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Original Article

Lung cancer-targeting treatment of iRGD-LP-DOX/Rg3 through integrin receptor-medicated endocytosis

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Abstract: Objective: To prepare the specific ligand for integrin receptors, iRGD-LP-DOX/Rg3 (liposomes co-carrying cytotoxic drug doxorubicin DOX and antiangiogenic agent ginsenoside Rg3 modified by iRGD), and to investigate its targeting property and therapeutic effect for lung cancer by performing experiments *in vivo* and *in vitro*. Methods: Film dispersion method was used to prepare iRGD-LP-DOX/Rg3 and its physiochemical properties including particle size, potential and encapsulation efficiency were observed. MTT method was used to detect the effect of liposomes on the proliferation of A549 and HUVEC (human umbilical vein endothelial cells) cell lines. Fluorescence spectrophotometric method was used to detect the uptake of liposomes by A549 and HUVEC cells and confocal microscopy was used to qualitatively observe the uptake of RGD-LP-DOX/Rg3 by A549 and HUVEC cells. A heterotopic lung cancer model was established in nude mice to explore the inhibitory effect of iRGD-LP-DOX/Rg3 on tumors and its accumulative ability in *in vivo* tumor tissues. Results: The particle size and potential of iRGD-LP-DOX/Rg3 were 112.3 ± 11.5 nm and 4.2 ± 1.16 mV, respectively. The encapsulation efficiency of DOX and Rg3 were (89.5 ± 4.5)% and (82.6 ± 2.1)%, respectively. On the one hand, 48 h after administration in A549 cells, in comparison to PBS group, the cell viability of A549 cell of groups treated with LP-DOX/Rg3, iRGD-LP-DOX, iRGD-LP-Rg3 and iRGD-LP-DOX/Rg3 were 61.2%, 42.9%, 73.3% and 22.3%, respectively. The differences among groups were of statistical significance ($P<0.01$). On the other hand, the cell viability of HUVEC cell groups treated by LP-DOX/Rg3, iRGD-LP-DOX, iRGD-LP-Rg3 and iRGD-LP-DOX/Rg3 were 55.3%, 43.9%, 62.8% and 18.6%, respectively. The differences among groups were also of statistical significance ($P<0.01$). The uptake efficiency of iRGD-LP in A549 cells was 2.9 times more than that of regular liposomes (LP) and the uptake efficiency of iRGD-LP in HUVEC cells was 3.7 times more than that of LP. The differences were both of statistical significance ($P<0.01$). Results: of confocal microscopy and quantitative experiments were consistent and indicated that the modification of iRGD could improve the cell uptake efficiency of LP. Experiments performed in tumor-bearing nude mice demonstrated that the inhibitory effect of iRGD-LP-DOX/Rg3 on tumor growth was evidently stronger than that of other groups and the differences among groups were of statistical significance ($P<0.01$). Compared with other groups, iRGD-LP-DOX/Rg3 could extend the survival time of tumor-bearing nude mice effectively. *In vivo* near-infrared fluorescence imaging assay showed that the accumulative ability of iRGD-LP was obviously stronger than that of LP. Conclusion: iRGD-LP-DOX/Rg3, with good affinity to A549 and HUVEC cells, is a potentially highly efficient tumor and angiogenesis targeting drug-delivery system.

Keywords: Integrin receptor, angiogenesis, LP, lung cancer

Introduction

Lung cancer is the No.1 malignant tumor threatening human health. In China, about 500,000 cases is newly identified every year. It is predicted that there will be 1000,000 patients with lung cancer annually until 2025 [1, 2]. Clinicians, therapists for tumor in particular, are facing an extremely difficult and tough situation. Currently, the treatment of lung cancer is mainly based on surgery and partly based on combined chemotherapy. However, due to a lack of selectivity of chemotherapy with cytotoxicity, adverse reactions and toxicity are inevitable and patients have poor tolerance. Therefore, tumor targeting drugs become the hotspot of current anti-tumor researches.

