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Full Length Research Paper

Low-frequency low-intensity ultrasound with contrast agent for the treatment of subcutaneous tumors in mice

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To determine the effects of ultrasound exposure in the presence of microbubble contrast agent (SonoVue) on the subcutaneous tumor of nude mice. Nude mice bearing subcutaneous tumor were prepared and treated with ultrasound. These animals were divided into three groups: control group (without treatment), low-frequency ultrasound group (US) and low-frequency ultrasound + contrast agent group (US+UCA). UCA was microbubble contrast agent (SonoVue). The tumors were exposed to pulsed ultrasound with a 40% duty cycle and an intensity of 26 MW/cm^2 at a frequency of 20 kHz for 3 minutes using a digital sonifier once every other day for two weeks. The damage to vascular endothelial cells was assessed via transmission electron microscopy (TEM). The protein expressions of VEGF and COX-2 of vessels were detected by Western blot assay. The percentage of tumor shrinkage in US + UCA group was higher than US group and down-regulation of VEGF and COX-2 were detected in US + UCA group. After treatment, degeneration of endothelial cells, mitochondrial vacuolation and lumen occlusion were observed in the tumor of US + UCA group. These changes were seldom observed in the US group. Low-frequency ultrasound in the presence of contrast agent may exert therapeutic effect on subcutaneous tumor through destruct of the blood vessels in the tumor.

Key words: Ultrasonic biophysics, low-frequency, contrast agent, cavitations, thermal mechanism.

INTRODUCTION

The biological effects of ultrasound (US) include thermal and mechanical (non-thermal) ones. From the point of view of physics, the thermal effect is due to the absorption of US by the tissues and the mechanical one usually associated with the acoustic cavitation particularly relevant in the presence of encapsulated micro bubbles (EMBs) *in situ*. When the pressure amplitude is fairly low, bioeffects of US can often be attributed to the radiation force or acoustic streaming while in higher amplitudes, the effects are largely attributed to the inertial cavitation. Inertial cavitation, also known as "transient" cavitation, occurs if the acoustic pressure amplitude is sufficiently high and above a threshold. Under this condition, the EMBs may firstly grow in the volume, and then implode violently. Non-inertial cavitation, also known as "stable" cavitation, occurs when an EMB in a liquid is forced to oscillate with only a relatively small to moderate increase and decrease of radius (off-resonance regime), when the pressure amplitude of the external acoustic field is not high enough (Holland and Apfel, 1990).

It may take longer for non-inertial cavitation to exert bioeffects, while its advantage is that, it is easily controlled and seldom cause non-reparable injury to cells. Inertial cavitation can exert its effects faster but it is difficult to control and may generate permanent damage to cells, which is also known as non-reparable sonoporation (Kaddur et al., 2010). *In vitro* studies have shown that Ultrasound-mediated microbubble destruction increases the permeability of cell membranes (Tachibana et al., 1999; Schlicher et al., 2010; Karshafian et al., 2010). It is likely that cavitation depends on the cell state, pressure amplitude and experimental conditions. If the goal is to produce the inertial cavitation *in situ*, a sound field with strong focus and low driving frequency (for

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example 0.5 MHz instead of 2 MHz) is recommended (Wu and Nyborg, 2008). Low-frequency ultrasound is a novel tool for the treatment of several areas such as blood-tumor barrier (Shang et al., 2011; Fan et al., 2011), local gene delivery (Lin et al., 2010; Yang et al., 2010), tumor cell destruction (Lagneaux et al., 2002; Ward et al., 1999; Sergeeva et al., 2001). However, the *in vivo* environment where low-frequency ultrasound exerts effects is different from that *in vitro*. The present study aimed to explore the effects of low-frequency ultrasound on tumor *in vivo*.

MATERIALS AND METHODS

Sixteen males nude mice aged 4 weeks, were purchased from the Animal Center of Shanghai Jiaotong University, China. Animals were anesthetized with ketamine (50 mg/kg, i.p.) and medetomidine (0.3 mg/kg, i.p.). Prostate cancer cell suspension (DU145; 2×10^6 cells/ml) was subcutaneously inoculated into right abdomen and mice were allowed to recover. DU145 cells are a primary prostate cancer cell line prepared from humans, and it has been shown that inoculation of DU145 cells can spontaneously generate cancers at the injection site. In the present study, a solid and hard mass was observed at the injection site. One mouse without cancer was excluded from the experiment. Then each group has five mice. All animals were handled in accordance with Institutional Animal Use and Care Committee guidelines of Shanghai Jiaotong University. Tumor size was estimated by calipers. A low-frequency ultrasound machine was developed by Shanghai Jiaotong University and used to treat the murine cancer. The contrast agent was administered via the tail vein and ultrasound was applied at the site of cancer using a 20 kHz transducer with 4:6 duty and insonation energy level of 26 MW/cm^2 for 3 min. The dose of contrast agent is 0.2 ml per mouse. Low-frequency ultrasound was applied about three to five seconds after contrast injection because the contrast agent can flow into the blood vessel of the subcutaneous tumor of mice. Following ultrasound treatment, the cancers were collected for microscopy, electroscopy and detection of protein expressions of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor (VEGF). Blood flow images of the tumor were obtained using a 570FD scanner (Baisen Medical Company, Italy) by an experienced examiner. The frequency of the probe was 15 MHz.

