Dynamic Micro-Magnetic Resonance Imaging of Liver Micrometastasis in Mice with a Novel Liver Macromolecular Magnetic Resonance Contrast Agent DAB-Am64-(1B4M-Gd)$_{64}$

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**Abstract**

DAB-Am64-(1B4M-Gd)$_{64}$ is a newly synthesized macromolecular liver magnetic resonance imaging (MRI) contrast agent with a polypropyleneimine diaminobutane (DAB) dendrimer conjugated with a bifunctional diethylenetriaminepentaacetic acid (DTPA) derivative for complexing Gd(III) atoms. The characteristics of DAB-Am64-(1B4M-Gd)$_{64}$, which quickly accumulated in the liver, have been reported recently. In the present study, the dynamic micro-MRI with DAB-Am64-(1B4M-Gd)$_{64}$ was obtained in the mouse liver metastasis model using colon carcinoma cells to evaluate the ability to visualize the micrometastatic tumors compared with that using Gd-DTPA. The dynamic micro-MRI with DAB-Am64-(1B4M-Gd)$_{64}$ was able to homogeneously enhance the normal liver parenchyma and visualize micrometastatic tumors of 0.3-mm diameter in the liver of the mice with better contrast than that with Gd-DTPA. In conclusion, DAB-Am64-(1B4M-Gd)$_{64}$ is a new liver MRI contrast agent potentially useful for diagnosis of micrometastasis in the liver.

**Introduction**

SPIOs[^3] were the first clinically approved liver-specific contrast agents in Europe and the United States among a variety of cell/organ-specific MR contrast agents (1, 2). SPIO agents [e.g., AMI-25 (Advanced Magnetics, Cambridge, MA) and SH U 555A (Schering, Berlin, Germany)] efficiently accumulate in the liver (~80% of injected dose) and the spleen (5–10% of injected dose) within minutes of their administration (3–5). After sequestration by phagocytic cells, the agents significantly decrease T2-weighted signal in the liver, resulting in the visualization of the hypointense/dark liver on the T2-weighted image.

The presence of hydrophobic groups on the Gd(III) chelates can lead to hepatocellular uptake and excretion into the bile ducts, gall bladder, and intestines, resulting in the visualization of the hypointense/bright liver on the T1-weighted image. This type of liver MRI contrast agent, which contains gadolinium and enhances the signal of the normal liver parenchyma, has been actively investigated for many years (6, 7) and is currently used in clinical practice. The first gadolinium-based agent of this class, [Gd(DTPA)(H$_2$O)]$^{2-}$ (Multi-Hance), was approved in Europe (8–11). A related chelate, [Gd(EOB-DTPA)(H$_2$O)]$^{2-}$ (Eovist), is currently in Phase III clinical trials (12–14). This agent is excreted to a greater extent via the liver (roughly 50% for [Gd(EOB-DTPA)(H$_2$O)]$^{2-}$ versus 2–4% for [Gd(BOPTA)(H$_2$O)]$^{2-}$), resulting in significantly greater liver enhancement.

DAB-Am64-(1B4M-Gd)$_{64}$ was evaluated to accumulate in the liver at ~50% of the injected dose within 15 min after injection and homogeneously enhanced the hepatic parenchyma (15). Therefore, DAB-Am64-(1B4M-Gd)$_{64}$ appears to be a comparable agent to [Gd(EOB-DTPA)(H$_2$O)]$^{2-}$. In the present study, imaging of experimental liver micrometastases of LS174T tumors, which were unable to be visualized by other imaging modalities in living animals, was studied with dynamic micro-MRI using this liver MRI contrast agent, DAB-Am64-(1B4M-Gd)$_{64}$, to evaluate its ability compared with Gd-DTPA. To the best of our knowledge, no study has been performed relating serial imaging of metastatic liver tumors in living mice.

**Materials and Methods**

**Dendrimer**

A polypropyleneimine diaminobutyl (DAB) dendrimer [polypropyleneimine tetrahexacontaamine dendrimer, Generation 5.0 (DAB-Am64); Aldrich Chemical Co., Milwaukee, WI] with a DAB core, 64 terminal primary amino groups, and a molecular weight of M$_r$ 7168 (16) was used.

