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CD90-Targeted Liposomes Increase the Therapeutic Efficacy of a Retinoic Acid Derivative in Pulmonary Carcinoma

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Abstract

Title: The therapeutic efficacy of CD90-targeted liposomes in pulmonary carcinoma.

Background: In refractory lung cancer tissue, it has been reported that cell populations exist that exhibit resistance to existing therapeutics. These cells have characteristics such as high stemness and tumorigenic potential, and they are thought to contribute to relapse and metastasis. Differentiation inducer 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) carbamoyl] benzoic acid (Am80) has a growth inhibitory effect on pulmonary carcinoma cells, but it has been effective only in a high dose that causes damage to normal cells. We aimed to achieve a dose reduction by specific targeting of anaplastic pulmonary carcinoma cells and modification of antibodies on the liposome surface specific for membrane protein of undifferentiated cells because undifferentiated pulmonary cells have unique membrane proteins. CD90 is one of the undifferentiated markers expressed in pulmonary cells.

Methods and Findings: In this study, we examined the usefulness evaluation of Am80 liposomes conjugated with anti-CD90 antibody using Calu-6, a CD90-positive undifferentiated human pulmonary carcinoma cell line.

After the preparation of Am80 liposomes, the characterization and cell specificity, therapeutic efficacy of them were assessed. Am80 liposomes conjugated with antibody selectively combined with the surface of Calu-6 and had a significant influence on the inhibition of cell and tumor growth. The twice-weekly intratumoral administration of Am80 liposomes conjugated antibody (0.1 mg/kg as Am80) inhibited tumor growth to 3.64 ± 1.10 compared to empty liposomes (5.89 ± 1.61) in relative tumor volume at day 28.

Conclusions: We revealed that the cellular uptake of Am80 liposomes conjugated with anti-CD90 antibody by anaplastic pulmonary carcinoma cells was highly effective and efficient.

Keywords: Liposomes; Am80; Anti-CD90; Pulmonary carcinoma

Abbreviations: Am80: 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) carbamoyl] benzoic acid; APL: Acute Promyelocytic Leukemia; DDS: Drug Delivery System; DOX: Doxorubicin; ATRA: All-T*rans* Retinoic Acid; CYP: Cytochrome P450; RAR: Retinoic Acid Receptor

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Introduction

Pulmonary carcinoma is a leading cause of cancer-related deaths worldwide [1] and its incidence is increasing even today [2]. No drug can cure pulmonary carcinoma completely [3] and thus, it is urgently necessary to develop a novel therapeutic drug. Cancer stem cells can be considered a cause of cancer metastasis and relapse [4,5]. Chemotherapy and radiation therapy are current treatments for pulmonary carcinoma [6,7] but these therapies target cancer cells. It is thought that cancer stem cells survive and lead to metastasis and relapse because of the low effect of these therapies on cancer stem cells, which are undifferentiated

and are resistant to radiation or the oxidative stress of anticancer agents [8,9]. Therefore, it is necessary to develop a therapy that directly targets cancer stem cells. Differentiation-inducing therapy can be considered an effective therapy for cancer stem cells that cause cancer metastasis and relapse [10,11]. This therapy puts cancer cells on a track towards differentiation of cells by using a differentiation inducer to return the cancer cells to normal cells. It has attracted attention because of the small number of serious complications produced in comparison to hemorrhage and infection with chemotherapy [12,13].

Recently, it was reported that all-trans retinoic acid (ATRA) has effects on differentiation induction pathologically and on anti-proliferation of pulmonary carcinoma cells [14,15]. Because ATRA, which is an active metabolite of vitamin A, can highly induce cell differentiation, it has been used as therapy against acute promyelocytic leukemia (APL), which represents the most successful example of differentiation-induction therapy in clinical oncology [16]. However, low metabolic stability has been pointed out as a problem of ATRA [17,18]. There are several possible explanations for this problem, such as a decreased blood concentration of ATRA caused by an increase in cytochrome P450 (CYP) or oxidative enzyme activity or induced expression of cellular retinoic acid binding protein, an agent involved in ATRA metabolism [19]. Therefore, we focused on 4[(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) carbamoyl] benzoic acid (Am80), which is a derivative of ATRA. Am80 is a kind of synthetic retinoid that was developed to improve drug resistance against APL and reduce side effects. Although similar to ATRA, Am80 is an agonist of the retinoic acid receptor (RAR), and it has high selectivity in RAR receptors. While ATRA binds to all subtypes of RAR (RARα, RARβ, and RARγ), Am80 binds only to RARα and RARβ [20]. Furthermore, Am80 has more activity than ATRA and the differentiation ability against HL-60 human promyelocytic leukemia cells or NB-4 cells of Am80 is approximately 10 times higher than that of ATRA [21]. Therefore, Am80 has been used as a treatment for relapsed or refractory APL post-ATRA remission. In addition, it is highly stable against light and heat, is not easily oxidized, and has high metabolic stability. For example, regarding CYP, although Am80 is metabolized by CYP3A4 and ATRA, Am80 is not metabolized by CYP26, which is involved in the metabolism of retinoic acid [22]. It is reported that Am80 is less susceptible to metabolism than ATRA because of its lower affinity for cellular retinoic acid binding protein than ATRA [23].

We reported that Am80 has an antiproliferative effect against pulmonary carcinoma cells at a high dose [24] and systemic side effects such as retinoic acid syndrome can be a concern in clinical application. Retinoic acid syndrome is a fatal complication in which increased numbers of mature cells caused by simultaneous cell differentiation invade the lung and cause cellular damage such as that to the vascular endothelium, which leads to hypoxemia, heart failure, and other problems [25,26]. Therefore, the targeting to specific cell is needed.

