Distribution of Liposomes into Brain and Rat Brain Tumor Models by Convection-Enhanced Delivery Monitored with Magnetic Resonance Imaging

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ABSTRACT

Although liposomes have been used as a vehicle for delivery of therapeutic agents in oncology, their efficacy in targeting brain tumors has been limited due to poor penetration through the blood-brain barrier. Because convection-enhanced delivery (CED) of liposomes may improve the therapeutic index for targeting brain tumors, we conducted a three-stage study: stage 1 established the feasibility of using in vivo magnetic resonance imaging (MRI) to confirm adequate liposomal distribution within targeted regions in normal rat brain. Liposomes colabeled with gadolinium (Gd) and a fluorescent indicator, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-5,5'-disulfonic acid [DiI-DS; formally DiIC18(3)-DS], were administered by CED into striatal regions. The minimum concentration of Gd needed for monitoring, correlation of infused volume with distribution volume, clearance of infused liposome containing Gd and DiI-DS (Lip/Gd/DiI-DS), and potential local toxicity were evaluated. After determination of adequate conditions for MRI detection in normal brain, stage 2 evaluated the feasibility of in vivo MRI monitoring of liposomal distribution in C6 and 9L-2 rat glioma models. In both models, the distribution of Lip/Gd/DiI-DS covering the tumor mass was well defined and monitored with MRI. Stage 3 was designed to develop a clinically relevant treatment strategy in the 9L-2 model by infusing liposome containing Gd as Lip/Gd/DiI-DS, prepared in the same size as Lip/Gd/DiI-DS, with Doxil, a liposomal drug of similar size used to treat several cancers. MRI detection of Lip/Gd coadministered with Doxil provided optimum CED parameters for complete coverage of 9L-2 tumors. By permitting in vivo monitoring of therapeutic distribution in brain tumors, this technique optimizes local drug delivery and may provide a basis for clinical applications in the treatment of malignant glioma.

INTRODUCTION

Liposomes (phospholipid bilayers formed into spheres in the presence of water that can be made to incorporate a variety of agents) are a vehicle for administering therapeutic agents, including drugs and genes, to areas of the body afflicted with cancer (1, 2). Recently, efforts have been made to increase the vehicular efficiency of liposomes and to direct therapeutic agents to specific target sites (3, 4). Preclinical studies using drug-encapsulated liposomes have shown improvement in the sustained release of the drug, prolongation of the drug’s half-life, and an increase in the therapeutic index of corresponding drugs (5). Although immunoliposomes using antibody fragments for molecular targeting have shown promising results (6, 7), the potential effectiveness of such immunoliposomes in targeting tumors of the central nervous system (CNS) has not been established. Systemic administration may not achieve satisfactory penetration of the blood-brain barrier, and local injection cannot achieve optimum distribution.

Convection-enhanced delivery (CED) is a direct intracranial drug delivery technique that utilizes a bulk-flow mechanism to deliver and distribute macromolecules to clinically significant volumes of solid tissues (8, 9). This approach offers a greater volume of distribution than simple diffusion and is designed to direct a drug to a specific target site. As compared with systemic delivery, the CED of liposomes carrying chemotherapy drugs bypasses the blood-brain barrier (8), provides a larger distribution of liposomes within the target site, allows for a locally sustained release of drugs, and minimizes systemic exposure, thereby producing fewer side effects.

Despite advances in neurosurgical techniques and in radiation and drug therapies, the mean survival for patients who have a malignant glioma is less than 12 months, and only 20% of patients survive for more than 2 years. More than 80% of patients with this disease experience local recurrence of tumor, which leads to their death (10, 11). Therefore, development of new local management strategies such as the CED of therapeutic liposomes may provide a therapeutic advantage in the adjuvant medical management of gliomas.

To develop a CED method for the administration of liposomal therapeutics in the treatment of malignant glioma, our study was designed in three stages. Stage 1 studies were designed to establish the feasibility of using direct in vivo magnetic resonance imaging (MRI) of liposomal delivery to confirm adequate drug distribution within targeted CNS regions. MRI was used to visualize liposomes incorporating the contrast agent gadodiamide [a stable gadolinium (Gd) chelate], which were dispensed by CED to obtain robust distribution in the CNS. The following issues related to the successful application of this technology were assessed in normal brain parenchyma of intact rats: (a) monitoring of the CED infusion of liposomes containing Gd and a fluorescent indicator, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-5,5'-disulfonic acid [DiI-DS; formally DiIC18(3)-DS], into rat brain hemispheres to evaluate its feasibility and determination of the lowest concentration of contrast agent required to show the targeted distribution area; (b) evaluation of the liposome containing Gd and DiI-DS (Lip/Gd/DiI-DS) distribution when infusing a defined volume at intervals from 5 to 40 μl using MRI monitoring and histological detection of the fluorescence; (c) a time-course evaluation to determine retention of Gd-containing liposomes in the brain after infusion by CED as represented by the liposomal clearance of the magnetic resonance (MR) signal generated by Lip/Gd/DiI-DS; and (d) possible adverse effects of Lip/Gd/DiI-DS infused into the CNS by CED.