**Conjugation of the Chelating Agent to Dendrimers**

The dendrimer was concentrated to 10 mg/ml and diafiltrated against 0.1 M phosphate buffer at pH 9. The DAB-Am64 and PAMAM-generation 4 dendrimers were reacted with a 64-fold molar excess of 1B4M at 40°C, and the reaction solution was maintained at pH 9 with 1 M NaOH over the reaction time of 48 h. An additional equal amount of the 1B4M was added after 24 h as a solid. The resulting preparation was purified by diafiltration using a Centricon 30 (Amicon Co.). This resulted in >98% of the amine groups on the dendrimers reacting with 1B4M, as determined by a $^{153}$Gd labeling assay of the reacted samples as described previously (17).

**Preparation of Contrast Agent for MRI with Nonradioactive Gd(III)**

Approximately 3 mg of DAB-Am64-(1B4M)$_{64}$ conjugate (containing 4 μmol of 1B4M) were mixed with 10 μmol of nonradioactive Gd(III) citrate (Nakarai, Tokyo, Japan) in 0.3 M citrate buffer for 2 h at 40°C. The excess Gd(III) in DAB-Am64-(1B4M-Gd)$_{64}$ was removed by diafiltration using the Centricon 30 (Amicon Co.) while simultaneously changing the buffer to 0.05 M PBS at pH 7.4. The purified samples were diluted to 1 ml with 0.05 M PBS, and 200 μl of this solution were used per mouse. A replacement assay using $^{153}$Gd showed that 84–88% of the 1B4M chelators on DAB-Am64-(1B4M-Gd)$_{64}$ were indeed chelating Gd(III) atoms for each preparation. In brief, ~500,000 cpm of carrier-free $^{153}$Gd, which was purchased from NEN DuPont
inhalation, and the spleen was exteriorized through a short left subcostal incision. A single-cell suspension of 1 \times 10^6 LS174T cells in 50 \mu l of serum-free RPMI 1640 was slowly injected into the spleen through a 27-gauge needle, followed 2 min later by splenectomy. The left subcostal incision was closed with metal clips (19). With this procedure, all mice developed multiple liver metastases of \(-100\mu m\) in diameter within 1 week.

**MR Image Acquisition**

To verify visualization of intrahepatic micrometastasis, the dynamic 3D-micro-MR images of the liver in the mice were obtained with injection of 0.03 mmol of Gd/kg of DAB-Am64-(1B4M-Gd)_{64} or 0.1 mmol Gd/kg of dimeglumine-DTPA-Gd (Magnevist, Japan Schering, Osaka, Japan) using a 1.5-Tesla (T) superconductive magnet unit (Signa; General Electric Medical System, Milwaukee, WI). All images were obtained with the finger coil (birdcage type, 1-inch round surface coil) fixed by an in-house constructed coil holder. The tumor-bearing mice were anesthetized with \(-1.15\) mg of sodium pentobarbital (Dinabot) and placed at the center of the coils. For the dynamic study, the 3D-fast spoiled gradient echo technique (efgre3d; TR/TE 10.5/2.7; TI 31; flip angle 30°; scan time 1 min 38 s; 4NEX) with chemical fat suppression was used for all mice. The images were acquired at preinjection and 0 (immediately postinjection), 3, 6, 9, 12, 15, 20, and 25 min postinjection of the contrast agent. The axial images were reconstructed with 0.8-mm section thickness with 0.4-mm overlap. The field of view was 8 x 4 cm, and the size of matrix was 256 x 128. For the precise comparison between MRI images and histological sections, the delayed coronal and axial MR images were obtained with the 3D-fast spoiled gradient echo technique (efgre3d; TR/TE 10.5/2.7; TI 31 ms; flip angle 30°; scan time 2 min 02 s; 4NEX) with chemical fat suppression used for all mice after the dynamic study. The images were reconstructed with 0.6-mm section thickness with 0.3-mm overlap. The field of view was 6 x 3 cm, and the size of matrix was 256 x 128. In addition, the slice data were analyzed (Advantage Windows; General Electric Medical System). The three series of experiments that were performed are described in detail below.

