] 23 ms, 128×128 matrix, four steps per acquisition, 4×5 cm² field of view, 1 mm slice thickness).¹⁷

Multiple ¹H-MR image slices were taken in the transverse plane using a spin echo multislice (TR = 0.8 s, TE = 23 ms, matrix = 128×128, four steps per acquisition, field of view = 4×5 cm², 1 mm slice thickness). For determination of *T*₂ values of the IO nanoprobe in gliomas, a RAREVTR (rapid acquisition with relaxation enhancement) method was used with the following parameters: TE = 15 ms, TR = 3000 ms, eight echoes (TE_{effective} = 15, 30, 45, 60, 75, 90, 105, and 120 ms), number of averages = 2, matrix = 256×256, slice thickness = 1 mm, and an estimated total scan time of 25 min. *T*₂ maps were generated from the multiecho datasets. Rat brains were imaged at 0 (prenanoprobe [CLIO anti-VEGF-R2 nanoprobe] or precontrast agent [control CLIO-IgG] administration), 20, 40, 60, 120, and 180 min intervals postnanoprobe or IgG contrast agent injections. Rats were injected with a single dose intravenously via a tail vein catheter with either the anti-VEGF-R2 nanoprobe (anti-VEGF-R2 Abs [rabbit anti-rat] tagged with an IO-based contrast agent [CLIO based]; 200 μL/200 g rat; 1 mg Ab/kg; 0.05 mmol Fe⁺³/kg), or the normal rat IgG control contrast agent (same dose as anti-VEGF-R2 nanoprobe).

Multiple regions of interest (ROIs) (three ROIs within each of tumor and nontumor tissues) were selected from *T*₂-weighted images and *T*₂ maps to calculate relative changes in MRI signal intensities and *T*₂ values in C6 glioma-bearing rats administered either the CLIO-anti-VEGF-R2 nanoprobe (*n* = 2) or the CLIO-IgG contrast agent (*n* = 1).

FIG. 4. (A) Transmission electron microscopy of the CLIO nanoparticles averaging from 20 to 60 nm (larger sizes are aggregates). (B) Nanotrak particle size analysis of the CLIO nanoparticles (~120 nm). Color images available online at www.liebertonline.com/ten.

Particle size analysis of nanodispersions

A NanoTrac Ultra Particle Size Analyzer (Microtrac, Montgomeryville, PA) was used to measure the size and size distribution of nanoparticles in a stable aqueous dispersion. The NanoTrac Ultra Particle Size Analyzer is based on

FIG. 5. *T*₂-weighted MRI at pre- and 3 h postadministration of the anti-VEGF-R2 probe in horizontal (A) and cross-sectional (B) image orientations. SI difference images (pre-minus 3 h postadministration) in (A) and (B) depict bright areas (white dotted oval regions) indicating uptake of the anti-VEGF-R2 probe. (C) SI difference image obtained between pre- and 3 h postadministration of the IgG-control contrast agent, depicting minimal bright regions (white dotted oval), compared with the anti-VEGF-R2-administered animals (B, difference image). (D) Percentage (%) changes in *T*₂ and SI decrease in tumor and normal brain tissue regions when comparing preadministration and 3 h postadministration of the anti-VEGF-R2 probe. Both *T*₂ and SI are found to decrease significantly (*p* < 0.05), when compared with normal tissue values (mean ± standard deviation; *n* = 3 regions of interest (ROIs) (red circles) per group (tumor (right) and normal (left) brain tissues). Comparatively, % changes in *T*₂ and SI are relatively small in the tumor region of an IgG-control contrast agent-administered glioma-bearing rat. (E) Relative changes in *T*₂ relaxations in tumor and normal brain tissues of C6 glioma-bearing rats (mean ± standard deviation; *n* = 3). (F) Prussian blue staining of glioma region after administration of an anti-VEGF-R2-IO nanoprobe (i, ii), compared with a glioma following administration of a control IgG-IO contrast agent (iii, iv). (iii) Slide with only residual IgG-IO contrast agent present. (iv) A representative of all other slides taken. SI, signal intensity. Color images available online at www.liebertonline.com/ten.

dynamic light scattering for particle size measurements. Light has properties that can be used for determining particle size and particle size distributions. The NanoTrac uses an advanced power spectrum analysis of Doppler shifts to produce a full volume distribution of particle sizes, provided as a particle size distribution of volume (mass) percent and intensity. Calculation of the full particle size distribution, using the FLEX Application Software (Microtrac, ver. 10.3.14), provides maximum information regarding the sample dispersion.

TEM

CLIO nanoparticles were spread onto Formvar-coated 100 mesh nickel grids, air-dried, and examined on a Hitachi electron microscope.

