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Original Article

RTA inhibits liver tumor growth in mice through microvesicles delivery

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Abstract: Ricin chain A (RTA) is toxic protein with significant clinical values in the treatment of various tumors. Current drug delivery methods were limited by their own shortcomings. Therefore, our study aimed to develop microvesicles that can effectively deliver RTA expression vector to liver cancer. In this study, ten BALB/c mice (20 ± 2 g) were were randomly divided into two groups including control group and RTA group, 10 mice in each. 5×10^4 Hep G2 cells were injected into mice in different groups. When tumor volume reached 500 mm^3, 100 μL solutions containing about 5×10^5 microvesicles were injected into each tumor. Tumor volume was checked every 3 days for another 18 days to monitor the growth of tumor. Mice were sacrificed and the tumors were collected to measure the weight and for total RNA extraction. polyethyleneimine-cholesterol cationic lipopolymer (PEI-Chol) was synthesized to serve as the backbone of the microvesicles, and AFP promoter was used to carry the coding region of RTA in the vector. Results show RTA was only expressed in AFP positive cells; RTA expression driven by AFP promoter could only kill AFP positive cell lines and RTA expression in tumor cells could inhibit tumor growth in vivo. We found that PEI-Chol microvesicles that carrying RTA expressing vector driven by AFP promoter can effectively and specifically inhibit AFP positive tumor growth.

Keywords: Ricin chain A, microvesicles, drug delivery, liver tumor

Introduction

Liver cancer is one of the most common cancer in digestive system, and has become the second leading cause of cancer related deaths worldwide [1-3]. Although various treatment including precision radiotherapy, intra-arterial hepatic therapies, systemic therapy have been developed to treat liver cancer, liver resection and liver transplantation are still the most effective methods [4]. As to patients who were diagnosed at advanced stage, above mentioned surgical operations are suitable, which requires the development of novel treatment methods with higher efficiency.

As a type 2 ribosome-inactivating protein, ricin is consisted of an A-chain and B-chain connected by disulfide bond [5]. A-chain contributes most to toxic effects by promoting cell death through the inhibition of protein synthesis, while the main task of B-chain is to facilitate the internalization of A-chain [5]. With the toxic effects, ricin toxin chain A (RTA) has shown promising therapeutic effects in the treatment of various cancers [6]. However, RTA itself cannot bind to specific cell types, and normal cell death can usually happen without accurate drug delivery. Therefore, the application of RTA in the treatment of human diseases is still challenged by the lack of efficient delivery method [7]. In the meantime, target therapy aims to deliver RTA to specific cell types has shown promising therapeutic effects, but its short half-life (10-30 minutes) and strong immunogenicity limited clinical application [8]. Polyetherimide can form complex with DNA and mediate transfection through endocytosis or membrane fusion, but the toxicity of polyetherimide may induce cell death [9], polyethyleneimine-cholesterol cationic lipopolymer (PEI-Chol) can reduce the toxicity of polyetherimide to increase the safety [10].

In this study, PEI-Chol was synthesized to serve as backbone to make microvesicles carrying

RTA expressing vector driven by AFP promoter. The effects of microvesicles on cell survival of multiple cells lines and tumor growth in mice were investigated.

Materials and methods

Preparation of microvesicles carrying RTA expression vector

A 1207 bp EcoRV-EcoRV DNA sequence containing AFP promoter and coding region of RTA [11] was synthesize by Genepharma (Shanghai, China). This fragment was inserted into eukaryotic expression vector pcDNA3. Polyethyleneimine-cholesterol cationic lipopolymer (PEI-Chol) was synthesized. PEI-Chol was mixed with 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), DSPE-PEG-Biotin, and 1% ween 80/Span 60. The sulfur hexafluoride was injected into the mixture to make microvesicles. The vectors expression RTA was then mixed with microvesicles to make vectors bind to PEI. FITC-labeled microvesicles were then checked under optical microscope and fluorescence microscope. Every procedure was approved by the Animal Care and Use Committee of the Fujian Medical University Union Hospital and was in conformity with the guidelines of National Institute of Health (No81004).

Cell lines, cell culture and transfection

Normal live tissue cell line AML12, AFP positive liver cancer cell lines Hep G2 and Hep 3B, and AFP negative liver cancer cell lines SK-HEP-1 and RBE were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured according to the instructions provided by ATCC. Cells were collected during logarithmic growth period for subsequent experiments. Transfection was performed by ultrasound-targeted microbubble disruption (UTMD) using the same method described in a previous study [12].

