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Research Paper

Enhanced Intracellular Uptake of Sterically Stabilized Liposomal Doxorubicin *in Vitro* Resulting in Improved Antitumor Activity *in Vivo*

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Purpose. To investigate the correlation between the *in vitro* intracellular uptake and the *in vivo* antitumor activity of anticancer drugs delivered by sterically stabilized liposomes (SSL).
Methods. Arginine-glycine-aspartic acid (RGD) peptide or RGD mimetic (RGDm) was coupled onto the surface of SSL to obtain the cell-binding carrier to facilitate the intracellular delivery of the encapsulated drugs. DOX-loaded SSL (SSL-DOX), DOX-loaded RGD-modified SSL (RGD-SSL-DOX) and DOX-loaded RGDm-modified SSL (RGDm-SSL-DOX) were prepared by lipid film dispersion followed by remote loading of DOX. The intracellular uptake of DOX from the various liposomal formulations was evaluated *in vitro* with melanoma B16 cells, and the pharmacokinetics, biodistribution, and antitumor activity were compared in C57BL/6 mice carrying melanoma B16 tumors.
Results. *In vitro* intracellular uptake of DOX by B16 cells and *in vivo* antitumor activity in terms of tumor growth inhibition and mice survival time prolongation for various liposomal DOX were in the following order: RGD-SSL-DOX > RGDm-SSL-DOX > SSL-DOX. The mean survival time of the mice treated with RGD-SSL-DOX, RGDm-SSL-DOX, and SSL-DOX was 55, 49, and 44 days, respectively. The three liposomal DOX formulations produced very close DOX accumulation in tumor, which is significantly higher than that of free DOX. RGD- or RGDm-SSL-DOX demonstrated prolonged circulation time similar to that of SSL-DOX, whereas they showed significantly lower DOX level in blood and remarkably higher uptake by spleen than SSL-DOX.
Conclusions. Enhanced intracellular uptake of DOX encapsulated in SSL could produce an improved therapeutic effect for the melanoma B16 tumors. Enhancing intracellular delivery of the anticancer drugs encapsulated in SSL may be a promising strategy to improve their therapeutic efficacy for solid tumors.

KEY WORDS: doxorubicin; drug targeting; intracellular uptake; sterically stabilized liposomes; tumor therapy.

INTRODUCTION

Sterically stabilized liposomes (SSL) (i.e., PEG-liposomes) can increase the accumulation of the encapsulated anticancer drugs into solid tumors by the process of "passive targeting" due to the effect of enhanced permeability and retention (EPR). To achieve more specific targeting, the PEG-liposomes have been modified with various ligands (1). These targeted PEG-liposomes demonstrated an improved therapeutic efficacy compared to non-targeted ones, whereas they showed less enhancement in tumor accumulation (2–4). So it could be concluded that the accumulation of the ligand-modified SSL into solid tumors was independent of the ligand introduction, but dependent on the passive mechanism as SSL did. Probably, the function of the incorporated ligands was to actively deliver the accumulated SSL-drug package into tumor cells and thus caused an efficient drug delivery pathway. Moreover, SSL can preferentially accumulate in tumor tissue because of the high leaky vascular and impaired lymphatic drainage in the tumor tissues but not in normal tissues. Therefore, whether the incorporated ligands being tumor-specific or not seems not so important for the therapeutic efficacy for the solid tumors. We expect that the strategy that can facilitate the intracellular delivery of the accumulated liposome-drug package could lead to an improved antitumor activity for the solid tumors.

Integrins are a family of cell adhesion molecules composed of two non-covalently associated chains. Both

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ABBREVIATIONS: Chol, cholesterol; DOX, doxorubicin; DSPE-PEG, methoxypolyetheleneglycol (MW 2000)-distearylphosphatidylethanolamine; DSPE-PEG-BTC, 1,2-dioleyol-*sn*-glycero-3-phosphoethanolamine-*n*-[poly(ethyleneglycol)]-*N*-benzotriazole carbonate, PEG MW 3400; EPR, the effect of enhanced permeability and retention; PBS, phosphate-buffered solution; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid; RGD, arginine-glycine-aspartic acid; RGDm, RGD mimetic; RGD-SSL, RGD-modified SSL; RGD-SSL-DOX, DOX-loaded RGD-SSL; RGDm-SSL, RGDm-modified SSL; RGDm-SSL-DOX, DOX-loaded RGDm-SSL; SPC, soya phosphatidylcholine; SSL, sterically stabilized liposomes; SSL-DOX, DOX-loaded SSL.