After several conditions for MRI detection of Lip/Gd/DiI-DS were established in normal brain parenchyma of intact rats, stage 2 studies evaluated the feasibility of in vivo MRI of liposomal distribution in two morphologically dissimilar rat brain tumor models, C6 and 9L-2. These studies addressed the following issues: (a) comparison of Lip/Gd/DiI-DS distribution in normal brain and brain tumor tissues to assess the feasibility of using CED administration of liposomes for the treatment of malignant glioma; and (b) determination of the distribu-
tion of Lip/Gd/DiI-DS in brain tumors using MRI and correlated with the volume infused.

Stage 3 was designed to develop a clinically relevant treatment strategy in the 9L-rat brain tumor model by infusing liposome containing Gd (Lip/Gd), prepared as the same size liposome as Lip/Gd/DiI-DS, together with Doxil, a commercially available liposomal drug of similar size that is used clinically in the treatment of cancers other than malignant glioma. The goal of this experiment was to provide a basis for future applications coupling real-time MRI techniques during CED to the direct delivery of therapeutic liposomal agents into brain tumors.

MATERIALS AND METHODS

Liposome Preparation

Liposomes were prepared to contain a MR contrast agent, a fluorescent marker to visualize liposomal distribution histologically, or both of those agents. The paramagnetic contrast agent Gd diethylenetriaminepentaacetic acid (gadodiamide) was in the form of a commercial United States Pharmacopeia preparation of Omniscan (Amersham Health, Buckinghamshire, United Kingdom) that contains 0.5 mg gadodiamide in water. The fluorescent indicator DiI-DS was obtained from Molecular Probes (Eugene, OR); 1,2-dioleoyl-3-sn-glycerophosphocholine and N-methoxy-poly(ethylene glycol)-1,2-distearoyl-3-sn-phosphoethanolamine (M(2000) were obtained from Avanti Polar Lipids (Alabaster, AL); and cholesterol (Chol) was obtained from Calbiochem (San Diego, CA).

For all liposomal preparations, 1,2-dioleoyl-3-sn-glycerophosphocholine and cholesterol (molar ratio, 3:2) and N-methoxy-poly(ethylene glycol)-1,2-distearoyl-3-sn-phosphoethanolamine (5 mol%) were codiluted in chloroform and brought to dryness in a vacuum by rotary evaporation to form a lipid film. The lipid film had been empirically derived previously on the same system and was known to have a value of 4.07 mm/s. The concentration of the encapsulated gadodiamide was then calculated with the following equation:

$$\text{Concentration of gadodiamide} = \frac{(1/T1_{\text{w/Gado}} - (1/T1_{\text{w/oGado}}))}{4.07}$$

Animals

Male Sprague Dawley rats weighing 300–350 g (Charles River Laboratories, Wilmington, MA) and male Fisher 344 rats weighing 200–250 g (Harlan, Indianapolis, IN) were housed under aseptic conditions, which included filtered air and sterilized food, water, bedding, and cages. The protocol used in these studies was approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Surgery and the CED Procedure

While under deep isofluorane anesthesia, rats were placed in a small animal stereotactic frame (David Kopf Instruments, Tujunga, CA). A sagittal incision was made through the skin to expose the cranium, and a burr hole was made in the skull at 0.5 mm anteriorly and 3 mm laterally from the bregma with a small dental drill (15). Infusions were performed at the depth of 4.5 mm from brain surface by using the CED method described previously (16, 17). Briefly, an infusion cannula connected to the Hamilton syringe (Hamilton, Reno, NV) was attached to a rate-controllable microinfusion pump (Bioanalytical Systems, Lafayette, IN). Slow-infusion CED was performed by controlling the infusion rate. For the volume determinations, we evaluated 5-, 10-, 20-, and 40-μl infusions of Lip/Gd/DiI-DS at the original concentration (20 mM phospholipid). The following ascending infusion rates were applied throughout the study to achieve the appropriate target volumes: (a) for a 5-μl volume, 0.2 μl/min (5 min) and 0.5 μl/min (4 min); (b) for a 10-μl volume, 0.2 μl/min (5 min), 0.5 μl/min (10 min), and 0.8 μl/min (2 min); (c) for a 20-μl volume, 0.2 μl/min (5 min), 0.5 μl/min (10 min), and 0.8 μl/min (15 min); and (d) for a 40-μl volume, 0.2 μl/min (5 min), 0.5 μl/min (10 min), and 0.8 μl/min (40 min).

