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Development, characterization and anti-tumor effect of a sequential sustained-release preparation containing ricin and Cobra venom cytotoxin

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Cobra venom cytotoxin (CVC) loaded in poly (lactide-co-glycolide) (PLGA) microspheres was mixed with ricin and encapsulated in a thermosensitive PLGA-PEG-PLGA hydrogel for this study. This sequential sustained-release preparation (SSRP) containing ricin and CVC could avoid burst release effect of CVC from microspheres. In addition, in SSRP, the two biotoxins have different drug release rates and antitumor mechanisms, which can be complementary to each other. Ricin has a faster release rate than CVC. It can combine with the tumor cell membrane and enter the cell, inhibiting protein synthesis within 2 weeks. Whereas CVC releases slowly in 5 weeks directly dissolving the tumor cell membrane and killing the cells which are less-sensitive to ricin. The *in vivo* experiments showed that intratumoral injection of SSRP could inhibit hepatocellular carcinoma growth significantly, and the tumor growth inhibition rate reached 73.5%. It appears that a new medicine preparation for cancer local treatment should be further studied for clinical applications.

1. Introduction

Clinical chemotherapy for cancers is applied mostly by using systemic administration. Because of their poor selectivity, chemotherapeutics used in systemic administration often lead to serious side effects. In addition, systemic administration can only provide a relatively low drug concentration to the target tumor, making complete removal of the tumor cells difficult. In recent years, sustained-released preparations with polymeric materials, such as liposomes, microspheres and hydrogels, have been studied and made available (Ahmad et al. 2006; Jain et al. 2007; Ghahremankhani et al. 2008). These new drug carriers cannot only improve the stability of the enclosed drugs and maintain a steady drug release, but also help more thoroughly kill tumor cells, as well as reduce the incidence of systemic side effects, by means of the tumor local delivery (Chen et al. 2010). Both ricin and CVC are biotoxins with potent cytotoxicity (Sandvig et al. 2000; Levtsova et al. 2009; Su and Wang 2011). They were used to prepare a new agent by encapsulating CVC in PLGA microspheres, followed by mixing with ricin and loading in a PLGA-PEG-PLGA hydrogel. Due to their different release rates, after an intratumoral injection, ricin and CVC could sequentially release to the target tumors maintaining a desired drug concentration for a complete tumor kill with much reduced systemic side effects.

2. Investigations, results and discussion

2.1. In vitro anti-tumor effect of purified biotoxins

After incubating $HepG_2$ with ricin for 8 h, the majority of the cells began to shrink, become round in shape, and shed eventually. At prolonged incubation, the cells became necrotic and most cells treated with high-doses died after 72 h. CCV killed $HepG_2$ mainly through dissolving the cell membrane. After 8 h incubation, $HepG_2$ occured cytolysis and the cell structure disappeared in the high-dosage group.

After 72 h treatment on the $HepG_2$, the 50% inhibiting concentration (IC_{50}) of ricin and CVC were 5.7×10^{-9} mol/L and 1.6×10^{-7} mol/L, respectively. Combination of ricin and CVC at the concentration of their IC_{50} against $HepG_2$ cells for 72 h *in vitro* indicated a synergistic anticancer effect and achieved an inhibition rate of 78.3%.

2.2. Characterization of CCV microspheres

At present, PLGA is commonly used for the preparation of sustained-release microspheres. It has many advantages, such as non-toxicity, good biocompatibility, biodegradability, relatively long degradation period, etc. In addition, microspheres of varying degradation rates can be prepared by adjusting the relative molecular weight and/or the LA/GA ratio (Nojehdehian et al. 2009; Yang et al. 2009; Zhu et al. 2009).

Under a scanning electron microscope, the prepared CVC microspheres (CVCM) appear to be uniformly round in shape with smooth surfaces, and separate from one another without coalescing or aggregation (Fig. 1). The average size was 3.7 μm, ranging from 2.1 to 6.8 μm, which is considered a normal distribution. The encapsulation efficiency and drug loading rate were determined to be 78.22 ± 8.46 % and 0.79 ± 0.10 %, respectively.

