Effect of Polyethylene Glycol Linker Chain Length of Folate-Linked Microemulsions Loading Aclacinomycin A on Targeting Ability and Antitumor Effect *In vitro* and *In vivo*

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ABSTRACT

Purpose: **To establish a novel formulation tumor-targeted drug carrier of lipophilic antitumor antibiotics, aclacinomycin A (ACM), folate-linked microemulsions were prepared and investigated both *in vitro* and *in vivo*.**

Experimental Design: **Three kinds of folate-linked microemulsions with different polyethylene glycol (PEG) chain lengths loading ACM were formulated with 0.24 mol% folate-PEG_{2000}-distearoylphosphatidylethanolamine (DSPE), folate-PEG_{5000}-DSPE, and folate-lipid (without PEG linker) in microemulsions. *In vitro* studies were done in a human nasopharyngeal cell line, KB, which overexpresses the folate receptor (FR), and a human hepatoblastoma cell line, [FR(−)] HepG2. *In vivo* experiments were done in a KB xenograft by systemic administration of folate-linked microemulsions loading ACM.**

Results: **The association of folate-linked microemulsions to KB cells could be blocked by 2 mmol/L free folic acid. Selective FR-mediated cytotoxicity of folate-linked microemulsions loading ACM was obtained in KB but not in HepG2 cells. The association of the folate-PEG_{5000}-linked microemulsion and folate-PEG_{2000}-linked microemulsion with the cells was 200- and 4-fold higher, whereas their cytotoxicity was 90- and 3.5-fold higher than those of nonfolate microemulsion, respectively. The folate-PEG_{5000}-linked microemulsions showed 2.6-fold higher accumulation in solid tumors 24 hours after i.v. injection and greater tumor growth inhibition than free ACM.**

Conclusion: **These findings suggest that a folate-linked microemulsion is feasible for tumor-targeted ACM delivery. This study shows that folate modification with a sufficiently long PEG chain on emulsions is an effective way of targeting emulsion to tumor cells.**

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INTRODUCTION

Aclacinomycin A (ACM) hydrochloride is an anthracycline antitumor antibiotic isolated from *Streptomyces galilaeus* MA-144-M1. ACM inhibits synthesis of nucleic acid, especially RNA, by acting as an inhibitor of not only topoisomerase II but also topoisomerase I (1). This drug is widely used for the treatment of stomach, lung, and ovarian carcinoma; malignant lymphoma; and acute leukemia, but its clinical use is still limited by its toxicity. To reduce the toxic responses and increase the antitumor effect of ACM, it should be effectively targeted to the tumor. To achieve this, polyethylene glycol (PEG)–coated (containing PEG-derivatized phospholipid) liposomes, long-circulating liposomes, are promising. ACM is a lipophilic drug that is not stable in water. Therefore, long-circulating microemulsions, which can contain lipophilic agents and have no inner water phase, may be a more suitable carrier for delivery of ACM. We previously reported a long-circulating microemulsion loading ACM that showed successful antitumor effects in animal experiments (2). The antitumor effect would be enhanced by combining long circulation with tumor targeting.

A variety of targeting ligands has been examined as tumor-targeted drug carriers. Folate receptor (FR) is abundantly expressed in a large percentage of human tumors, but it is only minimally distributed in normal tissues (3–6). There are three FR isoforms, α, β, and γ, with distinctive patterns of tissue distribution. FR-α is predominantly expressed in most normal and malignant epithelial tissues (3), FR-β in some nonepithelial malignancies (4, 5), and FR-γ in hematopoietic cells (6). Therefore, FR can serve as an excellent tumor marker as well as a functional tumor-specific receptor.

Folic acid, a high-affinity ligand for FR, retains its receptor-binding and endocytosis properties even if it is covalently linked to a wide variety of molecules. Therefore, the liposomes conjugated to the folate ligand via a PEG spacer have been used to deliver chemotherapeutic agents, oligonucleotide, and markers to receptor-bearing tumor cells, e.g., KB cells, which overexpress FR-α (7–14). Folate-linked microemulsions *in vitro* have also been reported (15), but not yet *in vivo*. From the result of folate-PEG-linked liposomes, it is already known that the length of the PEG linker chain is important for the liposomes to be internalized by recognition of FR (8).

Here, we report the targeting ability and antitumor effect of folate-linked microemulsions, loading ACM both in *in vitro* and *in vivo*, in terms of the PEG length of the folate-PEG linker chain. This study shows that folate modification with a

sufficiently long PEG chain on emulsions is an effective way of targeting an emulsion to tumor cells.

MATERIALS AND METHODS

Chemicals. PEG_{2000}-distearoylphosphatidylethanolamine (PEG_{2000}-DSPE, mean molecular weight of PEG: M_r 2,000) was supplied, and amino-PEG_{2000}-DSPE and amino-PEG_{5000}-DSPE (mean molecular weight of PEG: M_r 2,000 and 5,000) were kindly donated by NOF Corporation (Tokyo, Japan). ACM hydrochloride for injection (Aclacinon) was kindly donated by Mercian Corporation (Tokyo, Japan). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Lambda Probes & Diagnostics (Graz, Austria). Folic acid, cholesterol, and vitamin E were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Other reagents used in this study were reagent grade.