MRI Methods

Distribution of liposomes was visualized on coronal T1-weighted spoiled gradient-echo images acquired on a 1.5 Telsa Signa LX scanner (GE Medical Systems, Waukesha, WI) with a pelvic phased array surface coil. The imaging parameters varied according to the number of rats scanned [Repitition Time (TR)/Echo Time (TE)/flip angle = 40 ms/6 ms/30°, 2 number of excitations (NEX), matrix = 256×256 or 384×256, field of view (FOV) = 18–22 cm, slice thickness = 1.0 mm]. These parameters resulted in nominal voxel sizes that ranged from 0.33 to 0.74 mm3. Images were acquired approximately 2–4 h after surgery or, for the time-course study, at the indicated intervals. During the MRI examination, rats were anesthetized with an i.p. injection of sodium pentobarbital (50 mg/kg). Three to four rats were scanned during each MRI session.

Stage 1: Determination of Optimal Parameters for MRI Detection of Lip/Gd/DiI-DS in Normal Brain Parenchyma of Intact Sprague Dawley Rats

MRI Detection of Lip/Gd/DiI-DS after Slow-Infusion CED. Four rats were given 20-μl infusions of Lip/Gd/DiI-DS into the right hemisphere and of Lip/DiI-DS (control liposomes) into the left hemisphere. Two rats received, in each hemisphere, 20-μl infusions of gadodiamide solution in a vehicle (HEPES-buffered saline) containing the same concentration of Gd as Lip/Gd/DiI-DS. MR images were acquired approximately 2 h after the infusion. To address the detection limits of Gd when using MRI and to determine the lowest concentration of Gd required to show the targeted distribution area, the original 100% Lip/Gd/DiI-DS mixture was diluted with the vehicle to achieve concentrations of 10%, 20%, and 50%. The diluted mixture was infused into the striatum at a fixed volume of 20 μl/site (9 rats, 18 hemispheres; n = 6 for 100% and 20%; n = 3 for 10% and 50%).

Correlation between Volume of Administration and Volume of Distribution as Measured by MRI and Histological Analysis. To address the relationship between infused volume and volume of distribution inside the normal brain, 20 hemispheres (10 rats) each received 5, 10, 20, or 40 μl of Lip/Gd/DiI-DS by CED infusion; that is, 5 hemispheres received each volume.
MRI was performed about 2 h after surgery, and the MR image-based volume of Lip/Gd/DiI-DS distribution in the right and left hemispheres of each rat was quantified by using image analysis software developed at University of California, San Francisco. A subregion containing only one hemisphere and the surrounding skull from one rat was masked out from the main image. An automated contouring routine that outlines image regions with intensities greater than a specified threshold was used to generate a region of interest (ROI) that corresponded to the liposome distribution in each brain. To control for variations in image intensity arising from the position of each rat within the phased-array MRI coil, we used slightly different threshold intensities for liposome presence based on the mean background signal intensity in each rat. The background signal intensity was determined by averaging the intensity in circular ROIs placed in muscle regions outside of the skull on the same image slices that contained liposomes (Fig. 2B). The threshold for liposome presence was the mean background signal intensity + 4 SD. All ROIs generated automatically by the software were visually inspected to confirm accuracy. In some instances, multiple ROIs highlighting liposomes in different brain regions (e.g., white matter and cortex) were combined into a single ROI representing the total volume in one hemisphere. Small (<0.003 cm³) spurious ROIs located more than 1 mm distant from the primary ROI or in regions outside of the brain parenchyma were excluded from analysis.

For the histological evaluation of Lip/Gd/DiI-DS distribution, the rats were euthanized immediately after each MRI session. The brains were harvested, freshly frozen by using ice-cold isopentane, and cut into serial coronal sections (25 μm) with a cryostat. The fluorescent signal generated by DiI-DS was visualized with a fluorescence microscope, and a charged-coupled device camera with a fixed aperture was used to capture the image. The volume of Lip/Gd/DiI-DS distribution was analyzed by using a Macintosh-based image analysis system (NIH Image 1.62; NIH Bethesda, MD) as described previously (18). The volume of DiI-DS distribution, as determined by the fluorescent microscopy images, was compared with the volume of Gd distribution as detected with 1.5 Tesla MRI.

Liposomal Time-Course Study. Clearance of the MR signal generated by Lip/Gd/DiI-DS when infused by CED into the intact brain was analyzed in the hemispheres of six rats (n = 12). MR images were acquired every day [i.e., from 2 to 72 h after CED infusions of Lip/Gd/DiI-DS (20 μl/site)], and the signal intensity was analyzed. In addition, the hemispheres of two rats (n = 4) received the infusion by CED with gadodiamide solution (20 μl/hemisphere) in the same vehicle and at the same concentration (4.7 mM) as the Lip/Gd/DiI-DS preparation.