Histologic examination

At the end of experiment, mice were euthanized and the cancers were collected, fixed, embedded in paraffin, and cut into sections from the middle part of each cancer followed by hematoxylin and eosin staining and subsequent light microscopy. The histopathologist blind to the study evaluated the findings in microscopy.

Western blotting

Mice were sacrificed at the end of experiment and cancers were collected followed by detection of protein expressions of COX-2 and VEGF by western blot assay. Cancer tissues were lysed in RIPA buffer (150 mM NaCl, 100 mM Tris-HCl, 1% Tween-20, 1% sodium deoxycholate and 0.1% SDS) with 0.5 mM EDTA, 1 mM PMSF, 10 µg/ml aprotinin and 1 µg/ml pepstatin. Proteins were subjected to SDS-PAGE and transferred to PVDF membranes, which were then treated with primary and secondary antibodies. Visualization was carried out using enhanced chemiluminescence method (Amersham Bioscience, Boston, MA). The following primary antibodies were used: anti-COX-2 antibody (Cell Signaling, #4842, 1 µg/ml) and anti-VEGFB antibody (Cell Signaling, #2463, 1 µg/ml).

Transmission electron microscopy (TEM)

EM sample preparations were fixed in 2% glutaraldehyde fixative PBS 2 h 4°C followed by PBS buffer, washed twice for 10 min 4°C. After 1% osmium tetroxide PBS were fixed in 4°C for 2 h and dehydration with 30 to 50 to 70% ethanol for 10 minutes, the sample were embedded with propylene oxide 1 for 1 2 h and stained with lead citrate E staining. Finally, they were watched with TEM (Netherlands Philips CM-120).

Statistical analysis

The tumor size of each group and protein ratio of VEGF and COX-2 was expressed as a mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed to compare the tumor size and protein ratio among different group (MedCalc Software, Mariakerke, Belgium).A value of $P< 0.05$ was considered statistically significant.

RESULTS

Characteristics of cancers

Under the ultrasound exposure, the cancer without treatment presented evenly hypoechoic as compared to the adjacent tissues and had clear borders (Figure 1a). The blood flow signal was found in the cancer under the color Doppler ultrasound (Figure 1b). Following US+UCA exposure, the color flow signal was absent (Figure 1c).

Tumor size

The tumor size in the US + UCA group was smaller than that in the US group and control group (Figure 2).

Histological examination

In the absence of injection of microbubbles, the cancers had minor histological changes (Figure 3a). After injection of microbubbles, insonation of cancers for 3 min significantly altered the histologic features which were characterized by occlusion of capillaries and hemorrhage (Figure 3b). In control group, the vessels were intact (Figure 3c).

TEM

Endothelial damage was assessed via TEM. In the control group, the endothelial was intact. Under TEM, the cancers also had intact vascular lumen and normal

