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Development of a Macromolecular Dual-Modality MR-Optical Imaging for Sentinel Lymph Node Mapping

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Abstract

Objective—To evaluate the effectiveness of a dual magnetic resonance-near infrared fluorescence optical imaging agent, poly(L-glutamic acid)-DTPA-Gd-NIR813, for both preoperative and intraoperative visualization and characterization of sentinel lymph nodes (SLN) in mice.

Materials and Methods—Poly(L-glutamic acid) was conjugated with DTPA-Gd and NIR813 dye to obtain PG-DTPA-Gd-NIR813. To confirm drainage into the SLNs, this agent was injected subcutaneously into the front paw of nude mice followed by isosulfan blue (n = 6). Furthermore, PG-DTPA-Gd-NIR813 was injected subcutaneously at doses of 0.002 mmol Gd/kg (4.8 nmol eq. NIR813) and 0.02 mmol Gd/kg (48 nmol eq. NIR813) (n = 3/dose). To differentiate metastatic from non-metastatic lymph nodes, nude mice bearing human oral squamous cell carcinoma (DM14) were injected intralingually with 0.02 mmol Gd/kg PG-DTPA-Gd-NIR813 (n=3). Pre- and post-contrast images were taken using 4.7T Bruker MRI scanner and Xenogen optical imaging system. The status of lymph nodes resected under the guidance of optical imaging was confirmed by histologic examinations.

Results—PG-DTPA-Gd-NIR813 co-localized with isosulfan blue, indicating drainage to the SLN. After subcutaneous injection, axillary and branchial lymph nodes were clearly visualized with both T1-weighted MR and optical imaging within 3 min of contrast injection, even at the lowest dose tested (0.002 mmol Gd/kg). After intralingual injection in tumor-bearing mice, MR imaging identified 4 of the 6 superficial cervical lymph nodes, whereas NIRF optical imaging identified all 6 cervical nodes. The pattern of contrast enhancement of SLN visualized in MR images showed a characteristic ring-shaped appearance with a central filling defect, possibly resulting from nodal infiltration of metastatic lesions. Histopathologic examination of the SLNs resected under NIRF imaging guidance revealed micrometastases in all 6 SLNs identified by NIRF imaging.

Conclusion—The dual modality imaging method demonstrated in this study represents an effective technique for localization and characterization of SLN.

Introduction

Sentinel lymph node (SLN) biopsy is routinely used to determine whether cancer has spread beyond a primary tumor into the nearby lymph nodes for patients with melanoma, gastrointestinal cancer, and breast cancer (1,2). While SLN biopsy has reduced the morbidity of regional staging by avoiding unnecessary removal of entire nodal basins, the radionuclide technique has low spatial resolution and exposes patients and health-care personnel to ionizing radiation. In addition, in some cases such as in patients with head and neck cancer, the drainage pathways can be complex. It is sometimes difficult to accurately localize these SLNs with a hand-held gamma counter probe alone (3). Imaging techniques that provide a three dimensional view that enable the surgeon to determine whether SLNs are superficial along the external jugular vein or deeper and adjacent to the internal jugular vein would be very helpful. High-resolution anatomic imaging of SLNs before surgical dissection is therefore highly desirable.

To date, several imaging techniques, including ultrasound (4), computed tomography (5), and MR imaging (6–9), have been tested to minimize some of the limitations associated with the standard approach to SLN evaluation. In particular, MR imaging has been used not only to localize SLNs but also to detect lymph node abnormalities. For example, 24 hr after injection of Gd-DTPA-labeled polyglucose, T₁-weighted signal intensity was significantly enhanced in normal but not metastatic regional lymph nodes in a rabbit model of lymph node metastases (7). However, because it is difficult to use MR imaging for intraoperative real-time visualization, the use of MR imaging alone for lymphatic mapping and SLN biopsy is technically challenging and impractical.

NIRF optical imaging is a relatively new imaging modality that has several advantages, including high sensitivity and capacity for continuous data acquisition for real-time imaging during surgery. Several previous studies have shown that NIRF optical imaging with fluorescent quantum dots (QD) can be used not only to localize SLNs but also to guide the surgical removal of SLNs in real time (10–12). However, a few problems, including potential toxic side effects, need to be addressed before QD-based optical imaging technique can be applied to humans. In addition, optical imaging is not ideal for identification of lymph nodes located in deep anatomic structures (i.e. splanchnic and mediastinal nodes). In such a case when lymph nodes are not accessible by NIRF imaging, accurate localization and characterization of the SLN may be achieved preoperatively using MRI.