Histology analyses and VEGF-R2 expression assays

Cardiac perfusion with phosphate-buffered solution is performed while the rat is under anesthesia (isoflurane), for extraction of the brain. The sections of the brain were either frozen (western blot assays), or fixed by immersion fixation in 10% buffered formalin (histology samples) or in 4% paraformaldehyde (immunofluorescence staining). In each rat, the contralateral side of the brain was used as a control. For histology, 7- μ m-thick paraffin-embedded sections were stained with hematoxylin and eosin.

Western blot assays of VEGF-R2 expression in glioma tissues was conducted as follows: frozen tissue was weighed and sliced into thin pieces, and then thawed in lysis buffer containing proteases and phosphatase inhibitors. Tissue were disrupted and homogenized at 4°C, incubated on ice for 30 min, and centrifuged (10,000 g, 10 min, 4°C) to recover cell lysate in the supernatant. After determining protein concentrations, lysates were separated on SDS-PAGE (Bio-Rad, Emeryville, CA) and transferred to nitrocellulose membranes. Western analysis was performed using Abs against VEGF-R2. The secondary Abs were labeled with horseradish peroxidase. The ECL Advance Western Blotting Detection Kit (Amersham Bioscience, Piscataway, NJ) was used to detect immunoreactive proteins. NIH Image J was used to calculate the density regions in western blot bands.

Immunohistochemistry staining of VEGF-R2 was done in the extracted rat brains, fixed by immersion in 10% buffered zinc formalin, and embedded in paraffin. The brain sections were immunostained with a mouse mAb anti-VEGF-R2 (dilution of 1:200; Santa Cruz Biotech) followed by Rat HRP-Polymer system (Biocare Medical, Concord, CA) for detection and NovaRed (Vector Laboratories, Burlingame, CA) chromogen for visualization.

Prussian blue staining of the nanoparticles

Detection of the IO-based nanoparticles in tissue cryosections was done using Prussian blue staining, which involves the treatment of sections with acid solutions of ferrocyanides. The ferric ion (+3) present in the IO-based nanoparticles from tissue sections combines with the ferrocyanide and results in the formation of ferric ferrocyanide, visible as a blue pigment in bright field imaging. The stained tissue slices were viewed and photographed with a Nikon C1 confocal laser scanning microscope (Nikon Instruments USA, Melville NY).

Statistical analyses

Statistical differences in MRI signal intensities and T_2 relaxations, which indicated specific binding of the nanoprobe in glioma tissue, were analyzed between the treatment and control groups, and between tumor and nontumor regions, with an unpaired, two-tailed Student's *t*-test using commercially available software (InStat; GraphPad Software, San Diego, CA). A *p*-value of less than 0.05 was considered to indicate a statistically significant difference.

Results

The C6 glioma model has been fully characterized regarding tumor volumes and tumor growth rates within our laboratory. Figure 2 is a representative MR image of a C6 rat glioma at 19 days post cell implantation, with corresponding histological images from various regions. Western blot levels of VEGF-R2 in glioma tissue compared with normal brain tissue are shown in Figure 2E, with higher levels of VEGF-R2 in glioma tissue compared with normal tissue. The immunohistological stains for VEGF-R2 in the C6 glioma were both highly elevated (Fig. 3B, C), compared with levels in control brain tissue which are considerably less (Fig. 3A).

TEM was used to establish the average size of the dextran-coated nanoparticles, which were found to be between 20 and 60 nm in diameter (Fig. 4A). Light scattering (Nanotrak particle size analysis) was used to establish the hydrodynamic size of the nanoparticles, which were found to have an average size of ~120 nm (Fig. 4B).

In vivo detection of VEGF-R2 overexpression in a C6 glioma using the CLIO-anti-VEGF-R2 nanoprobe is shown in Figure 5, demonstrating the feasibility of the approach. The signal intensity difference image (pre- minus postcontrast 3 h after intravenous administration of IO-based anti-VEGF-R2 probe) indicates that there is a sustained decrease in signal intensity and T_2 relaxation 3 h following administration of the anti-VEGF-R2 probe. These regions of decreased signal intensity can be used to depict regions of nanoprobe uptake (bright regions). Analysis of the percent change in signal intensity and T_2 relaxation values (a decrease between pre-injection and 3 h postinjection values following the administration of the CLIO-anti-VEGF-R2 nanoprobe) in tumor and normal tissues indicates a substantial effect only in the glioma tumor (Fig. 5D). A sustained decrease in signal intensity within the tumor suggests that the tumor marker-directed nanoprobe specifically binds to the tumor. The signal intensity change in the tumor region of an IgG-IO control is negligible. Figure 5C is a difference image obtained between pre- and 3 h postadministration of the IgG control contrast agent, depicting minimal bright regions, that is, minimal retention. Figure 5E is the relative change in T_2 relaxation over the course of 3 h, depicting a change in T_2 only in tumor tissue, compared with normal brain tissue. T_2 measurements were obtained from ROIs depicted in Figure 5B. Figure 5F are histological tissue slides stained with Prussian blue for IO, depicting the presence of the CLIO-anti-VEGF-R2 nanoprobe in the glioma region (i and ii of Fig. 5F) of a C6-glioma bearing rat and substantially less uptake of the IgG contrast agent in the tumor of an IgG-IO control (iii of Fig. 5F). Figure 5F(iii) depicts the only sample with some staining for IO,

