Therapeutic efficacy of a polymeric micellar doxorubicin infused by convection-enhanced delivery against intracranial 9L brain tumor models

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Therapeutic efficacy of a polymeric micellar doxorubicin infused by convection-enhanced delivery against intracranial 9L brain tumor models

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Convection-enhanced delivery (CED) with various drug carrier systems has recently emerged as a novel chemotherapeutic method to overcome the problems of current chemotherapies against brain tumors. Polymeric micelle systems have exhibited dramatically higher in vivo antitumor activity in systemic administration. This study investigated the effectiveness of CED with polymeric micellar doxorubicin (DOX) in a 9L syngeneic rat model. Distribution, toxicity, and efficacy of free, liposomal, and micellar DOX infused by CED were evaluated. Micellar DOX achieved much wider distribution in brain tumor tissue and surrounding normal brain tissue than free DOX. Tissue toxicity increased at higher doses, but rats treated with micellar DOX showed no abnormal neurological symptoms at any dose tested (0.1–1.0 mg/ml). Micellar DOX infused by CED resulted in prolonged median survival (36 days) compared with free DOX (19.6 days; $p = 0.0173$) and liposomal DOX (16.6 days; $p = 0.0007$) at the same dose (0.2 mg/ml). This study indicates the potential of CED with the polymeric micelle drug carrier system for the treatment of brain tumors. *Neuro-Oncology 11, 151–157, 2009 (Posted to Neuro-Oncology [serial online], Doc. D08-00039, August 28, 2008. URL http://neuro-oncology.dukejournals.org; DOI: 10.1215/15228517-2008-068)*

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Convection-enhanced delivery (CED) is a promising local delivery technique using bulk flow to deliver low-molecular-weight and macromolecular drugs directly to targeted sites in clinically significant volumes of tissue and to achieve wider volumes of distribution compared with simple diffusion techniques.[1] CED bypasses the blood–brain barrier that prevents most anticancer drugs from penetrating into the CNS, delivers a high concentration of therapeutic agents to the targeted site, and minimizes systemic exposure, resulting in fewer side effects.[1] Many antineoplastic drugs,[2–5] including immunotoxins and boronated drugs,[6,7] have been administered using CED, with promising outcomes in animal studies. The problems include rapid drug clearance from the tumor interstitium,[6] no selective accumulation in targeted tissues,[7] and brain damage caused by highly cytotoxic agents with extensive distribution in the CNS.[2–5] Consequently, novel drug delivery systems are necessary to achieve the highest possible therapeutic index against tumor cells over healthy neuronal cells.[8,9]

Incorporation or attachment of low-molecular-weight anticancer drugs into drug carriers with high molecular

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weight and hydrophilicity may provide substantial inhibition of drug clearance from the tumor interstitium, in contrast to low-molecular-weight drugs that are cleared very rapidly by active transport via proteins such as P-glycoprotein as well as by passive diffusive transport through the lipid bilayer of the endothelium. The drug carrier systems also offer the advantage of sustained drug release, as prolonged exposure time is more important than the peak concentration factor for many anticancer drugs. However, only liposomes have been studied as drug carriers in combination with CED.[8–12]

Polymeric micelles are an assembly of synthetic polymers, most typically block copolymers with both hydrophobic and hydrophilic blocks. Polymeric micelle carrier systems were first studied for targeting solid tumors by intravenous injection.[13–16] Polymeric micelle carrier systems are electrically neutral and so have the so-called stealth property that evades rapid clearance at the reticuloendothelial systems,[17] which substantially improves targeting of murine solid tumors due to the enhanced permeability and retention effect that depends on the hyperpermeable vasculature and absence of effective lymphatic drainage that prevents efficient clearance of micromolecules in the solid tumor tissues.[18] Polymeric micelles incorporating micellar doxorubicin (DOX) were initially developed to enhance the safety and efficacy of conventional DOX.[13] Various micelle-encapsulated cytotoxic agents are currently undergoing clinical evaluation of systemic administration, including DOX,[9] paclitaxel,[19] cisplatin,[20] camptothecin,[21] and the camptothecin derivative SN-38.[22] In contrast, local delivery of polymeric micelle systems for the treatment of brain tumors remains relatively unexplored.