2.3. Phase transition temperature of PLGA-PEG-PLGA copolymer

The PLGA-PEG-PLGA hydrogel can be used for injection and is biodegradable. Below its phase transition temperature, the PLGA-PEG-PLGA aqueous solution had low viscosity and

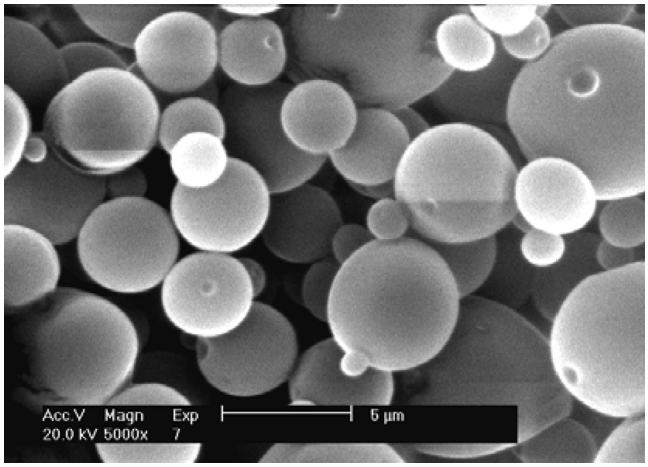

Fig. 1: Scanning electron microscopy of CVC-loaded PLGA microspheres: uniform and round in shape with smooth surfaces and no coalescing (× 5000)

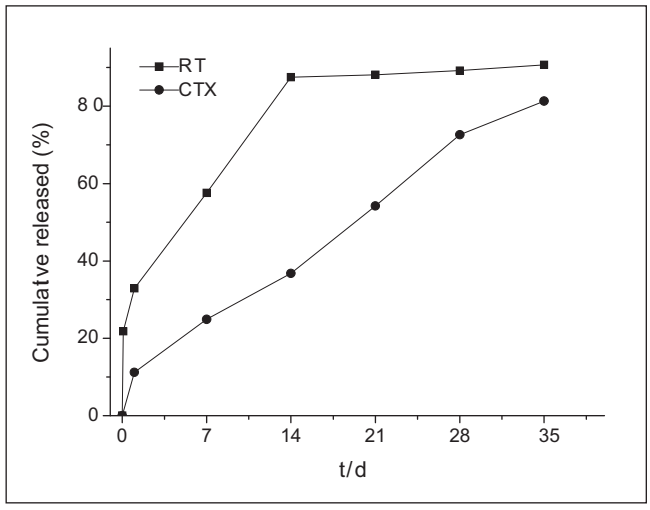

Fig. 2: *In vitro* release of SSRP: ricin enclosed in the gel coating was released first. The release was basically completed at the 14th day. The CVC in the microspheres inside the gel was released slowly and steadily in 5 weeks

could be sterilized by filtration with a 0.22 µm Millipore filter membrane. A variety of drugs can be carried by the copolymers simply by mixing the two ingredients. In addition, a relatively high drug-carrying rate can be obtained without the use of organic solvents, which is beneficial as far as preservation of the drug activity is concerned. After injecting the PLGA-PEG-PLGA aqueous solution into the body, the increased temperature will turn the solution into gel form rendering a desirable state for sustained drug release (Jeong et al. 1997; Zentner et al. 2001; Yu et al. 2011).

The molecular weight of polyethylene glycol (PEG) is the main factor that governs the copolymer's phase transition temperature. In this study, the copolymers were prepared with the same mass ratio of 1,000 and 1,500 molecular weight PEGs. When the concentration of copolymer solution was between 15% and 35%, it was reversely affected by heat - being a fluid when the temperature was below the gelling point, and a solid gel when the temperature was higher. If the temperature was further increased gradually, precipitation occurred. When the solution's concentration was 23%, the sol-gel phase transition temperature of the solution was 28 °C, which was suitable for injection applications.

2.4. In vitro release of SSRP

The *in vitro* drug release test for the ricin-and-CVCM-carrying SSRP preparation was also performed in a phosphate buffer solution. In 2 h, 21.8% of ricin released, and 32.9% within the first day of the test. In the subsequent 14 days, the drug release was steady and gradual with an accumulated release of 87.5%. The initial CVC release was relatively slow, accounting for 11.2% in the first day. A week later, it gradually increased for the following 5 weeks (Fig. 2). The double-encapsulation significantly reduced the burst release effect of CVC allowing a desirable drug release of the biotoxins.

