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Low intensity-pulsed ultrasound induced apoptosis of human hepatocellular carcinoma cells *in vitro*

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ABSTRACT

The present study was conducted to determine whether low intensity-pulsed ultrasound (LIPUS) could induce apoptosis of human hepatocellular carcinoma cells, SMMC-7721, and to define the mechanism of ultrasound-induced apoptosis, *in vitro*. MTT assay was used to measure cell proliferation. Apoptosis was investigated by multiple methods such as flow cytometry, DNA fragmentation, Ca^{2+} mobilizations, pro- and anti-apoptotic protein expression, and light as well as ultramicroscopic morphology. The results provide evidence that LIPUS induced a dose-dependent effect on cell viability and apoptosis of SMMC-7721 cells. Specifically, exposure of cells to >0.5 W/cm^2 intensity significantly increased cell apoptosis, caused shifts in cell cycle phase, and induced structural changes. Ultrasound significantly increased intracellular Ca^{2+} concentrations and modulated expression of caspase-3, Bcl-2 and Bax. The findings suggest that this novel technology can be used to induce SMMC-7721 apoptosis via the Ca^{2+}/mitochondrial pathway and could potentially be of clinical use for the treatment of hepatocellular carcinoma (SMMC-7721 cell line) and other cancers.

1. Introduction

The clinical use of ultrasound (US) for the treatment of cancers has been highlighted in recent reports, due to advances in diagnostic US and because of its therapeutic potential. Therapeutic ultrasound may be considered to be any application of ultrasound which instigates a direct improvement in disease outcome or enhances the activity of a co-applied conventional therapy. High intensity focused ultrasound (HIFU) involves exposing the target lesion to high ultrasound power densities/intensities for relatively short periods of time, including the thermal ablation of diseased tissue [1,2], cataract treatment by phacoemulsification [3], and the break-down of calculi such as kidney stones and gall stones so that they can be passed from the body, a process known as lithotripsy [4]. HIFU is effective for malignant tumor treatment and has been widely used in clinic.

Generally speaking, the low intensity ultrasound refers to the ultrasound intensities are less than 3 W/cm^2 or 5 W/cm^2 [5–7]. Low intensity ultrasound is used for stimulating tissue and bone repair [8] and to reversibly disrupt the blood–brain barrier [9]. However, the use of low intensity ultrasound for cancer treatment has been contraindicated because it is thought to promote tumor metastasis. There is no scientific evidence to support this premise. On the contrary, several studies have shown that low intensity-pulsed ultrasound (LIPUS) has an anticancer effect via multiple pathways [10,11] through the use of LIPUS alone or in combination with either radiation or chemotherapy.

In cancer therapy, induction of apoptosis is the preferred method of killing cancer cells. Radiation therapy, hyperthermia treatment, and chemotherapy are typically used in the treatment of cancer. In these methods, the level of apoptosis induction is used as an indicator of therapeutic effectiveness and therapeutic safety. Many investigators believe that the primary mode of cell killing by US is via apoptosis induction [10,12,13], where fairly intense US induces cell killing, cell lysis, loss of cell viability, and loss of clonogenicity [14]. The primary therapeutic effect of US in tissues is due to local periodic pressure oscillations of the medium [15]. Secondary effects include temperature elevation due to sound wave absorption, and generation of gaseous cavities (cavitation) [16]. Furthermore, *in vitro* experiments have demonstrated that US-induced hyperthermia with an intensity-dependent and the hyperthermia-induced apoptosis can be enhanced by US [17]. The therapeutic advantages of US are the focusing of sound wave, easily control of intensity, and ability to reach a sufficient tissue attenuation coefficient for deep tumor targets [18,19].

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However, the mechanism of LIPUS induced apoptosis was still not clarified. In this study, the objectives were to clear and define whether the LIPUS could cause apoptosis of SMMC-7721 and find the possible mechanisms.

2. Methods

2.1. Cells and cell culture

The human hepatocellular carcinoma cell line, SMMC-7721 (generously provided by the Liver Cancer Research Institute of Zhongshan Hospital, China) was cultured in DMEM culture medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA, USA) and 1% penicillin/streptomycin at 37 °C in humidified air with 5% CO_2. For the LIPUS experiments, cells were seeded 24 h prior to the experiment at T-25 cm^2 culture flasks (Corning, NY, USA). Cell viability before treatment was >95% as evaluated by trypan blue staining.

2.2. LIPUS

The ultrasonic apparatus (BTL-5825SL, BTL Industries Limited, Great Britain) was used in all LIPUS experiments. This device was equipped with a built-in digital timer, intensity regulator, duty factor (DF) controller. For the LIPUS procedure, the ultrasonic transducer (superficial area: 5 cm^2; center frequency: 1.0 MHz; duty factor: 25%; repetition frequency: 100 Hz) was fixed with a clamp attached to a metal stand to keep the transducer facing directly upward. All the parameters listed and different intensities used were read off the machine in order to enhance the accuracy, reliability and comparability of experiments. The ultrasonic coupling agent was also used in the interface between the transducer surface and plastic well to reduce the standing waves.