Recently, the nanocarriers have been developed in the drug delivery to the cancer cells [27]. In the present study, we aimed to reduce the amount of Am80 delivered by using with liposomes to reduce the side effects of Am80. Liposome is a closed endoplasmic reticulum comprising a lipid bilayer that consists of components similar to a biological membrane, such as phospholipid, cholesterol, protein, and glycolipid. It is easy to adjust the size of the small vesicles and the components of phospholipid, and it is possible to enclose a water-soluble drug, lipophilic drug, polymer, or other materials. Furthermore, liposomes can deliver the enclosed drugs to tumor tissue via the enhanced permeation and retention effect. This effect is a phenomenon in which a nanocarrier such as liposome can deliver the enclosed drugs to tumor tissue efficiently via blood vessels around the tumor tissue, which has many gaps in comparison to normal tissue [28]. In addition, liposomes leaking out from blood vessels can remain in tumor tissue for a long time because the lymphatic tissue surrounding tumor tissue is immature and cannot exclude foreign substances in tissue [29]. Liposomes are carriers suitable for localized drug delivery that make the reduction of side effects or the slow release of a drug possible. Doxorubicin (DOX) encapsulated in liposomes is an example whereby a reduction of side effects is achieved by using liposomes. In an evaluation of the therapeutic effect of DOX, which is a representative anticancer drug encapsulated in liposomes, on Meth-A-sarcoma-bearing mice, the mice treated with DOX encapsulated in liposomes did not die, whereas the mice treated with non-encapsulated DOX did [30]. This result showed that the serious cardiotoxic side effects of DOX were reduced. In this way, liposomes have been applied to a drug delivery system (DDS) as low-toxicity drug capsules with a structure similar to that of cells [31].

Furthermore, to add targeting functions to cells and tissue, the active targeting type of liposome, which has an antibody on its surface, is gathering attention as a next-generation DDS drug [32]. Because cancer cells have specific membrane proteins, the targeting of these proteins by anticancer drugs has been conducted. In other words, by specifically targeting cancer cells,

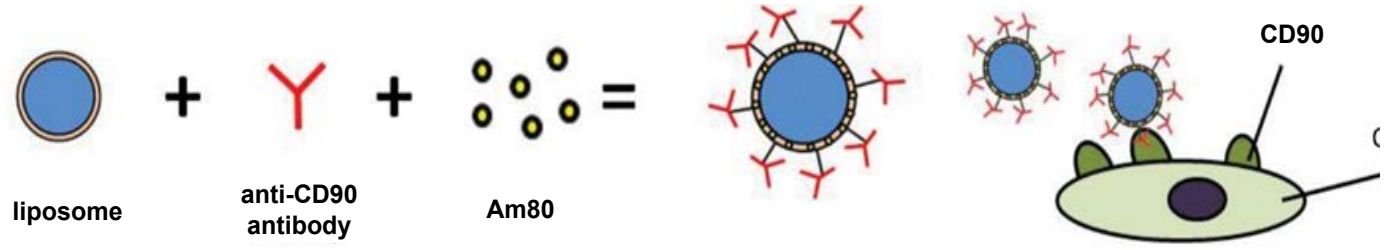

Figure 1 A graphical presentation of the nanocarriers.

a reduction in the amount of drug necessary has been achieved by modifying a specific antibody on the surface of liposomes against specific membrane proteins of the cancer cells, thus making the liposomes suitable for localized drug delivery [33,34]. It is reported that alveolar epithelial progenitor cells, which have the ability to differentiate into alveolar epithelial cells, were identified from normal human pulmonary tissue and that membrane protein CD90 is expressed on their surface [35]. Moreover, CD90 is reported as a candidate marker of lung cancer stem cells as well as CD133 and so on [36-40]. From these reports, we expect that that CD90 is also expressed on the surface of anaplastic pulmonary carcinoma cells such as Calu-6 cells, and we have targeted CD90 as a protein of pulmonary cancer stem cells.

In this study, we examine the usefulness evaluation and the preparation of Am80 liposomes conjugated with anti-CD90 antibody using Calu-6.

Materials and Methods

Ethics statement

All the animal experiments were conducted under the approval of the Tokyo University of Science ethics committee (Approval Number Y15042). The administration of Am80 was stopped when the average tumor volume reached over 600 mm^3 or when necrosis was induced in the tumor. Then, to ameliorate the pain and distress associated with tumor formation, the animals were euthanized, and the tumors were enucleated at the next scheduled date of administration.

Materials

Am80 was kindly provided by Prof. Shudo of the Itsuu Laboratory (Tokyo, Japan). Am80 was dissolved in ethanol and stored at -30°C until use. Cholesterol was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Hydrogenated soy phosphatidyl choline (HSPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-m- [methoxy (polyethyleneglycol)-2000] ($mPEG_{2000}$-DSPE) were kindly provided by Nippon Fine Chemical Co., Ltd. (Osaka, Japan). DSPE-PEG-MAL (SUNBRIGHT DSPE-020MA) was kindly provided by the NOF CORPORATION (Tokyo, Japan). *O*, *O*'-dietradecanoyl-*N*-(α-trimethyl ammonioacetyl) diethanolamine (DC-6-14) was kindly provided by Sogo Pharmaceutical Co., Ltd. (Tokyo, Japan). Rat anti-mouse CD90.2 was kindly provided by Prof. Abe of the Tokyo University of Science. Goat anti-human CD90 (Thy-1) polyclonal antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Donkey anti-goat IgG-fluorescein isothiocyanate was purchased from Millipore (CA, USA). Donkey anti-rabbit IgG H&L (Alexa Fluor 594) was purchased from abcam (Cambridge, UK). DAPI (4', 6-diamidino-2-phenylindole) was purchased from Roche Diagnostics (Mannheim, Germany), and coumarin-6 was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

Cell line

Calu-6 cells, an established human pulmonary carcinoma cell line (#HTB-56), were purchased from the American type culture collection (Rockville, MD, USA).

Cell culture

Calu-6 cells were cultured in Eagle's minimal essential medium (EMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, and 1 mM sodium pyruvate at 37°C in a humidified 5% CO_2 atmosphere. The established human pulmonary carcinoma cell line A549 was provided by Prof. Nishioka of Tokushima University Graduate School. A549 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% FBS at 37°C in a humidified 5% CO_2 atmosphere.

