Magnetic Resonance Lymphography of Profundus Lymph Nodes with Liposomal Gadolinium-Diethyleneetriamine Pentaacetic Acid

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Lymphography, especially imaging of profundus lymph nodes, is a useful tool for diagnosis of cancer metastases in lymph nodes. However, positive imaging agents for magnetic resonance lymphography (MRL) have not been available, since the positive imaging agents so far introduced are low-molecular-weight materials that are not trapped in lymph nodes. For the purpose of improved positive enhanced MRL, we employed liposomes as carriers of a positive enhancer, gadolinium-diethyleneetriamine pentaacetic acid (Gd-DTPA). Magnetic resonance (MR) imaging was performed after subcutaneous injection of Gd-liposomes into the hind feet of rabbits which had reactive enlarged retroperitoneal lymph nodes. As a result, not only popliteal but also profundus retroperitoneal lymph nodes were positively enhanced by Gd-liposomes, especially after 20 min massage of the injected sites. Gd-Liposomes containing dipalmitoylphosphatidylglycerol were more effective than Gd-liposomes containing palmityl-d-gluconuride, a type of long-circulating liposomes, suggesting that liposomal accumulation in lymph node is at least partly, mediated by the trapping of liposomes by macrophages. These data show that liposomes modified with Gd-DTPA are effective for positive enhancement of both regional and profundus lymph nodes in MR lymphography.

Key words: magnetic resonance imaging; liposome; lymphography; contrast enhancement; gadolinium

Imaging of profundus lymph nodes, such as retroperitoneal lymph nodes, is thought to be a useful method for diagnosis of metastases. Positive contrast of normal lymph nodes may enable imaging of metastatic sites as cold spots. Liposomes have been used as carriers of positive contrast paramagnetic agents for magnetic resonance (MR) imaging, since they can deliver the bulk of these contrast agents to the appropriate sites, where they cause an enhancement of the bulk water relaxation rate. In early studies, water soluble paramagnetic molecules, such as gadolinium-diethyleneetriamine pentaacetic acid (Gd-DTPA), were encapsulated into liposomal internal aqueous phase and liposome-encapsulated contrast agents enhanced MR contrast of the liver and spleen. These positive enhancing agents, however, should be released from liposomes for enhancement of the MR image, or water should quickly exchange across the liposomal membrane. Furthermore, the encapsulation efficiency of soluble paramagnetic molecules in liposomes is quite low. Therefore, many attempts have been made to incorporate the paramagnetic molecules in the liposomal membrane, either by covalent linkage to lipids or by chelation to a ligand which is anchored to the liposomal membrane. These liposomal paramagnetic molecules have been revealed to be useful for MR lymphography, although most of cases have described imaging of primary lymph nodes. In this study we examined the feasibility of profundus MR lymphography for reactive enlarged lymph nodes in the retroperitoneum of rabbits using various liposomal formulations.

MATERIALS AND METHODS

Materials: Diethyleneetriamine-N,N,N′,N″,N‴-pentaacetic acid (DTPA) was purchased from Sigma (St. Louis, MO), and DTPA-stearylamine (DTPA-SA) was synthesized according to a reported method. Dipalmitoylphosphatidylcholine (DPPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and dipalmitoylphosphatidylglycerol (DPPG) were the products of Nippon Fine Chemical (Hyogo, Japan). Cholesterol and GdCl₃ were obtained from Sigma (St. Louis, MO). ¹⁵³GdCl₃ was purchased from Daiichi Pure Chemicals (Chiba, Japan). InCl₃ was purchased from Nihon Medi-physics (Hyogo, Japan). Palmityl-d-gluconuride (PGlcUA) was synthesized as described previously.

Preparation of Liposomes: Gd-Liposomes were prepared as follows: DPPC, POPC, cholesterol, DTPA-SA, and charged lipid, DPPG or PGlcUA, (10:10:10:16.5) dissolved in chloroform/methanol were dried under reduced pressure and stored in vacuo for at least 1 h. Liposomes were produced by hydration of the thin lipid film with 0.15M sodium glutamate at pH 5.5 and sonicated for 15 min with 75% duty cycle (Branson Model 250) after freeze-thawing three times with liquid nitrogen. GdCl₃, with a trace of ¹⁵³GdCl₃ dissolved in 0.15M sodium glutamate at pH 5.5 was then added to the liposomal solution and incubated overnight with gentle shaking. Resulting Gd-liposomes were extruded through a polycarbonate membrane filter with a 200-nm pore size (Nucleapore, Costar Co., Cambridge, MA), and precipitated by centrifugation at 180000×g for 20 min (Hitachi, CS120EX) for removal of unbound Gd. Liposomes were resuspended in an appropriate concentration, and the pH of the liposomal solution was adjusted to 7.4. Liposomal size was determined by use of Nicomp (Model 250) and was 164nm with a S.D. of 53 nm. The recovery of Gd was determined by use of ¹⁵³Gd and was more than 90% during the preparation.