2.5. Inhibition rate of tumor growth

Ricin and CVC have different cytotoxic mechanisms. The former is effective mainly through the inhibition of protein synthesis, while the latter through the destruction of tumor cell membrane. Therefore, combining the two biotoxins could conceivably complement each other in cancer therapy, similar to the clinical practice of the combined use of penicillin and streptomycin. Combined drugs can achieve a more thorough germicidal effect and less likelihood for the development of drug resistant strains. In SSRP, ricin and CVC were encapsulated in different carriers and had different release rates. Ricin was contained in the outer gel coating and released firstly upon injection. The released ricin could quickly enter the tumor cells through the cell membrane and kill most of the cells. On the other hand, CVC was enclosed in the microspheres, which were wrapped with ricin in the outer gel. Potential burst release effect of the drugs was thus avoided, and a stable and long-lasting drug release was ensured for a thorough tumor kill.

After administration, the tumors on the nude mice in model group and blank carrier group continued to grow. Comparing to model group, ricin-gel group and CCVM group had slower tumor weight increases, while SSRP group indicated a stronger anti-tumor effect with the smallest tumor weight among all treatment groups ($P < 0.01$). The tumor growth inhibition rate calculated at 28 days after the administration was 73.5% for SSRP group, which was significantly higher than those of ricin-gel group and CCVM group (Table).

After the local injections of the various drugs, the nude mice in all treatment groups were generally in good condition without any apparent toxic symptoms. The ricin dosage applied in this study was about 1/6 and CVC about 1/4 of the medial lethal dose for the mice. Moreover, since the two biotoxins were enclosed in the sustained-release encapsulations and injected directly into the target tumor, the total result should significantly reduce the systemic side effects.

3. Experimental

3.1. Chemicals

PEG (Mr 1,000 and 1,500) was purchased from Shanghai Chemical Reagent Co., Ltd., Sinopharm Group, Shanghai, China. PLGA (Mr 10,000, LA/GA = 50/50) and polyvinyl alcohol (PVA) were purchased from Chengdu Organic Chemistry Co., Ltd. of the Chinese Academy of Sciences,

Table: *In vivo* antitumor effect of SSRP ($\bar{x} \pm s$)

Group	Mean tumor weight (g)	IR (%)
Model	4.8±1.0*	–
Blank carrier control	4.7±0.9*	2.1
Ricin-gel	2.5±0.5*	47.2
CCVM	2.9±0.6*	39.3
SSRP	1.3±0.3	73.5

* $P < 0.01$, *vs* SSRP group

Chengdu, China. DL-lactide and glycolide were purchased from Beijing Yuanshengrong Technology Co., Ltd., Beijing, China. Stannous octoate, D-galactose and methyl thiazolyl tetrazolium (MTT) were purchased from the Sigma Company, USA. Ricin polyclonal antibody was purchased from Abcam Inc., England.

3.2. Cell culture

The human hepatocellular carcinoma cell line $HepG_2$ was purchased from Shanghai Cell Bank of the Chinese Academy of Science, Shanghai, China. The cells were conventionally sub-cultured in RPMI-1640 culture medium (containing 10% fetal bovine serum) at 37 °C under 5% CO_2 and saturated humidity conditions.

3.3. Animals

The male SPF grade, BALB/C nude mice, which were 4–5 weeks old with a body weight ranging from 17 to 18 g, were purchased from Shanghai Slack Experimental Animal Co., Ltd., Shanghai, China. The mice were kept in a designated pathogen-free animal room and allowed to feed and drink freely. Handling of the animals was conducted strictly in accordance with the guidelines for animal experiments stipulated by Fujian Medical University, Fuzhou, Fujian, China.

3.4. Toxins purification

Ricin was purified as previously reported (Nicolson and Blaustein 1972). Briefly, castor beans were used to extract the protein with phosphate buffer solution after fat removal. After salting out the protein extract with saturated ammonium sulfate, solution was placed in the Sepharose-4B gel column for affinity chromatography. The eluted protein solution was purified using a Sephacryl S-100 column and collected ricin. The purity of the protein determined by HPLC was 95.3%.
Cobra venom was purified on a Sephadex G-100 column followed by CM-Sepharose FF, collected, and added to a Sephasil peptide C18 column for further purification (Joubert and Taljaard 1978). The peak protein was collected as CVC, which showed a purity of 97.2%.