subunits, α and β, traverse the membrane and "integrate" the extracellular matrix with the intracellular compartment (5). The RGD (Arg-Gly-Asp) sequence is known to serve as a recognition motif in multiple ligands for several different integrins (6). Integrin-mediated cell attachment and internalization are exploited by a variety of bacteria and viruses for cell entry (7,8). It is also suggested that the RGD-containing peptide can be internalized into cells by integrin-mediated endocytosis (9–12). Recently, integrin-mediated carriers, such as RGD-modified liposomes, nanoparticles, conjugates, have been investigated as gene vehicles to enhance gene transfection (13–15). It is reasonable that RGD-peptide could be used to modify the liposomes to facilitate the intracellular delivery of the entrapped anticancer agents.

In the current study, we developed PEG-modified liposomes with RGD or RGD mimetic (RGDm) conjugated to the distal terminal of the PEG chain and encapsulated doxorubicin (DOX) into these liposomes. RGD- or RGDm-modified SSL has the characteristics of passive tumor accumulation and efficient intracellular delivery, which is typical of the targeted liposomes above mentioned. The intracellular uptake of DOX for RGD-SSL-DOX, RGDm-SSL-DOX and SSL-DOX is evaluated with melanoma B16 cells *in vitro*. Pharmacokinetics, biodistribution and therapeutic efficacy of these liposomal DOX formulations are investigated in C57BL/6 mice bearing B16 tumor. Emphasis is given to the correlation between the *in vitro* intracellular uptake and *in vivo* therapeutic effect.

MATERIALS AND METHODS

Materials

DSPE-PEG_{3400}-BTC[1,2-dioleyol-*sn*-glycero-3-phosphoethanolamine-*n*-[poly(ethyleneglycol)]-*N*-benzotriazole carbonate, PEG MW 3400] was purchased from Shearwater Polymers Inc. (Huntsville, AL, USA). Methoxypolyetheleneglycol (MW 2000)- distearylphosphatidyl-ethanolamine ($DSPE_{2000}$-PEG) was obtained from NOF Co. (Tokyo, Japan) and soya phosphatidylcholine (SPC) from Lucas Meyer (Hamburg, Germany). Doxorubicin hydrochloride (DOX) was kindly provided as a gift by Haizheng Pharmaceutical Co. (Zhejiang province, China). Arginine-glycine-aspartic acid tripeptide (RGD) was obtained from Calbiochem Co. (Darmstadt, Germany). L-arginyl-6-aminohexanoic acid was synthesized as the RGD mimetic (RGDm) according to literature (16). The murine melanoma cells B16 were obtained from the Basic Medical Cell Center, Chinese Academy of Medical Science (CAMS, Beijing, China) and were cultured in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS in a humidified atmosphere containing 5% CO_2 at 37°C.

Animals and Tumor Models

Male C57BL/6 mice (6–8 weeks old), ranging from 18 to 22 g were provided by Vital River Laboratory Animal Center (Beijing, China). All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care. C57BL/6 mice were inoculated subcutaneously 1×10^6 B16 cells in the right flank of the mice to obtain melanoma models. Without treatment, most mice died of melanoma tumors 25–35 days postinoculation.

Preparation of RGD or RGDm-Modified Liposomes

RGD or RGDm were separately conjugated with DSPE-PEG_{3400}-BTC in 0.01 M isotonic HEPES buffer (pH 7.5) under the conditions of reaction (4 h at 4°C, gentle stirring and 1:2 molar ratio of peptides to DSPE-PEG_{3400}-BTC). The reaction was traced by TLC till the peptide was completely consumed. The mixture was then dialyzed against water, and lyophilized. The resulting conjugate (DSPE-PEG_{3400}-RGD or DSPE-PEG_{3400}-RGDm) was then used for preparing liposomes without further purification.

