MRI contrast agent for targeting glioma: interleukin-13 labeled liposome encapsulating gadolinium-DTPA

Xiaoli Liu, Achuthamangalam B. Madhankumar, Patti A. Miller, Kari A. Duck, Susan Hafenstein, Elias Rizk, Becky Slagle-Webb, Jonas M. Sheehan, James R. Connor, and Qing X. Yang

Department of Neurosurgery, Pennsylvania State University College of Medicine, Hershey, Pennsylvania (X.L., A.B.M., K.A.D., E.R., B.S.-W., J.M.S., J.R.C., Q.X.Y.); Department of Radiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania (X.L., P.A.M., Q.X.Y.); Department of Microbiology, Pennsylvania State University College of Medicine, Hershey, Pennsylvania (S.H.)

Corresponding Author: Qing X. Yang, PhD, Department of Radiology, 500 University Drive, H066, Hershey, PA, 16802 (qyang@hmc.psu.edu).

Background. Detection of glioma with MRI contrast agent is limited to cases in which the blood-brain barrier (BBB) is compromised as contrast agents cannot cross the BBB. Thus, an early-stage infiltrating tumor is not detectable. Interleukin-13 receptor alpha 2 (IL-13Rα2), which has been shown to be overexpressed in glioma, can be used as a target moiety. We hypothesized that liposomes conjugated with IL-13 and encapsulating MRI contrast agent are capable of passing through an intact BBB and producing MRI contrast with greater sensitivity.

Methods. The targeted MRI contrast agent was created by encapsulating Magnevist (Gd-DTPA) into liposomes conjugated with IL-13 and characterized by particle size distribution, cytotoxicity, and MRI relaxivity. MR image intensity was evaluated in the brain in normal mice post injection of Gd-DTPA and IL-13-liposome-Gd-DTPA one day apart. The specificity for glioma detection by IL-13-liposome-Gd-DTPA was demonstrated in an intracranial glioma mouse model and validated histologically.

Results. The average size of IL-13-liposome-Gd-DTPA was 137 ± 43 nm with relaxivity of 4.0 ± 0.4 L/mmole-s at 7 Tesla. No significant cytotoxicity was observed with MTS assay and serum chemistry in mice. The MRI signal intensity was enhanced up to 15% post injection of IL-13-liposome-Gd-DTPA in normal brain tissue following a similar time course as that for the pituitary gland outside of the BBB. MRI enhanced by IL-13-liposome-Gd-DTPA detected small tumor masses in addition to those seen with Magnevist-enhanced MRI.

Conclusions. IL-13-liposome-Gd-DTPA is able to pass through the uncompromised BBB and detect an early stage glioma that cannot be seen with conventional contrast-enhanced MRI.

Keywords: glioma, liposome, IL-13, blood-brain barrier (BBB), targeted MRI contrast agent.
Materials and Methods

All animal experimental procedures were carried out in accordance with institutional guidelines and approved by the Animal Care and Use Committee of the Pennsylvania State University College of Medicine.
and 120 minutes and 24 hours. The signal-to-noise ratios (SNRs), graphed as time course against SNR of preinjection and postinjection images were evaluated from 2 regions of interest: (i) the posterior pituitary gland where the BBB is absent as an internal reference and (ii) the brain parenchyma on the same image slice.

**Glioma Mouse Model and Histological Chemistry**

For testing IL-13-Lip-Gd-DTPA as a targeted contrast agent for glioma, the intracranial tumor model was prepared as previously described. The tumor formation and volume were monitored weekly with Magnevist-enhanced T1-weighted MRI starting at 2 weeks post tumor induction. When a tumor reached a size of 5–10 mm in diameter, the mice were imaged with the T1-weighted scan after injection of Magnevist as a control and FITC-labeled liposome (IL-13-Lip(FITC)-Gd-DTPA). Then, the mice were euthanized at ~2 hours post injection of IL-13-Lip(FITC)-Gd-DTPA, and the brains were harvested and frozen immediately at −20°C. The brain tissue was sectioned at a 12 μm thickness at −20°C and mounted on slides. The selected neighboring brain tissue slides were stained separately with hematoxylin and eosin (H&E) for tumor tissue identification, or DAPI for FITC-labeled liposomes accumulation in the glioma or with immunostaining for observation of IL-13Rα2 differential expressions between glioma and adjacent normal tissue following the standard protocol (See details in Supplementary Materials).