Integrins, a group of cell adhesion molecules, are widely expressed on the surface of nucle-

ated cells [3, 4]. Among them, integrin $\alpha_v\beta_3$ is highly expressed on the surface of various tumor cells like neuroglioma [5], melanin [6] and ovarian cancer [7], as well as that of endothelial cells [8-10] associated with tumor. It is closely related to tumor angiogenesis, metastasis and radiotherapy [11-13]. Hence, integrin $\alpha_v\beta_3$ is usually used as a specific target spot for tumors.

Tumor-targeting cell-penetrating peptides, with targeting property, can deliver drugs through cell surface receptor-mediated penetrating effect when they arrive at specific spots [14]. iRGD is a kind of tumor-targeting peptide. Its sequence is CRGDRGPDC, among which RGD can target integrins and thus target tumor sites where integrins are highly expressed. Then, the R residue produced after incision by a specific enzyme near the tumor will interact with NRP-1 receptors located on the surface of tumor cells, leading to cell penetrating effect [15-17]. For this reason, iRGD, this multifunctional polypeptide, has received much attention and been investigated.

With the development of modern oncomolecularbiology, more and more therapeutic methods for tumors appear, among which targeted inhibition of tumor angiogenesis is an effective one. Vessel-targeting antitumor therapy deprives the supply of nutrients and oxygen by blocking blood supply and thus causes avascular necrosis [18-21]. Recent studies indicated that ginsenoside Rg3 could inhibit the growth of lung cancer cells and angiogenesis in mice effectively [22]. Nevertheless, administration of antiangiogenic agents alone easily led to drug resistance. Therefore, cytotoxic drugs were combined with antiangiogenic agents so as to enhance antitumor effect. Studies suggested that integrin receptors were highly expressed on the surface of tumor cells and vascular endothelial cells [23-25]. Therefore, in this study, iRGD-LP-DOX/Rg3 was prepared by using liposomes as carriers to explore the therapeutic effect of this drug delivery system.

Material and methods

Experimental materials

Fetal calf serum and DMEM culture medium were purchased from Thermo-Fisher Biochemical Products (Beijing, China) Co., Ltd.; Doxorubicin (DOX) from Jiangsu Hengrui Medicine Co., Ltd.; MTT kit and ginsenoside Rg3 from Sigma; iRGD from GL Biochem (Shanghai) Co., Ltd.; Soyabean lecithin, DSPE-PEG2000 and DSPE-PEG2000-MAL from Avanti Polar Lipids (the US). The rest of reagents were analytically pure. A549 cell line was purchased from ATCC and female nude mice from Dashuo Experimental Animal Center of Chengdu (Sichuan, China).

Methods

Cell culture: A549 cells were cultured in a DMEM culture medium with 100 mg/L fetal calf serum, 5% CO_2 and saturation humidity at 37°C. When cell confluence reached up to 0.8-0.9, it was digested with 2.5 mg/L pancreatin and sub-cultured. Cells during logarithmic growth phase were colleted for study.

HUVEC cells were cultured in a DMEM culture medium with 100 mg/L fetal calf serum, 5% CO_2 and saturation humidity at 37°C. When cell confluence reached up to 0.8-0.9, it was digested with 2.5 mg/L pancreatin and sub-cultured. Cells during logarithmic growth phase were colleted for study.

The preparation of liposomes: iRGD-PEG2000-DSPE was synthesized according to methods described in literature [26, 27]. 12.05 mg soyabean lecithin, 0.96 mg cholesterol, 1.10 mg iRGD-PEG2000-DSPE, 0.33 mg DOX and 30.30 mg ginsenoside Rg were weighed carefully and dissolved in chloroform. The resultant solution underwent reduced pressure distillation in an eggplant-shaped bottle for film formation. Then, the organic solvent was removed and the rest was placed overnight in a vacuum drier to ensure intensive drying. Afterwards, 1 ml PBS was added for hydration and iRGD-LP-DOX/Rg3 was obtained by ultrasonography with probes (80 W, 10 s, 10 s, 5 times). iRGD-LP-DOX, iRGD-LP-Rg3 and LP-DOX/Rg3 were obtained in the same way.