To optimize the ratio of GdCl₃ to liposomal DTPA, liposomes incubated with various concentrations of ¹⁵³GdCl₃, and extruded through a 200-nm pore-sized filter were applied to a Sephadex G25 column (PD-10, Amersham Pharmacia Biotech, Tokyo, Japan). Liposomal Gd and free Gd were eluted with acetate-buffered saline, pH 5.5, and the radioactivity of each fraction was determined in an auto γ-counter (Beckman, 5500).

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Liposomes were prepared in a similar way to the [153Gd]liposomes.

**Animal Models** Japanese white rabbits (body weight 3.0 kg, Kanamaru Jikken Dobutsu, Tokyo, Japan) were used for the experiment. Egg yolk (0.05 ml/kg) obtained from fresh hen eggs was injected into the bilateral femoral muscles transcutaneously under light anesthesia with secobarbital. The injections were repeated 4 d later. These treatments provided reactive hyperplasia of the retroperitoneal lymph nodes. MR imaging studies were started 3 d after the second injection.

For popliteal lymphography, 0.05 ml/kg egg yolk was injected s.c. twice into hind footpad 7 and 3 d before imaging to obtain reactive enlarged lymph nodes.

**MR Imaging** Under anesthesia induced by intravenous injection of secobarbital (30 mg/kg, Yoshitomi Seiyaku, Osaka, Japan) and intraperitoneal injection of urethane (0.75 g/kg, Sigma, St. Louis, MO), Gd-PGleUA-liposomes or Gd-DPPG-liposomes (10 μmol/kg as Gd) were injected into both thighs (5 μmol Gd/kg, each). Following administration, massage was performed at the injection sites for 5 min to assist the lymph flow of the anesthetized animals. In the case of popliteal lymphography, Gd-PGleUA-liposomes (5 μmol/kg as Gd) were injected into the left hind foot.

MR imaging was performed before, and 5, 20 min and 2 h after administration of the agents with a 1.5T imager (Gyrosan S15HP, Philips, Netherlands). Coronal images were obtained with a field of view of 150 mm, a section thickness of 3 mm, and a 256×256 matrix. A head coil was used with 4 acquisitions and a half-encoding method. Fat suppressed T1-weighted images of spectral presaturation inversion recovery spin echo sequences with a repetition time of 270 ms and an echo time of 20 (SPIR-SE270/20) were obtained with ordinary T1-weighted images (SE270/20) as references. Evaluation was performed qualitatively and quantitatively by signal intensity ratio of the lymph nodes to the adjacent muscles as the internal standard, which were measured from the area of the marginal tracing cursor.

**γ-Imaging** [111In]DPPG-liposomes (10 μmol DPPG/kg) were injected into both hind feet of rabbits under anesthesia, and massage at the injection sites for 5 min was performed to assist the lymph flow of the anesthetized animals. γ-Imaging was performed for 90 s from just after administration, and 5, 20 min and 2 h after injection of the agents with a γ-camera (Arora, Omega-500). Tissue distribution was determined by the radioactivity of the region of interests in the γ-image, expressed as percent counts/cm². After the experiment, the animals were sacrificed and retroperitoneal lymph node was removed for determination of the radioactivity by an auto γ-counter.

**RESULTS AND DISCUSSION**

At first, the appropriate liposomal composition was determined as DPPC, POPC, cholesterol, DTPA-SA, and charged lipid (10:10:10:16:5) from the observations that PGleUA-modification could endow liposomes with long circulating character, recovery of liposomes after extrusion did not decrease, and the obtained liposomes could keep internal aqueous phase. Next, the ratio of GdCl₃ to liposomal DTPA was optimized. Liposomes containing DTPA-SA were incubated with various concentrations of [153GdCl₃, and were applied to a

![Fig. 1. Gel Filtration Profiles of 153Gd after Incubation with Liposomes](image1)

Liposomes composed of DPPC, POPC, cholesterol, DTPA-SA, and DPPG (10:10:10:16:5) were incubated with various concentrations of [153GdCl₃ overnight. The Gd/DTPA-SA (10:16:5) fraction was applied to a Sephadex G25 column (PD-10) and eluted with acetic buffered saline, pH 5.5. The radioactivity of each fraction was determined.

![Fig. 2. Popliteal MR Lymphography Enhanced by PGleUA-Liposomal Gd](image2)

Liposomes composed of DPPC, POPC, cholesterol, DTPA-SA, and PGleUA (10:10:10:10:16:5) with Gd were administered to the left hind foot (5 μmol/kg as Gd) of rabbits whose lymphs had been reactive enlarged by double s.c. injection of 0.05 ml/kg egg yolk as described in Materials and Methods. SPIR T1 weighted spin echo imaging was performed at 10 min after administration of Gd-liposomes. (a) transverse section; (b) coronal section.