Synthesis of the Folate-Lipid and Folate-Polyethylene Glycol-Lipid. Folate-lipid (direct folate conjugate of lipid), *N*-(*N*-folyl-6-aminohexyl)-3,5-bis(dodecyloxy)benzamide, was synthesized as follows (Fig. 1*A*). Folic acid (300 mg, 0.680 mmol) and dicyclohexylcarbodiimide (280 mg, 1.36 mmol) were dissolved in DMSO/pyridine (10 mL/4 mL) and the solution was stirred at room temperature for 1 hour. *N*-(6-Aminohexyl)-3,5-bis(dodecyloxy)benzamide (400 mg, 0.680 mmol; ref. 16) was added to the solution and the reaction was continued at room temperature for another 3 hours. The resulting mixture was concentrated to remove pyridine and cooled at 4°C. The resulting precipitate was collected by filtration and was washed with methanol to give *N*-(*N*-folyl-6-aminohexyl)-3,5-bis(dodecyloxy)benzamide (412 mg, 0.407 mmol) at a yield of 60%. ^{1}H nuclear magnetic resonance (600 MHz, DMSO-d_6/D_2O = 98:2) δ0.85 (6H, t, J = 6.9 Hz), 1.20 to 1.32 (36H, m), 1.39 (6H, m), 1.49 (2H, m), 1.69 (4H, quint, J = 6.8 Hz), 1.88 (1H, m), 1.99 (1H, m), 2.25 (2H, t, J = 7.9 Hz), 3.05 (2H, m), 3.20 (2H, m), 3.97 (4H, t, J = 6.8 Hz), 4.35 (1H, dd, J = 3.9, 3.4 Hz), 4.48 (2H, s), 6.56 (1H, s), 6.65 (2H, d, J = 8.6 Hz), 6.95 (2H, s), 7.64 (2H, d, J = 8.6 Hz), 8.64 (1H, s).

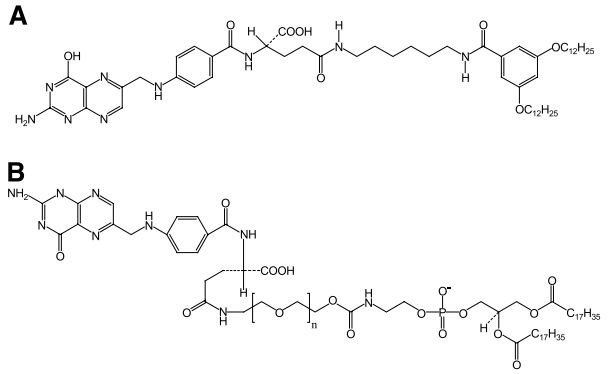

Fig. 1 Structure of the folate-linked lipids. *A,* folate-lipid, which is a folate direct conjugate of a double-chain lipid, was synthesized by dicyclohexylcarbodiimide-mediated coupling. *B,* folate-PEG_{5000}-DSPE and folate-PEG_{2000}-DSPE, which are conjugates of folate and PEG-DSPE, were also synthesized by dicyclohexylcarbodiimide-mediated coupling of folic acid to amino-PEG-DSPE. n ≈ 42 (PEG_{2000}), 113 (PEG_{5000}).

Folate-linked PEG_{2000}-DSPE (folate-PEG_{2000}-DSPE) and folate-PEG_{5000}-DSPE, which are conjugates of folic acid and amino-PEG-DSPE, were prepared as previously reported (Fig. 1*B*; ref. 8). The folate content of the product was determined by quantitative UV spectrophotometry of the product dissolved in a 10 mmol/L Tris-HCL buffer (pH 8.0) / methanol (1:1, v/v) at 285 nm. The yields of folate-PEG_{2000}-DSPE and folate-PEG_{5000}-DSPE were 86% and 75%, respectively. The chemical structure of the product was analyzed by electrospray ionization time-of-flight mass spectrometry. Electrospray ionization-ion trap mass spectrometry (methanol) calculated for $C_{155}H_{288}N_9O_{62}P$ (n = 46), $[M\text{-}2H]^{2-}/2$ 1,649.5, found 1,648.3; calculated for $C_{157}H_{292}N_9O_{63}P$ (n = 47), $[M\text{-}2H]^{2-}/2$ 1,671.5, found 1,669.8; calculated for $C_{159}H_{296}N_9O_{64}P$ (n = 48), $[M\text{-}2H]^{2-}/2$ 1,693.5, found 1,692.3.

Preparation of Microemulsions. Microemulsions were composed of PEG_{2000}-DSPE/cholesterol/vitamin E/ACM hydrochloride (M-ACM, 3:3:3:1, weight ratio; 7:48.3:43.3:1.5, molar ratio). Drug-free folate-linked microemulsions are expressed as F/M. Folate-PEG_{5000}-DSPE (F5), folate-PEG_{2000}-DSPE (F2), and folate-lipid (F0), at a mol% of 0.24, were added as a part of PEG_{2000}-DSPE of M-ACM (F5, F2, F0/PEG_{2000}-DSPE/cholesterol/vitamin E/ACM hydrochloride = 0.24:6.7:48.3:43.3:1.5, molar ratio; F5/M-ACM, F2/M-ACM, F0/M-ACM, respectively). Microemulsions were prepared by a modified ethanol injection method as described previously (2), except for F0/M-ACM. For F0/M-ACM, folate-lipid and cholesterol were dissolved in an appropriate volume of chloroform that was then removed. The subsequent process after being dissolved in ethanol was the same as for the other microemulsions. DiI-labeled F5/M, F2/M, F0/M, and microemulsion were prepared by the same protocol, but with post-addition of DiI at 1.33 mol% of the total lipids. The particle diameter distribution of microemulsions was measured by the dynamic light scattering method with ELS-800 (Otsuka Electronics Co., Ltd., Osaka, Japan) at 25°C by diluting the dispersion to an appropriate volume with water. The ACM-loading efficiency was taken as the percentage of ACM carried by the microemulsions and was determined by Sephadex G-50 chromatography and a fluorescence detector, as described previously (2). The ACM-loading efficiency was also confirmed by measuring the ACM amount of the microemulsion fraction using high-performance liquid chromatography as described in Biodistribution Studies in Tumor-Bearing Mice.