A proper amount of liposomes prepared was taken to measure its size and potential with a laser particle analyzer. Unencapsulated DOX and ginsenoside Rg3 were separated from liposomes through glucose gel column chromatography. Then, demulsification was conducted for these liposomes with methanol. After that, the content of DOX and ginsenoside Rg3 was detected by using HPLC method at 233 nm

Table 1. Characteristics of iRGD-LP-DOX/Rg3, iRGD-LP-DOX, iRGD-LP-Rg3 and LP-DOX/Rg3: particle size, size distribution, zeta-potential (*n*=5)

Formulation	Size/nm	PDI	Zeta-potential/mV	Encapsulation efficiency (DOX)	Encapsulation efficiency (Rg3)
iRGD-LP-DOX/Rg3	112.3 ± 11.5	0.130 ± 0.030	4.2 ± 1.16	89.5 ± 4.5	82.6 ± 2.1
iRGD-LP-DOX	115.4 ± 6.3	0.210 ± 0.040	5.8 ± 3.13	92.7 ± 3.6	/
iRGD-LP-Rg3	123.8 ± 5.5	0.150 ± 0.080	3.5 ± 2.23	/	80.5 ± 3.3
LP-DOX/Rg3	121.8 ± 7.5	0.110 ± 0.080	-2.9 ± 1.79	91.6 ± 3.9	83.1 ± 2.5

and 269 nm, respectively. At last, the encapsulation efficiency was calculated according to the following equation: $EE\%=W_{encapsulated}/W_{delivered}\times100\%$.

MTT assay: A549 and HUVEC cells were cultured and inoculated into a 96-well plate. When complete adhesion to wall was observed for cells in the plate and it came to the logarithmic growth phase, liposomes after aseptic filtration were added until the final concentration of each well was 5 μmol/mL. The plate was then placed into an incubator with CO_2 at 37°C. After being cultured for 24 h and 48 h, the plate was taken out. After 20 μL 5 mg/mL MTT solution was added in each well, it was placed into the incubator again for incubation for 4 h. After that, liquid in the plate was poured out and 200 μL DMSO was added in each well. After it was shaken for 15 min at 37°C in dark, the optical density (OD) of each well was measured at 490 nm with a microplate reader.

Cell uptake assay: Cells during the logarithmic growth phase were inoculated into a 6-well plate (density: 5×10^5 cells/well). After the plate was cultured for 24 h at 37°C, a proper amount of iRGD-LP-DOX and LP-DOX was added in each well until the concentration of liposomes in each well reached 0.2 mg/mL. Then, after plate being incubated for 4 h at 37°C, media with liposomes were removed and it was washed twice with cold PBS. Later, after digestion with 0.25% pancreatin, it was centrifuged and washed thrice with PBS. Thus, the fluorescence value was measured by Fluorescence spectrophotometric method.

In order to observe cell uptake of liposomes qualitatively, liposomes were incubated with cells for 4 h and these cells washed thrice with PBS. Then, 2 $\mu g.mL^{-1}$ DAPI solution was added and incubated at room temperature for 20 min. After that, the solution was washed with icecold PBS for three times. Later, 4% paraformaldehyde was added for fixation for 15 min. Then, paraformaldehyde was discarded and the resultant substance was stored with icecold PBS and placed under a laser confocal fluorescent inverted microscope for observation.