MTT assay

A total of 2×10^3 cells from each cell line were inoculated into each well of 96-well plate and incubated in an incubator (37°C, 5% CO_2) to reach 80-90% confluent. Then microvesicles were added and the density was adjusted to 1×10^4, 1×10^5, 1×10^6, 5×10^6, 1×10^7, 2×10^7, 5×10^7 and 1×10^8 per micro liter. After transfection using UTMD, cells were incubator in a incubator (37°C, 5% CO_2) for another 96 h, then 20 μL MTT solution (5 mg/mL, Sigma, USA) was added, followed by incubation for another 4 h. Supernatant was then removed and 100 μL of DMSO (Sigma, USA) was added. OD values at 490 nm were measured using a microplate reader.

Semi-quantitative PCR

Total RNA was extracted from in vitro cultured cells and tumor tissue using Trizol reagent (Invitrogen, USA). All RNA samples were tested by NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, USA), and only the ones showed a A260/A280 ratio between 1.8 and 2.0 were used in reverse transcription to synthesize cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, USA). Taq DNA polymerase (NEB, USA) was used to prepare PCR reactions. The following primers were used in PCR reaction: 5'-ACCAATGCATATGTGGTCGG-3' (sense) and 5'-GTTGGAAGCTGAGTGCCACC-3' (antisence) for RTA; 5'-CCGCATCTTCTTTTGCGTCGC-3' (sense) and 5'-CCATGTAGTTGAGGTCAATG-3'-(antisence) for GAPDH. PCR products were subjected to agarose gel electrophoresis, and the gels were checked using Gel Doc™ XR+ Imager (Bio-Rad, USA).

Mice transplantation

Ten BALB/c mice (20 ± 2 g) were provided by Guangdong Medical Laboratory Animal Center (Guangdong, China). All animal experiments have been approved by the Ethics Committee of Fujian Medical University Union Hospital. Mice were randomly divided into 2 groups including control group and RTA group, 10 mice in each. 5×10^4 Hep G2 cells were resuspended in 100 μL of matrigel/PBS mixture and were injected into mice in different groups. Tumor volume was measured daily using the following formula: volume (mm^3) = (length × $width^2$)/2. When tumor volume reached 500 mm^3, 100 μL solutions containing about 5×10^5 microvesicles were injected into each tumor. Tumor volume was checked every 3 days for another 18 days to monitor the growth of tumor. Mice were sacrificed and the tumors were collected to measure the weight and for total RNA extraction. Statistical analysis SPSS19.0 (SPSS Inc., USA) was used. All data were recorded by mean (SD)

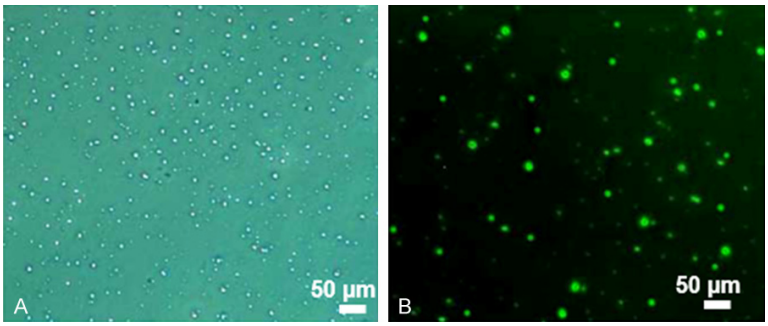

Figure 1. Observation of microvesicles. A. Observation under optical microscope; B. Observation under fluorescence microscope. Microvesicles were uniformly distributed and average diameter was (3.0 ± 0.4) μm. The diameter of microvesicles mostly ranged from 2 to 7 μm. Green fluorescence can be observed under fluorescence microscope.

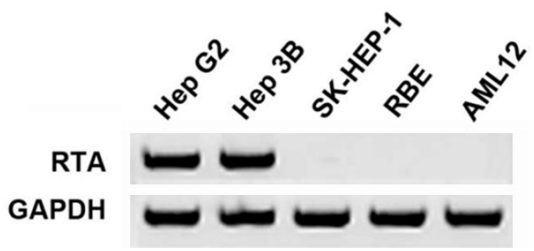

Figure 2. Expression of RTA in different cell lines after transfection. APDH was detected in all five cell lines; RTA was only detected in Hep G2 and Hep 3B but not in SK-HEP-1, RBE and AML12, indicating that RTA was only expressed in AFP positive cells.

and comparisons between two groups were performed by *t* test. $p<0.05$ was considered to be statistically significant.

