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Original Articles

Ultrasound-mediated destruction of paclitaxel and oxygen loaded lipid microbubbles for combination therapy in ovarian cancer xenografts

Li Liu [a,b,c,1], Shufang Chang [a,*], Jiangchuan Sun [a,1], Shenyin Zhu [d,1], Minyue Yin [a], Yi Zhu [a], Zhigang Wang [b], Ronald X. Xu [a,e]

[a] Department of Obstetrics and Gynecology, Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China
[b] Institute of Ultrasound Imaging, Second Affiliated Hospital of Chongqing Medical University, Chongqing 400010, China
[c] State Key Laboratory of Ultrasound Engineering in Medicine Co-founded by Chongqing and the Ministry of Science and Technology, Chongqing 400016, China
[d] Department of Pharmacy, First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China
[e] Department of Biomedical Engineering, The Ohio State University, Columbus, OH 43210, USA

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ABSTRACT

We have synthesized multifunctional oxygen and paclitaxel loaded microbubbles (OPLMBs) for ultrasound mediated delivery of combination therapy in an ovarian cancer xenograft model. In comparison with other therapeutic options, intravenous injection of OPLMBs followed by ultrasound mediation yielded a superior therapeutic outcome. Immunohistochemical analyses of the dissected tumor tissue confirmed the increased tumor apoptosis and the reduced VEGF expression after treatment. Western Blot tests further confirmed the decreased expressions of HIF-1α and P-gp. Our experiment suggests that ultrasound mediation of OPLMBs may provide a promising drug delivery strategy for the combination treatment of ovarian cancer.

Introduction

Ovarian cancer is one of the most deadly diseases threatening women's health [1]. Since the disease has common and non-specific symptoms, it is often overlooked by patients and healthcare professionals until at an advanced stage. The current standard of care for treating advanced ovarian cancer includes cytoreductive surgery and systemic chemotherapy [2]. However, clinical efficacy of chemotherapy is suboptimal, owing to dose limiting toxicity and the development of therapeutic resistance. Dose limiting toxicity is associated with non-specific biodistribution of the anticancer agents that leads to a low therapeutic index and serious side effects. Therapeutic resistance is caused by many contributing factors, including tumor angiogenesis and hypoxia. Tumor angiogenesis is the proliferation of a network of abnormal blood vessels that penetrates into cancerous growths. Impaired tumor vasculature, increased vascular permeability, interstitial hypertension, and increased flow resistance contribute to the low drug delivery efficiency at the tumor site [3–6]. Tumor hypoxia triggers an array of cellular defense mechanisms that induce the resistance of hypoxic tumor cells to various treatment modalities [7–11]. Therefore, oxygen treatment is commonly prescribed before radiotherapy or chemotherapy in order to boost tumor oxygenation, improve drug uptake, and enhance tumor response [10,12–14].

Microbubbles (MBs) offer a new opportunity for simultaneous delivery of oxygen and anticancer drugs in chemotherapy. Once used as a contrast enhancement agent in ultrasonography, MBs have been loaded with therapeutics for an ultrasound-targeted microbubble destruction (UTMD) process that enhances the drug concentration at the tumor site [15–19]. Stimulated fragmentation of these MBs may facilitate multiple mechanisms such as bubble implosion, microstreaming, shockwave generation, microjetting, and sonoporation that further enhance the therapeutic efficiency [20–22]. Meanwhile, perfluorocarbon (PFC) represents a group of compounds commonly used as filler materials for ultrasonic MBs. PFC has superior oxygen solubility and can be used as an artificial oxygen carrier [23,24]. Various oxygen-loaded MBs and nanobubbles have been fabricated for ultrasound-mediated oxygen delivery [25,26]. However, ultrasound mediation for simultaneous delivery of oxygen and therapeutics has not been widely explored yet. Considering the

Abbreviations: PLMBs, paclitaxel loaded lipid microbubbles; OPLMBs, oxygen and paclitaxel loaded MBs; PBST, PBS with 0.1% Tween-20.

* Corresponding author. Tel.: +86 23 6369 3279; fax: +86 23-65104238.
E-mail address: shfch2005@163.com (S. Chang).

[1] These authors contributed equally to this work.

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multifaceted functions of MBs as a contrast enhancement agent, an oxygen carrier, and a drug delivery vehicle, it is desirable to integrate these functions for simultaneous enrichment of oxygenation and selective delivery of drugs at the tumor site.