In vivo assay: A549 cells, after being digested with pancreatin and centrifugation, were suspended in DMEM culture solution. The concentration of this solution was adjusted to 5×10^7 cells/mL. Female nude mice weighing 20-25 g aged 4-6 weeks were taken out and A549 cell suspension prepared was inoculated at the back of these mice (0.1 mL per mice). 1-2 weeks after inoculation, when masses were found at their back, it meant that the inoculation was successful. Then, 50 tumor-bearing nude mice selected were randomized into five groups (10 mice/group) -LP-DOX/Rg3, iRGD-LP-DOX, iRGD-LP-Rg3, iRGD-LP-DOX/Rg3 and PBS group. These drugs were administered at days 1, 3, 6 and 9. Tumor size and the body weight of nude mice were recorded every two days. The survival time of each mouse was described and Kaplan-Meier curves were drawn.

Liposomes carrying near infrared fluorescence DIR were prepared according to Section 1.2.2 and were injected through caudal vein in tumorbearing nude mice. 8 h later, these mice were anesthetized with 10% chloral hydrate and fixed under a whole body optical imaging system in supine position for observation and photography (Ex=730, Em=790).

Statistical methods

Experimental data was expressed as mean ± SD. Data analysis was performed by using statistical software SPSS11.0. Comparison of me-

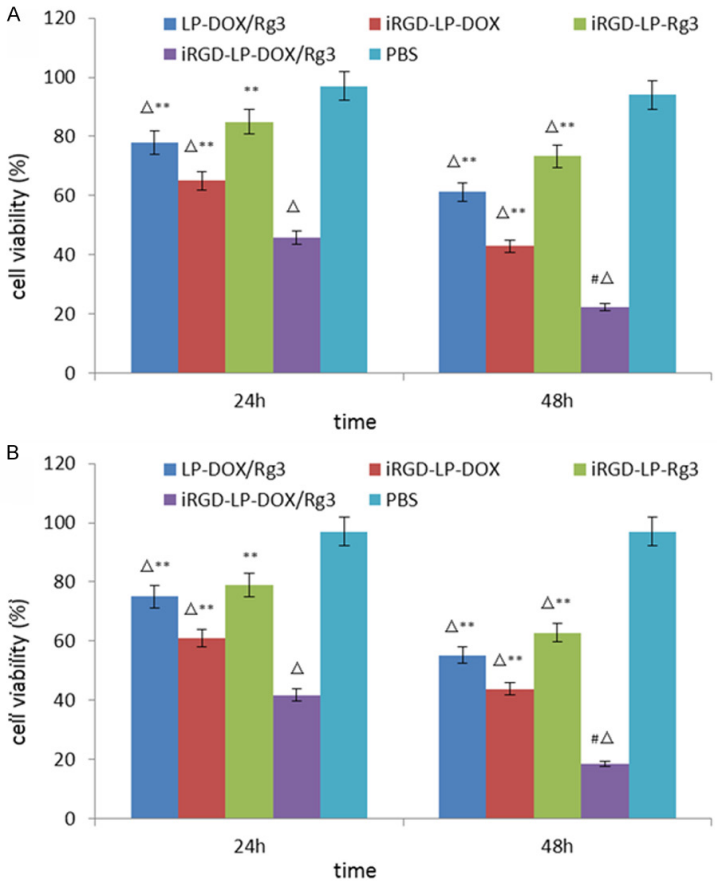

Figure 1. The survival rate of A549 cells (A) and HUVEC cells (B) after being treated with different liposomes. Compared with PBS group, $^{\Delta}$P<0.01; Compared with iRGD-LP-DOX/Rg3, **P<0.01; Compared with cell survival rate at 24 h, $^{\#}$P<0.01.

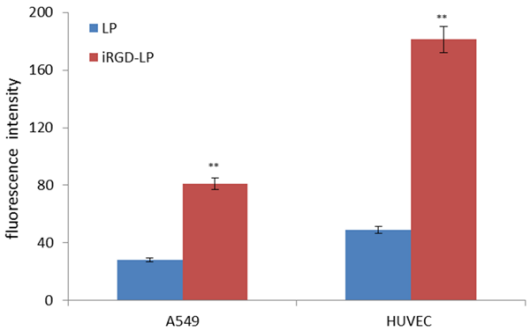

Figure 2. A549 and HUVEC cell uptake of liposomes. **P<0.01, the difference was of statistical significance.

ans was carried out by *t* test between two groups and by ANOVA among three or more groups, and posthoc multiple comparisons were conducted by SNK-*q* test. The inspection level α was 0.05.