Thus, the combination of MR imaging and optical imaging for SLN identification may overcome the limitations of either imaging method when used alone. MR imaging offers excellent spatial resolution and intrinsic soft-tissue contrast, which may facilitate the preoperative study of tumor lymphatic drainage and SLN abnormalities. On the other hand, NIRF optical imaging offers high sensitivity and the prospect of real-time intraoperative monitoring of SLN biopsy. Previously, Josephson and colleagues have used dual MR-optical imaging approach to define tumor margin and to detect apoptosis using cross-linked iron oxide nanoparticles (13,14). At the time of preparing this manuscript, Talanov et al. (15) reported a Gd-based dual modality MRI and fluorescence imaging probe based on a non-degradable polyamidoamine dendrimer.

In this communication, we report the synthesis and characterization of a biodegradable, macromolecular dual-modality MR-optical imaging contrast agent. We show that this agent has the potential not only to accurately define anatomic localization of SLNs with MRI, but also to allow real-time assessment of SLNs nodes during surgery with NIRF imaging.

Materials and Methods

Materials

Poly(L-glutamic acid) (PG) sodium salt, 1,3-diisopropylcarbodiimide, pyridine, 4-dimethylaminopyridine, trifluoroacetic acid (TFA), gadolinium (III) chloride hexahydrate, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), 2-morpholinoethanesulfonic acid buffer (MES), IR-783 dye, N-hydroxysuccinimide (NHS), N,N-diisopropylethylamine, isosulfan blue, and all the other reagents and solvents used were purchased from Sigma-Aldrich (St. Louis, MO). *N*-tert-butoxycarbonyl-1,5-diaminopentane toluenesulfonic acid salt was purchased from Novabiochem (San Diego, CA). 4-Mercaptobenzoic acid was purchased from TCI (Portland, Oregon). *p*-Aminobenzyl-diethylenetriaminepenta(acetic acid-*t*-butyl ester) (*p*-aminobenzyl-DTPA(*t*-butyl ester) was obtained from Macrocyclics (Dallas, TX). Spectra/Pro 7 dialysis tubing with a molecular weight cut-off of 10,000 and PD-10 columns were purchased from Amersham-Pharmacia Biotech (Piscataway, NJ).

Analytical methods

Gel permeation chromatography (GPC) was performed on a Waters (Milford, MA) high-performance liquid chromatography system consisting of a 600 controller, a 717 plus auto sampler, and a Viscotek E-Z^{PRO} triple detector (Viscotek, Houston, TX) that records refractive index, viscosity, and light-scattering signals. The samples were separated using a TSK-G4000PW 4.6 mm × 30 cm column (TosoHaas, Montgomeryville, PA) eluted with phosphate-buffered saline containing 0.1% LiBr at a flow rate of 1.0 mL/min. Number-average molecular weights of the polymer conjugates were calculated using Viscotek TriSEC GPC software. Matrix-assisted laser desorption ionization mass spectrometry (MALDI) was performed by the Proteomics Core Laboratory, Department of Molecular Pathology, the University of Texas M. D. Anderson Cancer Center. Elemental analysis was performed by Galbraith Laboratories, Inc. (Knoxville, TN).

Analytical high-performance liquid chromatography was carried out on an Agilent 1100 system (Wilmington, DE) equipped with a Vydac peptide and protein analytic C-18 column (Anaheim, CA). Sample was eluted with water and acetonitrile containing 0.1% TFA varying from 10% to 80% over 30 min. Fluorescence emission scan was measured using a Spex Fluorolog spectrofluorimeter (Jobin Yvon Inc, Edison, NJ), while the fluorescence intensity at different NIR813 loading of PG-DTPA-Gd-NIR813 was measured using the 800 nm channel of an Odyssey Imaging system (Li-Cor, Lincoln, Nebraska).

Synthesis of IR-783-S-Ph-CONH(CH₂)₅NH₂ (NIR813 dye)

NIRF dye containing a primary amine, IR-783-S-Ph-CONH(CH₂)₅NH₂, was synthesized in 3 steps (Fig. 1A). First, IR-783-S-Ph-COOH was synthesized according to the method of Strekowski et al. (16). Briefly, IR-783 (250 mg, 0.33 mmol) and 4-mercaptobenzoic acid were dissolved in 5 mL dimethylformamide (DMF). This solution was stirred overnight at room temperature. After the solvent was removed, the residue was dissolved in methanol and precipitated in ether. The solid was collected by filtration and further purified with flash chromatography using ethyl acetate and methanol as the mobile phase. IR-783-S-Ph-COOH was then conjugated to the *t*-Boc-protected heterodiamine *t*-BocNH(CH₂)₅NH₂ using activated ester. Thus, IR-783-S-Ph-COOH (150 mg, 0.18 mmol) and NHS (22 mg, 0.21 mmol) were dissolved in 5 mL DMF together with 1,3-diisopropylcarbodiimide (31 μL, 0.21 mmol) and 4-dimethylaminopyridine (2.5 mg, 0.02 mmol). The reaction proceeded at room temperature for 4 hr, thereafter the solvent was removed under vacuum and the residue washed with ether. The resulting IR-783-S-Ph-CO-NHS was reacted with BocNH(CH₂)₅NH₂ (42 mg, 0.21 mmol) for 4 hr in 5 mL of DMF containing 5% *N,N*-diisopropylethylamine. The product was then purified with flash chromatography. Finally, the *t*-Boc protection group in IR-783-S-Ph-CONH