Evaluation of Liposomal Toxicity. To evaluate possible adverse effects of Lip/Gd/DiI-DS on normal brain parenchyma, five Sprague Dawley rats were given 20-μl infusions of the original 100% Lip/Gd/DiI-DS concentration into their right hemisphere and Lip/DiI-DS into their left hemisphere by CED. Body weight was measured before the CED procedure and on day 7 and day 14 after the CED procedure. Two rats were euthanized on day 7 after the CED procedure for histological evaluation, and three rats were euthanized on day 14 after the CED procedure for histological evaluation. The rats were perfused with 10% formalin, and the brains were processed for histological examination with H&E staining.

Stage 2: In Vivo MRI of Liposome Distribution in Brain Tumor Models

Brain Tumor Models. Two established rat glioma cell lines (C6 and 9L-2) were obtained from the Department of Neurological Surgery Tissue Bank at University of California, San Francisco. Cells were seeded into tissue culture flasks approximately 2–3 days before they were implanted into the brains of rats and maintained as monolayers in a complete medium consisting of Earle’s MEM supplemented with 10% FCS and nonessential amino acids. Cells were cultured at 37°C in a humidified atmosphere containing 95% air and 5% CO₂. For the tumor models, cells were harvested by trypsinization, washed once with HBSS without Ca²⁺ and Mg²⁺, and resuspended in HBSS for implantation.

The C6 glioma cells were implanted into the CNS of three Sprague Dawley rats. The 9L-2 glioma cells were implanted into the CNS of nine Fisher 344 rats. For both tumor models, 5 × 10⁶ cells were stereotactically implanted into the right hemisphere of each rat by using the following coordinates: 0.5 mm anterior and 3 mm lateral from the bregma; and 4.5 mm deep from the brain surface.

CED of liposomal agents was performed on day 16 after tumor implantation for the C6 glioma model and on day 10 after tumor implantation for the 9L-2 glioma model.

Liposome Infusion in a C6 Glioma Brain Tumor Model. To evaluate the feasibility of this method for treating brain tumors, liposomal agents containing Gd/DiI-DS were slowly infused by CED into the C6 glioma brain tumor model in three Sprague Dawley rats. On day 16 after implantation of the tumor cells, 20 μl of Lip/Gd/DiI-DS were administered via intratumoral CED infusion, and a T₁-weighted coronal MR image was taken. Rats were euthanized after the MRI session, and the brains were processed for histological detection of fluorescent signal generated by DiI-DS.

Liposome Infusion in a 9L-2 Rat Brain Tumor Model. CED infusion of Lip/Gd/DiI-DS was evaluated in a 9L-2 tumor model in nine Fisher 344 rats. To test the effect of liposomes on the 9L-2 infiltrative brain tumor model, CED infusions were performed on day 10 after tumor implantation. Because this tumor was invisible without contrast enhancement, a baseline MR image was obtained with the i.p. administration of gadopentetate dimeglumine (Magnevist; Berlex Laboratories, Montreal, Canada) on day 9, before the administration of liposomes, to visualize the tumor size (Fig. 6B; Ref. 19). To maximize the signal:noise ratio for better visualization of the tumor volume, we used a T₁-weighted spin echo sequence (TR/TE = 500 ms/20 ms, 4 NEX, 256 × 256 matrix, 18 cm FOV, 1-mm slice thickness) instead of the gradient echo sequence used in the other experiments. On the next day, CED infusion of Lip/Gd/DiI-DS was performed in nine rats; of those, three received 20-μl infusions, and six received 40-μl infusions. Similarly to the study with the C6 glioma model, rats were euthanized after the MRI session, and the brains were processed.

Stage 3: Administration of Lip/Gd Together with Doxil

To develop a clinically relevant treatment strategy, a separate experiment addressed the feasibility of confining Lip/Gd, using the same size liposome as Lip/Gd/DiI-DS, with Doxil. Because Doxil (97.8 ± 30.3 nm) and Lip/Gd (77.1 ± 6.6 nm) share similar size characteristics, we hypothesized a similar distribution pattern. To verify this hypothesis, three intact Fischer 344 rats were given a 20-μl infusion of Lip/Gd/DiI-DS by CED into the left hemisphere (n = 3) and given the same dose of Doxil into the right hemisphere (n = 3). Because Doxil is a liposomal drug containing doxorubicin, which generates fluorescence with UV illumination, the distribution volume was calculated as the fluorescence area generated from Doxil and similarly from Lip/Gd/DiI-DS. Thereafter, 40 μl of a liposome mixture consisting of 20% Lip/Gd (without DiI-DS) and 80% Doxil were infused by CED into the 9L-2 brain tumor in three Fisher 344 rats on day 10 after tumor implantation. MRI was followed by histological examination including H&E staining. The volume of distribution was calculated from the fluorescence generated from Doxil in the 9L-2 tumor model and compared with area of distribution measure using MRI.