Lipid compositions of SPC:Chol:DSPE-PEG_{2000}:DSPE-PEG_{3400}-RGD or DSPE-PEG_{3400}-RGDm (20:10:1:1, mole ratio) and SPC:Chol:DSPE-PEG_{2000} (20:10:2, mole ratio) were used for RGD-SSL-DOX, RGDm-SSL-DOX and SSL-DOX, respectively. Briefly, lipids of above compositions were dissolved in chloroform, dried into a thin film in a round bottom flask on a rotary evaporator under vaccum at 37°C. The dried lipid film was rehydrated in 123 mM ammonium sulfate (pH 5.4) by bath sonication, and then sequentially extruded five times through a 0.2 μm pore size polycarbonate filter (Nulcepore, USA). For DOX-loaded liposomes, the drug was encapsulated by remote loading using an ammonium sulfate gradient (17). In short, the resulting liposomes were passed through a Sephadex G-50 column equilibrated with PBS (pH 7.4) to exchange the external phase. DOX was added to liposomes at a drug-to-lipid ratio of 1:15 (w/w) and incubated at 40°C for 10 minutes. The DOX-loaded liposomes were separated from the free DOX by a Sephadex G-50 column eluted with PBS (pH 7.4). The concentration of DOX was measured by spectrophotometry at 485 nm following dissolution in 0.1% Triton X-100. The mean liposomal diameter and particle size distribution were measured by photon-correlation spectroscopy on a Malvern Zetasizer 3000HS (Malvern Instruments, UK).

In Vitro Release of DOX from Liposomes

The *in vitro* leakage of DOX from liposomes was measured using a dialysis method. DOX encapsulated liposomes were passed over a Sephadex G-50 column immediately prior to use to remove any free DOX. The dialysis was conducted in cell culture medium containing 10% fetal bovine serum (FBS). Liposomes at a concentration of 0.5 mg DOX/ml diluted in the media were placed into a dialysis bag (MW cutoff 12,000–14,000) sealed at both ends with clips. The liposomes-loaded dialysis bag was then placed into a beaker containing 50 ml of the media, and incubated with stirring for 48 h at 37°C. At various time points, aliquots were withdrawn from the beaker and replaced with equal volume of the media. The DOX concentrations were then measured spectrophotometrically at 485 nm.

Cellular Uptake of DOX via RGD-SSL-DOX, RGDm-SSL-DOX, and SSL-DOX

B16 cells grown as a monolayer were suspended by brief treatment with trypsin and then washed once with fresh cul-

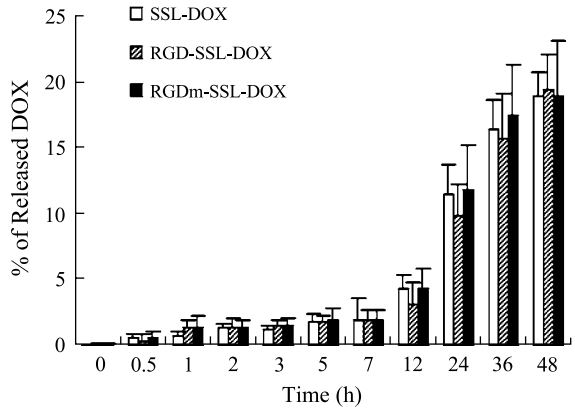

Fig. 1. *In vitro* release of DOX from liposomes in cell culture media containing 10% FBS. Liposomes at a concentration of 0.5 mg DOX/ml diluted in the culture medium were placed in a dialysis bag (MW cutoff 12,000–14,000) and incubated at 37°C in 50 ml of the media. At various time points, aliquots were withdrawn, and the DOX was measured spectrophotometrically as described in "Materials and Methods" (n = 3).

ture medium. Aliquots of the B16 suspension were incubated with RGDm-SSL-DOX, RGD-SSL-DOX or SSL-DOX (containing 20 μg/ml DOX) diluted in culture medium for the indicated time at 37°C. The cells were then washed three times with cold PBS and examined by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA, USA). Cell-associated DOX was excited with an argon laser (488 nm) and fluorescence was detected at 560 nm. Files were collected of 20,000 gated events and analyzed with the FACStation software program.