(CH₂)₅NHBoc was removed by treatment with 40% TFA in dichloromethane. After solvent removal, the product was purified by precipitation from a methanol solution with ether. IR-783-S-Ph-CONH(CH₂)₅NH₂, was collected by filtration and dried by lyophilization. MALDI (*m/z*) calcd. for C₅₀H₆₅N₄O₇S₃⁺: 929.40; found 929.43 (M⁺). The fluorescence emission maximum for IR-783-S-Ph-CONH(CH₂)₅NH₂ was 813 nm (Fig. 2). Consequently, IR-783-S-Ph-CONH(CH₂)₅NH₂ is termed NIR813 dye throughout the rest of this paper.

Synthesis of PG-DTPA-Gd

PG-*p*-aminobenzyl-DTPA-Gd (PG-DTPA-Gd) was synthesized according to previously reported procedures (17). Briefly, *p*-aminobenzyl-DTPA(*t*-butyl ester) (2.1 g, 2.79 mmol) was conjugated to PG (M_n, 41,400; 1 g, 7.75 mmol of carboxylic unit) in DMF using 1,3-diisopropylcarbodiimide (403 mg, 3.1 mmol) as a coupling agent. The *t*-butyl protecting groups were removed by treatment with TFA at 4°C overnight to yield PG-DTPA. To chelate PG-DTPA with Gd³⁺, a solution of GdCl₃·6H₂O in 0.1 M sodium acetate was added into a solution of PG-DTPA in 0.1 M sodium acetate (pH 5.5) in small fractions. The solution was then dialyzed extensively against water (molecular weight cut-off, 10,000) until no free Gd³⁺ was detected, and lyophilized to yield 1.22 g of white powder (81%). The compound contained 10.8% (w/w) gadolinium.

Synthesis of PG-DTPA-Gd-NIR813

The scheme for the synthesis of PG-DTPA-Gd-NIR813 is shown in Figure 1B. For the synthesis of PG-DTPA-Gd-NIR813 containing 1% (mole/mole repeating unit) dye, NIR813 (4.17 mg, 0.0045 mmol) dissolved in 200 μL of DMF was added to a solution of PG-DTPA-Gd (90 mg, 0.698 mmol Glu) in 0.1 M MES buffer (2 mL) in the presence of EDC (10 mg, 0.005 mmol). The reaction mixture was stirred at 4°C overnight while protected from light, filtered through a 0.2-μm filter, dialyzed against phosphate-buffered saline and water sequentially, and lyophilized. The yield was 64.6 mg (72%). The conjugate contained about 4.4% (w/w) or 1% (mole dye/mole repeating unit) NIR813.

Other PG-DTPA-Gd-NIR813 conjugates with different NIR813 loading were synthesized similarly.

Relaxivity

Solutions of PG-DTPA-Gd-NIR813 were prepared in water at gadolinium concentrations of 0.005, 0.01, 0.02, 0.04, 0.08, and 0.16 mM. Spin lattice (T₁) and spin-spin (T₂) relaxivities were measured at room temperature with a 4.7 Tesla Bruker Biospec small animal MRI system (Bruker Biospin Corp., Billerica, MA) using inversion recovery and multi-echo pulse sequences. Relaxivities (R₁ or R₂ in mM⁻¹s⁻¹) were obtained from linear least square determination of the slopes of 1/T₁ vs [Gd] or 1/T₂ vs [Gd] plots.

Cell line and animals

Human DM14 squamous carcinoma cells were a soft agar clone derived from Tu167 cells (a gift from Dr. Gary Clayman, The University of Texas M. D. Anderson Cancer Center). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and L-glutamine (GIBCO, Grand Island, NY).

