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Anti-tumor efficacy of ultrasonic cavitation is potentiated by concurrent delivery of anti-angiogenic drug in colon cancer

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ABSTRACT

This study investigated the efficacy of concurrent delivery of an anti-angiogenic drug and ultrasonic cavitation therapy in a mouse model of human colon cancer. A biotinylated form of the anti-angiogenic drug Endostar was conjugated to a streptavidin-coated microbubble (MB). Mice bearing subcutaneous tumors (HT29) were divided into 4 groups. Group 1 served as an untreated control. Group 2 served as a cavitation control and received naked microbubbles and sham ultrasonic cavitation (MB + sham cavitation). Group 3 received naked microbubbles and ultrasonic cavitation (MB + cavitation). Group 4 received Endostar loaded microbubbles and ultrasonic cavitation (Endostar-MB + cavitation). Ultrasonic cavitation was performed using a high-power custom built sonicator. Contrast-enhanced ultrasound imaging (CEUS) was used to measure tumor blood flow before and after ultrasonic cavitation. In vivo fluorescence imaging was performed to monitor changes in tumor volume. Immunohistochemistry was performed to assess CD31, VEGFR-2 and alpha-v beta-3 integrin expression within the tumor. Apoptosis of the tumor cells was determined by TUNEL assay, and ultrastructural changes within the tumor were examined by electron microcopy. Ultrasonic cavitation with Endostar-MB demonstrated a significantly greater inhibition of tumor blood flow on day 7 and tumor growth on day 16 compared with naked MB and control groups. The Endostar-MB treated mice showed significantly decreased expression VEGFR-2 and alpha-v beta-3 integrin, and increased apoptosis of tumor cells and degradation of the tumor ultrastructure. Our findings indicated that the anti-vascular and anti-tumor effects of ultrasonic cavitation could be potentiated by simultaneously delivering an anti-angiogenic drug in colon cancer.

1. Introduction

Although it has traditionally been perceived as a tool for diagnostic imaging, ultrasound is emerging as a highly effective, low-cost and truly noninvasive tool for therapy [1–3]. Of particular note is high-intensity focused ultrasound (HIFU), which is now used for noninvasive thermal ablation of deep-seated solid tumors [4–6]. More recently, the introduction of microbubble contrast agents into the therapeutic ultrasound process has received increasing attention [7–12] due to their potential for localized drug delivery and potentiation of cavitation effects. Ultrasound contrast agents are highly echogenic microbubbles with many unique properties with relevance to ultrasound-mediated therapy. At high acoustic pressures (typically beyond those used for imaging), ultrasound causes acoustic cavitation and microbubble destruction, and can produce extremely high mechanical stresses [13–15] over a very localized region. Some preclinical studies have demonstrated that cavitation therapy with microbubbles can induce a reduction or complete abolishment of blood flow within the tumor [16,17]. Although the precise mechanism(s) at work have not been elucidated, it is possible that anti-angiogenic effects caused by cavitation-induced ischemia are responsible for the observed anti-tumor effect.

The use of targeted microbubbles may enable concentration of the treatment at the desired location upon activation with ultrasound [18–20], and various targeting ligands have been conjugated to the surface of microbubbles to achieve site-specific accumulation

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[21–23]. We hypothesized that local delivery of an anti-angiogenic drug may potentiate the anti-vascular effect of cavitation therapy, and lead to a sustained anti-tumor effect.

Endostar, an anti-angiogenic drug, is a modified version of recombinant human Endostatin and has been approved by the SFDA (State Food and Drug Administration) for the treatment of non-small cell lung cancer in China [24–26]. In the present study we describe an ultrasonic cavitation strategy with Endostar-loaded microbubble able to target tumor angiogenesis and release its payload upon focal ultrasound treatment for anti-tumor efficacy. The efficacy of this strategy was evaluated in a colon cancer model using real-time quantitative CEUS and in vivo fluorescence imaging.

2. Materials and methods

2.1. Cell culture

A human colon cancer cell line HT-29 transfected with green fluorescence protein gene (HT-29-GFP) was obtained from AntiCancer, Inc., (San Diego, CA). Cells were cultured in RPMI 1640 (GIBCO Life Technologies, New. York, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT) at 37 °C in 5% CO_2 saturated humidity. All media was supplemented with penicillin/streptomycin (Gibco-BRL, Grand Island, NY).