**Results**

**Characterization of IL-13-Lip-Gd-DTPA**

The average size of IL-13-Lip-Gd-DTPA was 137 ± 43 nm, which was consistent for four 4°C different batches. Fig. 1A shows an example of the size distribution, with a polydispersity index of 0.1–0.2 nm indicating that the size of the liposome was uniformly produced. The TEM image in Fig. 1B provides visualization of the spherical morphology of IL-13-Lip-Gd-DTPA. The loading efficiency of gadolinium in IL-13-Lip was within the range of 4.0–10.0 mg/L determined by measuring concentration of gadolinium in IL-13-Lip-Gd-DTPA using ICP-AES. The
conjugation of IL-13 with the liposomes was confirmed through immunoblotting analysis as shown in Lane 2 in Fig. 1C. The dark band in lanes 1, 2, and 3 represent the original recombinant IL-13 protein, its conjugated complex with liposomes, and the intermediate species (2-Iminothiolane), respectively, indicating the existence of IL-13 in each form. The Western immunoblotting analysis shown in Fig. 1D revealed that the IL-13Rα2 Ab was overexpressed in glioma U251 and T3691 cells, as anticipated.

**IL-13-Lip-Gd-DTPA Toxicity Assessment**

The cytotoxicity of the liposomes was not observed in either the in-vitro MTS assay or the in-vivo serum chemistry data. As shown in Fig. 2A and B, U251 and T3691 cell exposure to Gd concentrations in liposomes from 15 μg/mL – 60 μg/mL did not produce a significant difference in cell cytotoxicity when compared with the controls using MTS assays. In addition, the blood serum chemistry study between the control (injection of Magnevist) and the injection of IL-13-Lip-Gd-DTPA into mice did not show any significant toxicity in terms of blood urea nitrogen mg/dL, total bilirubin mg/dL, creatinine mg/dL (Fig. 2C), AST U/L, and ALT U/L (Fig. 2D). However, alkaline phosphatase U/L, appeared to be lowered in the mice treated with liposomes, which was likely caused by the minor perturbations of nutritional uptake.

**IL-13-Lip-Gd-DTPA Uptake**

Figure 3 shows confocal microscopy images captured after exposure of FITC labelled IL-13-Lip-Gd-DTPA to the cell cultures. The FITC green color in Fig. 3A–C indicated the presence of liposomes in the cytosol of glioma U251, U87, and T3691 cells, respectively, thus demonstrating the uptake of IL-13-Lip-Gd-DTPA by all 3 cell lines.

**IL-13-Lip-Gd-DTPA Crossing BBB in-vitro Model**

Figure 4 shows the Gd concentration transported from the apical to the basal chamber of the BBB in-vitro model at 2, 4, and 24 hours. At 2 hours, the Gd concentration of free Gd-DTPA and IL-13-Lip-Gd-DTPA did not show a marked difference. But at 4 hours, the Gd concentration from the IL-13-Lip-Gd-DTPA was 6000 μg/L, which is 3-fold greater than that from free Gd-DTPA. This difference in Gd concentration in the basal chamber suggested that IL-13-Lip-Gd-DTPA facilitated Gd crossing the BBB in the in-vitro model. Rhodamine-labeled dextran,
present on the apical chamber of the model, served as an indicator of the integrity of the tight junctions and was not observed in the basal chambers.27