Male athymic nude mice were obtained from the National Cancer Institute (Frederick, MD). All animal studies were carried out in accordance with institutional guidelines and approved by animal care and use committee. To produce mice with lymph node metastases, 2 × 10⁵ DM14 cells suspended in 50 μL of Hank's balanced salt solution were injected submucosally

in the anterior portion of tongue of male athymic nude mice ($n = 3$) using a 30-gauge needle attached to a 1-ml tuberculin syringe (Hamilton Co., Reno, NV). A previous study in such mice demonstrated that mice develop metastases in the cervical lymph nodes by 2 weeks after inoculation (18). In the current dual modality imaging study, experiments were conducted on day 15 after tumor cell inoculation.

MR and optical imaging

Prior to imaging, mice were anesthetized with 2% isoflurane gas in oxygen. During imaging, mice were maintained in an anesthetized state with 0.5%–1.5% isoflurane in oxygen. For optical imaging, an IVIS imaging system (200 series) (Xenogen Corp., Alameda, CA) was used with ICG filter sets (excitation/emission, 710–760/810–875 nm). The field of view was 13.1 cm in diameter. The fluency rate for NIRF excitation light was 2 mW/cm². The camera settings included maximum gain, 2×2 binning, 640 × 480 pixel resolution, and an exposure time of 0.8 sec. MR imaging was performed on a 4.7-Tesla Bruker Biospec scanner. Axial and coronal images were obtained using a 950-mT/m, 5.7-cm-inner-diameter actively shielded gradient coil system (19,000 mT/m-s slew rate) and a 3.5-cm-inner-diameter volume radiofrequency coil. T₁-weighted MR images (TE = 8.5 ms, TR = 1000 ms) were acquired with a 4×3 cm field of view, 1-mm section thickness, 0.25-mm gap, and a 256×192 matrix.

Comparison of PG-DTPA-Gd-NIR813 and isosulfan blue dye localization

To compare the localization of PG-DTPA-Gd-NIR813 and isosulfan blue, the gold standard for SLN detection, 6 male athymic nude mice weighing 20–25 g each were injected subcutaneously in the left front paw with 10 µL of PG-DTPA-Gd-NIR813 (0.002 mmol Gd/kg, 4.8 nmol eq. NIR813/mouse) in phosphate-buffered saline. Optical images were obtained before and at 5 min after contrast injection. Then 10 µL of 1% isosulfan blue (17.6 mM) was injected into the same sites where PG-DTPA-Gd-NIR813 had been injected. Animals were killed 1 h later, and the skin was removed in the area where a fluorescence signal had been detected to permit direct visualization of the dye. Nodes that appeared blue-stained under bright light were resected and imaged again with a NIRF camera. These nodes were then processed for histologic examinations.

Dual MR-optical imaging detection of axillary and brachial lymph nodes

Six male athymic nude mice were injected subcutaneously in the left front paw with PG-DTPA-Gd-NIR813 at a dose of 0.02 mmol Gd/kg (48 nmol NIR813/mouse) (3 mice) or 0.002 mmol Gd/kg (4.8 nmol NIR813/mouse) (3 mice). Precontrast images were obtained with both optical and MR imaging. T₁-weighted MR images were acquired every 3 minutes for 30 minutes after contrast injection, after which the mice were imaged again with a NIRF camera. Axillary and brachial lymph nodes were removed under NIRF guidance. The fluorescence intensities of the resected lymph nodes were measured with a Xenogen camera using LIVINGIMAGE v.2.11 software. The data were expressed as flux (photons/s) per lymph node. Student's *t* test was used to compare difference in fluorescence intensity between two groups of mice, with *p* values less than 0.05 considered significant. The resected nodes were processed for histologic examination.

For analysis of MR signal enhancement in the axillary and brachial lymph nodes, the same region of interest, encompassing all of the enhanced axillary lymph nodes, was drawn on each of the consecutive transaxial MR images. Image J software (<http://rsb.info.nih.gov/ij/>) was used to analyze the MR imaging data. Signal-to-noise ratio (SNR) was calculated for each time point by dividing the average signal intensity of the region of interest of each lymph node by the root mean square of the background. The relative increase in SNR (SNR%), calculated according to the formula $\text{SNR}\% = (\text{SNR}_{\text{post}} - \text{SNR}_{\text{pre}}) / \text{SNR}_{\text{pre}} \times 100\%$, was plotted as a

function of time. SNR% value at each time point was compared between the two dose groups using the Student's *t* test with $p < 0.05$ considered significant.