2.2. Animal care

48 BALB/C male nude mice, aged 4–6 weeks and weighing 20–25 g, were purchased from the Beijing Kelihua laboratory animal center (Beijing, P.R. China). All mice were maintained in a HEPA-filtered environment at 24–25 °C and humidity was maintained at 50–60%. All animals were fed with autoclaved laboratory rodent diet. Animal experiments were approved by the Animal Committee of Nanjing Origin Biosciences, China.

2.3. Xenograft mouse tumor model

A mouse model of human colon cancer (cell line HT29-GFP) was used to assess the efficacy of drug loaded ultrasound contrast agents with ultrasound treatment for inhibition of tumor growth. Stocks of HT-29-GFP tumors were established by subcutaneously injecting 5×10^6 HT-29-GFP cells in the flank of nude mice. Colon tumors, grown s.c. in nude mice, were harvested at the exponential growth phase and resected under aseptic conditions. Strong GFP expression of the HT-29-GFP tumor tissue was confirmed by fluorescence microscopy. Necrotic tissues were removed and viable tissues were cut with scissors and minced into 1-mm^3 pieces. Animals were anesthetized by injection of 0.02 ml of solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. Two pieces of tumor fragment were transplanted to the flank of nude mice with 8–0 surgical sutures. All surgical procedures and animal manipulations were conducted under HEPA-filtered laminar-flow hoods with a ×8 surgical microscope (Shanghai Precision Instruments, YZ20P5, Shanghai, China).

2.4. Preparation of drug loaded ultrasound contrast agent

Targestar®-SA (MB, Targeson Inc., San Diego, CA; distributed in China by Origin Bioscience) was used as the microbubbles in this study. Targestar-SA is an ultrasound contrast agent composed of a perfluorocarbon gas core encapsulated by a lipid shell. The outer shell is derivatized with streptavidin, which binds biotinylated ligands at a density of $80–220 \times 10^3$ molecules per microbubble. The agents are suspended in aqueous saline at a concentration of approximately 1×10^9 particles per mL, and have a mean diameter of approximately 2.0 μm. Endostar (Simcere Pharmaceutical, Nanjing, P.R. China) was used as the therapeutic payload. Endostar is a small molecule inhibitor of angiogenesis based on Endostatin. Endostar was biotinylated by Signalway Antibody (Nanjing, P.R. China). Microbubbles were incubated with biotinylated Endostar at room temperature for 20 min at a ratio of 0.7 nmoles of biotinylated Endostar per 10^9 microbubbles. The unreacted Endostar was removed from the microbubbles by centrifugal washing, per the manufacturers recommended protocol.

The presence of microbubble-bound Endostar was assessed using fluorescence microscopy and flow cytometry. Endostar was conjugated to the MBs and fluorescently labeled using a rabbit anti-Endostatin antibody and a FITC conjugated anti-rabbit IgG secondary antibody (Abcam). The conjugated microbubble concentration was counted with a hemocytometer. The payload of Endostar was determined by quantitation of microbubble-bound Endostar by BCA Protein Assay (Pierce, Rockford, IL, USA). Naked Targestar-SA microbubbles were used directly from the vial without the addition of Endostar.

2.5. Administration of ultrasound contrast agents

48 Tumor-bearing mice were randomly divided into 4 groups of 12 mice once the average tumor size had reached 120 mm^3. Mice were anesthetized with ketamine, acepromazine and xylazine and then placed in a supine position on a heated stage. A dose of 1×10^8 microbubbles in 70 μl per mouse was administered by retro-orbital injection with a 27 gauge needle. Group 1 served as an untreated control. Group 2 received naked microbubbles and a sham ultrasonic cavitation (MB + sham cavitation). Group 3 received naked microbubbles and ultrasonic cavitation (MB + cavitation). Group 4 received Endostar loaded microbubbles and ultrasonic radiation (Endostar-MB + cavitation).