Results

The size, potential and encapsulation efficiency of liposomes

The size of iRGD-LP-DOX/Rg3 was 112.3 ± 11.5 nm and potential 4.2 ± 1.16 mV. The encapsulation efficiency of DOX and Rg3 was (89.5 ± 4.5)% and (82.6 ± 2.1)%, respectively. They were presented in **Table 1**.

The inhibitory effect of iRGD-LP-DOX/Rg3 on the proliferation of A549 and HUVEC cells

Results of MTT assay was shown in **Figure 1**. On the one hand, 48 h after administration in A549 cells, compared with PBS group, the cell viability of A549 cell of groups treated with LP-DOX/Rg3, iRGD-LP-DOX, iRGD-LP-Rg3 and iRGD-LP-DOX/Rg3 were 61.2%, 42.9%, 73.3% and 22.3%, respectively. The differences among groups were of statistical significance (P<0.01). Further, the inhibition ratio of each group for the proliferation of A549 cells at 48 h was obviously higher than that at 24 h and the difference was of statistical significance (P<0.01). On the other hand, the cell viability of HUVEC cell groups treated by LP-DOX/Rg3, iRGD-LP-DOX, iRGD-LP-Rg3 and iRGD-LP-DOX/Rg3 were 55.3%, 43.9%, 62.8% and 18.6%, respectively. The differences among groups were of statistical significance (P<0.01). Further, the inhibition ratio of each group for the proliferation of HUVEC cells at 48 h was obviously higher than that at 24 h and the difference was of statistical significance (P<0.01).

A549 and HUVEC cells uptake of liposomes

Integrin receptors were highly expressed on the surface of A549 and HUVEC cells. Quantitative results of cell uptake assay were shown in **Figure 2**: The A549 cell uptake efficiency of iRGD-LP was 2.9 times more than that of regular LP and the difference was of statistically significance (P<0.01); The HUVEC cell uptake efficiency of iRGD-LP was 3.7 times more than that of LP and the difference was of statistically sig-

