research article

The therapeutic effect of ultrasound targeted destruction of schisandrin A contrast microbubbles on liver cancer and its mechanism

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Background. The aim of the study was to explore the therapeutic effect of ultrasound targeted destruction of schisandrin A contrast microbubbles on liver cancer and its related mechanism.

Materials and methods. The Span-PEG microbubbles loaded with schisandrin A were prepared using Span60, NaCl, PEG-1500, and schisandrin A. The loading rate of schisandrin A in Span-PEG composite microbubbles was determined by ultraviolet spectrophotometry method. The Walker-256 cell survival rate of schisandrin A was determined by 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di- phenytetrazoliumromide (MTT) assay. The content of schisandrin A in the cells was determined by high performance liquid chromatography. Ultrasound imaging was used to evaluate the therapeutic effect *in situ*. Enzyme linked immunosorbent assay (ELISA) was used to measure the content of inflammatory factors in serum. Hematoxylin-eosin (HE) staining was used to observe the pathological changes of experimental animals in each group. Immunohistochemistry was used to detect the expression of hypoxia inducible factor-1a (HIF-1a), vascular endothlial growth factor (VEGF) and vascular endothelial growth factor receptor 2 (VEGFR-2) in tumor tissues, and western blot was used to detect the protein expression of phosphoinositide 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway in tumor tissues.

Results. The composite microbubbles were uniform in size, and the particle size distribution was unimodal and stable, which met the requirements of ultrasound contrast agents. The loading rate of schisandrin A in Span-PEG microbubbles was 8.84 ± 0.14%, the encapsulation efficiency was 82.24±1.21%. The IC50 value of schisandrin A was 2.87 μg/mL. The drug + microbubbles + ultrasound (D+M+U) group had the most obvious inhibitory effect on Walker-256 cancer cells, the highest intracellular drug concentration, the largest reduction in tumor volume, the most obvious reduction in serum inflammatory factors, and the most obvious improvement in pathological results. The results of immunohistochemistry showed that HIF-1a, VEGF and VEGFR-2 protein decreased most significantly in D+M+U group ($P < 0.01$). WB results showed that D+M+U group inhibited the PI3K/AKT/mTOR signaling pathway most significantly ($P < 0.01$).

Conclusions. Schisandrin A had an anti-tumor effect, and its mechanism might be related to the inhibition of the PI3K/AKT/mTOR signaling pathway. The schisandrin A microbubbles could promote the intake of schisandrin A in tumor cells after being destroyed at the site of tumor under ultrasound irradiation, thus playing the best anti-tumor effect.

Key words: ultrasonic targeted destruction; schisandrin A contrast microbubbles; liver cancer; mechanism of action

Introduction

Ultrasound contrast agent microbubbles were microparticles used to enhance the contrast of ultrasound images.[1] The latest generation of ultrasound contrast agents had started to use microbubbles to carry drugs or genes, which could improve the effect of ultrasound imaging and achieve the purpose of treating diseases.[2]

Liver cancer is a common malignant tumor of the digestive system and the second most common human malignant tumor after lung cancer.[3] At present, although the research on liver cancer had made a great breakthrough, it was still an important risk factor that seriously affected the life and quality of life of patients. At present, the treatment of liver cancer was mainly based on the stage and the age of the patient. The commonly used treatment methods include surgery, radiotherapy, chemotherapy, vaccines, and so on.[4] In the chemotherapy of liver cancer, cisplatin-based combination chemotherapy was commonly used in clinical practice.[5] Liver tumors were highly sensitive to platinum drugs and had good curative effect. However, the side effects and drug resistance of platinum drugs were also important factors affecting the application of platinum drugs.[6] Despite the continuous improvement of surgery, radiotherapy and technology, and the emergence of chemotherapy drugs, the survival time and quality of patients had not been fundamentally improved.

Ultrasound-targeted microbubble destruction (UTMD) was a technology that improved the efficacy of targeted drugs by increasing the absorption of targeted drugs into cells.[7] This technology mainly used microbubbles to localize "explosive" ultrasound irradiation and released the drugs they carried.[8] At the same time, the shock caused by ultrasound and microbubble rupture increased the local cell permeability, generates reversible sonopores, and promotes drug entry into the nucleus, which could improve the efficiency of drug intervention in tumor cells.[9] Secondly, the protection of microbubbles could prevent the drug from being metabolized and degraded by the body, thereby reducing the bioavailability of the drug, so that it could reach the target organ or tissue directly through the blood circulation.[10] Many preclinical studies and few clinical studies reported the use of microbubble-assisted ultrasound for the delivery of wide range of therapeutics into primary liver tumors or liver mets.[11] Schisandrin A is a bioactive lignan isolated from the traditional Chinese medicine *Fructus schisandrae chinensis*. Studies showed that schisandrin A had many pharmacological effects, such as anticancer, hepatoprotection, antiinflammation, which was worthy of further research and development in the future.[12] Our previous study found that Schisandrisin A significantly reduced the inflammation level of HepG2 cells; improved the oxidative stress state; downregulated transforming growth factor beta 1 (TGF-β1), vascular endothlial growth factor (VEGF), phosphoinositide 3-kinase (PI3K), and Akt mRNA levels; inhibited the expression of the PI3K-Akt signaling pathway, and had a significant anti-tumor effect on tumor cells with high activity and small molecular weight, which was an ideal candidate for the production of contrast-enhanced ultrasound microbubbles.[13] Therefore, in this study, Schisandin A was loaded into Span-PEG microbubbles to make ultrasound contrast agent and to play an anti-tumor effect on the lesions of liver cancer, which was rarely reported. This study would provide a new reference for the treatment of liver cancer.