The present study evaluated the therapeutic possibilities of micellar DOX in a 9L syngeneic rat brain tumor model.

Materials and Methods

Preparation of Agents

Doxorubicin hydrochloride was purchased from Mercian Corp. (Tokyo, Japan). Stock solutions of free DOX were prepared by diluting DOX in dimethyl sulfoxide to a concentration of 50 mg/ml. The infusion solution of free DOX was made by diluting the stock solution with phosphate-buffered saline (PBS). Liposomal DOX (Doxil) was obtained from Alza Pharmaceuticals (Mountain View, CA, USA). Micellar DOX was prepared by the previously reported method in a slight modification only in high-performance liquid chromatography (HPLC) analysis conditions.[14–16] In brief, DOX was chemically conjugated to the aspartic acid residue of poly(ethylene glycol)-b-poly(aspartic acid) block copolymer. The hydrophobicity of the DOX-conjugated poly(aspartic acid) block results in the formation of a polymeric micelle structure. This conjugate block copolymer was used to form empty polymeric micelles in this study because this type of micelle does not contain unbound DOX that is important in cytotoxic activity. Free DOX was incorporated into empty polymeric micelles to form pharmacologically active polymeric micelles that contain free DOX in the micelle inner core. The poly(ethylene glycol) block had a molecular weight of 12 kDa and contained 22 aspartic acid units as determined by ^{1}H-nuclear magnetic resonance spectrum in D_2O. DOX was chemically conjugated to 59% of the aspartic acid residues, and the micelles contained 13 wt% free DOX. The amounts of the chemically conjugated DOX and the physically entrapped DOX were determined by a reverse-phase HPLC according to methods previously reported.[15,16] The empty polymeric micelles (DOX was not physically entrapped) chemically conjugated DOX molecules to 68% of the aspartic acid residues. The infusion solution for micellar DOX, PBS, had no toxicity when 20 µl was infused by CED (preliminary data not shown).

Tumor Cell Lines

9L gliosarcoma cells (American Type Culture Collection, Rockville, MD, USA) were maintained as monolayers in a complete medium consisting of Eagle's minimal essential medium supplemented with 10% fetal calf serum, nonessential amino acids, and 100 U/ml penicillin G. Cells were cultured at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2.

Animals and Intracranial Syngeneic Transplantation Technique

All protocols utilized in the animal studies were approved by the Institute for Animal Experimentation of Tohoku University Graduate School of Medicine. Male Fischer 344 rats weighing 150–200 g and normal male Sprague-Dawley rats weighing approximately 150–200 g were purchased from Charles-River Laboratories (Charles-River Japan Inc., Tsukuba, Japan). For the intracranial syngeneic tumor model, 9L gliosarcoma cells were harvested by trypsinization, washed once with Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS), and resuspended in HBSS for implantation. Cells (5×10^5) in 10 µl HBSS were implanted into the striatal region of the rat brains as follows. The rats under deep isoflurane anesthesia were placed in a small-animal stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). A sagittal incision was made to expose the cranium, followed by a burr hole in the skull at 0.5 mm anterior and 3 mm lateral from the bregma using a small dental drill. Cell suspension (5 µl) was injected over 2 min at a depth of 4.5 mm from the brain surface; after a 2-min wait, another 5 µl was injected over 2 min at a depth of 4.0 mm, and after a final 2-min wait, the needle was removed and the wound was sutured.

CED Infusion

CED of PBS, free DOX, liposomal DOX, and micellar DOX was performed as described previously.[9,11] The infusion system consisted of a reflux-free step design infusion cannula[23] connected to a loading line (containing 20 µl PBS, free DOX, liposomal DOX, or micellar

DOX solutions) and an olive oil infusion line. A 1-ml syringe (filled with oil) was mounted onto a microinfusion pump (BeeHive; Bioanalytical Systems, West Lafayette, IN, USA) to regulate the flow of fluid through the system. Based on chosen coordinates, the infusion cannula was mounted onto a stereotactic holder and guided to the target region of the brain through burr holes made in the skull (see below). The infusion rates followed the following ascending pattern to deliver the total 20-µl infusion volume: 0.2 µl/min (15 min) + 0.5 µl/min (10 min) + 0.8 µl/min (15 min).