Cell Culture. KB cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). HepG2 cells were obtained from the Riken Cell Bank (Ibaraki, Japan). Both cells were cultured in folate-deficient MEM (originally prepared based on Eagle's MEM) with 10% heat-inactivated fetal bovine serum (Invitrogen Corp., Carlsbad, CA) and 50 μg/mL kanamycin sulfate (Wako Pure Chemical Industries) as a 5% CO_2 at 37°C.

RNA Isolation and Reverse Transcription-PCR. Total RNA was isolated from KB and HepG2 cells, respectively, using NusleoSpin RNA II (Macherey-Nagel, Dueren, Germany). RNA yield and purity were checked by spectrometric measurement at 260 and 280 nm and RNA electrophoresis, respectively. First-strand cDNA was synthesized from 5 μg total RNA after denaturation for 5 minutes at 65°C using 50 pmol random primer, 0.5 mmol/L deoxynucleotide triphosphate, and 5 units

of AMV Reverse Transcriptase XL (Takara Shuzo Co., LTD., Japan). The reaction was done at 41°C for 1 hour in 20 μL volume. For reverse transcription-PCR, a 25 μL reaction volume contained the following: 1 μL synthesized cDNA, 10 pmol of each specific primer pair, and 0.25 units Ex Taq DNA polymerase (Takara Shuzo) with a PCR buffer containing 1.5 mmol/L $MgCl_2$ and 0.2 mmol/L of each deoxynucleotide triphosphate. The profile of PCR amplification consisted of denaturation at 94°C for 0.5 minute, primer annealing at 58°C for 0.5 minute, and elongation at 72°C for 1 minute for 25 cycles. PCRs of the housekeeping gene *β-actin*, *FRs* (*FR-α*, *FR-β*, *FR-γ*), and *reduced folate carrier* were done at the same cycle run for all samples. The PCR products for FRs, reduced folate carrier, and β-actin were analyzed by 1.5% agarose gel electrophoresis in a Tris-borate-EDTA buffer. The products were visualized by ethidium bromide staining.

Flow Cytometry Analysis. Cells were prepared by plating 3×10^5 cells in a 12-well culture plate 1 day before the assay. Cells were incubated with DiI-labeled microemulsions [F5/M, F2/M, F0/M, and microemulsion (M-ACM without ACM) containing 90 μg total lipid] diluted in 1 mL serum-free medium for 1 hour at 37°C. In free folate competition studies, 2 mmol/L folic acid was added to the medium. After incubation, cells were washed thrice with cold PBS (pH 7.4) to remove unbound microemulsions, detached with 0.02% EDTA-PBS, and then suspended in PBS containing 0.1% bovine serum albumin and 1 mmol/L EDTA. The suspended cells were directly introduced to a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 488 nm argon ion laser. Data for 10,000 fluorescent events were obtained by recording forward scatter, side scatter, and 585/42 nm fluorescence. The autofluorescence of cells was taken as a control; cells were incubated with serum-free medium without drugs for 1 hour.

Confocal Microscopy. Cell cultures were prepared by plating 3×10^5 cells in 35 mm culture dishes 1 day before the experiment. Medium was replaced with 1 mL fresh serum-free medium containing either F2/M-ACM or M-ACM (containing 0.5 μg ACM/mL). Following 1-hour incubation, each dish was rinsed five times with 1 mL cold PBS and then fixed with 1 mL 20% neutral-buffered formalin (Mildform 20 N, Wako Pure Chemical Industries) for 1 hour at room temperature. Cell-associated fluorescence was imaged using a Radiance 2100 confocal laser-scanning microscope (Bio-Rad Laboratories, Inc., Hercules, CA). Maximum excitation was done with the 488 nm line of an argon laser and fluorescence emission was observed at >570 nm (using 560DCLP and E570LP filters).

Cytotoxicity Study. Briefly, 5×10^4 cells were plated into 96-well culture plates 1 day before the experiment. KB and HepG2 cells were then incubated for 1 hour at 37°C with 100 μL F5/M-ACM, F2/M-ACM, M-ACM, or free ACM (containing 0.004, 0.04, 0.4, or 4 μg ACM) diluted in 1 mL serum-free medium. The medium was then replaced with fresh medium and incubated for a further 72 hours. The cytotoxicity was determined by a WST-8 assay (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan). The number of viable cells was then determined by absorbance measured at 450 nm on an automated plate reader.

***In vitro* Drug Release.** The release of the drug in M-ACM in PBS (pH 7.4) was monitored by a dialysis method. The dialysis was done at 37°C using seamless cellulose tube membranes (Viskase Sales Corp., Willowbrook, IL) with molecular weight cutoff of M_r 14,000 as the sink solution. The initial concentration of M-ACM was 20 μg/mL. The sample volume in the dialysis bag was 1 mL and the sink volume was 100 mL. The concentration of drug was analyzed at various time points during the dialysis process. The concentration of drug was monitored using high-performance liquid chromatography as described in Biodistribution Studies in Tumor-Bearing Mice.

Animals and Inoculation of Tumor Xenografts. Specific pathogen-free female athymic BALB/c *nu/nu* nude mice (7 weeks of age on arrival, Oriental Yeast Co., Ltd., Tokyo, Japan) were maintained on a folate-deficient diet (AIN-93M-based Folate-Deficient Rodent Diet, Oriental Yeast) on arrival and for the duration of the study. To generate tumor xenografts, a KB cell suspension of appropriate concentration in PBS was injected s.c. in the right hind crus of the mice. The animal experiments were done under ethical approval from our Institutional Animal Care and Use Committee.