3.5. Determination of cytotoxic effect in vitro

$HepG_2$ cells were inoculated on a 96-well plate, and drug-containing medium was added to the wells. After 72 h of drug treatment, 5 $mg \cdot mL^{-1}$ MTT were added 20 μL to each well and incubation was continued for 4 h. A microplate reader was used to determine light absorption of the samples at 570 nm. Cell growth inhibition rate, and IC_{50} were determined.

3.6. Preparation and characterization of CVCM

CVCM were prepared with a double emulsion-solvent evaporation method as follows (Karal-Yilmaz et al. 2011; Yang et al. 2011). CVC aqueous solution 0.2 mL 5 $mg \cdot mL^{-1}$ was placed in 1 mL PLGA methylene chloride solution (100 $mg \cdot mL^{-1}$) and homogenized at 10,000 rpm for 1 min. The initial emulsion was quickly infused into 2 mL 3% PVA solution followed by homogenization at 10, 000 rpm for 2 min. Deionized water (30 mL) was added and stirred at 400 rpm for 4 h to completely evaporate dichloromethane prior to drying in a freeze-dryer.
CVCM were spread on a double-sided tape and coated with gold by an ion sputtering instrument. The superficial morphology and particle size of the spheres were observed using a scanning electron microscopy. The drug loading rate and encapsulation efficiency were determined by HPLC. Actual drug loading = drug (enc.)/[drug (tot.) + polymer]. Theoretical drug loading = drug (tot.)/[drug (tot.) + polymer]. Encapsulation efficiency (%) = (actual drug loading/theoretical drug loading) × 100%.

3.7. Synthesis and in vitro drug release of PLGA-PEG-PLGA hydrogel

PEG 1000 and PEG 1500 (3 g each) were dried for 3 h at 90 °C in a vacuum dryer followed by further drying for 30 min after addition of 11.6 g DL-lactide and 3 g glycolide. Stannous octoate (50 mg was added as catalyst) and allowed to react under 150 °C nitrogen protection for 8 h. The product was dissolved in 4 °C water, and heated to 80 °C and precipitate was dried to a constant weight in a freeze dryer. The sol–gel phase-transition temperature was determined using the turnover tube method (Qiao et al. 2008).
23% PLGA-PEG-PLGA aqueous solution (1 mL) was placed in a 5 mL round-bottom centrifuge tube, ricin and CVCM were added, and mixed well before transferring the mixture to a 37 °C constant temperature oscillation incubator. After the gel was formed, added 2 mL phosphate buffer solution and the solution was sampled at each time point for CVC content determination by BCA protein assay as well as enzyme-linked immunosorbent assay for ricin content. Data were used to construct a release curve.

3.8. Model of hepatocellular carcinoma xerograft

The amplified $HepG_2$ cells were applied to make a saline solution with a cell density of $1 \times 10^7 \cdot mL^{-1}$ for a 0.2 mL injection on the right armpit of each nude mouse. Growth of the subcutaneously transplanted tumor on the nude mice was regularly monitored.

3.9. Animal grouping and drug administration

Three weeks after the subcutaneous inoculation of the tumor cells, 25 nude mice that had tumors with diameters between 1.5 cm and 2.0 cm were randomly divided into five groups of 5 each. To the mice in different treatment groups, various drugs, were intratumorally injected as listed below, once for each mouse. The injection was done in a manner to ensure an even drug distribution throughout the tumor as much as possible.
Group A: The model group.
Group B: The blank carrier control, in which the mice were intratumorally injected with 0.5 mL 23% thermosensitive hydrogel carrying blank microspheres.
Group C: The ricin-gel group, in which the mice were intratumorally injected with 0.5 mL ricin-in-hydrogel at the ricin dosage of 5 μg/kg.
Group D: The CVCM group, in which the mice were intratumorally injected with the CVCM in 0.5 mL physiological saline at the CVC dosage of 0.5 mg/kg.
Group E: The SSRP group, in which the mice were intratumorally injected with 0.5 mL SSRP at the CVC dosage of 0.5 mg/kg and ricin of 5 μg/kg.

3.10. Inhibition rate of tumor growth

Twenty eight days post-administration, the mice were sacrificed and the tumors were removed and weighed to calculate the tumor growth inhibition rate according to the formula: IR (%) = (1-mean tumor mass of treatment group/mean tumor mass of model group) × 100%.

3.11. Statistical analysis

Presented data as mean ± standard deviation using SPSS 16.0 statistical software package, carried out single-factor analysis of variance, and set $P < 0.01$ to indicate significant difference

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