Identification of cervical lymph nodes and detection of metastases following imaging-guided nodal resection

Three male athymic nude mice bearing human DM14 squamous cell carcinoma were injected interstitially around the primary tumor with PG-DTPA-Gd-NIR813 at a dose of 0.02 mmol Gd/kg (48 mmol NIR813/mouse). Each mouse was examined by optical and MR imaging before contrast injection. Optical images were acquired at 10 min, 30 min, 2 h, 4 h, 6 h, and 24 h after contrast injection. T₁-weighted MR images were then acquired at 2 h and 24 h after contrast injection. At the end of the last imaging session, NIRF imaging-guided resection of SLNs was performed, and the fluorescence intensities of the resected lymph nodes were measured as described before. The nodes were further processed for histologic examination. Student's *t* test was used to compare difference in fluorescence intensity between normal mice and tumor-bearing mice with $p < 0.05$ considered significant.

For histopathologic examination, nodal tissues were embedded in Optimal Cutting Temperature compound (Sakura Finetek USA, Torrance, CA), snap-frozen, cryosectioned into 10- μ m slices, and stained with hematoxylin and eosin (H&E). Consecutive unstained sections were photographed on a Leica fluorescence microscope (Leica Microsystems, Bannockburn, IL). The microscope was equipped with a 75-W Xenon lamp, differential interference contrast optical components, 775/845 nm (excitation/emission) filter sets (Chroma Technology, Brattleboro, VT), a Hamamatsu black and white chilled charge-coupled device camera (Hamamatsu Photonics K.K., Hamamatsu City, Japan), and Image-Pro Plus 4.5.1 software (Media Cybernetics, Silver Spring, MD).

Results

Synthesis and Characterization of PG-DTPA-Gd-NIR813

There were approximately 3 NIR813 dyes per polymer chain for PG-DTPA-Gd-NIR813 containing 1% NIR813 dye (mole/mole repeating unit). The fluorescence intensity of each PG-DTPA-Gd-NIR813 conjugate (1% mole/mole) was approximately 32% stronger than that of each NIR813 molecule. Increasing the loading of NIR813 dye caused gradual decrease in fluorescence intensity of the polymeric conjugate (data not shown). Almost complete quenching of fluorescence signal was observed when more than 15 dye molecules were conjugated to each PG chain (9% mole/mole repeating unit) (Figure 2A). Therefore, PG-DTPA-Gd-NIR813 containing an average of 3 NIR813 molecules per polymer chain (1%) was selected for further studies.

The physicochemical properties of PG-DTPA-Gd and PG-DTPA-Gd-NIR813 are summarized in Table 1. By GPC analysis, PG-DTPA-Gd-NIR813 had a hydrodynamic diameter of 46 ± 6 nm, whereas the hydrodynamic diameter of PG-DTPA-Gd was 11 ± 0.4 nm. For comparison, the theoretical number-average molecular weight calculated on the basis of the starting molecular weight of PG is also listed in Table 1. Conjugation of NIR813 to PG-DTPA-Gd resulted in a significant increase in the apparent size of the resulting polymeric conjugate, which could not be accounted for by the addition of NIR813 to PG-DTPA-Gd alone. This result suggests the presence of intermolecular hydrophobic interaction among polymer chains. About 51 of the 274 glutamic acid units per PG chain were attached to DTPA-Gd.

The excitation/emission wavelengths in methanol solution were 766/798 nm for IR783 and 766/813 nm for NIR813. Therefore, NIR813 had a greater Stokes shift (47 nm) as compared to that of IR783 (32 nm). A comparison of fluorescence emission spectra of NIR813 and PG-

DTPA-Gd-NIR813 acquired at the same equivalent dye concentration is presented in Figure 2B. Both compounds had an emission maximum of 813 nm when excited at 766 nm. However, the fluorescence intensity of PG-DTPA-Gd-NIR813 on the basis of equivalent NIR813 dye concentration was reduced to approximately 44% of that of unconjugated NIR813, which suggests the presence of intramolecular interaction among NIR813 dyes attached to PG in aqueous solution.

Co-localization of PG-DTPA-Gd-NIR813 with isosulfan blue dye

When PG-DTPA-Gd-NIR813 was injected subcutaneously into the front paw of a mouse, it entered the lymphatics and migrated to the axillary and brachial lymph nodes within 5 minutes. Isosulfan blue injected at the same site colocalized with the NIR fluorescence signal ($n = 6$, Fig. 3A–3D). These brightly fluorescent areas were resected and proved on histologic examination to be lymph nodes. No residual fluorescence signal was observed in the surrounding areas. Analysis of resected fluorescent tissues demonstrated that PG-DTPA-Gd-NIR813 was completely trapped in the lymph nodes and was not present in the surrounding tissues (Fig. 3E–3H).