Serum Folate Concentration. Tumor-inoculated mice were first maintained on a regular diet (MF Rodent Certified Diet, Oriental Yeast) for 3 weeks on arrival and then either continued on this diet or switched to a folate-deficient diet for 1 week. Folate concentration in sera was measured using a competitive enzyme immunoassay kit, AIA-Pack Folate (Tosoh Corporation, Tokyo, Japan).

Biodistribution Studies in Tumor-Bearing Mice. Mice were s.c. inoculated with 2×10^6 cells and then grouped at random. Seven days after inoculation, mice were given F5/M-ACM, M-ACM, and free ACM by i.v. injection via the lateral tail vein at a single dose of 5 mg ACM/kg. After 24 hours, the mice were anesthetized by ether inhalation and immediately sacrificed by cervical dislocation. Blood was collected in heparinized tubes and immediately centrifuged to separate plasma from blood cells. The liver, spleen, kidneys, heart, lung, and tumor were removed, rinsed in physiologic saline, weighed, and frozen at −20°C. They were homogenized by adding 0.2 mol/L phosphate buffer (pH 7.0). ACM was extracted from the biological samples as previously reported (17). The high-performance liquid chromatography system was composed of an LC-10AS pump (Shimadzu Co., Ltd., Kyoto, Japan), an RF-10AXL fluorescence detector (excitation = 435 nm, emission = 505 nm; Shimadzu), and a YMC-Pack ODS-AA-302 column (150 × 4.6 mm internal diameter, YMC Co., Ltd., Kyoto, Japan). The mobile phase was acetonitrile/methanol/1.5% aqueous H_3PO_4 (40:90:70, v/v) and the flow rate was 1.0 mL/min (18). The concentration of ACM in each sample was determined using a calibration curve.

Antitumor Effects. To evaluate the antitumor effects of microemulsion, mice were inoculated with 1×10^6 cells s.c. in the hind crus and were then grouped at random (day 0). Three days after inoculation, treatment was started at a dose of 5 mg ACM/kg: (*a*) F5/M-ACM, (*b*) F2/M-ACM, (*c*) M-ACM, (*d*) free ACM, and (*e*) saline as a control. The drugs were administered by i.v. injection via the lateral tail vein on every 3rd day (on day 3, 6, 9, 12, and 15). Tumor size was measured with vernier calipers on every 3rd day eight times (on day 3, 6, 9, 12, 15, 18, 21, and 24). Tumor volume was

calculated by the following equation: volume = $\pi/6 \times$ width $\times$ height $\times$ length.

Statistical Analysis. The statistical significance of the data was evaluated by the Student's t test. $P \leq 0.05$ was considered significant.

RESULTS

Characterization of Microemulsions. Four kinds of ACM-loading microemulsions were formulated with M-ACM and 0.24 mol% of folate in F5/M-ACM, F2/M-ACM, and F0/M-ACM. All microemulsions were coated with PEG_{2000}-DSPE to prolong the circulation time in the bloodstream. A schematic diagram shows that conjugated folate is located outside the coating PEG layer in F5/M-ACM, on the surface of the PEG-layer in F2/M-ACM, and inside the PEG-layer in F0/M-ACM (Fig. 2).

The average particle diameter of each microemulsion in water was ~120 nm and the ACM-loading efficiency was ~80% (data not shown). The average diameter and the ACM-loading efficiency of microemulsions did not change significantly for at least 1 month at 4°C in the dark. M-ACM was stable, showing the attenuated drug release, <50% release of drug for 48 hours in PBS (data not shown).

Expression of Folate Receptor Messenger RNA. We investigated the expression of FRs in the cell lines by reverse transcription-PCR. There were three FR isoforms, α, β, and γ, with distinctive patterns of tissue distribution. FR-α mRNA was strongly expressed in KB cells, but not HepG2 cells. FR-β and FR-γ mRNAs were not expressed in the cell lines (data not shown). This suggested that the cellular uptake of folate-linked microemulsion in KB cells was mediated via FR-α.

Cellular Association Determined by Flow Cytometry. We examined the selectivity of folate-linked microemulsions for delivery into KB cells using microemulsions labeled with DiI, a nonexchangeable fluorescent membrane probe, by flow cytometry.

As shown in Fig. 3, flow cytometry analysis represented a shift in the curve, indicating a clear increase in cellular association of F5/M and F2/M after 1-hour exposure. The mean fluorescence intensities of F5/M and F2/M had ~200- and 4-fold greater association than nonfolate microemulsion, respectively. In contrast, F0/M showed a similar curve to nonfolate microemulsion. These results correspond well with the idea that conjugating folate to a shorter PEG polymer reduces folate exposure by interference with the ability of liposome to interact with FR (8). Additionally, these increased associations of F5/M and F2/M could be completely blocked by adding 2 mmol/L free folic acid to the medium (Fig. 4). From Fig. 2, one can assume that in F0/M-ACM, steric hindrances created by the emulsion surface or by long PEG chains attached to the emulsion interfere with FR recognition, whereas in F5/M-ACM and F2/M-ACM, there are no obstacles for such interaction. These findings are consistent with those previously reported on the receptor-dependent cellular uptake of folate-linked liposome (7, 19). These results suggest that microemulsions conjugated with folic acid outside the PEG layer can associate with the cells via FR.