Sephadex G25 column (PD-10). Free [153GdCl₃, without liposomes was recovered at a peak fraction number of 14 (data not shown). As shown in Fig. 1, when the amount of [153Gd
increased, the recovery percent of $^{153}$Gd in the liposomal fraction decreased. At a molar ratio of Gd/DTPA-SA=10:16, 98.4% of radioactivity was recovered from the liposomal fraction (fraction 6—8), while there was almost no free Gd. Thus, the molar ratio of Gd/DTPA-SA=10:16 appeared to be favorable for preparing Gd-liposomes, since, if DTPA-SA is evenly distributed between outer and inner leaflets of the liposomal membrane, a molar ratio of Gd/DTPA-SA=8:16 means full binding of Gd to DTPA-SA at the outer leaflet of the liposomal membrane without free Gd. Therefore, the data indicated that most of the DTPA-SA outside the liposomes was occupied with Gd. In this paper, we used 200-nm sized liposomes, which are known to accumulate in lymph node.\(^{14}\) The following experiment was done using liposomes prepared with this Gd/DTPA-SA ratio.

Prior to MR imaging of retroperitoneal lymph nodes, MR imaging of popliteal lymphs was performed with Gd-PGlcUA-liposomes administered s.c. into the dorsal footpad of rabbit. As a result, Gd-liposomes enhanced the contrast of popliteal lymph node compared with the node of the other leg (Fig. 2). Thus the liposomes prepared here were effectively accumulated in the regional lymph node and had contrast enhancing activity.

Next, MR imaging of retroperitoneal lymph nodes was examined by use of Gd-liposomes. As shown in Figs. 3a—e, Gd-liposomes also enhanced the contrast of retroperitoneal lymph nodes. Since conventional liposomes tend to be trapped in the reticuloendothelial system (RES) after i.v. administration, various researchers have attempted to avoid RES-trapping of liposomes. Modification of liposomes with a glucuronic acid derivative\(^{15}\) or polyethylene glycol (PEG)\(^{16,17}\) resulted in liposomes with a longer circulation time in the bloodstream. Furthermore, since these liposomes tend to accumulate in tumor tissues due to the leaky endothelia of the tissues, tumor imaging is also favorable using these liposomes.\(^{20}\) We compared conventional and long-circulating liposomes as carriers of contrast agent for interstitial MR lymphography. As shown in Fig. 3 (compare Fig. 3c with 3f), both conventional liposomes, i.e., DPPG-liposomes containing Gd-DTPA-SA, and long-circulating liposomes, i.e., PGlcUA-liposomes containing Gd-DTPA-SA, enhanced signal intensities of the enlarged retroperitoneal lymph nodes. The long-circulating, PGlcUA-modified, liposome, however, showed less effects on contrast enhancement than the conventional liposome. Surface modification with glucuronic acid also decreased liposomal adhesion to and phagocytosis in macrophage-derived cells.\(^{19}\) Since the trapping of liposomes by phagocytic cells in lymph node may contribute to the high accumulation of Gd-DPPG-liposomes, PGlcUA-liposomes may not be favorable compared to conventional liposomes for MR lymphography. On the contrary, liposomes containing phosphatidylinerine, that are easily recognized and phagocytized by macrophages,\(^{19}\) were reported to show high accumulation in lymph nodes.\(^{14}\)

On the other hand, liposomes modified with PEG are reported to highly enhance the lymph node-to-muscle MR signal intensity.\(^{20}\) It is possible that the behavior of PEG-liposomes is different from PGlcUA-liposomes in lymph fluid, although both types of liposomes have long-circulating characteristics in blood stream.

Figure 4 shows the time-intensity curves of contrast in retroperitoneal lymph nodes after injection of Gd-liposomes with conventional lipid composition. High contrast enhanced images were obtained at 5 to 20 min after administration of the agent. The signal intensity ratio of node-to-muscle was more than three-fold. This result suggests that distinction
of metastatic lymph nodes in MR lymphography may be achieved by enhancement of the non-metastatic (normal or reactive enlarged) nodes since metastatic nodes lack phagocytic activity and would not be enhanced.

Finally, liposomal trafficking after s.c. injection was determined by use of $[^{111}\text{In}]$DPPG-liposomes. $\gamma$-Imaging showed specific accumulation of radioactivity around the sites of popliteal and retroperitoneal lymph node. Around the site of liver was also strongly imaged in a time dependent manner (data not shown). Time–activity curves obtained are shown in Fig. 5. This figure suggests $^{111}\text{In}$ accumulated initially in popliteal lymph nodes, and secondly in retroperitoneal lymph nodes, and finally in liver. The accumulation of $^{111}\text{In}$ in retroperitoneal lymph nodes 2 h after injection was 1.01±0.02% of injected dose.

In this report, we demonstrated liposomal trafficking after s.c. injection. Liposomes migrate from the injection site into lymphatic vessels and distribute to proximal lymph nodes, then distribute to distal lymph nodes depending on the flow of lymph. This result enables positive enhancement of MR lymphography of not only proximal but also distal lymph nodes by use of Gd-liposomes, since the liposomes tended to accumulate in lymph nodes. A possible reason for the accumulation of Gd-liposomes in lymph nodes is the trapping of liposomes by phagocytic cells, since Gd-liposomes which are not trapped by phagocytic cells showed less accumulation. The strategy shown here may be useful for diagnosis of metastasis.

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