Expression of CD90 in Calu-6

Immunostaining: Cells were seeded onto 8-well chamber slides (6×10^3 cells/cm^2) and washed twice with PBS and fixed by 4% paraformaldehyde in PBS for 15 min. The cells were washed three times with PBS, blocked using 10% bovine serum albumin at room temperature for 60 minutes and then incubated with the primary antibody against CD90 at a 1:200 dilution at 4°C overnight. After incubation at room temperature for 2 h, the samples were washed three times with PBS containing 0.1% polysorbate 80 and then incubated with Alexa Fluor 594 donkey anti-rabbit IgG at 1:200 dilution and DAPI, which is used for the staining of nuclei, at room temperature for 2 h. Slides were washed three times with PBS containing 0.1% polysorbate 80, and after mounting of the sections, the slides were observed using a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Western blot analysis: Cells were washed with PBS, and lysed in 2× sample buffer [0.5 M Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 6.8, with 10% sodium dodecyl sulfate (SDS), 2-mercaptoethanol, glycerol, and bromophenol blue] and denatured at 95°C for 10 min. Protein concentrations in cell lysates were determined by the Bradford assay (Bio-Rad). Equivalent total protein amounts were loaded onto each lane of an SDS-18% polyacrylamide gel electrophoresis gel, followed by transfer to a polyvinylidene fluoride (PVDF) membrane and blocking in 5% nonfat dry milk containing 0.1% polysorbate 20 at room temperature for 1 h. The blotted membrane was incubated with appropriate primary antibody against CD90 (Goat anti-human CD90 antibody) at a 1:500 dilution in Can Get Signal Immunoreaction Enhancer Solution (TOYOBO Co., Ltd., Osaka, Japan) at room temperature for 2 h and then at 4°C overnight. After six washes with PBS containing 0.1% polysorbate 20, the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (donkey anti-goat IgG-HRP) at a 1:1000 dilution in can get signal immunoreaction enhancer solution (TOYOBO Co., Ltd., Osaka, Japan) at room temperature for 2 h. After six washes with PBS containing 0.1% polysorbate 20, the immunoreactions were visualized by immobilon western chemiluminescent HRP Substrate (Merck Millipore Co., Darmstadt, Germany) with an ImageQuantLAS4000EPUVmini (FUJI FILM, Tokyo, Japan).

Preparation of Am80 liposomes conjugated with anti-CD90 antibody

The composition of the liposomes was as in a previously reported method [41]. After dissolving lipid (HSPC:cholesterol:mPEG$_{2000}$-DSPE: DC-6-14:DSPE-PEG-MAL = 2:1:0.1:2:0.1) and Am80 in 100% EtOH, we injected the solution into HEPES-buffered saline (HBS) with a syringe and prepared the liposomes. After the centrifugation for 40 min at 64200 rpm, anti-CD90 antibody that had been subjected to a reduction treatment (rat anti-mouse CD90.2, 1 mg/mL) was added to the solution, and the antibody-modified liposome surface was formed by incubation for 16 hours at room temperature. Finally, any unbound antibody was removed by centrifugation to prepare the Am80 liposomes conjugated with anti-CD90 antibody. And we prepared the anti-CD90 Empty liposome by same method without Am80.

Particle size and zeta potential of liposomes

The particle size distribution and zeta potential of the liposomes were determined by dynamic light scattering technique with an ELSZ2P analyzer (Otsuka Electronics Co., Ltd., Osaka, Japan).

Percentage entrapment of Am80 in the liposomes

Percentage drug encapsulation in the liposomes was determined by centrifugation. The solution of Am80 liposome conjugated with anti-CD90 antibody and 2% Triton-X (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were mixed each in equal quantity and then centrifuged at 20,000 rpm for 15 min. The supernatants were collected, and the Am80 concentration was measured by high-performance liquid chromatography (HPLC). The entrapment efficiency of Am80 in the liposomes was estimated as: $E=(W_{Encapsulated}/W_{First\ added}) \times 100\%$, where E is the encapsulation efficiency of liposomes, $W_{encapsulated}$ is the measured amount of Am80 in the liposome suspensions after centrifugation, and $W_{first\ added}$ is the measured amount of Am80 at first addition.

Am80 was quantified by a COSMOSIL 5CN-R Packed Column (NACALAI TESQUE, INC., 250×4.6 mm) and detected by HPLC, which was performed with an LC-20AD solvent delivery system equipped with an SPD-20AV UV/VIS detector (both, Shimadzu, Kyoto, Japan). The mobile phase was composed of 1) 1% ammonium acetate solution and 2) acetonitrile, using a gradient elution and performed at 40°C with a flow rate of 1.0 mL/min. The eluate was monitored by a UV detector at 285 nm.

Percentage of antibody modified

The solution of Am80 liposome conjugated with anti-CD90 antibody was centrifuged at 64,200 rpm for 20 min. The supernatants were removed, and then 2% Triton-X 10 µL was added to create blotting samples. PVDF membrane was incubated with 100% ethanol for 15s, then with ultrapure water for 2 min, and subsequently with transfer buffer for 5 min. After drying the surface of membrane, the 2 µL blotting samples were blotted to the membrane. The blotted membrane was dried completely and washed once with PBS containing 0.1% polysorbate 20 and incubated with horseradish peroxidase-conjugated secondary antibody (Goat anti-mouse IgG H&L) at a 1:100000 dilution in can get signal immunoreaction enhancer solution (TOYOBO Co., Ltd., Osaka, Japan) at room temperature for 2 h. After two washes with PBS containing 0.1% polysorbate 20, the immunoreactions were visualized by immobilon western chemiluminescent HRP Substrate (Merck Millipore Co., Darmstadt, Germany) using ImageQuantLAS4000EPUVmini (FUJI FILM, Tokyo, Japan).

Stability of Am80 liposomes conjugated with anti-CD90 antibody

To assess the stability of the Am80 liposomes conjugated with anti-CD90 antibody in HBS buffer and culture medium (10% FBS/EMEM), we added Am80 liposomes (prepared as described) to 4°C HBS and 37°C culture medium, respectively. After 0, 12, 18, 24, 36, and 48 h of incubation, the average size of the liposomes was determined by dynamic light scattering with an ELSZ2P analyzer.