Pharmacokinetics and Biodistribution Studies in Tumor-Bearing Mice

Tumor-bearing mice were prepared by inoculating murine B16 melanoma cells (1×10^6) into the right flank of the mice, and the tumor was allowed to grow for approximately 15 days when the tumor volume reached to 1 cm^3. The DOX solution or liposomal DOX was injected intravenously via tail veil at a dose of 5 mg DOX/kg body weight. At the desired times, blood (1 ml) was collected form retro-orbital sinus after ether anesthesia and serum was separated. Mice were immediately sacrificed by cervical dislocation, and their major organ were excised, washed in cold physiological saline, dried over filter paper, and weighed. Serum and organs were either processed immediately as described below or kept frozen at −20°C until analysis. These tissues as well as 0.2 ml serum were homogenized and extracted with chloroform/methanol (4:1, v/v), the extracts were then subjected to HPLC assay according to the method of Shinozawa *et al.* (18). The pharmacokinetic parameters were calculated from the average blood concentrations by the pharmacokinetic software 3P87 (the Chinese Society of Mathematical Pharmacology, Shanghai, China).

Therapeutic Efficacy in Tumor-Bearing Mice

Treatment were given at 24 h postinoculation and consisted of (a) saline control, (b) SSL-DOX (5 mg/kg in DOX), (c) RGD-SSL-DOX (5 mg/kg in DOX) and (d) RGDm-SSL-DOX (5 mg/kg in DOX). The drug was given by intravenous injection via tail vein on every seven day (q7d) for four doses (days 1, 7, 14, and 21). Tumor size was measured on every other day with a caliper in two dimensions and animal survival was monitored daily. Tumor size was calculated using the following equation: volume = $d1 \times (d2)^2 \times 0.5$. Survival data were presented in a Kaplan–Meier plot.

RESULTS

Characterization of Liposomes

DOX loading efficiency obtained by this procedure is found to be more than 95% for the three liposomal DOX formulations. The mean particle diameter was ~120 nm for all liposome preparations (polydispersity, 0.30). The zeta-potential was −1.8, −2.8, −1.4 mV for RGD-SSL-DOX, RGDm-SSL-DOX and SSL-DOX, respectively.

In Vitro Release from Liposomes

The results of *in vitro* DOX release experiments are presented in Fig. 1. SSL-DOX, RGD-SSL-DOX and RGDm-SSL-DOX showed very similar DOX leakage in culture medium within 48 h of incubation. There were no pronounced differences in DOX release from the three types of liposomes at every time point evaluated. These DOX encapsulated liposomes showed minimal DOX leakage in culture medium within 12 h of incubation, and more than 95% of the encapsulated DOX was still retained in liposomes after 12 h of incubation at 37°C.

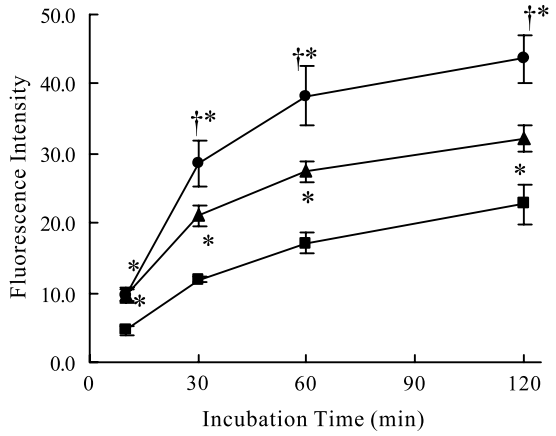

Fig. 2. DOX association with B16 cells when incubated with RGD-SSL-DOX (●), RGDm-SSL-DOX (▲), or SSL-DOX (■). Melanoma cells B16 (1×10^6 cells/well) were incubated with various liposomal DOX at a final concentration of 20 μg DOX/ml diluted in culture medium at 37°C. At various time points, aliquots of the cells were withdrawn, washed with PBS (pH 7.4), and cell-associated DOX was evaluated using flow cytometry (n = 3). *$p < 0.01$, RGD- or RGDm-SSL-DOX *vs.* SSL-DOX; †$p < 0.01$, RGD-SSL-DOX *vs.* RGDm-SSL-DOX.