Evaluation of Distribution of Micellar DOX in Normal Rodent CNS

Normal Sprague-Dawley rats (five rats in each group) received CED using free DOX (2 mg/ml DOX in 20 µl solution), liposomal DOX (20 µl solution containing 2 mg/ml DOX equivalent), and micellar DOX (20 µl solution containing 2 mg/ml physically entrapped DOX and 4.3 mg/ml chemically conjugated DOX equivalent), and empty polymeric micelles (20 µl solution containing 7.9 mg/ml chemically conjugated DOX equivalent) and were euthanized immediately after CED. The brains were harvested, frozen in isopentane chilled in dry ice, and cut into serial coronal sections (25 µm) with a cryostat. DOX fluoresces under UV illumination, so the areas of distribution could be visualized by fluorescence microscopy and captured with a charged-coupled device camera with a fixed aperture. The empty polymeric micelles were also fluorescent in a similar manner to the micellar DOX, since the empty polymeric micelles contained chemically conjugated DOX molecules that were almost equivalent in their fluorescent behavior to physically entrapped DOX of the micellar DOX. The volume of distribution was analyzed with a Macintosh-based image-analysis system (NIH Image 1.62; NIH, Bethesda, MD, USA) as described previously.[24]

Evaluation of Distribution of Micellar DOX in Rats with 9L Intracranial Tumors

Fischer 344 rats (four rats in each group) with 9L intracranial tumors received CED using micellar DOX and free DOX (20 µl solution containing 2 mg/ml DOX equivalent) 7 days after tumor cell implantation. Rats were euthanized immediately after CED. The brains were harvested, frozen in isopentane chilled in dry ice, and cut into serial coronal sections (25 µm) with a cryostat.

Toxicity Tests of Micellar DOX

Normal Sprague-Dawley rats (five rats in each group) received a single CED infusion of free DOX, liposomal DOX, micellar DOX, and empty polymeric micelles (20 µl solutions containing 0.1, 0.2, 0.4, or 1.0 mg/ml free DOX equivalent). Rats were monitored daily for survival and general health (alertness, grooming, feeding, excreta, skin, fur, mucous membrane conditions, ambulation, breathing, and posture) and weekly for weight. The rats in each group were euthanized 3 weeks after the CED treatment, and their brains were removed, fixed, cut into sections (5 µm), and stained with hematoxylin and eosin (H&E).

Survival Studies

Fifty-two Fischer 344 rats with 9L tumor cells were randomly assigned to four groups: (1) the control group that received PBS (20 µl solution; $n = 17$), (2) free DOX (0.2 mg/ml DOX in 20 µl solution; $n = 10$), (3) liposomal DOX (20 µl solution containing 0.2 mg/ml DOX equivalent; $n = 14$), and (4) micellar DOX (20 µl solution containing 0.2 mg/ml physically entrapped free DOX equivalent; $n = 11$). Seven days after tumor cell implantation, a single CED infusion was performed for each group. Rats were monitored daily for survival and general health, and weekly for weight. The study was terminated 90 days after tumor implantation. The surviving animals were euthanized and their brains stained with H&E. Survival was expressed as a Kaplan-Meier curve. Survival between the treatment groups was compared with a log-rank test.

Results

Evaluation of Distribution of Micellar DOX in Normal Rodent CNS

Compared with free DOX (Fig. 1a), intrastriatal administration of liposomal DOX (Fig. 1b), micellar DOX (Fig. 1c), and empty polymeric micelles (Fig. 1d) via CED (20 µl volume) produced extensive and diffuse distribution in the striatum. The mean volumes of distribution of free DOX, liposomal DOX, micellar DOX, and empty polymeric micelles in normal rat brains were 13.91 ± 1.23 mm^3 (range, 12.12–15.32 mm^3), 64.25 ± 7.83 mm^3 (54.76–77.40 mm^3), 60.54 ± 5.71 mm^3 (54.40–64.25 mm^3), and 54.74 ± 4.39 mm^3 (50.30–58.97 mm^3), respectively (Fig. 1e). A significant difference was observed between free DOX and micellar DOX ($p = 0.009$), but not between liposomal DOX and micellar DOX ($p = 0.465$).