Cytotoxicity Studies. FR-targeted microemulsions were evaluated for *in vitro* cytotoxicity in FR-α (+) KB and FR-α (−) HepG2 cells by WST-8 assay. F5/M-ACM showed higher cytotoxicity than free ACM, M-ACM, and F2/M-ACM (Fig. 5*A*). M-ACM showed a slightly higher antitumor effect than free ACM in the FR-α (+) KB cells. In contrast, no therapeutic advantageous effect on cytotoxicity was observed with FR-targeted microemulsions in the HepG2 cell line (Fig. 5*B*), which is FR-α (−). Superior cytotoxicity of folate-linked microemulsion over M-ACM was observed in the FR-α (+) KB cells, but not in the FR-α (−) HepG2 cells.

ACM concentrations leading to 50% cell death (IC_{50}) by the microemulsions in KB cells were determined from concentration-dependent cell viability curves. Calculated IC_{50} values of F5/M-ACM, F2/M-ACM, and M-ACM were 0.8, 20, and 71 ng/mL, respectively, which were 150-, 6-, and 1.7-fold lower than free ACM (120 ng/mL). F5/M-ACM and F2/M-ACM were found to be 90- and 3.5-fold more cytotoxic than M-ACM, respectively. These results suggest that the microemulsions internalized into the cell release ACM and show an antitumor effect.

Serum Folate Concentration. The effect of dietary folic acid on serum concentration was investigated. In mice on a regular diet, serum folate remained at a relatively high level (203 nmol/L). Meanwhile, mice that were switched to a folate-deficient diet showed a significantly reduced serum folate concentration (70 nmol/L). Mice on a folate-deficient diet were actually able to maintain a plasma folate level around the human physiologic range (14-51 nmol/L; ref. 11).

Biodistribution of Microemulsions in Tumor-Bearing Mice. To reveal the tumor-targeting ability of folate-linked microemulsion-loading ACM, biodistribution of microemulsions was evaluated in mice bearing KB tumor xenografts. As shown in Fig. 6, the majority of microemulsions accumulated in the

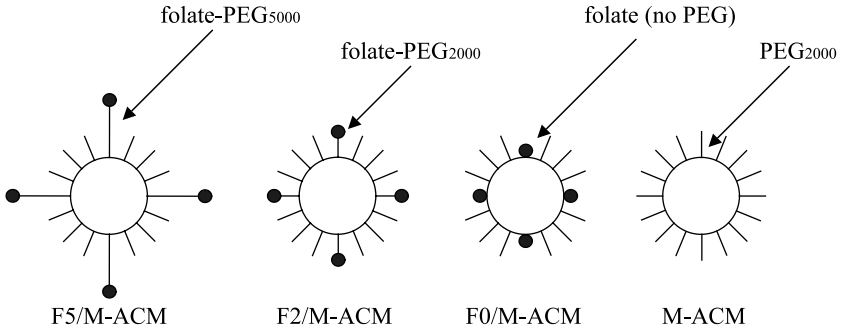

Fig. 2 Schematic diagrams of the microemulsion and folate-linked microemulsions loading ACM. Folate-linked microemulsions loading ACM consisted of folate-linked lipids, PEG_{2000}-DSPE, cholesterol, vitamin E, and ACM hydrochloride (0.24:6.7:48.3:43.3:1.5, molar ratio, F5/M-ACM, F2/M-ACM, and F0/M-ACM) were prepared by a modified ethanol injection method. For nonfolate microemulsions (M-ACM), folate-linked lipid was substituted with the same number of moles of PEG_{2000}-DSPE. Closed circles, folate; straight lines, PEG chain.

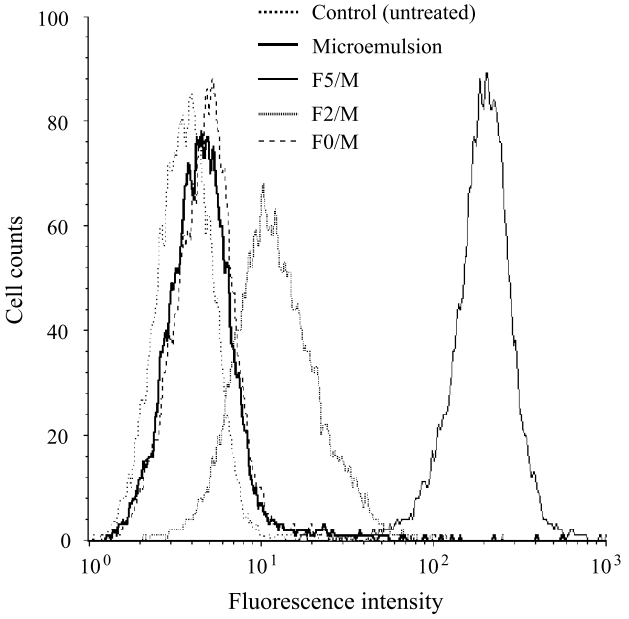

Fig. 3 Association of DiI-labeled F5/M, F2/M, F0/M, and microemulsion with KB cells. Cellular association was examined using DiI-labeled microemulsions (F5/M, F2/M, F0/M, and microemulsion) by flow cytometry. F5/M, F2/M, and F0/M represent folate microemulsion corresponding to F5/M-ACM, F2/M-ACM, F0/M-ACM without ACM, and microemulsion represents nonfolate microemulsion corresponding to M-ACM without ACM. Cells were incubated with microemulsions in serum-free medium at 37°C and analyzed by flow cytometry. Control indicates autofluorescence of untreated cells. Each analysis was generated by counting 10^4 cells.

spleen rather than in the tumor. The ACM concentrations of M-ACM in the plasma were ~1%, but that of F5/M-ACM and free ACM were not detected 24 hours after injection (data not shown). In each organ, ACM concentration was significantly higher in the order of F5/M-ACM, M-ACM, and free ACM, except in the lung. F5/M-ACM and M-ACM tended to accumulate in solid tumor regions 2.6- and 2.1-fold more than free ACM. These differences in tumor uptake reached statistical significance in F5/M-ACM to free ACM. There was no significant difference in tumor uptake between the folate- and nonfolate-linked microemulsions. This finding suggests that folate-linked microemulsions loading ACM circulated longer than free ACM. High tumor accumulation of ACM from microemulsion may require 48 to 72 hours after i.v. injection (12).