RESULTS

Stage 1: Determination of Parameters for MRI Detection of Lip/Gd/DiI-DS in Normal Brain Parenchyma of Intact Rats

MRI Detection of Lip/Gd/DiI-DS after Slow-Infusion CED. Using MRI, the gadodiamide concentration in Lip/Gd/DiI-DS was determined to be 4.7 mM. Infused into a rat brain, this Lip/Gd/DiI-DS (20 μl) generated a clear signal that was detected by MRI and could be monitored at 2 h after infusion. In contrast, Lip/DiI-DS, which contained no Gd, generated no MRI-detectable signal (Fig. 1A, ii). Distribution of the gadodiamide solution (4.7 mM) after CED (Fig. 1A, iii) differed substantially from that of Lip/Gd/DiI-DS. Whereas the MR signal generated from the Lip/Gd/DiI-DS-treated hemisphere was well defined and confined to regions near the infusion site (Fig. 1A, ii), the tissue distribution pattern of gadodiamide solution within the striatum was more diffuse (Fig. 1A, iii). Because of the robust MR signal generated by Lip/Gd/DiI-DS containing 4.7 mM Gd, serial dilutions of Lip/Gd/DiI-DS were made to evaluate the sensitivity of the imaging system after CED. Fig. 1B shows the signal generated from the stock 100%
Magnetic resonance imaging (MRI) was performed 2 h after infusion. The signal from gadodiamide solution revealed more diffuse distribution. Lip/Gd/DiI-DS containing 4.7 mM Gd was used for the study. The volume of distribution as calculated from MRI of the rats receiving a 20% concentration of Lip/Gd/DiI-DS (n = 6) was almost the same as that of the rats receiving a 100% concentration (n = 11).

Lip/Gd/DiI-DS solution and the 10%, 20%, and 50% dilutions. Signal intensity was observed with the 50% and 20% diluted mixtures of Lip/Gd/DiI-DS, but signal intensity from the 10% Lip/Gd/DiI-DS dilution was insufficient to generate a consistent MRI signal. On the MR images, comparisons showed a similar volume of distribution between the rats receiving the 20% solution and those receiving the Lip/Gd/DiI-DS stock 100% solution (Fig. 1C).

Correlation between Volume of Administration and Volume of Distribution as Measured by MRI and Histological Analysis. The volume of liposomal distribution in normal brain was estimated by using histological detection of fluorescence generated from DiI-DS and MR signals. Fig. 2A shows representative sections from rats given 10 μl of Lip/Gd/DiI-DS into the left hemisphere and 20 μl of Lip/Gd/DiI-DS into the right hemisphere by CED infusion. Areas of fluorescence were delineated using the NIH Image analysis system, and the volume of distribution was calculated. Correlations between the volume of distribution and the infused dose were made and confirmed by $T_1$-weighted MR images (Fig. 2B). Not surprisingly, the volume of Lip/Gd/DiI-DS distribution as measured by histological techniques was greater than that obtained with MRI, probably due to increased levels of sensitivity. Both methods, however, detected a strong correlation between the volume of Lip/Gd/DiI-DS administration and the volume of distribution (Fig. 2).

Liposomal Time-Course Study. Representative images of Lip/Gd/DiI-DS distribution at 2, 24, 48, and 72 h after CED are shown in Fig. 3A. Fig. 3B shows the distribution of gadodiamide solution, dispensed at the same concentration as in the Lip/Gd/DiI-DS preparation, at 2 and 24 h after CED. Evaluation of the change of MR signal over time for the gadodiamide solution infusion and Lip/Gd/DiI-DS preparations showed that the gadodiamide solution started to distribute rapidly and also washed out earlier. For the gadodiamide solution, MR signals were diffuse as early as 2 h after CED, and they appeared to disappear at 24 h after infusion. In contrast, the MR signal generated from Lip/Gd/DiI-DS was robust at 2 h and was slightly to moderately diffuse at the infused loci at the 24 and 48 h time points. Even after 72 h, the signal from infused Lip/Gd/DiI-DS was clearly observed. Fig. 3C shows the change of MR signal over time after Lip/Gd/DiI-DS infusion, standardized by background MR signal intensity. A correlation between the MR signal intensity and time elapsed was observed.