Dual MR-optical imaging detection of axillary and brachial lymph nodes

To examine whether axillary and brachial lymph nodes could be detected by both MR and NIRF optical imaging, mice were given a single subcutaneous injection of PG-DTPA-Gd-NIR813 at a dose of 0.02 mmol Gd/kg or 0.002 mmol Gd/kg. On NIRF imaging, at both dose levels, the axillary and brachial nodes were readily visualized (Fig. 4A–4D). At 24 h time point, the fluorescence intensity of the resected lymph nodes were 7.73×10^9 and 16.2×10^9 photons/sec at the injected dose of 0.002 mmol Gd/kg and 0.02 mmol Gd/kg, respectively ($p = 0.005$). On MR imaging, at the higher dose level, both axillary and brachial nodes were already identifiable at 3 min after contrast injection (Fig. 4E). However, at the lower dose level, only the axillary nodes could be visualized (Fig. 4F). Percent increase in MR signal intensity for the axillary nodes was dose-dependent; signal intensities at a dose of 0.02 mmol Gd/kg were significantly higher than those at a dose of 0.002 mmol Gd/kg from 6 min after injection and throughout the 30-min study period ($p < 0.05$, Fig. 5).

Dual MR-optical imaging detection of cervical lymph nodes containing metastases

Next, we investigated whether dual MR-optical imaging could also be used to detect metastatic SLNs preoperatively and postoperatively (following imaging-guided resection) in an orthotopic head and neck tumor xenograft model. In mice without tumors, both MR and NIRF imaging readily detected uptake of PG-DTPA-Gd-NIR813 in the superficial cervical lymph nodes after interstitial injection into the tongue at a dose of 0.02 mmol Gd/kg (Fig. 6A–6D). In each of the 3 mice with orthotopic human DM14 squamous cell carcinoma tumors, both superficial cervical nodes were visualized using NIRF imaging (Fig. 6E–6H). However, 2 of the 6 superficial cervical nodes visualized with NIRF were not visualized with MR imaging. The pattern of enhancement of the 4 nodes that were revealed by MR imaging was different from that observed in normal cervical tissue: in general, the lymph nodes showed less enhancement, and the enhancement was located at the rim of the lymph node (compare Fig. 6A and 6E). Histopathologic examination confirmed metastases in all 6 draining lymph nodes visualized with NIRF imaging (Fig. 6J). Interestingly, micrometastases were noted in the lumen of a vascular structure in the tongue of one of the tumor-bearing mice (Fig. 6K). No statistical significant difference was found in fluorescence intensity of the resected lymph nodes between normal mice and tumor-bearing mice.

Discussion

The present study demonstrates that dual MR-optical imaging using a water-soluble polymeric carrier may facilitate pre- and intraoperative detection of SLNs. This approach combines the merits of MR and optical imaging and permits acquisition of complementary information after administration of a single imaging agent. In theory, similar information could be obtained by co-injection of two separate macromolecular imaging agents. However, such an approach is less favorable because the two agents may not necessarily possess the same pharmacologic and pharmacokinetic behaviors. In addition, the same set of experiments would have to be performed to validate the use of each of the two monofunctional agent. In cases where only superficial or only deep nodes were to be imaged and only one modality is suffice, it is still feasible to use dual-labeled imaging agent, provided that it exhibits a similar toxicity profile as its corresponding monofunctional imaging agents.

The findings of our study suggest that MR imaging may facilitate preoperative identification of lymph node metastases. Recently, new MR imaging agents consisting of polyamidoamine dendrimers have been developed for preoperative characterization of lymphatic drainage and lymph node metastases from mammary tumors (8,9). These studies demonstrated that the superior temporal and spatial resolution of micro-MR imaging facilitates the identification of lymphatic metastasis in experimental animals. In the present study, T₁-weighted MR images obtained after injection of a dual-modality contrast agent in mice with lymph node metastases allowed differentiation of normal and metastatic nodes. The observed differences in pattern of lymph node enhancement may be due to differences in macrophage uptake of macromolecular contrast agents between normal and metastatic lymph nodes, as has been shown to be the case for superparamagnetic iron oxide nanoparticles (USPIO) (19–21). In the case of USPIO, the contrast agent was injected intravenously, and the metastatic lesions in the lymph nodes were detected as a result of the negative contrast of the surrounding normal nodal tissue. Another possibility is the blockade of lymphatic drainage by in-transit micrometastases, which may restrict delivery of the contrast agent to lymph nodes of tumor-bearing mice. Such in-transit micrometastases were observed in the current study in the lumen of a lymphatic vessel in a specimen from a mouse with a DM14 tongue tumor. Finally, non-uniform distribution in the nodal sinus system may also explain the patterns of contrast uptake in the lymph nodes. Clearly, because of limited number of animals used in this study, additional studies are necessary to investigate the differential MR imaging pattern between normal and metastatic lymph nodes, and the role of macrophage uptake and/or in-transit micrometastases in contrast enhancement of SLNs after interstitial injection of PG-DTPA-Gd-NIR813.