Cellular uptake of liposomes

Calu-6 (6×10^3 cells/cm^2) was seeded onto 8-well chamber slides and overnight pre-cultured. Coumarin-6 liposomes conjugated with anti-CD90 antibody and coumarin-6 liposomes without conjugation (500 µM, 100 µL/well) were added, respectively, and were treated for 16 min. After treatment with the liposomes, cells were washed twice with PBS and fixed by 4% paraformaldehyde in PBS for 15 min. The cells were washed three times with PBS, blocked using 10% bovine serum albumin at room temperature for 60 min, and then incubated with the primary antibody against CD90 at a 1:200 dilution at 4°C overnight. After incubation at room temperature for 2 h, the samples were washed three times with PBS containing 0.1% polysorbate 80 and then incubated with Alexa Fluor 594 donkey anti-rabbit IgG at 1:200 dilution and DAPI, for staining of the nuclei, at room temperature for 2 h. Slides were washed three times with PBS containing 0.1% polysorbate 80, and after mounting of the sections, the slides were observed with a BZ-9000 fluorescence microscope (Keyence, Osaka, Japan).

Cell specificity of liposomes

Coumarin-6 liposomes conjugated with anti-CD90 antibody (500 µM, 100 µL/well) were added to Calu-6 cells seeded onto 8-well chamber slides and overnight pre-cultured, respectively. After 0, 2, 4, 8, 16, 32, and 64 min of incubation, cells were washed twice with PBS and fixed by 4% paraformaldehyde in PBS for 15 min. The cells were washed three times with PBS and then incubated with DAPI at room temperature for 2 h. Cell membranes were labeled by Cell Light Plasma Membrane-RFP, BacMam 2.0 according to the kit manufacturer's protocol (Life Technologies Japan Ltd., Tokyo, Japan). Slides were washed three times with PBS containing 0.1% polysorbate 80, and after mounting of the sections, the slides were observed with a BZ-9000 fluorescence microscope at 400 nm (for DAPI), 495 nm (for coumarin-6), and 565 nm (for cell membranes).

Cell viability assay

The cellular viability of Calu-6 cells after Am80 treatment was assessed using a CellTiter-Glo Luminescent Cell Viability Assay

(Promega KK, Tokyo, Japan) according to the manufacturer's protocol. Cells (625 cells/cm^2) were seeded in 100 µL of medium in 96-well flat-bottom plates and incubated overnight at 37°C. After exposure to drugs for 2-6 days, the plates were assayed using an Envision Plate Reader (PerkinElmer Japan Co. Ltd. Kanagawa, Japan). The medium containing Am80 was exchanged every 48 h.

In vivo tumorigenesis assay

Five-week-old male congenital athymic BALB/C nude (nu/nu) mice were purchased from Sankyo Labo Service Corporation (Tokyo, Japan). A total of 1.3 × 10^6 Calu-6 cells were suspended in 100 µL of PBS (50% Matrigel; Becton Dickinson & Co., San Jose, CA) and injected subcutaneously into both the left and right flanks of the nude mice. When the tumors reached over 50 mm^3 in volume, the mice were divided randomly into three groups (HBS, anti-CD90-Empty liposome, and anti-CD90-Am80-liposome). The twice-weekly intratumoral administration of Am80 liposomes (0.1 mg/kg as Am80 and liposomes was prepared in HBS at 0.1 mg/mL as the liposome concentration), dissolved in a saline solution with up to 1% DMSO, was then initiated (day 0). The dose of Am80 was selected based on previous reports [42,43] and our previous preliminary experiments. The mice were weighed, and the tumor volume was determined using the standard formula L × W^2 × 0.5, where L is the longest diameter and W is the shortest diameter, as previously described [44].

Statistics

Normally distributed data are presented as the mean ± standard deviation (SD). The Student *t*-test was used for the comparisons of two groups. P values <0.01 or <0.05 were considered to indicate statistical significance.

Results

Expression of CD90 in Calu-6

To identify the anaplastic pulmonary cell protein, we tested the expression of CD90 as a marker of human alveolar epithelial progenitor cells in Calu-6. Calu-6 (reported as an anaplastic pulmonary carcinoma cell) and A549 (reported as a mature pulmonary carcinoma cell) were evaluated by immunostaining and Western blot analysis. Assessment of CD90 expression by fluorescence microscopy revealed that Calu-6 expressed CD90 more greatly than it expressed A549 **(Figure 2A)**. Western blot analysis showed the expression level of CD90 in Calu-6 to be significantly higher than that of A549 **(Figure 2B)**.

Characterization and stability of Am80 liposomes conjugated with anti-CD90 antibody

To evaluate the characterization of Am80 liposomes conjugated with anti-CD90 antibody, we conducted experiments on particle size, zeta potential, percentage of drug entrapment, and percentage of antibody modification **(Table 1)**. The particle size and zeta potential of the Am80 liposomes conjugated with anti-CD90 antibody were 116.4 nm and -5.97 mV, respectively. The particle size was increased by about 10 nm and the zeta potential was decreased by 2 mV compared with the pre-modification values. The percentage of drug entrapment was 15.5% and that of antibody modification was 21.7%.

After Am80 liposomes conjugated with anti-CD90 antibody were respectively incubated in 4°C HBS and 37°C culture medium, we