Antitumor Effect. Biological antitumor activities of microemulsions were evaluated. Mice were given F5/M-ACM, F2/M-ACM, M-ACM, free ACM, and saline as a control every 3rd day, 5 times. As shown in Fig. 7, rapid tumor growth was observed in control mice. In contrast, tumor growth suppression was partially observed in all drug-treated mice. All microemulsions showed higher antitumor activity than free ACM, probably due to their long circulation and tumor targeting. The antitumor activity of F5/M-ACM was higher than that of F2/M-ACM. F5/M-ACM on day 21 and M-ACM on day 24 showed significantly higher tumor suppression than free ACM. Thus, F5/M-ACM seemed to be more effective than free ACM and nonfolate microemulsions in inhibiting tumor growth.

DISCUSSION

Recently, many reports have revealed the potential of FR-mediated drug delivery using folate-conjugated carriers. This strategy is based on the tumor cell specificity derived from the abundant expression of FR in a large percentage of human cancers. FR mediates the physiologic uptake of folate by endocytosis, and similarly actively internalizes conjugation with folate. To investigate FR targeting ability of folate-linked microemulsions, we used the similar experimental design and evaluation method with folate-linked liposomes that were reported (7–12). In this study, three kinds of folate-linked microemulsions, F5/M-ACM, F2/M-ACM, F0/M-ACM, and a nonfolate microemulsion, M-ACM, were prepared. These microemulsions were investigated and compared to reveal the targeting ability of PEG length in the folate-PEG linker chain both *in vitro* and *in vivo*. This study showed that folate modification with a sufficiently long PEG chain on emulsions is an effective way of targeting the emulsion to tumor cells.

This microemulsion was formed from a novel formulation. PEG-DSPE was used to stabilize the surface of droplets together with cholesterol (2). The folate-PEG modification microemulsion uses the same approach with liposomes reported by Lee and Low

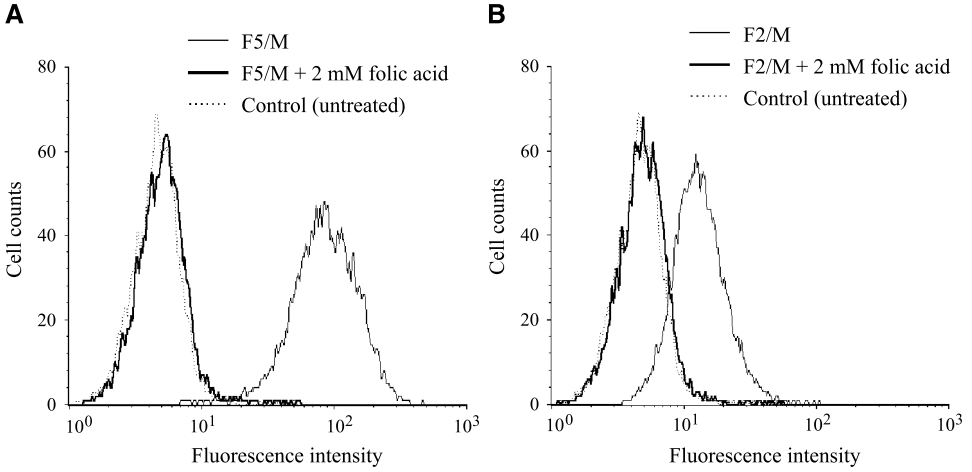

Fig. 4 Association of DiI-labeled F5/M (*A*) and DiI-labeled F2/M (*B*) with KB cells in the presence or absence of 2 mmol/L folic acid. Cells were incubated with DiI-labeled microemulsions in serum-free medium for 1 hour at 37°C. In free folate competition studies, 2 mmol/L folic acid was added to the culture medium. Control indicates autofluorescence of untreated cells in the absence of folic acid. Each analysis was generated by counting 10^4 cells. The curve shifts to the right, indicating an increase in the amount of DiI in the cells.