Compared to MRI, NIRF imaging has much higher sensitivity for the detection of lymph nodes. At an injected dose of 0.02 mmol Gd/kg, we were able to detect the same sets of lymph nodes as soon as 3 min after the injection of PG-DTPA-Gd-NIR813 with both MR imaging and NIRF optical imaging. However, at a reduced dose of 0.002 mmol Gd/kg, MR imaging was capable of detecting only axillary but not brachial lymph nodes, both of which could be reliably visualized with NIRF imaging. Moreover, NIRF imaging allowed detection of all 6 cervical lymph nodes containing micrometastases in mice with DM14 squamous carcinoma at all time points between 10 min and 24 h after contrast injection. In contrast, MR imaging revealed contrast enhancement only in 4 out of the 6 lymph nodes. These findings are consistent with lower detection sensitivity with MR imaging compared to NIRF imaging. For this reason and because of the technical difficulties associated with intraoperative use of MR imaging, we believe NIRF optical imaging is a more suitable imaging modality for guiding the intraoperative exploration and resection of SLNs.

In general, lymphangiographic agents for SLN mapping should be large enough to be confined in the lymphatic ducts but small enough to remain mobile for rapid transit within the lymphatic

tract. Contrast agents with a hydrodynamic diameter of 5–40 nm are thought to be suitable for SLN imaging (11,22). The hydrodynamic volume of PG-DTPA-Gd-NIR813 is 46 ± 6 nm. Therefore, PG-DTPA-Gd-NIR813 satisfies the size criteria as an imaging agent for SLN detection. In addition to size consideration, it is also desirable to obtain a biocompatible contrast agent that can be metabolized and eventually cleared from the body. The polymeric carrier for PG-DTPA-Gd-NIR813 is a biodegradable polymer, which has demonstrated excellent biocompatibility. In various studies in rodents, PG was used at doses from 200 to 800 mg/kg without causing apparent toxic effects after intravenous injection (23). In fact, polymeric anticancer agents based on PG have advanced into clinical trial studies. Because a large fraction of PG-DTPA-Gd-NIR813 injected interstitially would eventually be removed by lymph nodes with little if there is any entering systemic circulation, we expect that this agent should have acceptable toxicity profile in SLN mapping at doses that are 10- to 100-fold less than the dose of conventional MR imaging contrast agent used clinically for intravenous injection.

Using PG-DTPA-Gd-NIR813 and dual-modality imaging approach, the following hypothetical clinical scenario may be envisioned. First, MR imaging will be used for noninvasive detection of lymph node metastases. If the presence of lymph node metastases is confirmed unequivocally with MR imaging alone, surgery to remove the whole nodal basin should be performed, thus eliminating the SLN biopsy step and the associated waiting period. If MR imaging does not unequivocally confirm metastases, SLN mapping and subsequent SLN biopsy will be performed using pre- and intraoperative NIRF imaging. This should permit intraoperative dissection without the use of an ionizing radiotracer and cumbersome interventional MRI. Because of high detection sensitivity, NIRF imaging could also be used for inspection of the surgical site to ensure complete removal of SLNs.

It is anticipated that with respect to nodal staging of cancer patients, a systemic administration rather than interstitial injection would be more desirable. Therefore, future studies will include the evaluation of the biodistribution and lymph node uptake of PG-Gd-NIR813 after intravenous injection. In addition, an ideal regional sentinel node agent would localize only to first order nodes thereby minimizing ambiguity in sentinel node detection. A pass-through effect in first order nodes would cause enhancement of more distant nodes in the lymphatic chain. Additional studies are thus needed to determine the extent of PG-Gd-NIR813 distribution over time. Such data would help define an optimal imaging window to minimize regional contrast uptake by higher order nodes. Increasing the molecular weight of PG-Gd-NIR813 can be another effective method to reduce the pass-through effect.

In summary, we have developed a dual-functional macromolecular contrast agent suitable for both MR and NIRF optical imaging of SLNs. Such an agent may be useful not only for precise localization of SLNs and preoperative characterization of lymph node abnormalities using MR imaging but also for the SLN mapping and monitoring the success of complete resection of SLNs during surgery.

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Figure 1.
Reaction schemes for the synthesis of (A) NIR813 and (B) PG-DTPA-Gd-NIR813.

Figure 2.