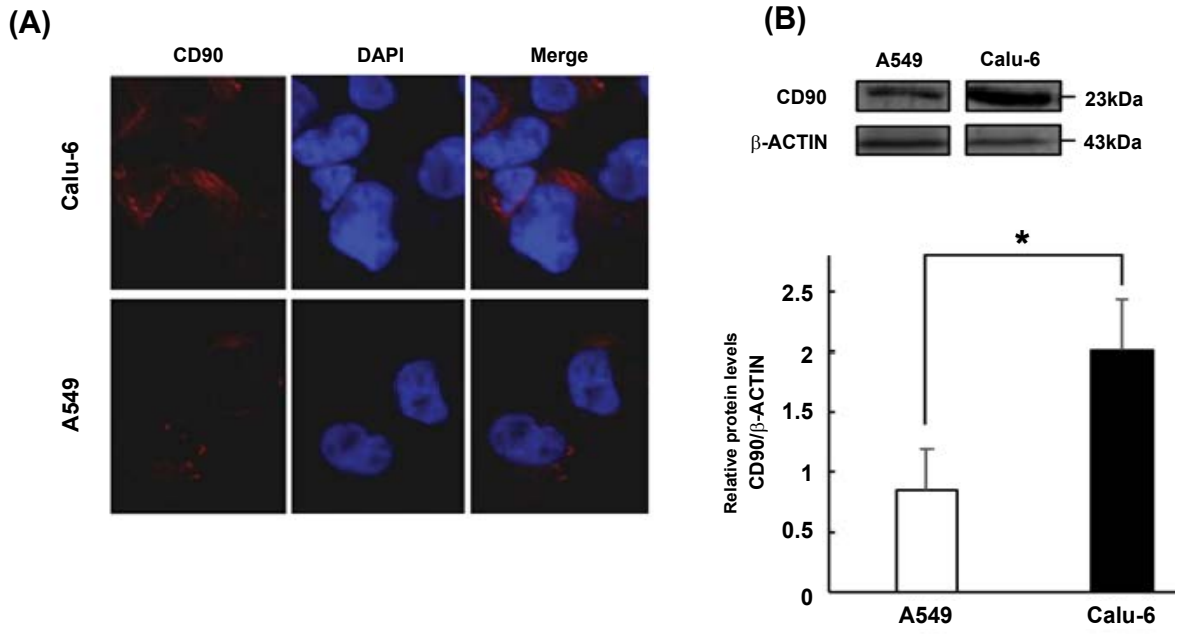

Figure 2 Expression of CD90 in Calu-6 and A549 cells. (A) The expression of CD90 in Calu-6 and A549 cells as indicated by immunostaining. (B) The expression levels of CD90 in Calu-6 and A549 cells as indicated by Western blotting. Data are given as mean ± SD from n=3. **p<0.01 compared with A549.

evaluated the stability of the liposomes by measuring particle size over time. The particle size of the liposomes was almost unchanged in both the 4°C HBS and 37°C culture medium, indicating that the liposomes were stable over a period of 48 h **(Figure 3).**

Cellular uptake of liposomes conjugated with anti-CD90 antibody

We compared the cellular uptake of liposomes in pulmonary carcinoma cells between the conditions of before and after CD90-antibody modification. Calu-6 was seeded, and liposome solution was added at 200 μM as a final concentration. After the solution was incubated for 16 min, we observed the cellular uptake of liposomes in Calu-6. The uptake of the liposomes conjugated with anti-CD90 antibody was greater than that of the non-conjugated liposomes **(Figure 4)**. We observed that many liposomes were cohering to the cell surface.

Cell specificity of liposomes conjugated with anti-CD90 antibody

To assess the specificity of the liposomes conjugated with anti-CD90 antibody against CD90-positive cells, Calu-6 and A549 cells were seeded and liposome solution was added at 200 μM as a final concentration After incubation, we observed the cellular uptake of the liposomes over time in both cell lines. The uptake in the Calu-6 cells was increased over that in the A549 cells **(Figure 5)**. We observed that compared with the A549 cells, many more liposomes were cohering to the membrane of the Calu-6 cells.

Cell growth inhibition of Am80 liposomes conjugated with anti-CD90 antibody against pulmonary carcinoma cells

To assess cell growth inhibition of the Am80 liposomes conjugated with anti-CD90 antibody against Calu-6, we evaluated cell viability after Am80 liposome treatment. Calu-6 was seeded and was exposed to Am80 and to Am80 liposomes conjugated with anti-CD90 antibody for 6 days, respectively. The results showed that 10 μM Am80 inhibited cell growth by about 11%, whereas 10 μM Am80 liposomes conjugated with anti-CD90 antibody inhibited cell growth by about 87%, indicating that Am80 encapsulated in liposomes inhibited cell growth significantly more than the non-encapsulated Am80 did **(Figure 6)**.

We then evaluated the effect of Am80 liposomes conjugated with anti-CD90 antibody on cell growth inhibition over time. After 2 d of treatment, 10 μM Am80 liposomes conjugated with anti-CD90 antibody significantly inhibited cell growth by about 38% compared with the non-encapsulated Am80 (Figure 7). These results showed that Am80 liposomes conjugated with anti-CD90 antibody have an effect of growth inhibition against Calu-6.

In vivo therapeutic efficacy of Am80 liposomes conjugated with anti-CD90 antibody

We evaluated the therapeutic efficacy of Am80 liposomes conjugated with anti-CD90 antibody in BALB/c nude (nu/nu) mice bearing Calu-6 cell subcutaneous tumors. After the tumors were palpated (50 mm^3), HBS and anti-CD90-Empty liposome were administered intratumorally into the mice in the control groups

Table 1 Characterization of Am80 liposomes conjugated with or without anti-CD90 antibody (n=3).

Liposome formulation	Particle size (nm), mean ± SD	Zeta potentials (mV)	Drug encapsulation efficiency (%), mean ± SD	Coupling efficiency (%), mean ± SD
Am80 liposome (without anti-CD90 TTantibody)	106.7 ± 24.9	-5.97	-	-
anti-CD90-Am80 liposome	116.4 ± 30.1	-8.18	15.5 ± 1.5	21.7 ± 1.27