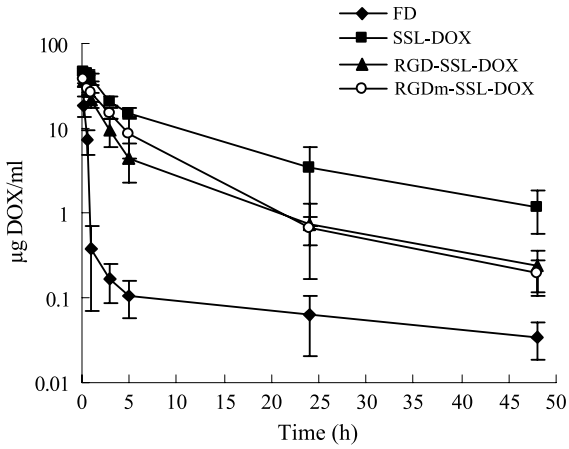

Fig. 3. Pharmacokinetics of DOX encapsulated in various liposomes in tumor-bearing C57BL/6 mice. Mice were treated with 5 mg/kg of RGD-SSL-DOX, RGDm-SSL-DOX, SSL-DOX or DOX solution by i.v. injection via tail vein. Serum DOX levels were determined at 0.25, 0.5, 1, 3, 5, 24, and 48 h for the DOX formulations after i.v. injection as described in "Materials and Methods." Each point represents the mean ± SD of 5 ~ 6 mice.

Uptake of RGD-SSL-DOX, RGDm-SSL-DOX, and SSL-DOX by Cultured B16 Cells

Cellular uptake of liposomal DOX was assessed by flow cytometry based on DOX fluorescence. Differences in uptake kinetics were observed between RGD-SSL-DOX, RGDm-SSL-DOX and SSL-DOX. As shown in Fig. 2, the uptake of DOX into B16 cells for RGD- or RGDm-SSL-DOX was faster than that for SSL-DOX and there was an increased DOX levels in cells after 30 min of incubation. There was a sharp increase in DOX uptake by B16 cells from RGD- or RGDm-SSL-DOX within 30 min of incubation, which apparently represents the binding of RGD- or RGDm-SSL-DOX to the surface of the cells. Further increase up to 120 min resulted in a slower linear accumulation of DOX probably representing internalization of them. DOX uptake into B16 cells for RGD-SSL-DOX was faster than that for RGDm-SSL-DOX, which resulted in an increased DOX levels in B16 cells after 30 min of incubation.

Pharmacokinetics and Tissue Distribution Studies in Tumor-Bearing Mice

The profile of DOX in the blood after tail intravenous injection of 5 mg/kg dose of RGD-SSL-DOX, RGDm-SSL-DOX, SSL-DOX, and DOX solution are shown in Fig. 3. All the DOX formulations displayed a biexponential clearance process characterized by two elimination half-lives, $t_{1/2}\alpha$ and $t_{1/2}\beta$ (Table I). As anticipated, the three liposomal DOX preparations showed a much greater systemic circulation time than DOX solution, which displayed a rapid clearance kinetics. The MRTs of the three types of liposomal DOX were significantly higher than that of the DOX solution. Mice treated with RGD- or RGDm-SSL-DOX displayed pronouncedly lower plasma concentrations at 1, 3, 5, 24, and 48 h compared to those with SSL-DOX ($p < 0.05$). There was a substantial decrease in the AUC and MRT for RGD- or RGDm-SSL-DOX versus the SSL-DOX. There was no significant difference observed in DOX clearance for RGD-SSL-DOX and RGDm-SSL-DOX.

The concentrations of DOX in the blood, spleen, liver, kidney, heart, lung and tumor at 5 h after tail intravenous injection of 5 mg/kg dose of RGD-SSL-DOX, RGDm-SSL-DOX, SSL-DOX, and free DOX are shown in Fig. 4. DOX levels in all the tissues for the three liposomal formulations were significantly higher than those for DOX solution with the heart as an exception. DOX levels in blood for RGD- or RGDm-SSL-DOX were lower than that for SSL-DOX to a larger extent, while DOX levels in spleen for them were significantly higher. It is noteworthy that DOX levels in tumor for SSL-DOX, RGD-SSL-DOX, and RGDm-SSL-DOX were very close at this time point, suggesting RGD- or RGDm-SSL-DOX showed less advantage in tumor accumulation over SSL-DOX.

Antitumor Activity

The tumor inhibitory activities of above treatment were evaluated in C57BL/6 mice bearing B16 melanoma tumor. As shown in Fig. 5, all the DOX formulations were effective in preventing tumor growth compared to the treatment with saline. Treatment with RGD- or RGDm-SSL-DOX displayed stronger tumor inhibition than treatment with SSL-DOX. RGD-SSL-DOX was more effective than RGDm-SSL-DOX in tumor growth inhibition.