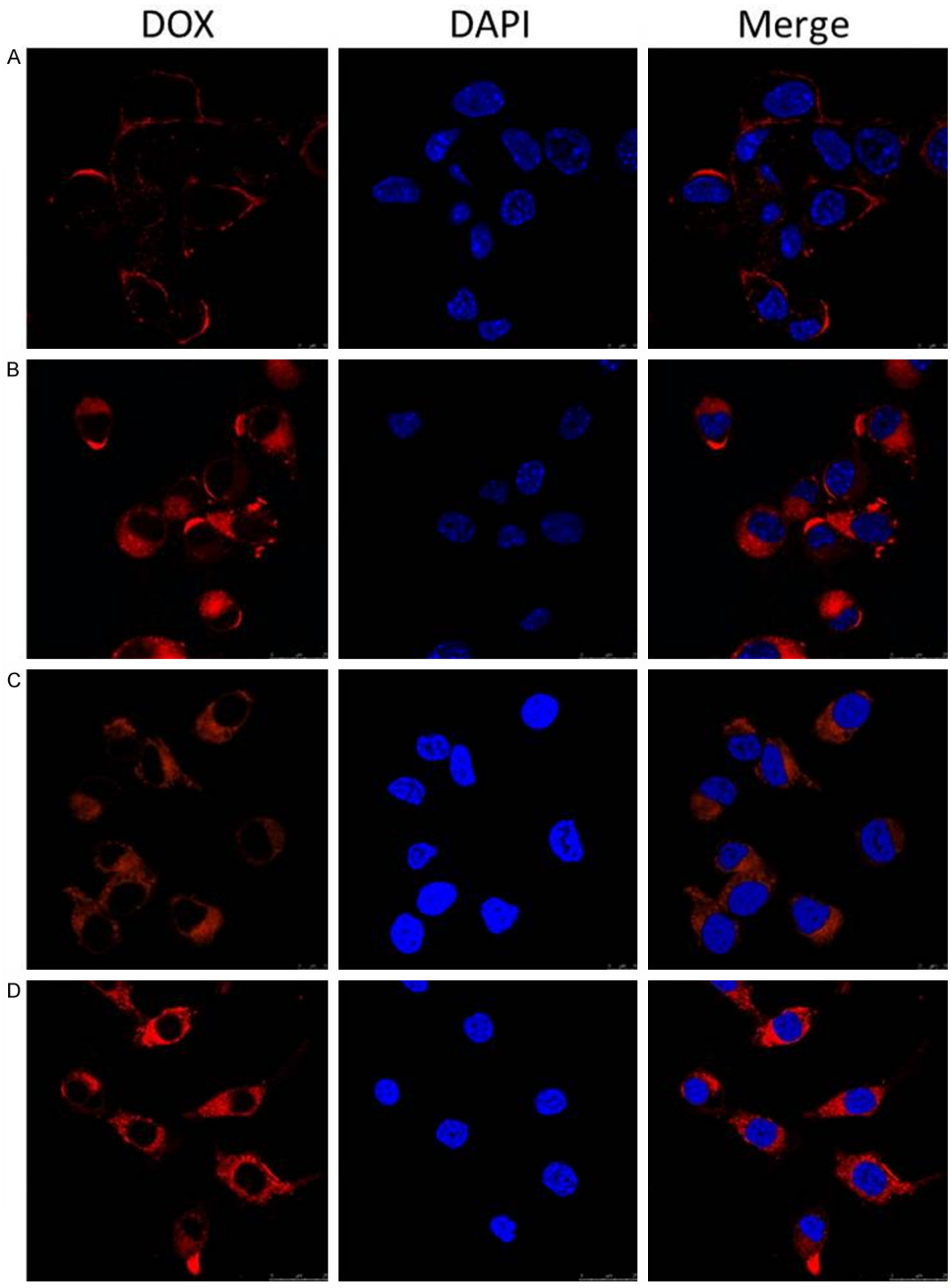

Figure 3. A549 (A, B) and HUVEC (C, D), cell uptake of LP (B, D) and iRGD-LP (A, C) observed under confocal microscopy.

nificance ($P<0.01$). Qualitative results of observation under laser confocal fluorescent inverted microscope were presented in **Figure 3**. The fluorescence intensity of iRGD-LP group in both