**Visualization and Histological Correlation of Intrahepatic Micrometastasis Using Dynamic 3D-Micro-MR Images with DAB-Am64-(1B4M-Gd)_{64}**

Dynamic MRI of Intrahepatic Micrometastasis with DAB-Am64-(1B4M-Gd)_{64} Two groups of mice \((n = 5)\) bearing LS174T intrahepatic micrometastases were used for the dynamic and delayed MRI with 0.03 mmol of Gd/kg of DAB-Am64-(1B4M-Gd)_{64} or 0.1 mmol of Gd/kg of dimeglumine-DTPA-Gd. The intensities of these regions of interest were measured: the hepatic and portal veins, the liver, and the tumors, and the time-intensity curves were made and analyzed (Advantage Windows; General Electric Medical System). The mice were killed with an injection of 10 mg of sodium pentobarbital into the tail vein immediately after the examinations and fixed in formaldehyde for longer than 2 weeks. The livers were sliced in the same planes as the MR sectional images, examined using a stereoscopic microscope (Photomakroskop; Wild, Heerbrugg, Switzerland), and correlated with the MR images.

Comparison of the 3D-Micro-MR imaging of Intrahepatic Micrometastasis between 0.03 mmol of Gd/kg of DAB-Am64-(1B4M-Gd)_{64} and 0.1 mmol of Gd/kg of Gd-DTPA in the Same Mouse. Dynamic contrast MR images of mice \((n = 5)\) bearing LS174T intrahepatic micrometastases with 0.1 mmol Gd/kg of dimeglumine-DTPA-Gd and 0.03 mmol of Gd/kg of DAB-Am64-(1B4M-Gd)_{64} were obtained at 1-day intervals, and the observations were compared. The mice were killed with injection of 10 mg of sodium pentobarbital into the tail vein immediately after the latter series of examinations and fixed in formaldehyde for \(>2\) weeks. The livers were sliced at 1-mm intervals in the same planes as the MR sectional images, examined using a stereoscopic microscope, and correlated with the serial MR images obtained every 0.4-mm intervals. In addition, the coronal histological sections of tumors with H&E staining were also correlated with corresponding MR images.

**Serial Follow-Up Study of Dynamic MRI to Evaluate the Growth of Intrahepatic Micrometastases.** To verify the serial change of intrahepatic micrometastasis, three to five serial dynamic contrast MR images of the same mice \((n = 5)\) bearing LS174T intrahepatic micrometastatic tumors were obtained at 1-week intervals using 0.03 mmol of Gd/kg of DAB-Am64-(1B4M-Gd)_{64}. The mice were killed with an injection of 10 mg of sodium pentobarbital into the tail vein immediately after the final series of examinations and fixed in formaldehyde for \(>2\) weeks. The livers were sliced at 1-mm intervals in the same planes as the MR sectional images and examined using a stereoscopic microscope (Photomakroskop; Wild, Heerbrugg, Switzerland) and correlated with the serial MR images obtained at every 0.4-mm interval.
addition, the axial histological sections of liver bearing metastatic LS174T tumors with H&E staining were also correlated with MR images to verify the metastatic tumors.

**Toxicological Study**

To evaluate the toxicity of DAB-Am64-(1B4M-Gd)_{64}, a group of four normal nude mice were injected with 0.3 mmol of Gd/kg of DAB-Am64-(1B4M-Gd)_{64} five times at 1-week intervals. The mice were observed for their body weight and appearance of the skin for 10 weeks. Thereafter, all of the mice were killed, and their organs were examined for their gross visually apparent changes and weight. As a control, another group of four normal nude mice were injected with PBS on the same schedule and placed together. All of the studies reported herein were approved by the Animal Care Committee of Kyoto University.