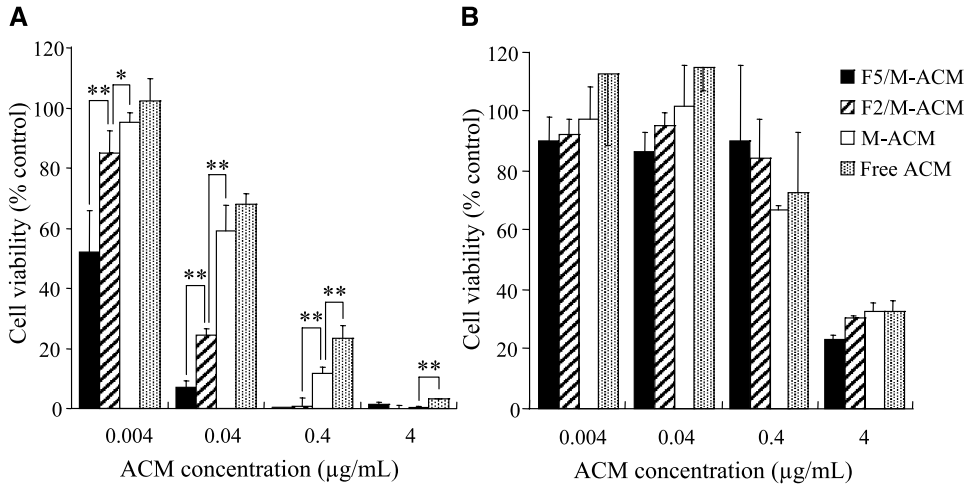

Fig. 5 Cytotoxicity of ACM delivered as F5/M-ACM, F2/M-ACM, M-ACM, or free ACM against KB cells (*A*) and HepG2 cells (*B*). Cells were incubated with drugs in serum-free medium for 1 hour, then in fresh medium without drugs for 72 hours at 37°C. Cell viability was assayed by the WST-8 method. Columns, mean; bars, SD (n = 5-6; *$P < 0.05$, **$P < 0.01$).

(7, 20). Folate-targeted liposomes contained 0.1 to 0.5 mol% folate-PEG_{2000}-DSPE or folate-PEG_{3350}-DSPE, and ~4 mol% PEG_{2000}-DSPE for PEG-coating of total liposome lipids (7–12). From this information, we decided to use 0.24 mol% folate-PEG_{2000}-DSPE or folate-PEG_{5000}-DSPE for targeting, and 6.7 mol% PEG_{2000}-DSPE for PEG coating in the forming emulsion. For nonfolate-linked microemulsions, PEG_{2000}-DSPE was used as M-ACM because increasing the PEG molecular mass from 1,900 to 5,000 had no effect for prolonged circulation (21). The folate-PEG–modified microemulsions were stable even after folate linked to microemulsion in the particle size and the amount of drug in the emulsion for at least 1 month at 4°C in the dark.

Folate-linked microemulsions were taken up by FR-mediated endocytosis. This was validated by the inhibition of association of folate-linked microemulsions in the presence of excess free folic acid and by selective FR-mediated cytotoxicity of folate-linked microemulsions loading ACM in KB and HepG2 cells.

When the results were compared among microemulsion formulations with cellular uptake by flow cytometry and cytotoxicity, the association of the folate-PEG_{5000}-linked microemulsion and folate-PEG_{2000}-linked microemulsion with the cells was 200- and 4-fold higher, whereas their cytotoxicity was 90- and 3.5-fold higher than those of nonfolate microemulsion, respectively. Other groups also reported both uptake enhancement and *in vitro* cytotoxicity enhancement in folate-linked liposomes. Lee and Low (7) reported that folate PEG_{3350}/PEG_{2000}-liposomes-entrapped doxorubicin showed ~1.6-fold higher uptake and 29-fold higher cytotoxicity than nonfolate liposomes in KB cells. Pan et al. (10) also reported that FR-β-targeted PEG_{3350}/PEG_{2000}-liposomes-entrapped doxorubicin showed a ~6.6-fold higher uptake by flow cytometry and a 25-fold higher cytotoxicity than the nonfolate one in KG-1 cells, respectively. It suggested that our folate-linked microemulsions were also highly FR-targeting carriers like liposomes.

PEG coating is mainly needed to extend systemic circulation *in vivo* to increase the possibility of FR targeting. The interference of the PEG coating with PEG_{2000} was more pronounced with folate-PEG_{2000}-DSPE than with folate-PEG_{5000}-DSPE. When the folate-PEG chain was PEG_{5000}, the association and cytotoxicity of emulsions were significantly increased compared with the nonfolate one as in the case of liposomes (8, 9). However, the following two findings in microemulsions showed differences from liposomes. First, nonfolate liposomes with a PEG-coating entrapped doxorubicin showed significantly lower cytotoxicity *in vitro* than the free drug because of the PEG coating (7, 9, 10). In contrast, the nonfolate microemulsion, M-ACM, showed a 1.7-fold higher cytotoxicity (IC_{50}) than free ACM. These findings are consistent with the fact that nonfolate emulsion loading paclitaxel showed a 1.2-fold higher level than the free drug in terms of cytotoxicity (15). Second, when the folate-PEG_{2000} chain length was the same in PEG_{2000}-DSPE for the PEG coating, the association of liposomes with the cells was comparable with the nonfolate one from the labeled marker (8), but that of emulsions (F2/M) showed a 4-fold increase compared with nonfolate microemulsion (Fig. 3). The

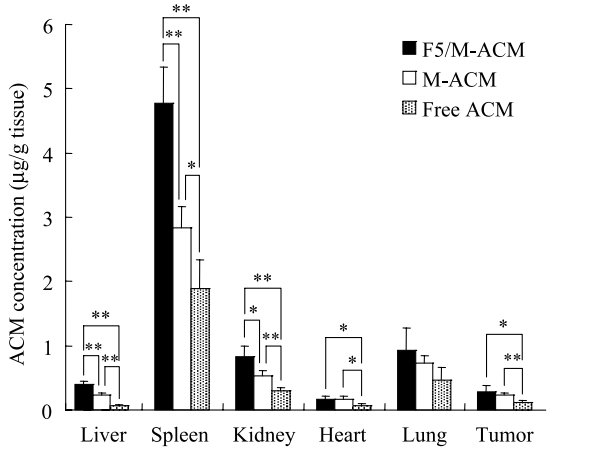

Fig. 6 Biodistribution of ACM 24 hours after i.v. injection of F5/M-ACM, M-ACM, and free ACM at a single dose of 5 mg ACM/kg into KB tumor–bearing BALB/c nude mice. Mice were inoculated s.c. with 2×10^6 cells into the right hind crus 7 days before the experiment. Mice were fed a folate-deficient diet from 1 week before tumor inoculation and for the duration of the study. Columns, mean; bars, SD (n = 3; *$P < 0.05$, **$P < 0.01$).