Survival of mice carrying B16 tumors in response to the above treatments was also determined. The results are represented in a Kaplan–Meier plot as indicated in Fig. 6. The mean survival time (MST) and the % increased life spans (ILS) for each treatment group are presented in Table II. The three DOX formulations were significantly more effective in prolonging mouse survival ($p < 0.01$) than saline. Mice treated with RGD- or RGDm-SSL-DOX showed significantly increased MST compared to those that received

Table I. Pharmacokinetics of DOX After i.v. Injection of SSL-DOX, RGD-SSL-DOX, RGDm-SSL-DOX or DOX Solution in Tumor-bearing Mice at a Dose of 5 mg DOX/kg (n = 5 ~ 6)

Formulations	MRT (h)	AUC (μg.h/ml)	Cls (ml/h)	k_{10} (h^{-1})	$t_{1/2\alpha}$ (h)	$t_{1/2\beta}$ (h)	Vss (ml)
DOX solution	3.38	11.48	0.436	3.96	0.22	0.53	0.11
SSL-DOX	9.15	337.0	0.015	0.15	1.85	15.38	0.10
RGD-SSL-DOX	6.10	112.3	0.042	0.34	1.18	11.58	0.13
RGDm-SSL-DOX	5.29	143.9	0.033	0.25	2.08	12.67	0.13

MRT, mean retention time; AUC, area under the plasma concentration-time curves (0–∞); Cls, total body clearance; k_{10}, elimination rate constant from the central compartment; $t_{1/2\alpha}$, distribution half-life; $t_{1/2\beta}$, elimination half-life; Vss, volume of distribution.

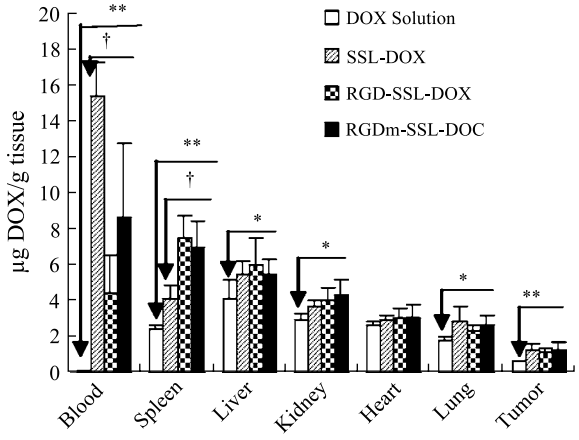

Fig. 4. Tissue distribution of DOX at 5 h postinjection into tumor-bearing mice of RGD-SSL-DOX, RGDm-SSL-DOX, SSL-DOX, and DOX solution at a dose of 5 mg DOX/kg. Tumors had an approximate size of 1 cm^3. Samples were collected and DOX levels per gram tissue were determined by HPLC as described in "Materials and Methods." Data are expressed as means ± SD (n = 5 ~ 6). *$p < 0.05$, **$p < 0.01$, *vs.* free DOX; †$p < 0.01$, *vs.* SSL-DOX.

SSL-DOX ($p < 0.01$), and RGD-SSL-DOX was more effective in prolonging mouse survival than RGDm-SSL-DOX ($p < 0.05$).

DISCUSSION

Results in this study have shown for the first time that RGD- or RGDm-modified liposomes could be used to improve the therapeutic efficacy of DOX in a melanoma tumor model without introducing excessive toxicity to the animals (19). Specifically, the RGD- or RGDm-modified liposomal DOX has been found to be significantly more effective in tumor growth inhibition and survival prolongation than the non-modified liposomal DOX in the murine melanoma xenografts model.

Although many studies have been carried out on tumor-targeting delivery of anticancer drugs using various specific ligands, the correlation of the *in vitro* intracellular uptake and *in vivo* antitumor activities is less described. Results in this study clearly demonstrated a strong correlation between the *in vitro* intracellular uptake and *in vivo* therapeutic efficacy for the encapsulated DOX, namely, increased intracellular uptake of liposomal DOX will result in improved antitumor activity. Obviously the increased intracellular uptake of DOX for RGD- or RGDm-SSL-DOX over SSL-DOX can not be caused by the extra leakage from them because all the liposomal DOX demonstrated similar release when incubated with cell culture (Fig. 1). Neither should it be ascribed to the electrical interaction since the three liposomal formulations demonstrated very close negative zeta potential.

