Original Article
Therapeutic effect of hepatocellular carcinoma-targeting liposome delivery system loaded with c[RGDYk] modified combrestatin A-4 and adriamycin: a pharmacodynamics study

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Abstract: Objective: To investigate the efficacy of long-circulating liposome drug carrier systems encapsulated with c[RGDYk]-modified combrestatin A-4 (CA-4) and adriamycin for targeted treatment of liver cancer. Method: In vitro drug release was monitored using HPLC and UV spectrophotometer; cellular drug content was analyzed using flow cytometry and immune fluorescence spectrophotometry. Blood drug concentration in mice was measured to determine the clearance rate and immunohistochemical studies were performed to investigate micro vessel density (MVD). Mouse models were used to compare the effect of different drug loading systems on tumor inhibition and the weight of mice. Results: The in vitro release rate of CA-4 was faster than that of adriamycin; adriamycin encapsulated in liposomes could improve the cell’s uptake of the drug, delay its clearance in mice, but did not affect the normal metabolism of encapsulated adriamycin. c[RGDYk]-L[CD] encapsulated adriamycin significantly inhibited angiogenesis and generated the strongest tumor-inhibitory effect with the least impact on the body weight of mice. Conclusion: c[RGDYk]-L[CD] can reduce angiogenesis in tumor, increase intracellular drug concentration, enhance anti-tumor activity and reduce drug toxicity.

Keywords: Hepatocellular carcinoma, targeted drug delivery, adriamycin

Introduction
Primary hepatocellular carcinoma (HCC) is highly malignant. Patients with the disease often suffer recurrence and metastasis despite active surgical and interventional treatments. In China, the mortality of HCC ranks the second among the tumor mortalities. There are a number of options for HCC treatments, including surgical treatment, interventional therapy, radiation therapy, chemotherapy and targeted therapy. However, the overall treatment efficacy has not been satisfactory and the prognosis is poor [1, 2]. Nano liposome targeted drug delivery system is a new drug delivery system, which can improve drug targeting ability to achieve higher drug concentration in the targeted focus or cells with better therapeutic efficacy [3, 4]. Meanwhile, the target drug loading and delivery systems with multiple drugs for tumors are being increasingly studied and explored [5]. Arginine-glycine-aspartate peptide (Arg-Gly-Asp, RGD) is recognized by αvβ3 receptor. Magnetic c[RGDyK] nanoparticles have unique magnetic properties such as superparamagnetism and high saturation magnetization and excellent biocompatibility. They have broad applications in resonance imaging and as drug targeted drug carriers.

In this study, we constructed model drug delivery systems targeting for HCC and investigated efficacy and mechanism of c[RGDYk]-modified nano drug delivery system. The result would provide insights for rational drug design and delivery for better HCC treatment.

Materials and methods
Cells and mice
B16F10 cells were purchased from the Cell Bank, Chinese Academy of Sciences, Shanghai; nude mice were purchased from Dashuo Experimental Animals Inc., Chengdu, China. Ani-
mal experimental protocols were approved the research ethic committee of Tumor Hospital of Jiangxi Province.

In vitro release assay for L-[CD] and c[RGDyk]-L-[CD]

One mL of L-[CD] and c[RGDyk]-L-[CD] (American Peptide, USA) was dialyzed against PBS and mixed well at 37°C on a shaker for 48 h. The dialysis bags were added with 2 mL of 10% Triton X-100 Triton to destroy all liposomes, and used as an infinite sample. The fresh medium was used as a blank control. Combretastatin A-4 (CA-4, Sigma, USA) and Adriamycin (DOX, Sigma, USA) were detected using HPLC method and UV spectrophotometer at 480 nm, respectively. Release (%) was calculated as $(A_t/A_\infty) \times 100\%$.

Flow cytometry

B16F10 cells in monolayer culture were digested and centrifuged to prepare the single cell suspension. The cells were inoculated into the wells of 6-well plates and cultured for 24 h in DMEM medium containing 10% fetal bovine serum (FBS, Gibco, USA) at 37°C in a CO$_2$ incubator. L-[D] and c[RGDyk]-L-[D] were added to a final concentration of DOX at 20 μg/ml and cultured in serum-free for 2 h 37°C in a CO$_2$ incubator. The cells were then digested with trypsin, washed three times with cold PBS (pH7.4) buffer, loaded onto the flow cytometer (FACS Aria II, BD, USA) to determine fluorescence intensity of DOX at excitation wavelength of 488 nm and detection wavelength of 560 nm. The number of cells used in each analysis was not less than 500000 and the number of cells sorted was 10000.

Fluorescence detection of drug intake

B16F10 cells at logarithmic growth phase were digested and centrifuged to prepare the single cell suspension. After cultured as for flow cytometry study, the cells were stained with DAPI and observed under the fluorescence microscope for red fluorescence from DOX.

Immunohistochemistry and microscopy

Tissues embedded in paraffin sections were dewaxed and hydrated, washed with PBS and stained with primary antibodies (Beyotime, Beijing). After rinsing with PBS, the slides were reacted with the secondary antibodies and developed in DAB solution. The slides were then counter-stained with hematoxylin and fixed in neutral gum. Micro vessel density (MVD) was determined under high magnification microscope in five randomly-selected fields. Tissue drug distribution was examined with aid of color of stained drug in five randomly-selected fields.