Evaluation of Liposomal Toxicity. Sprague Dawley rats receiving 20-μl CED infusions of Lip/Gd/DiI-DS into their right hemisphere and the same infusion of Lip/DiI-DS into their left hemisphere showed no substantial evidence of adverse effects over a 14-day period. Daily observations revealed no clinical deficits during the study. The rats appeared healthy and gained or maintained body weight at the same rate as did normal intact rats (Fig. 4D). Histological evaluation from two rats euthanized on day 7 after infusion revealed some evidence of tissue inflammation in striatal regions proximal to the needle track on the site infused with Lip/DiI-DS (Fig. 4A). The contralateral hemisphere, infused with Lip/Gd/DiI-DS, showed a similar but slightly more intense reaction at the same site (Fig. 4B). This tissue reaction was observed only adjacent to the

Fig. 1. A, $T_1$-weighted axial magnetic resonance image of an intact normal rat (i), a rat given a convection-enhanced delivery infusion of Lip/Gd/DiI-DS into the right hemisphere (ii), and a rat given gadodiamide solution at the same concentration as that for Lip/Gd/DiI-DS into the right hemisphere (iii). Magnetic resonance imaging (MRI) was performed 2 h after infusion. The signal from gadodiamide solution was detected, whereas Lip/DiI-DS could not be detected. The signal from the gadodiamide solution revealed more diffuse distribution. B, the concentration of Lip/Gd/DiI-DS detectable with MRI was estimated. Dilutions down to 20% were detectable with a similar distribution volume; however, the signal intensity from 10% Lip/Gd/DiI-DS was insufficient. Lip/Gd/DiI-DS containing 4.7 mM Gd was used for the study. C, the volume of distribution as calculated from MRI of the right hemisphere (red-highlighted area) and the same infusion of Lip/DiI-DS into their left hemisphere and 20 μl of Lip/Gd/DiI-DS into the left hemisphere by CED infusion. Areas of fluorescence were delineated using the NIH Image analysis system, and the volume of distribution was calculated. Correlations between the volume of distribution and the infused dose were made and confirmed by $T_1$-weighted MR images (Fig. 2B). Not surprisingly, the volume of Lip/Gd/DiI-DS distribution as measured by histological techniques was greater than that obtained with MRI, probably due to increased levels of sensitivity. Both methods, however, detected a strong correlation between the volume of Lip/Gd/DiI-DS administration and the volume of distribution (Fig. 2).

Fig. 2. A, volume of liposome distribution was estimated by using histological detection of fluorescence generated from DiI-DS and MR signals. Fig. 2A shows representative sections from rats given 10 μl of Lip/Gd/DiI-DS into the left hemisphere and 20 μl of Lip/Gd/DiI-DS into the right hemisphere by CED infusion. Areas of fluorescence were delineated using the NIH Image analysis system, and the volume of distribution was calculated. Correlations between the volume of distribution and the infused dose were made and confirmed by $T_1$-weighted MR images (Fig. 2B). Not surprisingly, the volume of Lip/Gd/DiI-DS distribution as measured by histological techniques was greater than that obtained with MRI, probably due to increased levels of sensitivity. Both methods, however, detected a strong correlation between the volume of Lip/Gd/DiI-DS administration and the volume of distribution (Fig. 2).
plotted against the time after infusion. Line, regression line; \( R^2 = 0.9905 \).

Fig. 3. A, \( T_1 \)-weighted axial magnetic resonance (MR) image showing time-course changes after convection-enhanced delivery infusion of 20 \( \mu l \) of Lip/Gd/DiI-DS. Gd signals were detected even after 72 h of infusion. B, \( T_1 \)-weighted axial MR image showing time-course changes after infusion of gadodiamide solution. As compared with the Lip/Gd/DiI-DS, gadodiamide solution distributed rapidly initially and washed out earlier. C, change in MR signals after Lip/Gd/DiI-DS infusion. Mean MR intensity of the distributed area was standardized with the background (b) mean MR signal intensity and plotted against the time after infusion. Line, regression line; \( R^2 = 0.9905 \).

MRI showed a similar volume of distribution in the 9L-2 tumor model (Fig. 6F) and in normal brain (Fig. 2B).

Stage 3: Administration of Lip/Gd Together with Doxil

In assessing the potential clinical relevance of a treatment strategy based on these studies, we found that Lip/Gd/DiI-DS had a distribution pattern similar to that of the drug Doxil after CED infusion in normal brain (Fig. 7A). After CED infusion of Lip/Gd (in liposomes the same size as Lip/Gd/DiI-DS) and Doxil into rats with a 9L-2 brain tumor (Fig. 7B), MRI showed that almost the entire tumor mass was covered with the liposome mixture (Fig. 7C). The representative histological tumor section from the same rat obtained immediately after the MRI session (Fig. 7D) and the fluorescent signal generated from Doxil in the same section (Fig. 7E) correlated with MRI. Heterogeneous pattern of Doxil distribution was attributed to different tissue density in the tumor and surrounding area; however, both structures contained Doxil (Fig. 7, E and F). The volume of distribution calculated from the fluorescent signal generated from Doxil in the 9L-2 tumor model was in the same range as expected from the data presented in Fig. 2A (histological data for Doxil not shown).