Results

Basic characteristics of microvesicles

Microvesicles carrying vectors expressing RTA were prepared. The appearance of microvesicles showed milky white, and the density was about $(5.2 \pm 0.1)\times10^8$/mL. Observation under optical microscope showed that microvesicles were uniformly distributed and average diameter was (3.0 ± 0.4) μm. The diameter of more than 82.4% of microvesicles ranged from 2 to 7 μm. Green fluorescence can be observed under fluorescence microscope (**Figure 1A**, **1B**).

Expression of RTA in different cell lines after transfection

Two AFP positive liver cancer cell lines Hep G2 and Hep 3B, AFP negative liver cancer cell lines SK-HEP-1 and RBE, and normal live tissue cell line AML12 were used in this study. Expression of RTA was detected by semi-quantitative PCR. As shown in **Figure 2**, GAPDH was detected in all five cell lines, indicating the success of PCR reaction. However, RTA was only detected in Hep G2 and Hep 3B but not in SK-HEP-1, RBE and AML12, indicating that RTA was only expressed in AFP positive cells.

Effects of different densities of microvesicles on survival of different liver cancer cell lines

Different densities of microvesicles were used to treatment different liver cell lines and the survival of each cell line was detected by MTT assay. As shown in **Figure 3A** and **3B**, the densities below 1×10^6/ml showed no significant effects on cell survival of Hep G2 and Hep 3B, and a density of 1×10^7/ml killed about 80% of the cells, and further increases in density of microvesicles showed no significant effects in reducing cell survival rate. Microvesicles carrying empty vectors (control) group showed no significant effects on cell survival. Besides that, microvesicles carrying RTA expressing vectors showed no significant effects on the survival of SK-HEP-1 and RBE (**Figure 3C** and **3D**). Those data suggest that RTA expression driven by AFP promoter can only kill AFP positive cell lines.

Effect of microvesicles on tumor growth in mice

Hep G2 cells were inoculated into the subcutaneous tissue of mice. Microvesicles carrying RTA expression vectors were injected into each tumor when tumor volume reached 500 mm. Bases on the results of cell survival assay, a density of about 5×10^6/ml is enough to kill about half of the cells. Therefore, the density of microvesicles was set at 5×10^6/ml in this experiment. As showed in **Figure 4A**, the volume of tumor decreased gradually in RTA group (microvesicles carrying RTA expression vectors), while the volume of tumor increased gradually in control group (microvesicles carrying empty vectors), significant differences in tumor