Tumor inhibition assays

0.1 mL B16F10 cells (10$^7$ cells/L) were inoculated in the right armpit of mice. Seven days after the inoculation when the tumors reached a volume of about 50 mm$^3$, the mice (n=6) were tail vein-injected with PBS (control), L-[D], L-[C], L-[C]+L-[D], L-[CD] and c[RGDyk]-L-[CD] on days 8, 10, 12, 14 and 16. The doses of CA-4 and DOX was 20 and 2 mg/kg, respectively. The dimensions of tumor and weight of mice were measured the day following the drug injection, and the volume was calculated as previously reported [6]. Two days after the last administration, the mice were sacrificed, weight-
ed and isolated for the tumor. The tumors were weighted and pictured.

Statistical analysis

Data were expressed as means ± s.d. and statistically analyzed using SPSS17.0 software. Difference was tested using t-test and value with \( P < 0.05 \) was considered statistically significant.

Results

Characterization of in vitro drug release

Measurements showed that about 50% and 70% of CA-4 encapsulated in L-[D] and c[RGDyk]-L-[CD] were released in 4 h and 12 h, respectively, while less than 30% of DOX was released in 48 h (Figure 1).

\( c[RGDyk]\)-L-[D] increased cellular DOX content

Flow cytometry showed that, compared with the L-[D], B16F10 cells had stronger DOX fluorescence intensity when incubated with c[RGDyk]-L-[D], indicating that there were more DOX uptake into the cells or bond to the cells (Figure 2A). Similarly, fluorescence assays showed that compared with unmodified liposomes, \( c[RGDyk]\)-modified liposomes generated stronger red fluorescence in the B16F10 cells (Figure 2B), indicating that more DOX was uptaken.

\( c[RGDyk]\)-L-[CD] had stronger tumor inhibitory and less toxic effect

Although our experimental results showed that all drug-loaded liposomes tested inhibited the
growth of tumor as compared with control (PBS) (Figure 4A), the inhibition was in order of c[RGDyk]-L-[CD]>L-[C]+L-[D]>L-[CD]>L-[D]=L-[C]>PBS, as measured with the volume of tumor at the end of treatment. Due to the growth of tumor, the weights of drug-treated mice were reduced after removing the tumors (Figure 4B). However, the weight reductions were less than the weight reduction of PBS-treated control mice. Among them, c[RGDyk]-L-[CD]-treated mice had the least weight reduction, suggesting that modification with the integrin ligand could not only improve anti-tumor activity, but also to reduce the toxicity of drugs (Figure 4B).

c[RGDyk]-L-[CD] enhanced anti-angiogenesis activity

We then investigated the MVD in tumor bearing mice after injected with DOX encapsulated in modified liposomes. The result showed that MVDs were 20.20 ± 2.86, 14.40 ± 2.97, 9.60 ± 0.55, 8.40 ± 0.55, 7.40 ± 0.55 and 4.60 ± 0.55 when the liposomes were modified with PBS, L-[D], L-[C], (L-[D]+L-[C]), L-[CD] and c[RGDyk]-L-[CD] 10 days after the treatments (Figure 5). Statistical analysis showed that the MVD in
c[RGDyk] and HCC

Discussion

Radiotherapy and chemotherapy are major options for cancer therapy in addition to surgical treatment. However, due to the lack of targeting and specificity to tumor, these treatments also have considerable damage to normal tissue under normal therapeutic doses [7, 8]. While HCC is being treated with a numbers of methods such as surgery, interventional therapy, radiotherapy and chemotherapy, the outcomes with drug treatment for HCC have not been desirable [4, 9].

Nano targeted drug delivery system uses nano drug loading systems to improve the selectivity of drug to the drug target with increased drug concentration in the target lesion [10, 11]. Tumor-targeting drug delivery systems have been shown to be able to deliver the drugs to target cells while reducing damage to normal cells, resulting in increased efficacy and reduced toxicity [9, 12, 13].

At present, in tumor cell-targeting drug delivery system, antigens or antibodies on tumor cell surface are targeted for drug delivery [14-16]. C[RGDyk]-modified liposome targeting drug delivery system is one of the most common targeting drug delivery systems [14]. DOX is one of the most common chemotherapy agents for liver cancer [15]. In this study, we found that the in vitro release of DOX was much slower longer than CA-4 (Figure 1). At the same time, when modified with c[RGDyk], the uptake of DOX was significantly increased by the cancer cells (Figure 2). These are similar to the results obtained in an early study [14]. Furthermore, encapsulation of drug in liposome has been shown to slow in vivo clearance of the encapsulated drug [14]. This is consistent with our results with CA-4 (Figure 3). However, the clearance of DOX was not affected as reported earlier [16].

The therapeutic efficacy studies showed that among the drugs tested, c[RGDyk]-L-[CD] was most effective in inhibiting tumor growth and had significantly reduced toxicity, a conclusion similar to what is observed previously [17, 18]. This might be due to greater anti-angiogenesis activity of c[RGDyk]-L-[CD] DOX (Figure 5).

Taken together, we demonstrate that c[RGDyk]-encapsulated DOX has better therapeutic efficacy and less toxicity, the effect is likely results from targeted delivery of the drug to cancer cells and tissues. More works are needed to further validate the results with human cancer and more drugs, and to elucidate the mechanism underlying the efficacy.

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Disclosure of conflict of interest

None.

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