DISCUSSION

The blood-brain barrier, while otherwise protecting the brain, restricts the delivery of systemically administered agents for treating brain tumors. Although partial disruption of blood-brain barrier is noted in brain tumors, efficient systemic drug delivery throughout the tumor is very difficult. In addition, infiltrating cells that are often outside of main tumor mass could not be targeted via systemic delivery. Therefore, CED techniques, introduced in 1994, as a method to circumvent the blood-brain barrier and enhance distribution of

Stage 2: In Vivo MRI of Liposome Distribution in Brain Tumor Models

Two rat brain tumor models with quite different characteristics were used in this study.

C6 Rat Glioma Model. Histological evaluation of the C6 brain tumor model used in our studies showed a heterogeneous tumor with multiple necrosis sites inside and a relatively clearly encapsulated border (Fig. 5A; Ref. 20). Liposomes, when infused into this heterogeneous tumor, became distributed in an irregular shape and leaked into the encapsulated tumor margin (Fig. 5, B and C). This finding was consistent in all three rats we tested. After infusion (Fig. 5D), the distribution pattern of fluorescence generated from Lip/Gd/DiI-DS was the same as that observed with MRI (Fig. 5E). The volume of liposomal distribution, calculated by histological analysis, achieved within the brain tumor mass was almost the same as that achieved in normal brain (Fig. 5F).

9L-2 Rat Gliosarcoma Model. Histological evaluation of the 9L-2 rat brain tumor model used showed a relatively homogeneous tumor with invasive characteristics (Fig. 6A). \( T_1 \)-weighted MRI with contrast enhancement obtained a day before liposome infusion detected a tumor mass (Fig. 6B). After CED infusion of Lip/Gd/DiI-DS, almost the whole tumor mass was covered with Lip/Gd/DiI-DS (Fig. 6C). Histological evaluation of the tumor after the infusion showed the tumor mass (Fig. 6D), and the whole brain tumor together with surrounding normal brain was covered with Lip/Gd/DiI-DS (Fig. 6E).

Fig. 4. Toxicity analysis. A–C, H&E stainings of paraffin sections (magnification, \( \times 50 \)). Five rats received a 20-\( \mu l \) infusion of Lip/Gd/DiI-DS into the right hemisphere and a 20-\( \mu l \) infusion of Lip/DiI-DS into the left hemisphere. After 7 days of infusion, inflammation noted in the brain receiving Lip/Gd/DiI-DS (A) was a little greater than inflammation of the brain receiving Lip/DiI-DS (B); however, the only inflamed tissue was adjacent to the needle tract and was resolving after 14 days (C). Arrow shows the needle tract found in these sections. D, gain in body weight of the rats receiving Lip/Gd/DiI-DS and Lip/DiI-DS infusions was compared with that of intact normal rats (data point: \( n = 5 \) for day 7; \( n = 3 \) for day 14).
therapeutic agents by local administration (8), represent promising technique for brain tumor therapy, and the safety and feasibility of CED have been well established (16, 17, 21). However, to deliver cytotoxic agents as therapy for brain tumors, which often reside in such critical regions of the brain as the motor cortex, CED administration must meet several requirements. The anticancer agent must be distributed as completely as possible within the tumor and the tumor margins to achieve maximum therapeutic response, but if the agent does not specifically target tumor cells, its distribution has to be restricted to the target tissue to limit nonspecific toxicity. In addition, as shown in our result with infusion in C6 glioma model, tissue distribution can be heterogeneous, with therapeutic agent leaking into an undesirable area. Tissue distribution after CED can be significantly reduced when the catheter tip is placed in the proximity of a large blood vessel, white matter tracts, or a resection cavity due to fluid escape along path of less resistance. Moreover, receptor distribution in the brain tumor and resident cells also will limit tissue penetration of the compounds that possess affinity to these receptors (18, 22). Thus, the only way to ensure efficient delivery of therapeutic agents with CED is to use a vehicle for the compound that will permit visualization of its tissue distribution by using real-time imaging (23). Liposomes have been extensively studied as a carrier of drugs for cancer therapy, including surrogate markers for visualization, and are a good candidate to fill this role (24).

Fig. 5. Convection-enhanced delivery infusion of Lip/Gd/DiI-DS in the C6 glioma rat brain tumor model. A, histological image of a C6 glioma implanted into the brain of Sprague Dawley rat shows a heterogeneous tumor with necrosis inside and a relatively clearly encapsulated border (H&E staining). B, $T_1$-weighted coronal magnetic resonance image after the infusion of 20 µl of Lip/Gd/DiI-DS into a C6 glioma on day 16 after implantation. C, illustration depicting B: the brain is the light blue area; the C6 glioma tumor is gray; and the infused liposome is shown as the black region. Liposomes, when infused into this heterogeneous C6 glioma tumor, became distributed in an irregular shape and leaked into the encapsulated tumor margin. D histological image (H&E staining) made after infusion shows a large tumor. E, fluorescence generated from DiI-DS in the same section. The distribution pattern and shape were comparable with that observed in B. F, the volume of distribution in the tumor mass, as calculated from histological analysis and compared with that achieved in normal brain, showed almost the same volume of distribution in tumor and normal brain.