Evaluation of Distribution of Micellar DOX in Rats with 9L Tumors

Examination of representative rat brain sections at 1-mm intervals confirmed successful formation of the tumor tissue. Fluorescent detection of DOX in the same sections revealed poor distribution of free DOX (Fig. 2a) but distribution of micellar DOX over almost the entire tumor mass, including the surrounding tumor margins (Fig. 2b). The findings were consistent in all four rats examined from this group.

Toxicity of Micellar DOX in Normal Rodent CNS

Rats euthanized 3 weeks after infusion with 0.2 (Fig. 3a, center), 0.4 (Fig. 3a, right), and 1.0 (data not shown) mg/ml micellar DOX showed tissue damage at the infusion

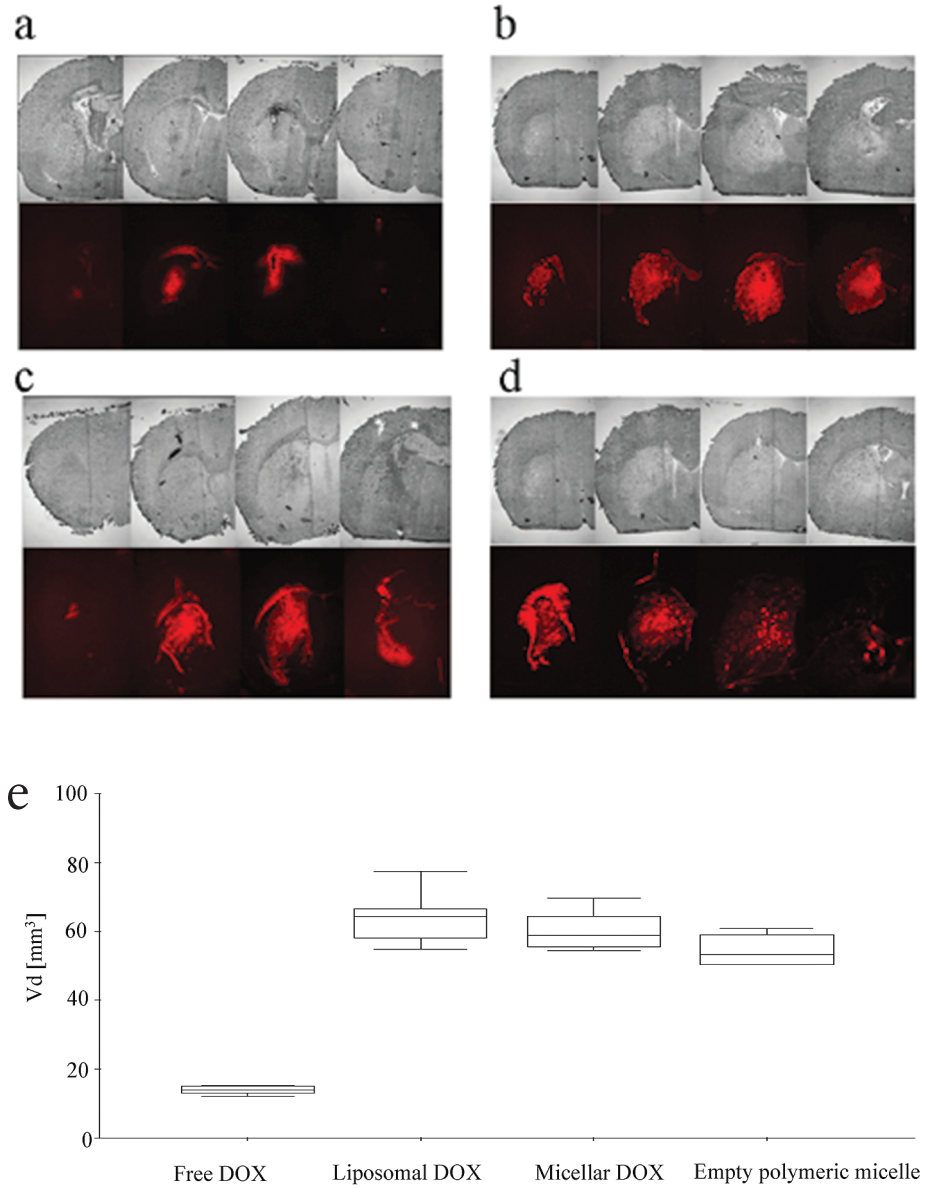

Fig. 1. Evaluation of distribution of micellar DOX in normal rats. Sequential hematoxylin and eosin-stained sections 25 μm thick at 1-mm intervals reveal the tumor (top). The sections were examined with a fluorescence microscope to detect the fluorescence generated by DOX (bottom). Compared with free DOX (a), liposomal DOX (b), micellar DOX (c), and empty polymeric micelles (d) produced extensive and diffuse distribution in the striatum. (e) Median values and the 75% quartiles of volume distribution (Vd) after infusion of free DOX, liposomal DOX, micellar DOX, and empty polymeric micelles. The difference in the