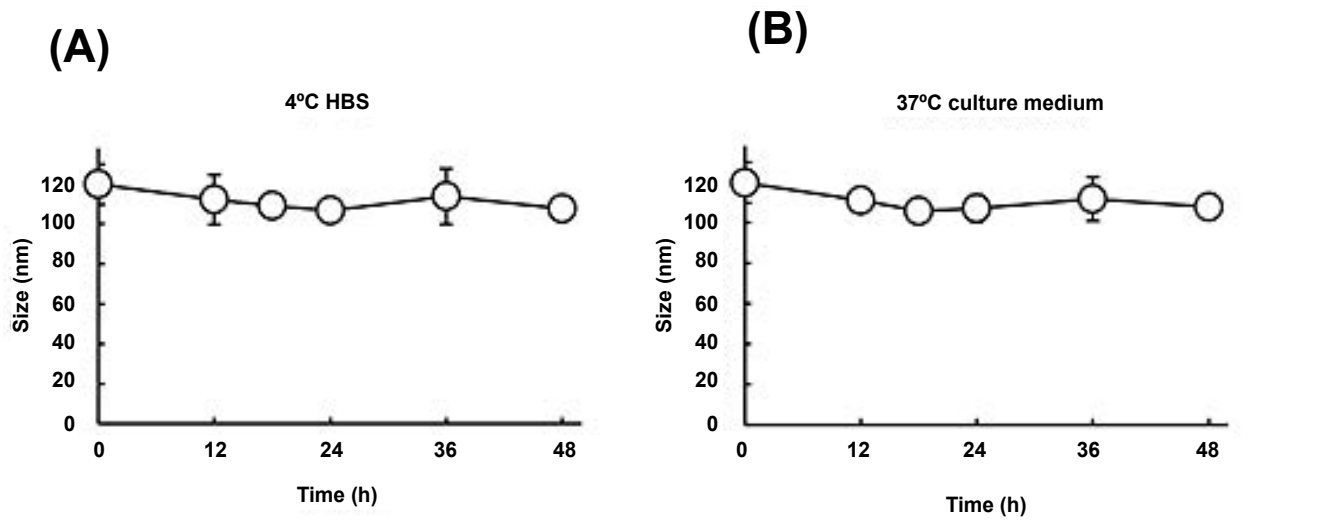

Figure 3 Stability of liposomes in buffer. Size of the Am80 liposomes measured for 48-h incubation in at 4°C HBS (A) and 37°C culture medium (B). Data are given as mean ± SD from n=3.

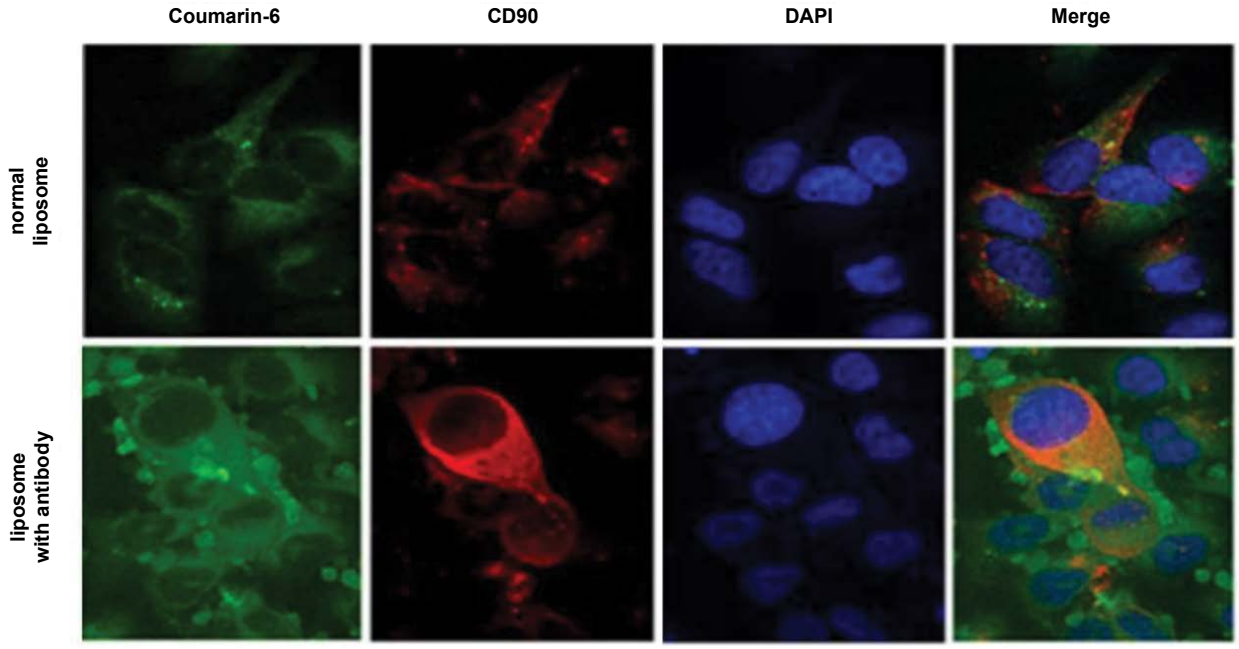

Figure 4 Immunofluorescence analysis of cellular uptake in Calu-6 cells. Immunofluorescence analysis of Calu-6 cells shows nucleus, coumarin-labeled liposomes, CD90, and merged images by fluorescence microscopy. The intracellular uptake of coumarin-6 liposomes conjugated with or without anti-CD90 antibody was observed within Calu-6 and A549 cells after 16 min of incubation.