**Statistical Analysis**

Statistical analysis was performed using Student’s t test (StatView; SAS Institute Inc., Cary, NC).

**Results**

**Visualization and Histological Correlation of Intrahepatic Micrometastasis Using Dynamic 3D-Micro-MR Images with DAB-Am64-(1B4M-Gd)_{64}**

Dynamic MRI of Intrahepatic Micrometastasis with DAB-Am64-(1B4M-Gd)_{64}. In the dynamic MRI study with administration of 0.03 mmol of Gd/kg of DAB-Am64-(1B4M-Gd)_{64} (Fig. 1a), the signal intensity in the liver was constantly high up to 25 min after injection. The signal intensity in the blood showed the highest value, which was similar in the liver, within the first 3 min after injection, and rapidly decreased over time (Fig. 2a). The signal intensity in the blood gradually increased with time. In contrast, on the dynamic MRI study with 0.1 mmol of Gd/kg of dimeglumine-DTPA-Gd (Fig. 1b), the signal intensity in the liver was much lower than that with 0.03 mmol of Gd/kg of DAB-Am64-(1B4M-Gd)_{64}, and the signal intensity in the tumors was comparable with DAB-Am64-(1B4M-Gd)_{64} (Fig.
Dendrimers are a recently synthesized class of highly branched spherical polymers. Two types of dendrimers, PAMAM (20) and DAB, are commercially available (15, 16). They are highly soluble in aqueous solution and have a unique surface of primary amino groups (20, 21). Compared with many other types of dendritic macromolecules that have recently been synthesized, these dendrimers are of a class of macromolecules that are unidispersed and show high positive charge densities restricted to the surface of the molecule (16, 22). The defined structure and large number of available surface amino groups of these dendrimers have led to their use as substrates for the attachment of large numbers of chelating agents to a single antibody molecule (21, 23–26). They have also been used for the preparation of MRI contrast agents (17, 27–30).

MRI contrast agents with PAMAM dendrimer cores were mainly used for vascular imaging because of their prolonged retention in the circulation compared with Gd-DTPA and their enhanced relaxivities (30). Although the PAMAM dendrimer cores themselves accumulated in the liver, when DTPA derivatives were conjugated with the PAMAM dendrimer, their hepatic accumulation decreased and their retention in circulation was prolonged (30). We reported recently that the conjugation of polyethylene glycol with the PAMAM dendrimer DTPA conjugates showed an additional decrease in hepatic accumulation, resulting in increased blood retention (18). When designing macromolecular MRI contrast agents, one notes that increased hydrophobicity enhances accumulation of molecules in the liver and that this observation provides a guide for the preparation of novel liver MRI contrast agents (31).

In the present study, the delayed images obtained 30 min after injection showed a homogeneously high signal intensity in the liver. The signal intensity of the liver quickly increased within 2 min. At that time, although the metastatic tumors showed low signal intensity, the blood vessels still showed similar high intensity to the liver.
Although the tumor:liver signal intensity ratio was still high at later time points, the blood vessels also showed a significantly lower signal intensity than the liver parenchyma, making it sometimes difficult to distinguish the vessels from small metastatic tumor. Therefore, the early images in the dynamic study were more useful to detect these smaller tumors as distinguished from hepatic vessels. In addition, the gradually increased signal intensity of the tumors might distinguish metastatic tumor from liver cysts, although the T2-weighted image usually play a major role for this purpose.

SPIOs, which are the only clinically approved, liver-specific contrast agents, function as negative contrast agents of the liver that lower usually play a major role for this purpose. In contrast, DAB-Am64−(1B4M-Gd)64 is a positive contrast agent of the liver. Positive contrast agents, function as negative contrast agents of the liver that lower signal:noise ratio, permitting MR images with better resolution or for shorter acquisition times.

In conclusion, dynamic micro-MRI using DAB-Am64−(1B4M-Gd)64 was useful for evaluating hepatic micrometastatic tumors as small as ~0.3 mm in diameter and for repeatedly following the progression of tumor growth in mice.

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References
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