2.6. Cavitation therapy and contrast-enhanced ultrasound imaging

The animals in group 3 and 4 received 3 consecutive daily cavitation therapies, using same acoustic conditions. For ultrasonic cavitation, the transducer from a sonicator equipped with a 1 cm^2 transducer cone tip (Haiying Medical Electronic Instrument Company, Wuxi, China) was placed over the tumor and coupled using acoustic coupling gel. Ultrasonic cavitation was performed at a frequency of 238 kHz, 400 mV, 0.5 MPa 60-s sonication duration, 10 pulses with 10-ms pulse length and 50% duty cycle. The animals in group 2 received 3 consecutive daily administrations of ultrasound contrast agents and sham cavitation, in which the microbubbles were injected and the transducer was placed over the tumor with acoustic coupling gel but not powered on.

Contrast-enhanced ultrasound imaging (CEUS) was used to analyze tumor vasculature before the cavitation and day 1 and 7 after the third cavitation therapy. This technique enables real-time evaluation of active microvascular perfusion in the intact tumor. CEUS was performed on a Mylab90 ultrasound scanner with 4.0–11.0 MHz LA332 linear transducer (Esaote, Genova ITALY). The transducer was coupled to the skin by covering the tumor with acoustic coupling gel. Imaging was performed in CnTI mode at a mechanical index (MI) of 0.04 and transmission frequency of 8 MHz. Imaging gain settings were optimized and held constant during the experiment. Right after injection of microbubbles, ultrasound images were captured to obtain the contrast signal from the tumor tissue as well as from adherent and freely circulating microbubbles. Digital raw data were stored as cine loops up to 2 min for analysis. One board-certified abdominal radiologists (CZ with 14 years of CEUS experience) reviewed and analyzed the data offline using the perfusions software QONTRAST (Bracco, Italy). A region of interest (ROI) was drawn freehand around the peripheral margin of the tumor using an electric-cursor, being careful to avoid the surrounding non-tumor tissue. A time-intensity curve (TIC) for the selected tumor tissue was derived automatically by the scanner software, and the following parameters were generated: peak intensity (PI), which is maximum signal intensity reached during the transit of the microbubble bolus; regional blood volume (RBV), which is proportional to the area under the time-intensity curve; mean transit time (MTT); and regional blood flow (RBF), which is the ratio of the RBV to MTT.

2.7. In vivo fluorescence imaging

Tumor bearing mice were monitored by real-time whole-body fluorescence imaging for tumor growth. The imaging was performed before the cavitation and on day 3, 7, 11 and 16 after cavitation therapy. Tumor size was measured and volume was calculated using the formula $(L \times W^2) \times ½$, where W and L represent the perpendicular minor dimension and major dimension, respectively. A fluorescence stereo microscope (MZ650; Nanjing Optic Instrument Inc., China) equipped with a D510 long-pass and HQ600/50 band-pass emission filters (Chroma Technology, Brattleboro, VT) and a cooled color charge-coupled device camera (Qimaging, BC, Canada) was used to image intact tumors in live mice. Selective excitation of GFP was produced through an illuminator equipped with HQ470/40 and HQ540/40 excitation band-pass filters (Chroma Technology, Brattleboro, VT). Images were processed and analyzed with the use of IMAGE PRO PLUS 6.0 software (Media Cybernetics, Silver Spring, MD).

2.8. Immunohistochemistry

At the end of the study, all mice were sacrificed on day 16 after the cavitation therapy and the tumors were removed and weighed. The parts of tumor sample were fixed in 10% buffered formalin and paraffin-embedded. For immunohistochemistry, sections were incubated with primary antibodies against CD31, VEGFR-2 and alpha-v beta-3 integrin (all from BD Biosciences, San Diego, CA) overnight at 4 °C after permeabilization with a solution of 0.1% sodium citrate and 0.1%Triton X-100 and blocking with 10% rabbit serum. After washing in PBS, the slices were incubated with horseradish peroxidase-labeled secondary antibody (1:200, Maixin Bio-Tech Co., Ltd., Fuzhou, China) for 30 min at room temperature. After color development using diaminobenzidine (Maixin Bio-Tech Co., Ltd.), the slices were counterstained in hematoxylin and mounted with a neutral resin medium. The whole slide was first viewed at 100-times magnification in order to identify a "hot spot" representing the area of the highest vessel density. The field was then switched to ×400 magnification for analysis. For each slide, the microvessel