Previous studies have shown that the extravasation of PEG-grafted liposomes from the vascular compartment into the tumor interstitium was size-dependent, and also dependent on the type of tumor, and mainly limited by their ability to diffuse through the 100–1,200 nm pores in the vessel wall (20). Although there is a substantial increase in the cellular association of DOX for RGD- and RGDm-SSL-DOX with B16 cells *in vitro*, less improvement in tumor accumulation was observed for them compared to SSL-DOX. This result confirms observations by other authors that ligand-modified liposomes did not show any advantages in tumor accumulation compared to non-modified liposomes (2–4). It would appear that the accumulation of targeted liposomes in solid tumors is governed by the same processes that govern the tumor accumulation of non-modified liposomes, that is, passive diffusion and extravasation, but independent of the ligand introduction.

Because these PEG-liposomes demonstrated very close DOX accumulation in tumor tissue, the difference existed in their antitumor activities will inevitably be relate to their intracellular uptake by the tumor cells. Once i.v. administered,

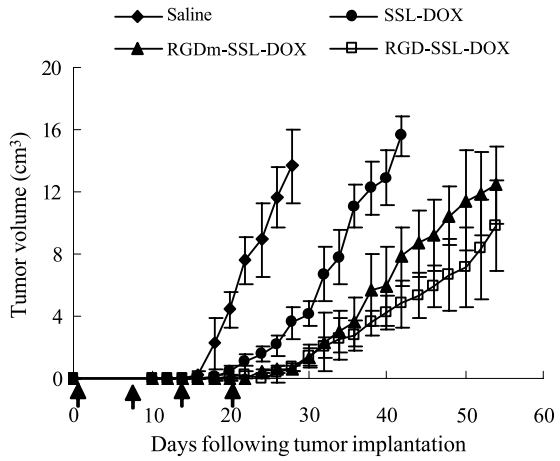

Fig. 5. Tumor growth inhibition by various DOX formulations in tumor-bearing C57BL/6 mice. C57BL/6 mice were inoculated with 1×10^6 B16 cells and treated with a series of four i.v. injections (given on every seventh day, as indicated by the arrows) of RGD-SSL-DOX, RGDm-SSL-DOX, SSL-DOX or normal saline as a control treatment. Tumor size was measured for each animal every 2 days starting from the day of the initial treatment (n = 8 to 12).

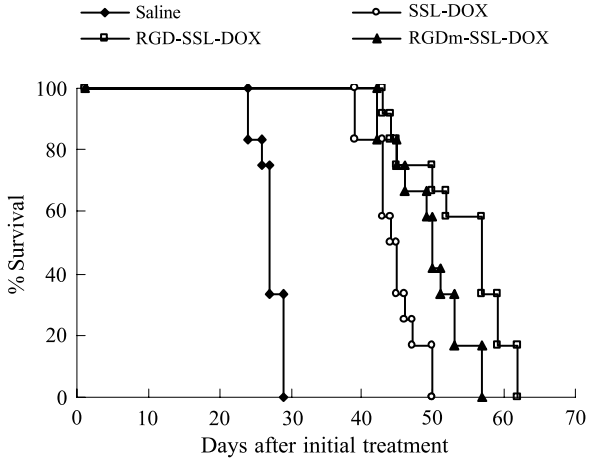

Fig. 6. Survival of C57BL/6 mice inoculated with 1×10^6 B16 cells, and treated with various DOX formulations. Treatments include RGD-SSL-DOX, RGDm-SSL-DOX, SSL-DOX, and saline. Mice were given four i.v. injections of the DOX formulations containing 5 mg/kg DOX, at 24 h, 7 days, 14 days, and 21 days postinoculation (12 mice per group). Animal survival was recorded starting from the day of initial treatment.

Table II. The Effect of Various DOX Formulations Treatment on the Survival of C57BL/6 Mice Inoculated with B16 Cells (n = 12)

Formulation	Single injection dosage (mg/kg)	Total dosage[a] (mg/kg)	MST[b] (days)	Median (days)	ILS[c] (%)
Saline control	0	0	27.1 ± 1.8	27	–
SSL-DOX	5	20	44.5 ± 3.5	44	63.0
RGDm-SSL-DOX	5	20	49.6 ± 5.1	49	85.2
RGD-SSL-DOX	5	20	54.5 ± 6.4	55	100.0

[a] The drug was injected i.v. on every seventh day for a total of four times.
[b] MST denotes mean survival time.
[c] ILS, increased life span ((T/C − 1) × 100 (%)), where T and C represent the mean survival time (days) of the treated and control animals, respectively.