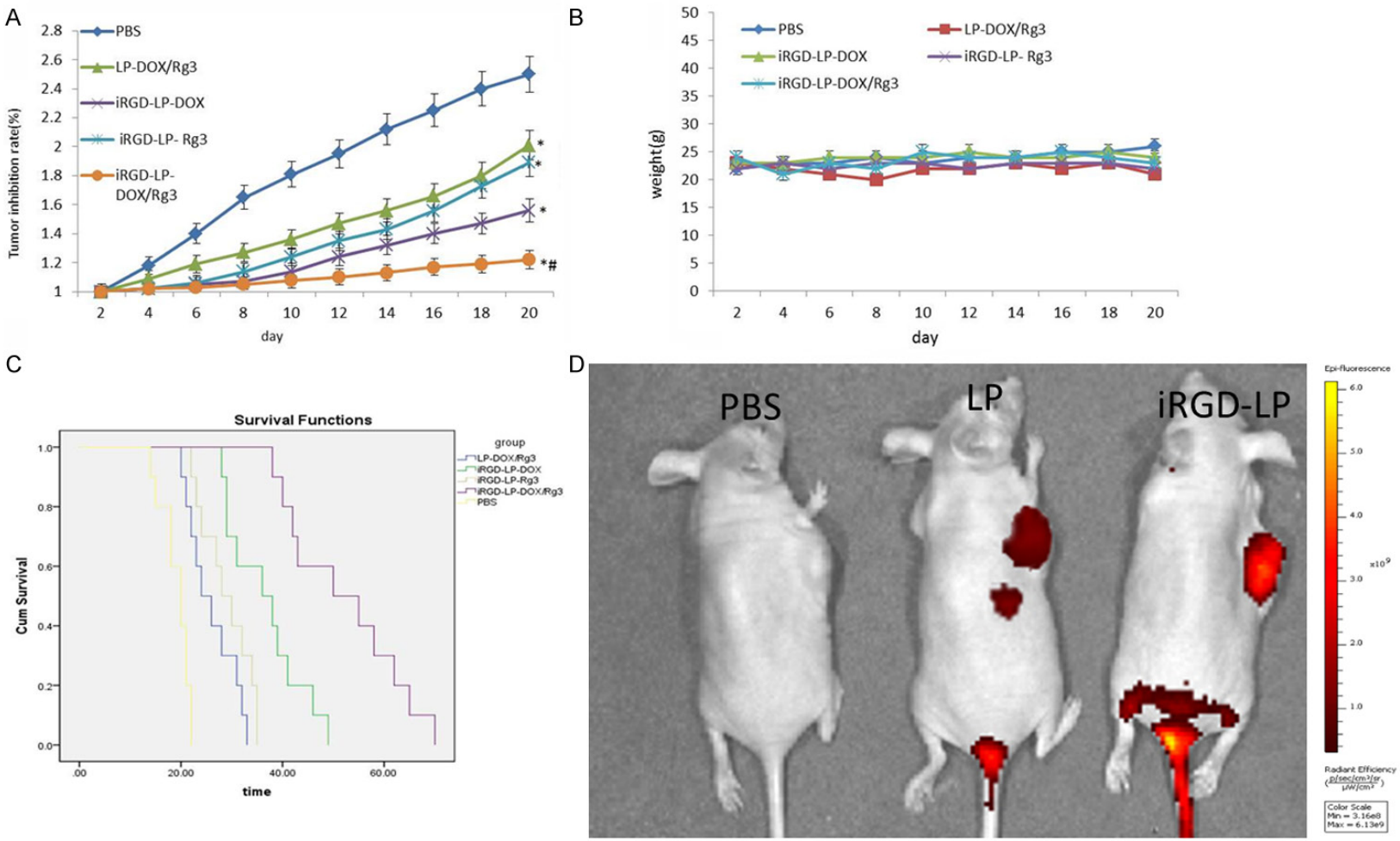

Figure 4. A. The volume (x ± s) of lung cancer tissues after being treated with different liposomes; B. The changing curve of body weight in each group; C. Kaplan-Meier curve; D. The representative in vivo images of A549 tumor-bearing BALB/C mice after injection of DiR-labeled liposomes. Compared with PBS group, $^{**}P<0.01$; Compared with LP-DOX/Rg3, iRGD-LP-DOX and iRGD-LP-Rg3 group, $^{\Delta}P<0.01$. The difference was of statistical significance.

A549 and HUVEC cell uptake assays was obviously stronger than that of LP, which was consistent with results of quantitative assay.