We have synthesized an oxygen and paclitaxel (PTX) loaded MB (OPLMB) agent for UTMD mediated drug delivery of combination therapy in an ovarian cancer xenograft model. Our *in vivo* experiments have verified our hypothesis that the UTMD process will facilitate controlled delivery of oxygen and drugs for the enhanced therapeutic efficiency. To the best of the authors' knowledge, ultrasound mediation of OPLMBs for simultaneous delivery of oxygen and PTX to ovarian cancer xenografts has not been reported elsewhere and is considered innovative.

Materials and methods

Cell lines and culture

Human ovarian cancer SKOV3 cells were obtained from School of Life and Health Sciences, Chongqing Medical University (Chongqing, China). The cells were maintained in a HyClone RPMI 1640 medium (Fisher Scientific, Shanghai, China) supplemented with 10% fetal bovine serum and 0.1% gentamicin sulfate at 37 °C in a humidified incubator containing 5% CO_2. For *in vivo* injection, cells in logarithmic growth phase were trypsinized and centrifuged at 800 *g* for 5 minutes, washed twice, reconstituted in PBS at a concentration of 2×10^7 cells/ml for 200 μl subcutaneous injections.

Animal model preparation

Four- to five-week-old female BALB/c nude mice were provided by the Chinese Academy of Medical Sciences (Beijing, China). The animals were housed in microisolator cages in a pathogen-free animal bio-safety level-2 facility at 22 ± 2 °C. Tumors were established by subcutaneous injection of the above SKOV3 cells into the left hind dorsal flank area of the mice. Tumors were permitted to grow until they reached approximately a volume of 70 to 100 mm^3 at the 14th day after inoculation. These cancer xenograft mice were used for investigations of tumor imaging, *in vivo* drug release, and antitumor activity. All procedures involving the use and care of mice were approved ethically and scientifically by the university in compliance with the Practice Guidelines for Laboratory Animals of China.

Preparation and characterization of oxygen and PTX loaded MBs (OPLMBs)

Detailed procedures for fabrication and characterization of OPLMBs, PTX loaded MBs (PLMBs), and bare MBs without oxygen and PTX have been described elsewhere [27] and will not be redundantly elaborated here. The above three kinds of MBs were washed with phosphate-buffered solution (PBS) three times and sterilized by 60Co irradiation. The OPLMBs and the PLMBs were dispersed in PBS buffer for morphology characterization by a bright field microscope, concentration detection by a blood cell count plate, and size and zeta potential measurement by a Malvern Zetasizer Nano ZS unit (Malvern Instrument, UK) [20]. The amount of PTX incorporated in the MBs was detected by an Agilent 1260 high performance liquid chromatography (HPLC) system. The drug entrapment efficiency and the drug-loading capacity were calculated by the following equations respectively: drug entrapment efficiency = (total PTX − free PTX)/total PTX × 100%; drug-loading capacity = amount of PTX loaded into the MBs/total amount of lipid material × 100%.

In vivo *tumor imaging*

A total of 4 cancer xenograft mice were used for the ultrasound imaging study. The imaging session was carried out by a Mylab 90 ultrasound system (Esaote North America, Indianapolis, IN) using a 10 MHz ultrasound probe. Prior to the imaging session, each animal was restrained on a flat platform and anesthetized with 10% hydral. The ultrasound probe was placed gently on the top of the tumor, with the index matched by commercial diagnostic ultrasound gel and the orientation aligned along the tumor's longest axis. Before, during and after single bolus injection of 0.2 ml OPLMBs or PLMBs (1×10^9/ml) through the tail vein for 20 minutes, the process was monitored by ultrasonography continuously. Ultrasound images were acquired before the injection of MBs and at the time of maximum contrast enhancement after the injection.

In vivo *drug delivery and biodistribution analysis*

On the 14th day after inoculation, thirty SKOV3 tumor-bearing mice were randomly divided into the following six treatment groups (five mice per group): (a) applying PTX only (i.e., "PTX only"); (b) applying PTX followed by ultrasound destruction (i.e., "PTX+US"); (c) applying PLMBs only (i.e., "PLMBs only"); (d) applying PLMBs followed by ultrasound destruction (i.e., "PLMBs+US"); (e) applying OPLMBs only (i.e., "OPLMBs only"); and (f) applying OPLMBs followed by ultrasound destruction (i.e., "OPLMBs+US"). All the mice received the same single dose of PTX (20 mg/kg) through the tail vein for 7 consecutive days. For the treatment groups (b), (d), and (f), a piezoelectric ceramic unfocused transducer (model CGZZ, Ultrasonographic Image Research Institute, Chongqing Medical University, Chongqing, China) with a diameter of one centimeter and a frequency of 300 kHz was used for UTMD mediated drug release and later antitumor experiments. The ultrasound transducer was positioned on the tumor site, with the index matched by commercial diagnostic ultrasound gel. The tumor was exposed to the ultrasound pulses for six minutes at an average intensity of 1 W/cm^2, a duration of 10 seconds, and a duty cycle of 50%. Each animal was sacrificed at 4 hours after the final treatment session. The tissue samples were collected from the tumor and the selected organs (i.e., heart, liver, spleen, and lung), weighed, and homogenized with saline. The tissue homogenates were extracted by acetonitrile, vortex-mixed for 30 seconds, and centrifuged at 3000 *g* for 10 minutes. 20 μl supernatant was then collected from each homogenate for HPLC study of the PTX concentration [28]. The rest of tumor tissue was snaps frozen for lysate preparation and Western Blot testing of HIF-1α and P-gp expression.