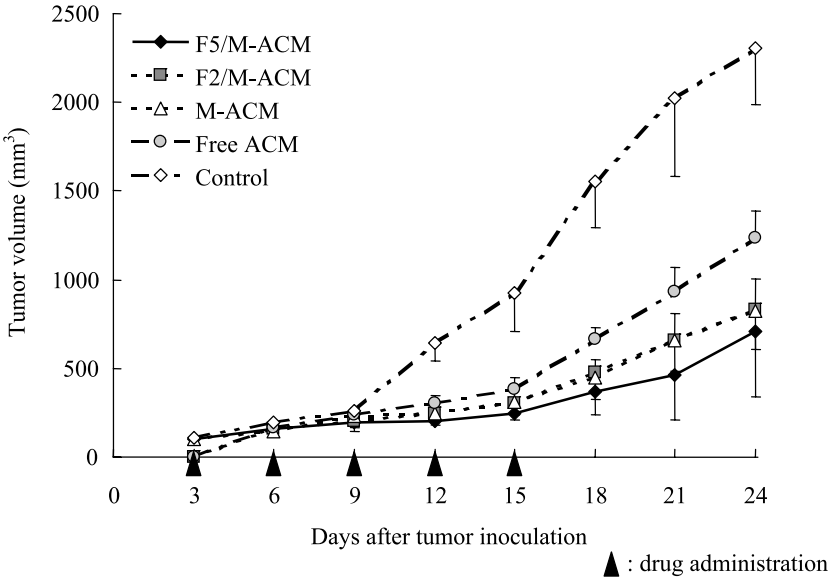

Fig. 7 *In vivo* antitumor effect by i.v. injection of F5/M-ACM, F2/M-ACM, and M-ACM at a dose of 5 mg ACM/kg in KB tumor-bearing BALB/c nude mice evaluated by solid tumor growth. Mice were inoculated s.c. with 1×10^6 cells into the right hind crus 3 days before the first administration of drugs. Mice were fed a folate-deficient diet from 1 week before tumor inoculation and for the duration of the study. Microemulsions were injected i.v. five times (day 3, 6, 9, 12, and 15) at a dose of 5 mg ACM/kg. The control was given the same volume of saline. Points, mean; bars, SD (n = 3-6).

mechanism for this difference between liposomes and microemulsions is unclear but this result implies that the interaction of PEG in carriers with the cells, and the release of a drug in them, might be different in spite of the similar PEG content (4 mol% in liposomes and 6.7 mol% in emulsions). PEG-DSPE in microemulsion was supposed to prolong circulation time *in vivo* as stabilizer and to form an injectable emulsion as emulsifier. Higher cytotoxicity of M-ACM than ACM suggests that M-ACM have some mechanism by which it achieves a high antitumor effect, such as circumvention of the efflux pump (9), because the release of ACM from M-ACM was attenuated (data not shown). Higher uptake of F2/M-ACM than M-ACM suggests that FR-targeted microemulsions are adsorbed and internalized quickly. Higher intracellular ACM fluorescence than M-ACM after 1-hour exposure of F2/M-ACM was also observed by confocal microscopy (data not shown).

The results from the biodistribution study revealed that the majority of i.v.-injected microemulsions accumulated in the liver and spleen rather than the tumor 24 hours after injection. The ACM concentrations of M-ACM in the plasma were ~1%, but F5/M-ACM and free ACM were not detected 24 hours after injection (data not shown). The level of ACM in the tumor of F5/M-ACM was shown to be similar to that of M-ACM and significantly higher than free ACM (Fig. 6). The higher accumulation of F5/M-ACM than that of M-ACM in the spleen might indicate that the folate residue counteracts the PEG-coating effect because the spleen constitutes a reticuloendothelial system. This result suggests the natural tendency of free ACM to accumulate in the spleen (22, 23).

In vivo experiments indicate that F5/M-ACM is also efficient *in vivo*. However, the advantages of F/M-ACM in terms of biodistribution and antitumor effect were relatively smaller than in *in vitro* experiments. Serum folate is not likely to have a significant inhibitory effect on the binding of folate-linked microemulsions to tumor cells expressing FR, because it was lower than the level required to produce significant competition. For folate-linked microemulsions to bind FR, they require extravasation from the blood vessel in the tumor region, pass through the intercellular space, and then reach the FR on the cell surface. F5/M-ACM showed higher antitumor activity than M-ACM that showed significantly higher than free ACM on day 24. It was suggested that the circulation time was long enough for the present folate-linked microemulsion formulation to target tumors. In this way, one of the reasons for this tendency may be that FR uptake could be a rate-limiting factor due to the easy saturation of FR as reported in folate-targeted liposomes (24, 25).

In summary, this study suggests that folate-linked microemulsions offer a promising approach for tumor-targeted ACM delivery into FR-positive tumors. Extending the PEG chain length of the folate-PEG lipid from 0 to M_r 2,000 and 2,000 to M_r 5,000 dramatically improved the selective FR-mediated association, and cytotoxicity of folate-linked microemulsions loading ACM *in vitro*. Also, folate-linked microemulsions had a higher tumor-targeting efficacy and antitumor effect *in vivo*. Although these findings show that an improved therapeutic regimen of folate-linked microemulsions is needed, the novel folate-linked microemulsions will provide targeted tumor delivery of hydrophobic drugs *in vivo*.

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