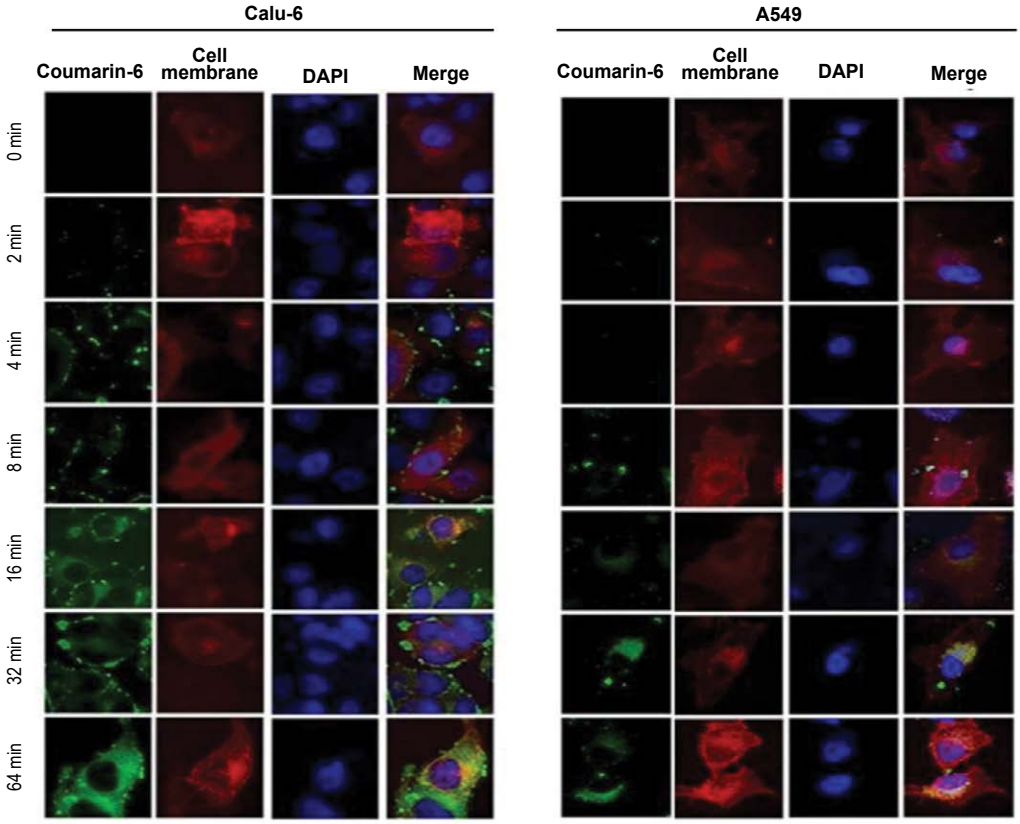

Figure 5 Cell specificity of liposomes by fluorescence microscopy analysis in Calu-6 and A549 cells. Fluorescence microscopy analysis shows nucleus, coumarin-6-labeled liposomes, cell membrane, and merged images. The intracellular uptake of liposomes was compared in Calu-6 (CD90-positive) and A549 (CD90-negative) cells after 2-64 min of incubation.

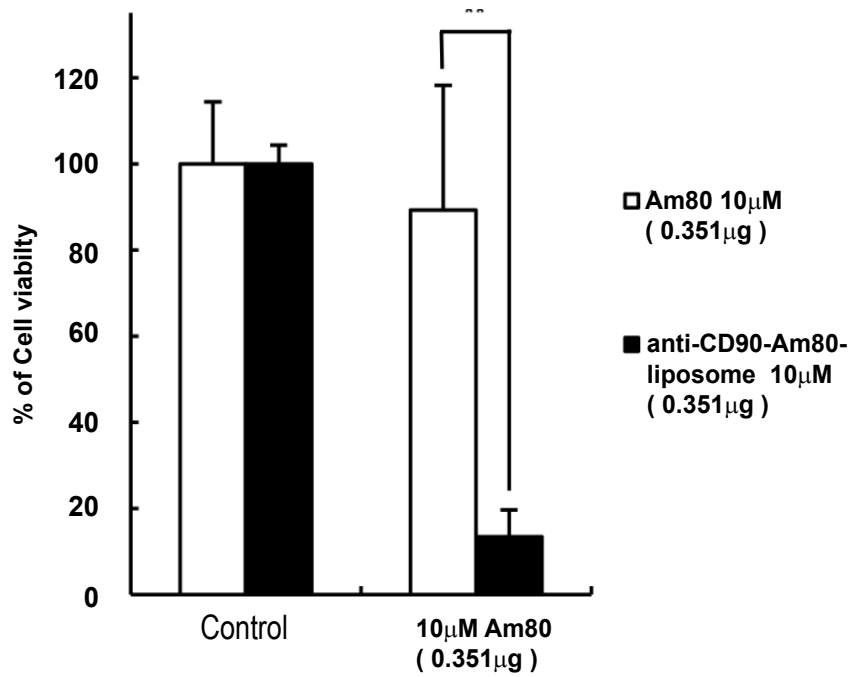

Figure 6 Cell growth inhibition with Am80 liposomes conjugated with anti-CD90 antibody. Cells were treated with 10 μM (0.351 μg) Am80 or anti-CD90-Am80-liposome for 6 days. Data are given as mean ± SD from n=3. **p<0.01 compared with Am80 treatment group. For plots of cell survival rate, the mean basal value (control) of each group is set at 100%.

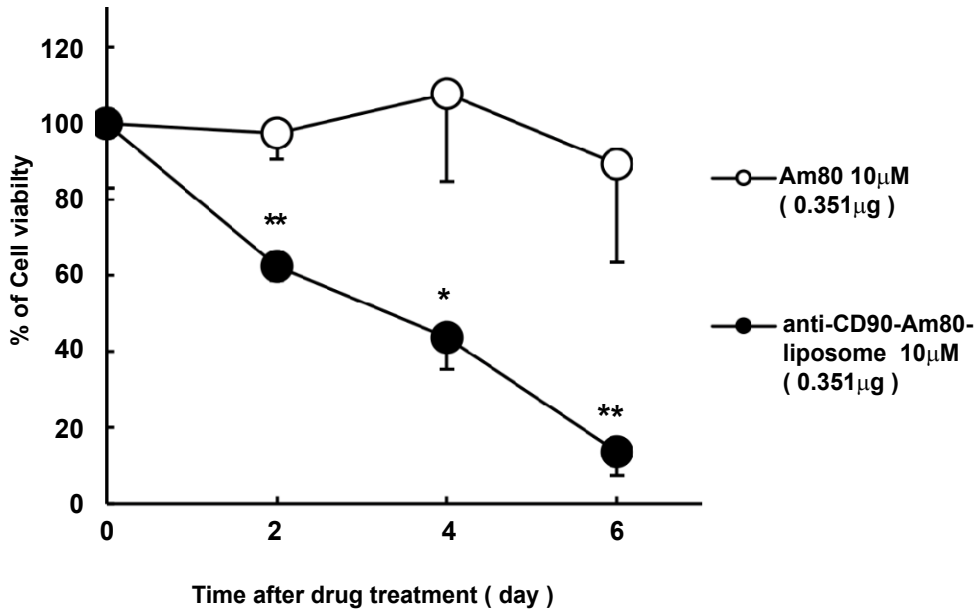

Figure 7 Time-dependent cell growth inhibition with Am80 liposomes conjugated with anti-CD90 antibody. Cells were treated with 10 μM (0.351 μg) Am80 or anti-CD90-Am80-liposome for 2, 4, 6 days. Data are given as mean ± SD from n=3. *p<0.05, **p<0.01 compared with the Am80 treatment group. For plots of cell survival rate, the mean basal value (day 0) of each group is set at 100%.

twice a week. Anti-CD90-Am80-liposome (Am80 liposomes conjugated with anti-CD90 antibody) was administered intratumorally in the treatment group twice a week. Tumor growth was significantly inhibited in anti-CD90-Am80-liposome treatment group compared with the control group from day 10 **(Figure 8A)**. However, we could not confirm the weight change of the mice between the control and treatment group **(Figure 8B)**. The results showed that Am80 liposomes conjugated with anti-CD90 antibody had an antitumor effect against the pulmonary carcinoma cells subcutaneously transplanted in mice.