the drug-containing liposomes were mainly found in tumor-resident macrophages or in perivascular areas (21,22). The encapsulated drugs may be released from liposomes and passively diffuse into cells, or the drug-liposome package may be directly internalized by endocytosis. The passive diffusion is dependent on drug release rate from the liposomes and cell permeability to the drugs, so its delivery efficiency could be limited to a larger extent. Internalization by endocytosis was considered a necessary process for improved therapeutic efficacy of targeted-liposomal drugs (23). The intracellular drug delivery by this mechanism may be highly efficient because entry of the drug into the cells is not limited by such factors as drug leakage, the process of passive diffusion and so on. From this point of view, the occurrence of increased tumor growth retardation and prolonged survival for the RGD- or RGDm-SSL-DOX relative to SSL-DOX can be well explained.

Because most of the integrins were universally expressed by tumor cells as well as normal cells, and the RGD ligand used here could not be highly tumor-selective, this raises the concern of potential toxicity on normal cells by RGD- or RGDm-modified liposomes as the carrier for anticancer agents. It is well-known that fast growing tumor tissues need a tremendous amount of oxygen and nutrients supplied by blood vessels. They release special growth factors including vascular endothelial cell growth factors (VEGF) to facilitate neo-vascularization. As a result, many new vessels are formed, but their cell junctions are not as tight as those of normal tissues (24). DOX-containing liposomes having a size of about 110 nm were likely to freely to pass through the endothelial junctions of the capillaries in tumor tissue, but not in normal tissues. Taken into consideration of the impaired lymphatic drainage in tumor tissue, it is reasonable that PEG-liposomes could preferentially accumulate in solid tumors rather than in normal tissues. So the toxicity caused by the increased DOX uptake into normal cells by integrin-mediated internalization will be limited to a large extent. In our study, the toxicity of RGD- or RGDm-SSL-DOX seemed not to be serious since the mice health was good, as judged by observations of maintained activity and body weight. Moreover, we found that RGD- or RGDm-SSL-DOX did not increase DOX accumulation in liver, lungs or kidney and, more importantly, in heart, which is most sensitive to DOX toxicity. Therefore, regarding cardiotoxicity, RGD-SSL-DOX should be equivalent to SSL-DOX.

The increased splenic uptake should be responsible for the rapid clearance of RGD- or RGDm-SSL-DOX from the circulation. In the spleen, RGD- or RGDm-modified liposomes were probably associated with macrophages, which express α_v integrins and depend on them to remove apoptotic cells from the circulation (25,26). It is likely that α_v-mediated uptake by macrophages in the spleen is also responsible for the uptake of RGD- or RGDm-SSL-DOX. Weather the increased uptake of RGD- or RGDm-SSL-DOX into the spleen will cause undesired toxicity on spleen needs to be addressed. The liver or spleen accumulation seems to be shared by various ligand-modified liposomes (27,28). The increased DOX accumulation in spleen or liver for ligand-modified liposomal DOX has also been reported (29). Because no serious toxicity on spleen was reported in these studies, it seems that the spleen could be not so sensitive to DOX toxicity. However this need to be evaluated thoroughly in the future research.

Tumor vascular damage could contribute to the tumor growth inhibition for RGD- or RGDm-modified liposomal DOX. It is well known that integrin αvβ3 is up-regulated in the endothelial cells of solid tumor vessels (30). RGD-peptides showing high affinity with αvβ3 have been tested to achieve tumor vascular targeting (31). It is suggested that RGD peptides could bind to tumor vascular endothelial cells as well as tumor cells (32). Vascular cells will be first exposed to the i.v. injected liposomes for a longer time compared to other cells, so the tumor endothelial cells could have more chances to arrest the RGD-liposomes by integrin-mediated binding and lead to vascular damage. However, to what extent this mechanism was involved in the antitumor activity needs to be determined in the future.

In conclusion, we demonstrated that RGD- or RGDm-modified liposomes loaded doxorubicin can increase the intracellular uptake of doxorubicin *in vitro* and lead to an improved antitumor activity for the melanoma B16 tumors. The combined effect of tumor accumulation and enhanced cellular uptake could be the main reason for their improved therapeutic efficacy. Enhancing the intracellular delivery could be a promising strategy to improve the antitumor activity for the anticancer drugs encapsulated in SSL.

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