**IL-13-Lip-Gd-DTPA Crossing BBB in Normal Mice**

The encapsulation of contrast agent into the liposomes made it possible to assess the ability of liposomes to cross the intact BBB in vivo by MRI by injection of IL-13-Lip-Gd-DTPA into normal mice. For histological analysis, the liposomes used in this study were labeled with FITC. As illustrated in Fig. 5A, the signal changes in the pituitary gland (blue trapezoid) and the brain tissues (red ellipse) were measured on the T1-weighted MRI of the same slice post injection of either Magnevist or IL-13-Lip-Gd-DTPA. The signal changes of the pituitary gland were used to make certain that the contrast agents IL-13-Lip-Gd-DTPA (Fig. 5A-a–e) or Magnevist (Fig. 5A-c–f) entered the blood stream. Since the pituitary gland does not have a BBB, the signal increased for about a 2 hour period in the pituitary gland for both cases, indicating that the liposomes encapsulated with Gd-DTPA were capable of inducing similar MRI contrast as Magnevist. Accordingly, the signal change in brain parenchyma provided an indication whether IL-13-Lip-Gd-DTPA or Magnevist crossed the intact BBB. As shown in the time courses in Fig. 5A-a–b, the signal of brain parenchyma followed similar temporal characteristics as that of the pituitary gland, with an increase up to 15% at 30 minutes post injection of IL-13-Lip-Gd-DTPA. As anticipated with injection of Magnevist, the signal from brain parenchyma shown in Fig. 5A-c–d remained unchanged because the Magnevist was blocked by the intact BBB. These data provided the first direct in-vivo evidence that IL-13-Lip-Gd-DTPA penetrated an intact BBB and entered the brain parenchyma of a living mouse.

To validate the in-vivo findings, histology was performed on the animals. Fig. 5B shows confocal microscopy images from a brain tissue section of a mouse after injection of FITC labeled IL-13-Lip-Gd-DTPA Fig. 5B-(a–f) and a control Fig. 5B-(g–i), respectively. The confocal images confirmed the in-vivo results with a widespread FITC distribution in the brain tissue compared with the complete absence of FITC in brain tissues without injection of IL-13-Lip(FITC)-Gd-DTPA. It is also important to note that the FITC-labeled liposomes were sparsely distributed. The magnified image (63X) in Fig. 5B-e indicated that the liposomes remaining in the brain were intracellular. The confocal microscopy images in Fig. 5C showed DAPI, FITC-liposomes, Ab GFAP-rhodamines, and co-localization of IL-13-Lip(FITC)-Gd-DTPA in a, b, c and d, respectively, which also confirmed that IL-13-Lip(FITC)-Gd-DTPA entered the brain tissue and co-localized with one of the astrocytes markers, GFAP.

**Detection of Glioma by IL-13-Lip-Gd-DTPA**

To further test the functionality of IL-13-Lip-Gd-DTPA as an MRI contrast agent for the detection of glioma, Fig. 6A shows an example of T1-weighted MR images acquired at the identical location from the same mouse with an intracranial tumor after

---

**Fig. 3.** Confocal microscopy images for IL-13-Lip-Gd-DTPA uptake in cell culture, (A) U251, (B) U87, and (C) T3691, respectively. Blue color indicates the nucleus stained with DAPI, and green color indicates FITC-labeled liposomes. The overlay for the 2 images in the left column shows the internalization of the FITC-labeled IL-13-Lip-Gd-DTPA in glioma tumor cell lines.