distribution between free DOX and micellar DOX was statistically significant ($p = 0.009$, as analyzed by Mann-Whitney U-test), but differences in the distribution among liposomal DOX, micellar DOX, and empty polymeric micelles were not.

site but did not develop neurological symptoms. Rats that received 0.1 mg/ml micellar DOX (Fig. 3a, left) showed negligible tissue damage and survived without neurological symptoms. Rats infused with 0.1 (Fig. 3b, left) and 0.4 (Fig. 3b, right) mg/ml free DOX showed tissue damage without neurological symptoms. Rats infused with 0.1 (Fig. 3c, left) and 0.4 (Fig. 3c, right) mg/ml liposomal DOX revealed less tissue damage. Therefore, the maximum tolerated dose of micellar DOX was established as below 0.2 mg/ml. Rats treated with empty polymeric micelles showed no toxicity (data not shown).

Antitumor Efficacy of Micellar DOX Treatment

Rats in the control group (Fig. 4a) were all euthanized at 10–21 days after tumor cell implantation due to neurological symptoms indicative of tumor progression. Median survival for this group was 16.9 days. Rats that received CED of free DOX (Fig. 4b) were euthanized due to neurological complications indicative of tumor progression at 16–33 days after tumor cell implantation. Median survival for this group was 19.6 days. Rats that received CED of 0.2 mg/ml liposomal DOX