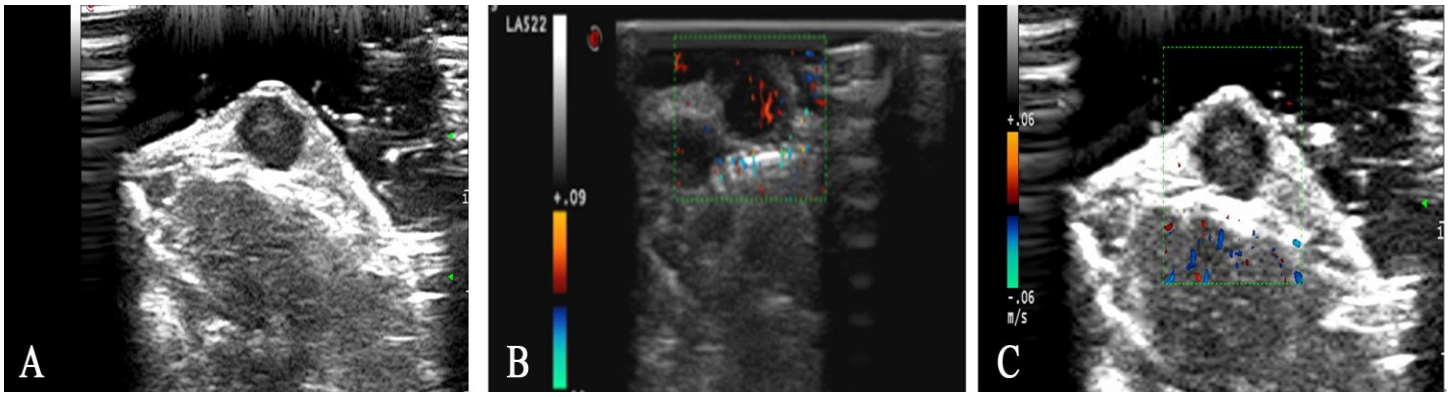

Figure 1A. Subcutaneous tumor of nude mice clearly visible in the ultrasonography. **B.** Internal blood flow signal in the color Doppler ultrasonography. **C.** Internal blood flow signal disappeared after treatment.

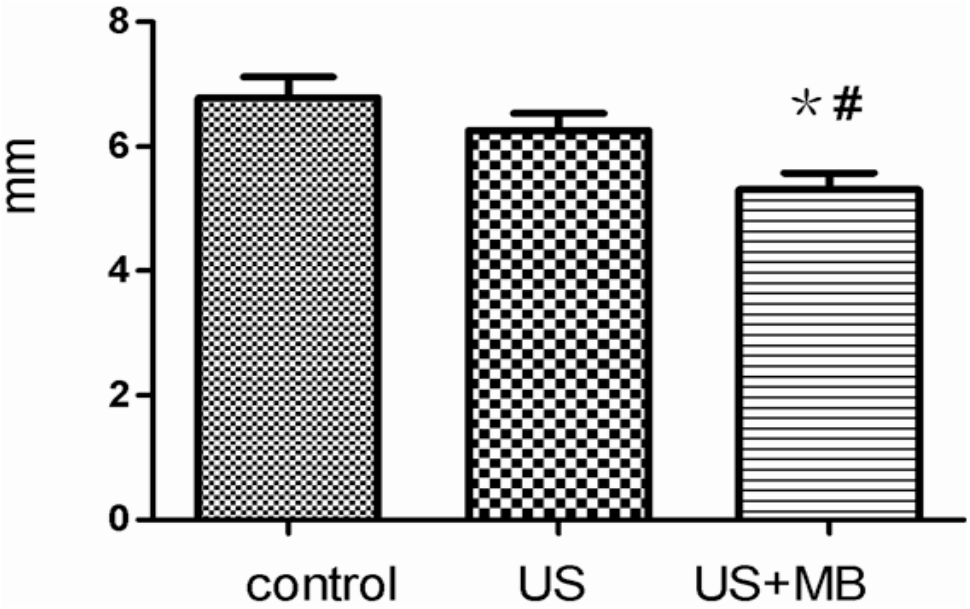

Figure 2. The tumor size among control, US and US + UCA groups; * the size was smaller in US + MB(UCA) than control group; # the size was smaller in US + MB(UCA) than US.

erythrocytes in the blood vessels on the cross-sections following exposure to US alone (Figures 4a,1 to 2). However, after ultrasound exposure in the presence of microbubbles, degeneration of endothelial cells, mitochondrial vacuolation and lumen occlusion were observed in these vessels (Figure 4b, 1 to 3). The cellular changes included cytoplasmic vacuolation, presence of granular endoplasmic reticulum and dilatation of perinuclear cisternae (Figure 4c).

Western blot assay

The protein expressions of VEGF and COX-2 in the US + UCA group were significantly lower than those in the US group and control group (Figure 5).

DISCUSSION

Numerous studies have been conducted to investigate the targeted treatment using low-frequency ultrasound to date (Wu and Nyborg, 2008; Pitt et al., 2011; Reuter et al., 2010). The application of therapeutic ultrasound in combination with contrast agent is a new concept and has elicited interest in various medical fields (Postema and Gilja, 2011; Burke and Price, 2010). Ultrasound can enhance the anti-tumor effects through different