In vivo antitumor study

Results of treatment experiments in tumor-bearing nude mice were shown in **Figure 4A**. The tumor inhibition ratios of LP-DOX/Rg3, iRGD-LP-DOX, iRGD-LP-Rg3 and iRGD-LP-DOX/Rg3 were higher than PBS group, respectively ($P<0.01$). The tumor inhibition ratios of iRGD-LP-DOX/Rg3 was higher than other liposomal groups, respectively ($P<0.01$). There was statistically significant difference between iRGD-LP-DOX/Rg3 group and other groups ($P<0.01$). **Figure 4B** indicated that, during treatment, the body weight of nude mice in each group had no big change, suggesting that liposomes prepared in this study were able to reduce toxicity effectively. The survival time of nude mice in each group was described and results were shown in **Figure 4C**. The median survival period was 20 days, 29 days, 37 days and 53 days for PBS group, iRGD-LP-Rg3 group, iRGD-LP-DOX group and iRGD-LP-DOX/Rg3 group, the difference was statistically significant ($P<0.01$). iRGD-LP-DOX/Rg3 could extend the survival time of tumor-bearing mice remarkably.

The in vivo biodistribution and tumor accumulation profiles of DIR-loaded liposomes were clearly visualized by monitoring the whole body NIRF intensity in subcutaneous xenograft bearing nude mice model (**Figure 4D**). Obviously, the tumor accumulation of iRGD-LP was higher than LP. These results implied that the iRGD-LP could efficiently target to solid tumors and decrease non-specific accumulation in normal organs such as livers, lungs and kidneys. Control animals injected with PBS produced no fluorescence signals, which confirmed that the observed fluorescence signal was truly from the liposomes.

Discussion

Studies revealed that ginsenoside Rg3 could inhibit the proliferation of various tumor cells, including lung cancer [28], osteosarcoma [29], ovarian cancer [30] and esophagus cancer [31]. Also, it could inhibit tumor angiogenesis effectively. Liposomes were bilayer spherical structures composed of phospholipids. In the past thirty years, they were widely studied. With good biocompatibility, liposomes were an ideal drug delivery system for tumors [2]. In 1971, Folkman [32] et al. pointed out that since tumor growth and metastasis relied on angiogenesis, inhibiting tumor angiogenesis could be used as a new strategy in treating cancer. Anti-angiogenetic therapy thus came into being and scholars had made numerous studies in this field [33]. It was demonstrated that integrin receptor $\alpha_v\beta_3$ was highly expressed on the surface of tumor cells and vascular endothelial cells [34]. iRGD was a kind of polypeptide which could combine specifically with integrin receptors. In this study, iRGD was attached to the surface of liposomes encapsulating DOX and ginsenoside Rg3 so as to target and inhibit the growth of lung cancer cells and tumor angiogenesis. Up to now, the drug delivery system co-carrying DOX and ginsenoside Rg3 has rarely been reported.

iRGD-LP-DOX/Rg3 was prepared successfully in this study. The A549 and HUVEC cell uptake of liposomes was then investigated via cell uptake assay. Results indicated that the endocytosis efficiency of liposomes modified by iRGD was obviously higher than unmodified ones. The same results were also obtained in the tumor penetration assay. The reason was endocytosis mediated by integrin receptors which were highly expressed in lung cancer cells and vascular endothelial cells. MTT assay explored the inhibitory effect of liposomes on the proliferation of lung cancer cells and vascular endothelial cells. Results suggested that modification of iRGD could evidently enhance cytotoxicity. They were consistent with those of cell uptake assay. That is to say, cell uptake of liposomes had an effect on their cytotoxicity. In addition, a heterotopic lung cancer model was established in nude mice to investigate the inhibitory effect of different liposomes on tumor growth. Results obtained were consistent with those of *in vitro* studies. The inhibitory effect of iRGD-LP-DOX/Rg3 was evidently stronger than other groups. It was demonstrated in a near infrared fluorescent imaging assay that liposomes modified by iRGD could improve their accumulation in tumor tissues to prevent tumor growth.

In conclusion, iRGD-LP-DOX/Rg3 was prepared in this study. It got to tumor tissues through EPR effect and endocytosis was enabled by taking advantage of integrin receptors highly expressed on the surface of tumors and vascu-

lar endothelial cells. With good tumor and tumor angiogenesis targeting property, it is a potentially highly efficient drug delivery system for treating tumors and angiogenesis.

Disclosure of conflict of interest

None.

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