Expression of HIF-1α and P-gp after in vivo *treatment*

The design of the treatment groups in this study is similar to that of the previous biodistribution study except that a control group (g) (i.e., PBS 200 μl i.v.) was added. After each treatment, the tumor tissue was harvested and the expression of protein-HIF-1α and P-gp was determined by western blot analysis in order to evaluate the drug resistance. Briefly, samples in each treatment group were homogenized and centrifuged at 12000 *g* for 30 minutes. The supernatant was collected and the protein concentration of the lysate was determined by a Bradford protein assay (Bio-Rad, Hercules, CA, USA). For western blot analysis, equal amounts of protein were loaded for sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE). The protein was then transferred to a nitrocellulose membrane, blocked with PBST (PBS with 0.1% Tween-20) solution containing 5% nonfat milk, and incubated overnight at 4 °C with a primary antibody against HIF-1α and P-gp (polyclonal, 1:1000, Abcam, UK), respectively. The antibody was detected by a horseradish peroxidase-conjugated secondary antibody (1:5000) after one-hour incubation and developed with an enhanced chemiluminescence detection kit. Equal loading was confirmed by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) detection. A Labworks 4.6 software package was used for densitometric analysis.

In vivo *antitumor efficacy*

UTMD mediated delivery of oxygen and PTX for ovarian cancer treatment was demonstrated in an ovarian tumor xenograft model. Thirty-five female BALB/c mice were subcutaneously inoculated with 4×10^6 SKOV3 cells on the 14th day after tumor inoculation, and the mice were randomly divided into the following seven treatment groups (five mice per group): (a) control (Saline 200 μl i.v.); (b) applying PTX only (i.e., "PTX only"); (c) applying bare MBs followed by ultrasound destruction only (i.e., "MBs+US"); (d) applying PLMBs only (i.e., "PLMBs only"); (e) applying OPLMBs only (i.e., "OPLMBs only"); (f) applying PLMBs followed by ultrasound destruction (i.e., "PLMBs+US"); (g) applying OPLMBs followed by ultrasound destruction (i.e., "OPLMBs+US"). Before and after each treatment, animals were weighted by an electronic balance. For each treatment group, an equivalent PTX dose of 20 mg/kg was administered once a day for 7 consecutive days. For the treatment groups (c), (f), and (g), the ultrasound pulses were applied immediately post injection following the same protocol as described before. After each treatment, the tumor size of each animal was measured by an operator using a dial caliper once a day until the 8th day. Since the operator didn't differentiate between the treatment group and the control group, the measurement was considered in a blind manner. The subcutaneous tumor volume was estimated by the following formula: Tumor volume ≈ largest diameter × smallest $diameter^2/2$. The tumor inhibition rate (*IR*) was calculated by the following formula: *IR* = 100% × [mean mass weight of control group − mean mass weight of treatment group]/mean mass weight of control group. All mice were sacrificed 24 hours after the last day of treatment and the tumors were harvested. After the tumor masses were weighed, the tissue specimens were dissected, placed in 4% paraformaldehyde for paraffin cline preparation, and snap frozen for lysate preparation.

Tumor apoptosis analysis after in vivo *treatment*

For quantitative assessment of apoptosis, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) was carried out with an *in situ* Cell Death Detection Kit (Roche) following the manufacturer's protocol [15]. The cells with brown-stained nuclei were apoptotic. Microscope images were acquired with a CoolSnap digital camera. Image Pro-plus software was used for image analysis. The integrated optical density (IOD) of the fluorophore was evaluated by measuring 100 mm^2 throughout the area in a blind manner without the operator's intervention. The results from individual experimental groups were normalized with respect to those of the control specimens.