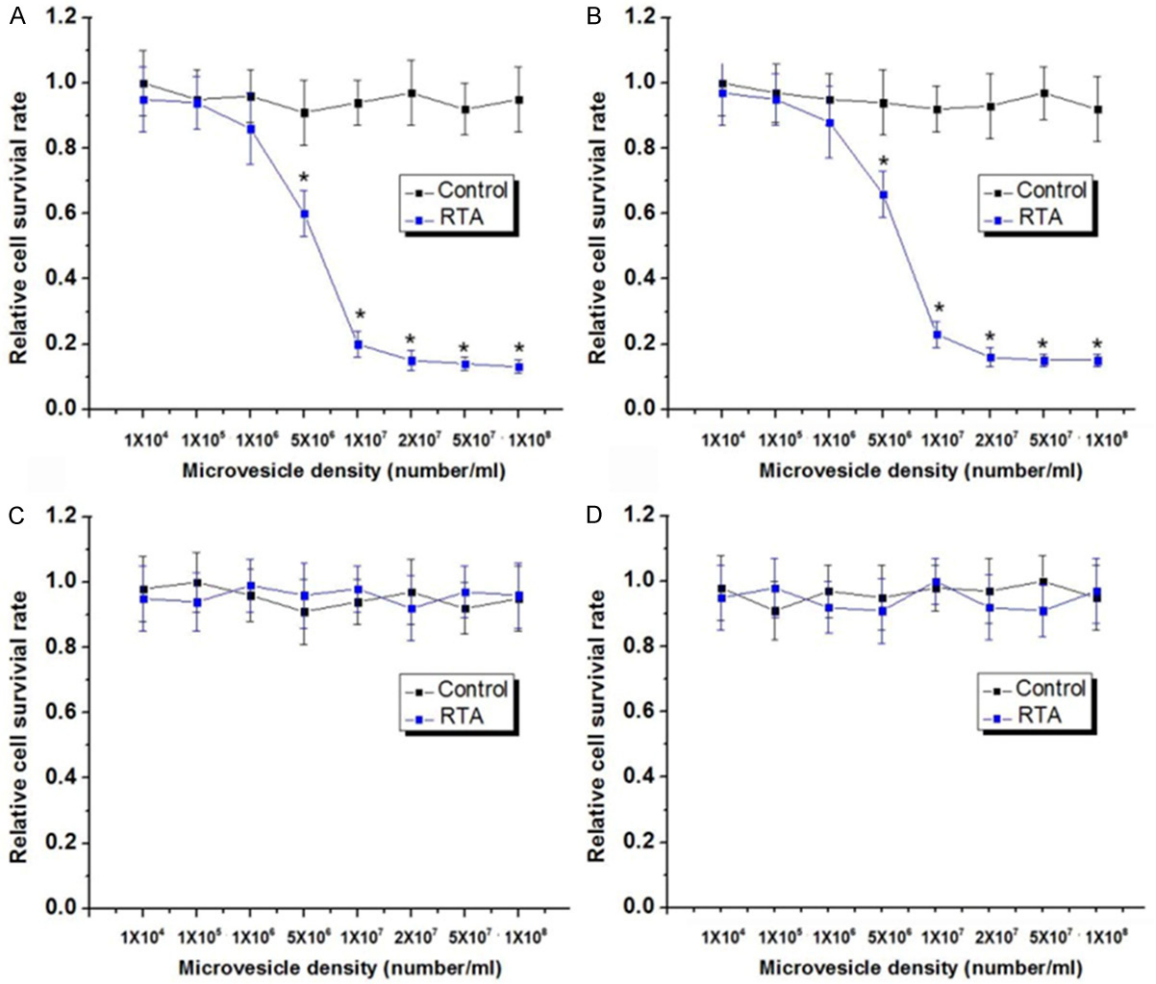

Figure 3. Effects of different densities of microvesicles on survival of four liver cancer cell lines. A. Effects of different densities of microvesicles on survival of Hep G2; B. Effects of different densities of microvesicles on survival of Hep 3B; C. Effects of different densities of microvesicles on survival of SK-HEP-1; D. Effects of different densities of microvesicles on survival of RBE. Notes: *Compared with control group, $p<0.05$.

volume were found between two groups at different time points ($p<0.05$). As shown in **Figure 4B** and **4C**, tumor weight was also significantly lower in RTA group than in control group ($p<0.05$). As shown in **Figure 5A** and **5B**, expression of RTA was only detected in RTA group but not in control group. Those data suggest that RTA expression in tumor cells can inhibit tumor growth in vivo.

Discussion

With the strong cytotoxicity, ricin has shown promising therapeutic effects in the treatment of various cancers [13-15]. In spite of the high efficiency of killing tumor cells, the application of ricin in the treatment of cancer is still limited by the lack of accurate and effective delivery method [6].

Alpha-Fetoprotein, which is also called AFP, is a protein mainly produced by yolk sac and the liver tissue in developing fetus [16]. Level of AFP is usually low in adults, but the production of AFP is increased in about two-thirds of liver cancer. Therefore, gene expression driven by AFP promoter provided a way to express certain proteins with therapeutic effects in liver cancer cells, gave insights about how to delivery ricin in proper way. In a recent study, Cre/LoxP-switched RNA interference driven by AFP promoter has been proved to be able to specifically act on liver cancer tissue [17]. In our study, RTA driven by AFP promoter was found to be only expressed in AFP positive liver cancer cell lines Hep G2 and Hep 3B, but not in AFP negative liver cancer cell lines SK-HEP-1 and RBE, and normal live tissue cell line AML12. Those data suggest that the drug delivery strategy devel-