Fig. 6. Convection-enhanced delivery infusion of Lip/Gd/DiI-DS into a 9L-2 glioma rat brain tumor model. A, histological image of a 9L-2 glioma grown in the brain of Fisher 344 rat shows a relatively homogeneous tumor with invasive characteristics (H&E staining). B, on day 9, this $T_1$-weighted spin echo magnetic resonance image was obtained after i.p. injection of a contrast agent. C, on day 10, 40 µl of Lip/Gd/DiI-DS were infused by convection-enhanced delivery. Almost the whole tumor mass was covered with Lip/Gd/DiI-DS. D, histological image of the tumor after infusion of liposomes (H&E staining). E, DiI-DS distribution was detected in this section. Almost the whole brain tumor and the surrounding normal brain were covered with Lip/Gd/DiI-DS. F, the volume of distribution detected by magnetic resonance imaging in the 9L-2 tumor-bearing rat brain. Three rats received a 20-µl infusion, and 9 rats received a 40-µl infusion. The distribution was similar to that in normal brain, as shown in Fig. 2B.
In this study, to develop an effective method of liposomal brain tumor therapy administered with CED, we established a method for using MRI to monitor the CED of liposomes in the brain. Although there were small differences between the volumes calculated from fluorescent images and MR images, they could be attributed to the greater sensitivity of the histological methods as compared with evaluation of MR images, and monitoring with MRI gave precise information about distribution pattern. MRI monitoring also enabled us to monitor the difference of distribution between different tumor models, i.e., C6 gliomas and 9L-2 gliomas, which suggests the importance of real-time imaging when clinical application is considered. The tumor model with 9L-2 cells, which is a subline derived from commonly used 9L gliosarcoma cells, used in this study has invasive properties, thus it can be considered as a proper model for drug delivery. CED effectively distributed the liposomes in the tumor and the surrounding normal brain tissue that contained isolated invasive tumor cells, implying the potential of a valuable therapeutic advantage in using CED for the treatment of human glioblastoma. In addition, the correlation between the infused volume and the distribution of liposomes in the brain tumor models was almost identical to that observed in normal brain tissue, a finding that could be important in predicting the distribution volume after infusion. One more finding was that the distribution of Doxil infused into the 9L-2 brain tumor model could be monitored using MRI by mixing Doxil with Lip/Gd (marker liposome), which also implies potential anticancer effects of this type of drug administration.

On the other hand, liposome-encapsulated cytotoxic drugs have several potential advantages over corresponding unencapsulated agents (2, 25, 26). When infused systemically, the liposome prolongs the half-life of the drugs in circulation and alters the biodistribution pattern, such that drug deposition is increased in tumor tissue. Taking this advantage, many liposome-encapsulated cytotoxic agents are currently undergoing clinical evaluation, including doxorubicin (27, 28), vincristine (29), and cisplatin (30), and some of them have shown efficacy in patients with solid cancers. After CED of liposomes containing pharmaceutical agents, the majority of the drug is contained within the region of infusion. We believe that drugs may slowly leak into the interstitium after nonspecific intracellular endocytosis (31), thus prolonging exposure of the targeted tissue to the drug. Our recent data after CED of Doxil into rodent brain tumors suggest that doxorubicin is present in the tissue several weeks after a single administration. In addition, greater therapeutic response was detected after CED of Doxil than after systemic administration, further demonstrating therapeutic advantage of local administration of liposomes.

Our findings provide a foundation for real-time MRI monitoring of therapy delivered by liposomal administration through CED into the CNS. They indicate that liposomes containing an anticancer drug could be either loaded with gadodiamide or administered simultaneously with gadodiamide liposomes to provide direct evidence of the volume of distribution of the drug in tissues during CED. Several parameters of CED, such as precise placement of the cannula and control of the rate of infusion and volume of agent delivered, could be adjusted during the infusion to ensure the most safe and efficient local administration of the medication. Several key issues must be addressed, however, before clinical application of this method could be considered. Development of real-time MRI monitoring of liposome distribution during CED is now being developed in our laboratory using nonhuman primates. As for the liposomes, the best therapeutic drug for encapsulation must be identified; liposome constructs, including lipid composition and surface pegylation, must be evaluated, and there must be further development of immunoliposomes for targeting tumor cells. Nonetheless, we believe that the MRI-monitored in vivo detection of liposomes after CED is a first step toward successful clinical application of this technology.

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6 Unpublished data.
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Distribution of Liposomes into Brain and Rat Brain Tumor Models by Convection-Enhanced Delivery Monitored with Magnetic Resonance Imaging

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