**Fig. 4.** Concentrations of Gd at the bottom well of the in-vitro blood-brain barrier (BBB) model at 2, 4, and 24 hour time points. The concentration of Gd is doubled at 4 and 24 hours post exposure of IL-13-Lip-Gd-DTPA compared with Magnevist. Significant differences were found for all 3 time points (*P < .05, Student t test).
receiving the Magnevist (Fig. 6A-a, c) and IL-13-Lip-Gd-DTPA injections 24 hours apart (Fig. 6A-b, d). As seen in the MR images from the primary tumor site, IL-13-Lip-Gd-DTPA produced similar contrast as Magnevist (white arrows) and delineated the tumor masses. However, there were additional small tumor masses that could be identified by IL-13-Lip-Gd-DTPA proximal to the primary tumor site (red arrows in Fig. 6A-b). Similarly, as indicated in Fig. 6A-d, IL-13-Lip-Gd-DTPA detected several additional tumor masses, which Magnevist failed to delineate, in the brain slice about 2 mm posterior to the primary site in Fig. 6A-c. This finding was confirmed by histology of the same tissue slice shown in Fig. 6B. As indicated in the zoomed-in image of H&E stain in Fig. 6B-e, f, a tumor mass (dark purple color) was positively identified in the same location as in the MRI image in Fig. 6A-d. In this tumor site, high accumulation of FITC-labeled liposomes were observed in Fig. 6B-g, indicating the effective targeting of the liposome. Accordingly, the confocal immunohistochemistry image in Fig. 6B-h demonstrated IL-13Rα2 differential expressions in glioma and the adjacent normal tissues.

**Discussion**

We presented novel in-vivo data demonstrating that we have developed a nanotechnology platform capable of passing through the BBB and delivering MRI contrast directly to brain tumors. The BBB is the major obstacle in delivering chemotherapeutic agents to the brain. Current techniques for enhancing penetration of the BBB can be classified into 2 categories: (i) bypassing the BBB through convection-enhanced delivery and focused ultrasound-mediated delivery. These kinds of approaches can be effective for delivering drugs to...
known cancer sites, but convection-enhanced delivery is invasive, and ultrasound-mediated delivery requires the stratum corneum to be intact and is time-consuming to administer, all of which offer little help for cancer detection. (ii) BBB non-disruptive approaches such as receptor-mediated delivery with liposomes or other delivery vehicles. Surface-pegylated liposomes are often used for this purpose because pegylation improves liposome stability in the blood stream and subsequently enhances ability to deliver agents.38,39 Such liposomes, especially those of a smaller size (eg, 50–200 nm), are believed to be able to extravasate the relatively permeable vasculature of tumors and promote efficient accumulation in the tumor interstitium (a phenomenon known as the enhanced permeability and retention (EPR) effect). However, there had been no direct experimental evidence and method to assess such ability in vivo. By encapsulating MRI contrast agent into our liposome, the penetration of an intact BBB by the liposome is clearly indicated in vivo in normal mice. Furthermore, our liposomal approach provides a possibility for developing our nanotechnology into a theranostic delivery system by combining chemotherapeutic agents or molecular targets with the MRI contrast agent.

Previous in-vivo data demonstrating that the liposome was capable of penetrating the BBB were based on indirect measurements showing higher drug efficacy with the liposome in an intracranial tumor model.27 Since the BBBs of the intracranial tumor models are known to be compromised, the data obtained with tumor models are deemed to be inconclusive on the ability of the liposome to cross the BBB. In our data shown in Fig. 5A-a, the MRI signal was enhanced up to 15% in the brain tissue with IL-13-Lip-Gd-DTPA in normal mice without a tumor. The MRI signal enhancement in the brain followed the same temporal characteristics as the pituitary gland, which served as an intracranial reference outside the BBB. In addition, the in-vivo data were validated histologically from the same group of mice by fluorescently labeling the FITC. A certain amount of FITC was found to be sparsely distributed in the brain tissues in all animals treated with FITC-labeled liposome as shown in Fig. 5B-c. Co-localization of FITC-labeled IL-13-Lip(FITC)-Gd-DTPA with astrocyte marker GFAP is illustrated in Fig. 5C-d. IL-13-Lip(FITC)-Gd-DTPA entering the normal brain tissue. Taken together, our experimental results provided concrete evidence that IL-13-liposome facilitates Gd-DTPA passing through an uncompromised BBB.