(A) Effect of NIR813 loading (mole/mole) on the fluorescence intensity of PG-DTPA-Gd-NIR813 at 10% Gd (w/w) loading. Measurements were made in a sodium acetate buffered solution (pH 5.5) at an equivalent NIR813 concentration of 10 μM. Inset: NIRF images of PG-DTPA-Gd-NIR813 solutions in 96-microwell plate from which the fluorescence intensity was quantified. (B) Fluorescence emission spectra of NIR813 (1 μM, in methanol) and PG-DTPA-Gd-NIR813 (1 μM equivalent NIR813, in water). The solutions were excited at 766 nm. AU: arbitrary unit.

Figure 3.

Localization of PG-DTPA-Gd-NIR813 and isosulfan blue dye in healthy male athymic nude mice. (A–D) NIRF images demonstrating colocalization of PG-DTPA-Gd-NIR813 and isosulfan blue dye in axillary and brachial lymph nodes. Each mouse was injected subcutaneously with PG-DTPA-Gd-NIR813 (10 μ L, 4.8 nmol eq. NIR813/mouse) in the left front paw. (A) Pre-contrast-injection overlay of white light and NIRF images. (B) Overlay of white light and NIRF images 5 min after contrast injection. The arrows indicate the putative SLNs. (C) Photograph of the mouse shown in B showing the same lymph nodes (arrows) stained blue by isosulfan blue. (D) Resected lymph nodes showed bright fluorescence signal. No residual fluorescence was found in the area surrounding the resected nodes. (E–H) Microphotographs of a representative resected lymph node confirming the uptake of PG-DTPA-Gd-NIR813 in the node. (E) H&E-stained tissue section. (F) Differential interference contrast (DIC) image. (G) NIRF image. (H) Overlay of the DIC and NIRF images. NIRF signal is pseudocolored green, and DIC signal is pseudocolored red. Original magnification: 50 $\times$.

Figure 4. Dual MR-optical imaging of the axillary and brachial lymph nodes in healthy male athymic nude mice. (A–D) NIRF images. (A) Pre-contrast-injection overlay of white light and NIRF images. (B) Overlay of white light and NIRF images 1 hr after injection of PG-DTPA-Gd-NIR813 (0.002 mmol Gd/kg). (C) NIRF image of the mouse shown in B without skin. (D) Resected lymph nodes showed bright fluorescence signal. (E–F) Representative T_1 -weighted axial MR images obtained at different times after injection of PG-DTPA-Gd-NIR813 at a dose of 0.02 mmol Gd/kg (E) or 0.002 mmol Gd/kg (F). The arrows indicate lymph nodes.

Figure 5. Time course of lymph node enhancement after injection of PG-DTPA-Gd-NIR813 at doses of 0.02 mmol Gd/kg and 0.002 mmol Gd/kg in healthy male athymic nude mice. MR signal intensity increased with time in a dose-dependent manner. All data were expressed as mean $\pm$ SD.

Figure 6.

Visualization of cervical lymph nodes after interstitial injection of PG-DTPA-Gd-NIR813 (0.02 mmol Gd/kg) into the tongue of a normal mouse (A–D) and a mouse with a human DM14 squamous cell carcinoma in the tongue (E–H). (A, E): T₁-weighted coronal image acquired 2 hr after contrast injection. (B, F): Overlay of white light and NIRF images 24 hr after contrast injection. (C, G) NIRF images of mice without skin. (D, H) NIRF images of resected lymph nodes. The white arrows indicate SLNs, and red arrow indicates the primary tumor. (I–K) Microphotographs of H&E-stained sections of (I) normal lymph node, (J) metastatic lymph node, and (K) tumor-bearing tongue indicating the presence of micrometastases, presumably in-transit metastases in a lymphatic duct. Note the difference in the pattern of contrast distribution in the lymph nodes between normal and tumor-bearing mice in MR images.

Table 1

Physicochemical properties of PG-DTPA-Gd and PG-DTPA-Gd-NIR813.

Property	PG-DTPA-Gd	PG-DTPA-Gd-NIR813
Molecular weight ^a (number of repeating units)	60,080 (274)	62,813 (274)
Hydrodynamic volume (nm) ^b	11±0.4	46±6
% Gd (w/w) ^c	10.83	10.40
Number of DTPA per PG	51	51
% NIR813 (w/w) ^d	-	4.4
Number of NIR813 per 100	-	1
Repeating Units in PG		
Relaxivity (R1 mmol ⁻¹ s ⁻¹)	8.2	8.6
(R2 mmol ⁻¹ s ⁻¹)	23.1	25.1

^a Number-average molecular weight calculated on the basis of starting molecular weight (42,100 Da) and the percentage of substitution.

^b Hydrodynamic volume (nm in diameter) measured with gel permeation chromatography.

^c Percentage of Gd by weight was measured with elemental analysis.

^d Percentage of NIR813 was measured spectrophotometrically.