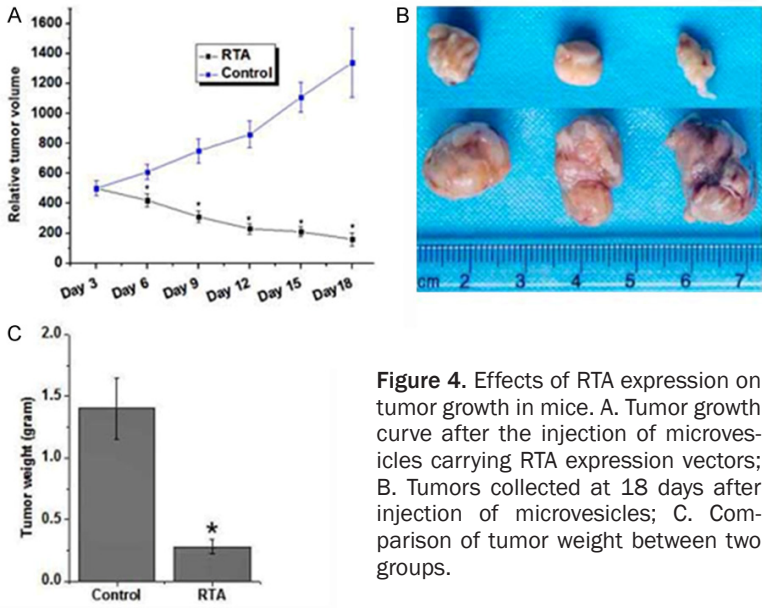

Figure 4. Effects of RTA expression on tumor growth in mice. A. Tumor growth curve after the injection of microvesicles carrying RTA expression vectors; B. Tumors collected at 18 days after injection of microvesicles; C. Comparison of tumor weight between two groups.

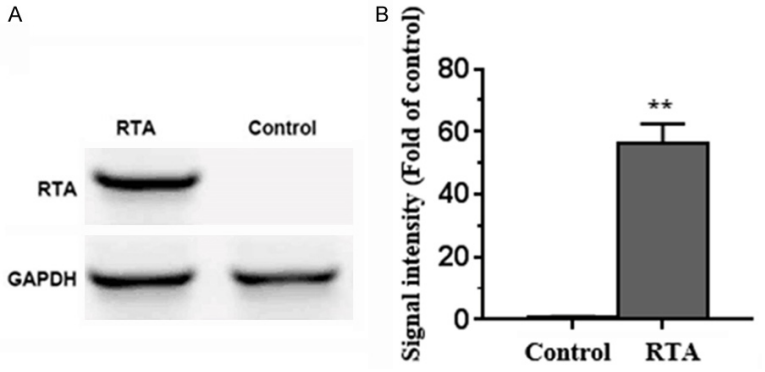

Figure 5. RTA expression in tumor. A. expression of RTA in tumor of two groups mice. B. Quantitation of signal intensities from Western blots. Notes: **Compared with control group, $p<0.01$.

oped in this study can specifically target positive liver cancer, which in turn increased specificity. Due to the high cytotoxicity for the application of polyetherimide in drug delivery [18], PEI-Chol could be used to reduce the toxicity of polyetherimide [19]. It is proved in our study that different densities of PEI-Chol microvesicles carrying empty vectors showed no significant effects on cell survival, indicating the high safety of PEI-Chol. In contrast, treatment with PEI-Chol microvesicles carrying RTA expressing vectors significantly reduced the survival rate of AFP positive liver cancer cell lines Hep G2 and Hep 3B, but AFP negative liver cancer cell lines SK-HEP-1 and RBE. Those results suggest that RTA expression driven by AFP promoter can specifically kill AFP positive liver tumors without bring significant effects on normal liver cells and AFP negative liver cancer cells, indicating the high safety.

Numerous in vitro studies have proved the high efficacy of ricin in killing tumor cells [20, 21]. In this in vivo study, microvesicles carrying RTA expressing vectors were injected into tumors in mice to investigate the effects of RTA expression on the growth of tumor. Results showed that RTA expression reversed to growth of tumor. Those results provided basis and references for further clinical studies to test the application of ricin in the treatment of human liver cancer.

In conclusion, microvesicles carrying RTA expressing vectors driven by AFP promoter were constructed. Transfection mediated by the microvesicles can achieve high efficient transfection and specific expression of RTA in AFP positive cells. RTA expression significantly reduced cell survival and reversed tumor growth in mice. Our study is limited by the small sample size. Future studies with bigger sample size are needed to further confirm our conclusions.

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Disclosure of conflict of interest

None.

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