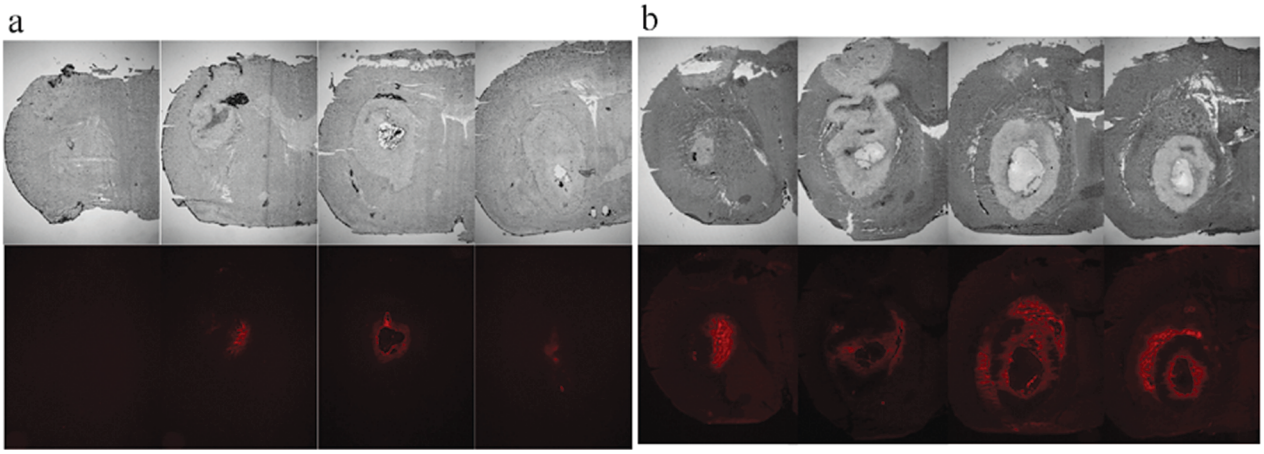

Fig. 2. Evaluation of distribution of micellar DOX in rats with 9L tumors. Seven days after tumor cell implantation, 2 mg/ml free DOX or micellar DOX was infused intratumorally by CED. Rats were euthanized 1 h after CED, and the brains were sectioned on a cryostat. Sequential hematoxylin and eosin-stained sections 25 μm thick at 1-mm intervals reveal the tumor (top). The same sections were examined with a fluorescence microscope to detect the fluorescence generated by DOX (bottom). Free DOX had poor distribution (a), whereas micellar DOX achieved coverage of almost the entire tumor mass, including the surrounding tumor margins (b). The findings were consistent in all four animals.

(Fig. 4c) were euthanized at 10–27 days after tumor cell implantation. Median survival for this group was 16.6 days. Formation of large tumors was verified in all rats euthanized in these three groups. Nine of the 11 rats that received CED of 0.2 mg/ml micellar DOX (Fig. 4d) were euthanized at 15–43 days after tumor cell implantation due to neurological symptoms, but the other two rats survived until termination of the study at 90 days. Median survival for this group was 36 days. The survival time after CED with 0.2 mg/ml micellar DOX was significantly greater than after CED with free DOX (p = 0.0173) or liposomal DOX (p = 0.0007). Although the rats in the control group had histological signs of tumor in the brain (Fig. 4e), only a small amount of brain damage was observed in the surviving two rats that received micellar DOX (Fig. 4f).

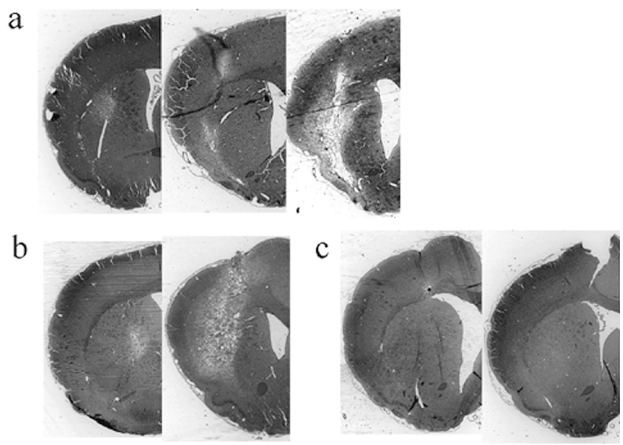

Fig. 3. Toxicity at different drug concentrations. (a) Rats infused with 0.1 mg/ml micellar DOX (left) survived without neurological symptoms and negligible tissue damage. Rats infused with 0.2 (center) and 0.4 (right) mg/ml micellar DOX showed significant tissue damage at the infusion site but did not develop neurological symptoms. (b) Rats infused with 0.1 (left) and 0.4 (right) mg/ml free DOX demonstrated tissue damage without neurological symptoms. (c) Rats infused with 0.1 (left) and 0.4 (right) mg/ml liposomal DOX revealed less tissue damage, which might reflect the poor drug release.

Discussion

The present study found that liposomal DOX and micellar DOX had similar extensive distributions in the normal rat brain and were far more widely distributed than free DOX. This study also showed distribution of micellar DOX over almost the entire tumor area, including the margins. CED distribution in CNS is significantly increased if the infusate is more hydrophilic, which implies less tissue affinity.[9,11] Furthermore, polyethylene glycol encapsulation provides steric stabilization, reduces surface charge, and achieves better distribution in brain.[9] The poorer brain distribution observed with hydrophobic or cationic infusate can be completely overcome by polyethylene glycol encapsulation.[9]