Discussion

In this study, we found that the CD90-targeted liposomes

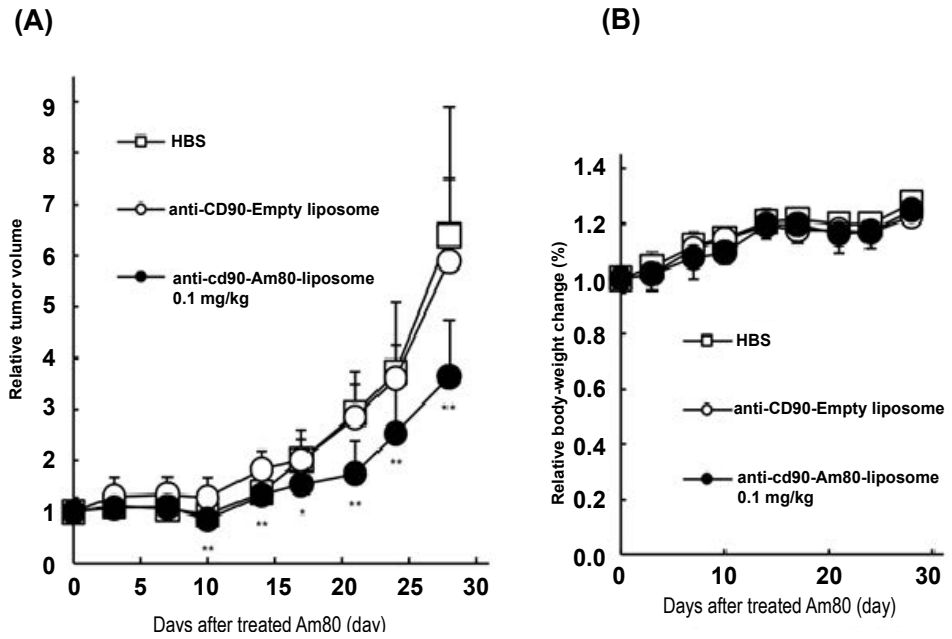

Figure 8 Therapeutic efficacy in the mice bearing Calu-6 cell subcutaneous tumors. (A) Average tumor growth curves from a representative experiment. Mice received intratumoral administration of Am80 liposomes conjugated with anti-CD90 antibody at an equivalent dose of 0.1 mg/kg on days 7, 10, 14, 17, 21, 24, and 28. (B) Body weight changes of the mice over the experimental period. Data are given as mean ± SD from n=10-12. **p<0.01 compared with the anti-CD90 Empty liposome group.

increased the therapeutic efficacy of Am80 in Calu-6 cells and tumors. These findings support the idea that CD90-targeted liposomes would be a novel antitumor DDS for the treatment of pulmonary carcinoma **(Figure 1)**.

We found that CD90 was expressed significantly more greatly in Calu-6 than in A549 cells **(Figure 2)**. CD90 is reportedly expressed in human alveolar epithelial progenitor cells [35]. Therefore, CD90 was expressed in Calu-6 anaplastic pulmonary carcinoma cells as well as in undifferentiated cells, whereas the expression of CD90 was low in A549, which is a differentiated pulmonary carcinoma cell line.

We observed increased uptake of the liposomes conjugated with anti-CD90 antibody compared with that of the non-conjugated liposomes by evaluating cellular uptake **(Figure 4)** and that the uptake in Calu-6 was increased over that in A549 cells by evaluating the cell specificity of liposomes **(Figure 5)**. Liposomes conjugated with anti-transferrin receptor antibody, which encapsulates DOX, are reported to specifically combine with transferrin receptor on the cell surface dependently against a DOX-resistant subline of human leukemia K562 cells of K562/ADM, and then the DOX is incorporated efficiently into the cell via endocytosis [45]. Because our results showed increased uptake of liposomes in Calu-6, as indicated by the high expression of CD90 in Calu-6 versus A549 we believe that the liposomes conjugated with antibody combined with the surface of the Calu-6 cells and endocytosis occurred.

We evaluated cell growth inhibition of Am80 liposomes conjugated with antibody against pulmonary carcinoma cells. Am80 encapsulated in liposomes significantly inhibited cell growth at a low dose and with high effect compared with the non-encapsulated Am80 **(Figure 6)**, suggesting the usefulness of Am80 liposomes conjugated with antibody. Our results suggested that the liposomes were internalized into cells by endocytosis after binding specifically to CD90, which is expressed on the surface of Calu-6 cells, and then Am80 was released into the cytoplasm. Therefore, it is suggested that Am80 encapsulated in liposomes was effectively internalized into the cells compared with the non-encapsulated Am80, thus producing a better effect.

Taking these results together, we then investigated the therapeutic efficacy of Am80 liposomes conjugated with anti-CD90 antibody *in vivo*. In the treatment group receiving Am80 liposomes conjugated with anti-CD90 antibody, tumor volume was significantly reduced compared with that in the control group **(Figure 8)**. In a previous study, after treatment with 0.1 mg/kg bufalin for 16 days in mice bearing melanoma subcutaneous tumors, the bufalin liposomes anchored to anti-CD40 antibody significantly reduced tumor volume to 70 mm^3 indicating an antitumor effect [46]. Likewise, we similarly revealed the usefulness of liposomes conjugated with anti-CD90 antibody.

By encapsulating differentiation-inducing drugs against anaplastic carcinoma into targeting liposomes conjugated with the appropriate antibody, the differentiation inducer can be delivered at a high dose specifically to the anaplastic carcinoma cells and not to normal cells. These studies provide the possibility of lightening the burden on patients by preventing the systemic side effects of drugs and contributing to the realization of pulmonary carcinoma therapy without metastasis or relapse. In addition, by changing the antibody that is conjugated on the surface of the liposome and the drug that is encapsulated within

liposome, it will be possible to apply therapy to other cancers in addition to pulmonary carcinoma.

Conclusions

Am80 liposome conjugated with anti-CD90 antibody significantly influenced the inhibition of the cell growth in a pulmonary carcinoma cell line. We revealed that the cellular uptake of Am80 liposomes conjugated with anti-CD90 antibody by anaplastic pulmonary carcinoma cells was highly effective and efficient compared with that of non-encapsulated Am80.

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Competing Interests

The authors declare no conflict of interest

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