The cell treatment protocol was performed as described previously [10]. Cell concentration of $\sim 2.0 \times 10^6$ cells/ml was use in this study, which was far below the cell density used to cause inhibition of free radical production and cell killing in that previous study [20]. The cells were gently mixed and 1.25 ml/sample of cell suspension was transferred to a 6-well polyethylene plate (Corning, NY, USA). Just before sonication, 1.25 ml freshly prepared culture medium was added to the cell suspension after gentle shaking.

For LIPUS treatment, cells were exposed to 1 MHz US at various intensities (0.3, 0.5, 1.0, 1.3, 1.5, 2.0 W/cm^2) for 1 min. All procedures were performed under sterile conditions inside a clean bench and the room temperature was maintained at 25 °C ± 2 °C. After sonication, cells were incubated for functional measurements and western blots analysis, respectively. Control cells were handled similarly but without US exposure. All processes were performed under sterile conditions. Each treatment was arranged for 5 independent replicates.

2.3. MTT assay for cell viability measurement

Viability of all the cells was determined by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) assay after incubated for 6 h with sonicated, as described by Nomikou et al. [21] with a few modification. Briefly, cells were cultured in 96-well plates and 20 μl (5 mg/ml) MTT (Beyotine, Shanghai, China) was added to each well under sterile conditions. Plates were incubated at 37 °C, 5% CO_2 for 4 h. The supernatant was then removed and dimethylsulfoxide (DMSO) (Sigma–Aldrich Corp. St. Louis, MO, USA) (150 μl per well) was added to dissolve the formazan product. Plates were then agitated on a plate shaker for 10 min. The absorbance value (AV) was measured at 490 nm using a microplate reader (Eppendorf, Hamburg, Germany). Cell viability was calculated using the following formula: % viability = (AV of experiment well/AV of control well) × 100.

2.4. Detection of apoptosis by flow cytometry

For analysis of apoptosis, flow cytometry (FCM) was performed using propidium iodide (PI) (Sigma–Aldrich Corp. St. Louis, MO, USA) and FITC-labeled annexin V (Life Technologies) to detect the expression of phosphatidyl serine (PS) on the plasma membrane of all the cells as described [22,23]. Cells were stained 6 h after sonication.

Cell cycle perturbations induced by sonication were analyzed by PI DNA staining, as described [24]. Briefly, exponentially growing SMMC-7721 cells were treated with 0.5, 1.3, 2.0 W/cm^2 US for 1 min followed by incubation for 6 h. All the cells (both the adhered and floating cells) were trypsinized, harvested, washed twice with PBS, fixed in 70% ethanol at 1×10^6 cells/ml and stored in −20 °C for at least 24 h. Ethanol-suspended cells were diluted with PBS then centrifuged at 1000*g* for 5 min to remove residual ethanol. For cell cycle analysis, the cell samples were suspended in 5 μl RNase (10 mg/ml) (Sigma–Aldrich Corp. St. Louis, MO, USA) for 1 h and 5 μl PI (10 mg/ml) for 30 min in the dark at room temperature. Stained cells were analyzed with a flow cytometer (BD Biosciences FACSCalibur, Franklin Lakes, NJ, USA).

2.5. Transmission electron microscope scanning

To identify apoptotic cells, the cells harvested at 6 h after sonication were washed with PBS and collected by centrifugation. Approximately 10^6 cells of each sample were immersion-fixed in cold 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) overnight. Cells were washed in PBS three times and resuspended in 10% FBS in PBS, followed by centrifugation at 600*g* for 10 min. The supernatant was removed and the cells were fixed in 1% osmic acid for

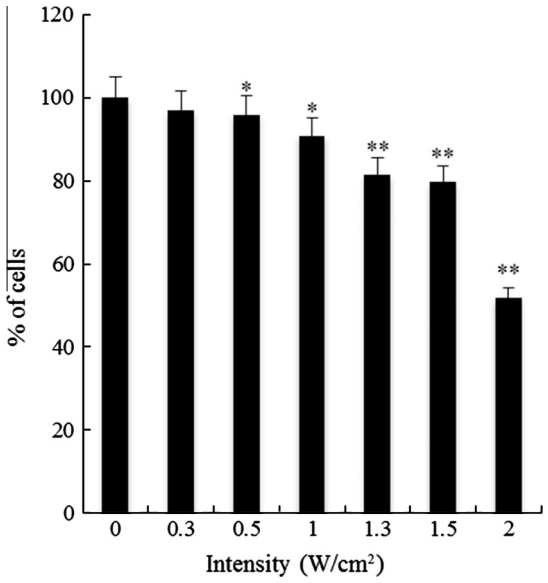

Fig. 1. Opimization of LIPUS for apoptosis induction. SMMC-7721 cells (at 25% confluence) were exposed to different intensities of ultrasound. Proliferation and viability were determined by MTT assay. There was no significant effect on cell viability in the 0.3 W/cm^2 group. However, at intensities of 0.5 W/cm^2 and above cell viability was significantly decreased. $^*p < 0.05$ versus control and $^{**}p < 0.01$ versus control. Data were averaged and presented as the mean ± standard deviation (S.D.), and each group consisted of 5 samples.