Delivery pattern of micellar DOX was expected to avoid the high peak concentrations of free DOX potentially associated with toxicity.[13,25] However, evaluation of the toxicity in normal rat brain found that 0.2 mg/ml micellar DOX caused a lesion similar to that caused by 0.2 mg/ml free DOX, although no difference in toxicity was observed. In contrast, 0.2 mg/ml liposomal DOX showed no toxicity, which might reflect poor release of the cytotoxic agent. These findings indicate that the safety of micellar DOX in CED could be achieved by optimization of drug release to avoid the high concentrations that trigger nonspecific toxicity. Micellar systems, relative to liposomal systems, offer the advantage of adjustable drug release based on the properties of the

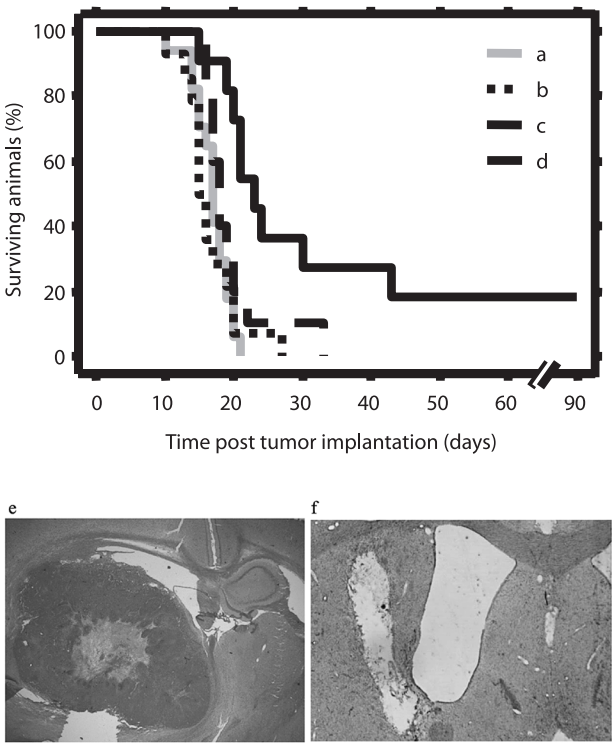

Fig. 4. Survival study. (a–d) Outcome for rats bearing 9L tumors with single CED infusion of saline (a), free DOX (b), liposomal DOX (c), and micellar DOX (d). Seven days after tumor implantation within the brain, rats were treated with 0.2 mg/ml of each agent. Median survival for the groups were 16.9 days (a), 19.6 days (b), 16.6 days (c), and 36 days (d). Statistically significant differences were observed between free DOX and micellar DOX ($p = 0.0173$) and between liposomal DOX and micellar DOX ($p = 0.0007$). (e and f) Histological examination found tumor formation in the control group (e) and brain damage in rats receiving micellar DOX (f).

micelle inner core,[13–16,25] but further development of micellar systems for use with CED is required.

The present study found that CED with 0.2 mg/ml micellar DOX significantly prolonged survival in rats with intracranial 9L glioma, compared with CED with 0.2 mg/ml free DOX and liposomal DOX, which is known to show efficient targeting to solid tumors by systemic injection like micellar DOX. Liposomal DOX does not release DOX efficiently because of excessive stable incorporation in the lipid bilayer,[17] which probably accounts for the absence of significant increase in efficacy against rapidly growing 9L syngeneics compared with free DOX.

The polymeric micelle system has three advantages as a drug carrier compared with the liposome drug carrier. First, polymeric micelles can incorporate hydrophobic drugs into the inner core phase, to high loadings such as 30 wt% without losing targeting potential. In contrast, the liposomal system may easily lose targeting ability at high drug loadings because the hydrophobic drug must be incorporated into the very thin lipid bilayer. Second, polymers may be based on many chemical structures with various physical characteristics such as crystalline and glassy, so the drug release rate can be adjusted in a very wide range from minutes to days. Finally, micelle diameters can be tightly controlled in a range from 10 nm to 100 nm, choosing the appropriate chemical structures and chain lengths of polymers. The polymeric micelle can obtain this size control among many types of drug carriers. Consequently, the micelle system has high potential for specific adjustment to the treatment of various CNS tumors by CED infusion. Furthermore, micellar systems could be used in monitoring drug distribution by using micelles containing a marker for imaging.[23,24] The present findings indicate the significant potential of micellar drugs for the treatment of malignant glioma.

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