Comparing the time course from in-vitro model, our in-vivo data indicated that the in-vitro model may not be suitable for the study of the kinetics of this process as it uses only gravitational force in thermal equilibrium to increase interactions with the endothelial cell layer, while the blood flow, blood pressure, vessel geometry, and morphologies for the in-vivo condition are all involved in the liposomal transport into the BBB. In future studies, it will be necessary to acquire quantitative data on IL-13-Lip-mediated transport of gadolinium through the BBB in order to understand the underlying mechanism and establish a kinetic model.

The average size and size distribution of the liposomes are important factors for their ability to cross the BBB and for systematic cytotoxicity.40 Thus, the size distribution and the lipid composition of the liposomes have been previously studied intensively.14–16,41 The average size of our liposome was 130 nm distributed within a narrow range of 40–300 nm (Fig. 1). Our liposome was composed of polyethylene glycol (PEG), which permitted the liposome to escape the immune system responses and increase its circulating time.42 With this formulation, no
detectable cytotoxicity was observed in either cell culture or serum blood chemistry.

As we discussed in the introduction, our liposome facilitates the MRI contrast agent passing through the BBB, but it is not sufficient if the contrast agent is not differentially accumulated in the tumor. As shown in the brain image of a normal mouse in Fig. 5A-a, the MRI signal from the brain parenchyma increased uniformly at the same time after injection of our IL-13-Lip-Gd-DTPA and yielded no visible image contrast change. We knew that the measurements of brain Gd-DTPA tissue concentrations using MRI and ICP-AES demonstrated a high degree of coincidence. Thus, in order to highlight tumors, a contrast agent must be able to preferentially target the tissue of interest. As shown by the comparison in Fig. 6A-a and Ab, IL-13-Lip-Gd-DTPA generated similar contrast as Magnevist. In this case, both contrast agents could perfuse into the brain tissue near the tumor site where the BBB was compromised. A closer look at the images in Fig. 6A-b and -d reveals several additional enhanced areas surrounding the primary tumor site. The areas enhanced only by IL-13-Lip-Gd-DTPA were infiltrating tumors. The advantage of our targeted contrast agent is undoubtedly demonstrated in Fig. 6A-b and A-d, where the tumor masses are small, distal to the primary site, and can be identified only with our liposomal contrast agent. As seen in Fig. 6B, the infiltrating tumor identified by our contrast agent presented the typical histological characteristics of GBM. In this case, the tumor masses had not caused significant damage to the BBB such that it could not be detected with Magnevist. Fig. 6A-c. The large amount of FITC found in this tumor site in Fig. 6B-g is additional confirmation that the contrast in the tumor produced by our agent was a result of its ability to pass through the BBB and preferentially deliver the MRI contrast agent to the glioma tissues. In addition, those tumor masses enhanced only by IL-13-Lip-Gd-DTPA preserved signal enhancement 24 hours post injection. The sustained signal enhancement by our targeted liposome in the infiltrating tumor sites behind the intact BBB was possible only if the contrast agent was in the intracellular space. This is consistent with the previous studies. In these studies, the targeting ability for glioma was determined indirectly by assessing its efficacy in inhibiting the tumor growth.26

Conclusion
We have developed and characterized IL-13-Lip-Gd-DTPA. We demonstrated in vivo that our agent is capable of delivering Gd-DTPA through the intact BBB in normal mice and preferentially targeting the glioma tissues in an intracranial mouse model. The in-vivo data were validated with postmortem histology from the same animal. The significance of this research is addressing an unmet need in diagnosis and treatment of glioma by developing a novel MRI contrast agent that directly targets specific receptors on the neoplastic cells, thereby enhancing uptake of the MRI contrast locally in the tumor tissues with greater specificity and sensitivity. Our approach provides feasibility for future development of a theranostic agent targeting glioma.

Supplementary Material
Supplementary material is available online at Neuro-Oncology (http://neuro-oncology.oxfordjournals.org/).

Funding
None declared.

Conflict of interest statement. There is no conflict of interest for any of the authors.

References