Materials and methods

Instrumentation

Ultrasonic cell crushing instrument (Ningbo Xinzhi Biological Technology Co., LTD.), Doppler ultrasound diagnostic instrument (Kunshan Ultrasonic Instrument Co., LTD.), Zeta potential/particle size instrument (British Malvern Instrument Co., LTD.), SW-CJ-1D single side vertical air supply purification table (Suzhou Zhijing Purification Equipment Co., LTD., Jiangsu Province), HZQA Constant temperature incubator (Jintan Shenglan Instrument Manufacturing Co., LTD.), LX-C50L vertical automatic electric heating pressure steam sterilizer (Beijing Sibo Shengda Technology Co., LTD.), scanning electron microscope (Japan Electronics Co., LTD.), Ultrasound imaging instrument (mindray M9cv, Superficial probe), Bio-Rad 680 iMark Microplate reader(American Bio-Rad Co., LTD.)

Reagents

Span60 (Tianjin BASF Chemical Co., LTD.), PEG1500 (Tianjin Guangfu Fine Chemical Research Institute), NaCl (Tianjin Beilian Fine Chemical Development Co., LTD.), schisandrin A (Chengdu Manst Biotechnology Co., LTD. HPLC ⩾ 98%), phenol and sulfuric acid (Tianjin Kemio Chemical Reagent Co., LTD.), Walker-256 (number: 399-88-2) was obtained from Shanghai Hongshun

Biotechnology Co., LTD. MEM (containing NEAA) basal medium (Procell PM150410) and fetal bovine serum (Procell 164210) was obtained from Pricella Biotechnology Co., LTD. Fixative solution (4% Paraformaldehyde, P1110) was obtained from Shanghai solarbio Bioscience & TechnologyCo., LTD. Enzyme linked immunosorbent assay (ELISA) kit tumor necrosis factor-α (TNF-α) (ab236712), interleukin-1β (IL-1β) (ab255730) and interleukin-6 (IL-6) (ab234570) was obtained from abcam Bioscience & TechnologyCo.,LTD. Hematoxylin-eosin (HE) Stain Kit (G1120) was obtained from Solarbio Bioscience & TechnologyCo.,LTD. Immunohistochemistry kit hypoxia inducible factor-1α (HIF-1α) (IHC0103715), VEGF(IHC0100011) and vascular endothelial growth factor receptor 2 (VEGFR-2) (IHC0102817) was obtained from Shanghai CaiYOU industrial Co., LTD. WB kit: Primary antibody p-PI3K (bs-6417R), PI3K(20584-1-AP), p-Akt (bs-0876R) was obtained from Bioss Co., LTD, AKT (60203-2-Ig), p- mammalian target of rapamycin (mTOR) (67778-1-Ig), mTOR (66888-1-Ig), was obtained from Proteintech Group, GAPDH was obtained from Hangzhou Hua 'an Biotechnology Co. LTD, Secondary antibody (SA00001-1) was obtained from Bioss Co., LTD.

Experimental cells

Walker-256 cell (Free of mycoplasma infection, cells were derived from ascites of liver cancer in rats) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and incubated at 37°C in 5%CO_2 incubator. The cells were routinely digested and subcultured with 2.5 g/L trypsin, and the logarithmic growth phase cells were used for experiments.

Experimental animals

36 SD (Sprague Dawley, male, 6 weeks, 180–200 g, wide type rats) rats were obtained from Henan Laboratory Animal Center. Animal experiment ethics was approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University (No. KY2023-006).

Preparation and analytical characterization of Span-PEG microbubbles loaded with schisandrin A

450 mg Span60, 900 mg NaCl, 450 mg PEG-1500 and 300 mg schisandrin A were weighed, placed in a mortar and thoroughly ground, dissolved in 40 mL PBS phosphate buffer solution, and heated to 80°C in a magnetic heating mixer, and stirred and dispersed evenly. Then, the solution was continuously sonicated at 570 W power for 6 min using an ultrasonic cell disruptor by acoustic cavitation method, while nitrogen gas was continuously injected into the above solution. A uniform milky yellow liquid mixture was prepared and centrifuged in an ultracentrifier at 2 000 g for 8 min. After centrifugation, a stratified solution was obtained. The upper and middle layers were removed and placed in a 250 mL separating funnel, washed with an equal volume of PBS phosphate buffer, and left to stand. The middle layer microbubbles were collected and freeze-dried to obtain Span-PEG ultrasound contrast agent microbubbles loaded with schisandrin A.[14] The size and shape of the microbubbles were observed by scanning electron microscopy (SEM). Detailed operation details were as follows: A small amount of microbubble powder was coated to one side of the double-sided glue and the other side was fixed on the stage of the scanning electron microscope. The surface morphology of the drug microbubbles was observed and photographed by scanning electron microscopy under a high voltage of 15 kV at a magnification of 5000. The particle size distribution and Zeta potential of the microbubbles were determined by ZS90 laser particle size analyzer. Detailed operation steps were as follows: Added pure water into the sample tank as the dispersing agent, turned on the ultrasonic disperser and set the intensity to 7, turned on pump switch after 2 minutes, adjusted the pump speed to 2680 r/min, specified water as the dispersing agent in the TAB, and other parameters were determined, clicked "Start", measured the sample, and saved the results. Then changed the measurement conditions and re-measured until the next measurement results were basically in line with the last measurement results, then the last measurement results are the particle size measurement results of the sample. This experiment was independently repeated three times with consistent results.

Determination of loading rate of schisandrin A in Span-PEG composite microbubbles

The loading rate of schisandrin A in Span-PEG microbubbles was determined by ultraviolet spectrophotometric method. The standard of schisandrin A was prepared at concentrations of