whereas all other samples (e.g., iv of Fig. 5F) did not have any Prussian blue staining.

Discussion

The study of angiogenesis with high-resolution imaging is somewhat constrained by the lack of suitable probes coupled to a noninvasive imaging modality that can detect markers for these processes in living animals.

Our laboratory has experience in using magnetic resonance angiography (MRA) to assess blood vasculature associated with gliomas¹⁷ and mMRI to assess molecular markers associated with glioma growth.¹⁸ Gliomas comprise the majority of primary brain tumors diagnosed annually in the United States.^{19,20} Important hallmarks of malignant gliomas are their invasive behavior and angiogenesis.²¹ Experimental rodent models, such as C6 gliomas, have emerged as useful systems for the study of human brain tumors.^{22,23}

MRI is the imaging modality of choice to assess gliomas, clinically²⁴ and in experimental rodent models.²⁵ With the use of MRI, tissue composition, morphology, and function can be studied *in vivo* and noninvasively, allowing serial studies in experimental animals and humans to be conducted.

mMRI technologies for specific molecular targeting have been developed, where an MRI contrast agent probe is targeted to a specific receptor or antigen by a monoclonal Ab (mAb). The advantage of using mMRI is that the spatial location of *in vivo* molecular marker overexpression can be readily visualized in heterogeneous deeply located tissues with good spatial resolution.

Magnetic IO-based nanocrystals are ideal as magnetic probes for MRI as a result of their signal-altering capabilities.²⁶ SPIO typically consist of two components, an IO core and a hydrophilic coating. For the hydrophilic coating we used dextran, which rendered 20–60 nm diameter as detected by TEM (Fig. 4A). Studies have shown that polymer-coated nanoparticles, such as the dextran-coated nanoparticles that were used in this study, have minimal impact on cell viability and function and have low toxicity.²⁷ The Ab component attached to the nanoparticles, which allows differentiation between specific binding to specific molecular targets and nonspecific binding in a 2–4 h time-frame, also may hinder successive monitoring in the 12–48 h window, as the Ab may stay bound for a couple of days.

The VEGF is one of the key regulators of tumor neoangiogenesis. VEGF acts through two types of high-affinity tyrosine kinase receptors (VEGF-R1 and VEGF-R2 expressed on endothelial cells).^{28,29} It has been found that the expression of VEGFRs correlates well with glioma tumor malignancy.^{28,29} We detected increased *in vivo* levels of VEGF-R2 associated with glioma tissue growth with the use of the anti-VEGF-R2 probe and mMRI (Fig. 5), as the decrease in signal due to a decrease in T_2 relaxation results from the presence of the anti-VEGF-R2 nanoprobe (Fig. 5A, B, D). This was also verified by Prussian blue staining for the presence of IO nanoparticles in tumor tissue following *in vivo* administration of the anti-VEGF-R2 probe (i and iii of Fig. 5F). The amount of residual nonspecific control IgG contrast agent (Fig. 5C, D) in the tumor may also reflect any lingering anti-VEGF-R2 probe that may not be specifically bound. There is a relative

uptake of the anti-VEGF-R2 probe over 3 h specifically in the tumor tissue, compared with normal brain tissue (Fig. 5E). Comparatively, a nonspecific control IgG contrast agent was found to be minimally taken up by tumor tissue, as detected by both mMRI (Fig. 5C, D) and Prussian blue staining of *ex vivo* tumor tissue (iii and iv of Fig. 5F, with iii being the only slide with any detected iron, i.e., iv is representative of all other slides). Of significance to tissue engineering, increased levels of VEGF-R2 are also detected in tissue-engineered grafts.^{1–3}

This is the first attempt at detecting *in vivo* expression of VEGF-R2 using IO-based nanoprobe in conjunction with mMRI. Our mMRI data, combined with microscopic detection of the anti-VEGF-R2 probe in glioma tissue, provide compelling evidence that this technique can be used to detect VEGF-R2 levels *in vivo* in gliomas, and that this method can be extended to tissue engineering applications.

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Disclosure Statement

No competing financial interests exist.

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