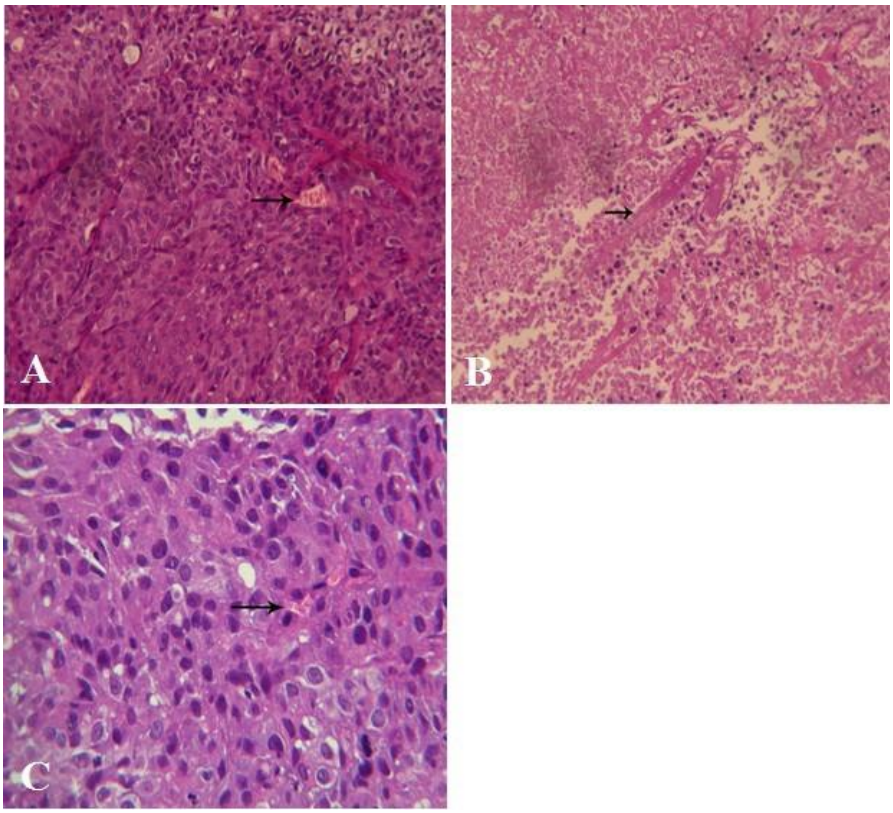

Figure 3. A: minor histologic changes in the tumors of US group; B: histologic findings were featured by an increase of capillary occlusion and hemorrhage (arrow) in the tumors of US + UCA group C, in control group, the vessel were intact (arrow).

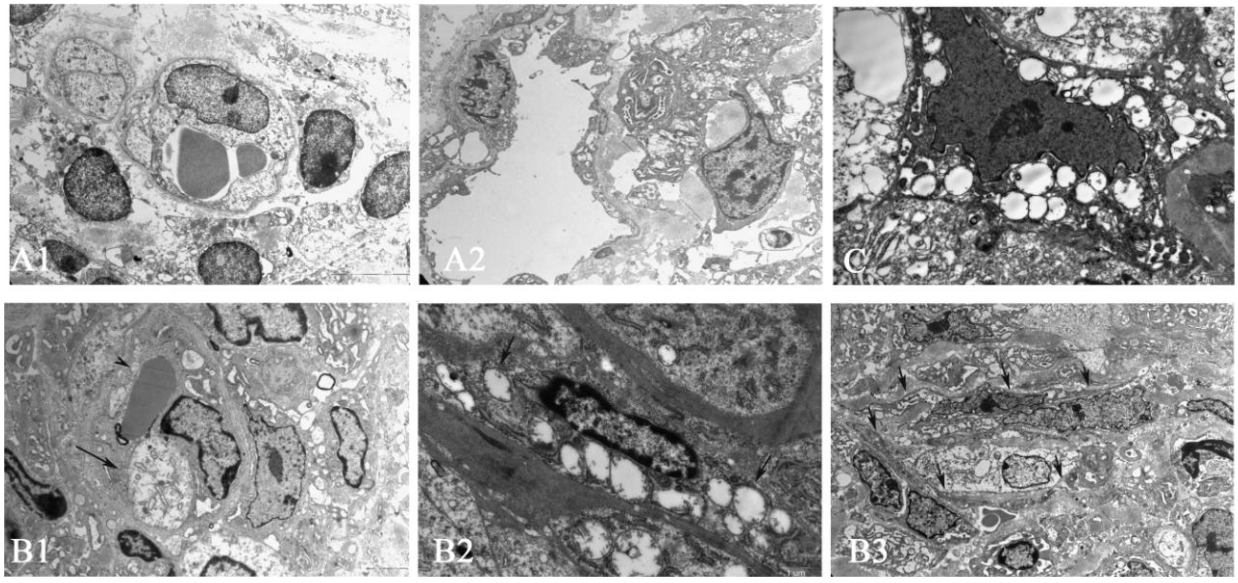

Figure 4A. (1) Normal erythrocytes observed in the vessels on the cross-sections of US group; **(2)** Complete vascular lumen was observed in the vessels of US group; **B: (1)** swelling endothelial cells (arrow) and narrowing lumen (arrow head) were observed in the vessels of US + UCA group; **(2)** Degeneration of endothelial cells and mitochondrial vacuolation (arrow) were observed in the vessels of US + UCA group; **(3)** Luminal occlusion (arrow) was observed in the vessels of US + UCA group; **C:** cellular changes included cytoplasmic vacuolation, the presence of granular endoplasmic reticulum and dilatation of perinuclear cisternae.

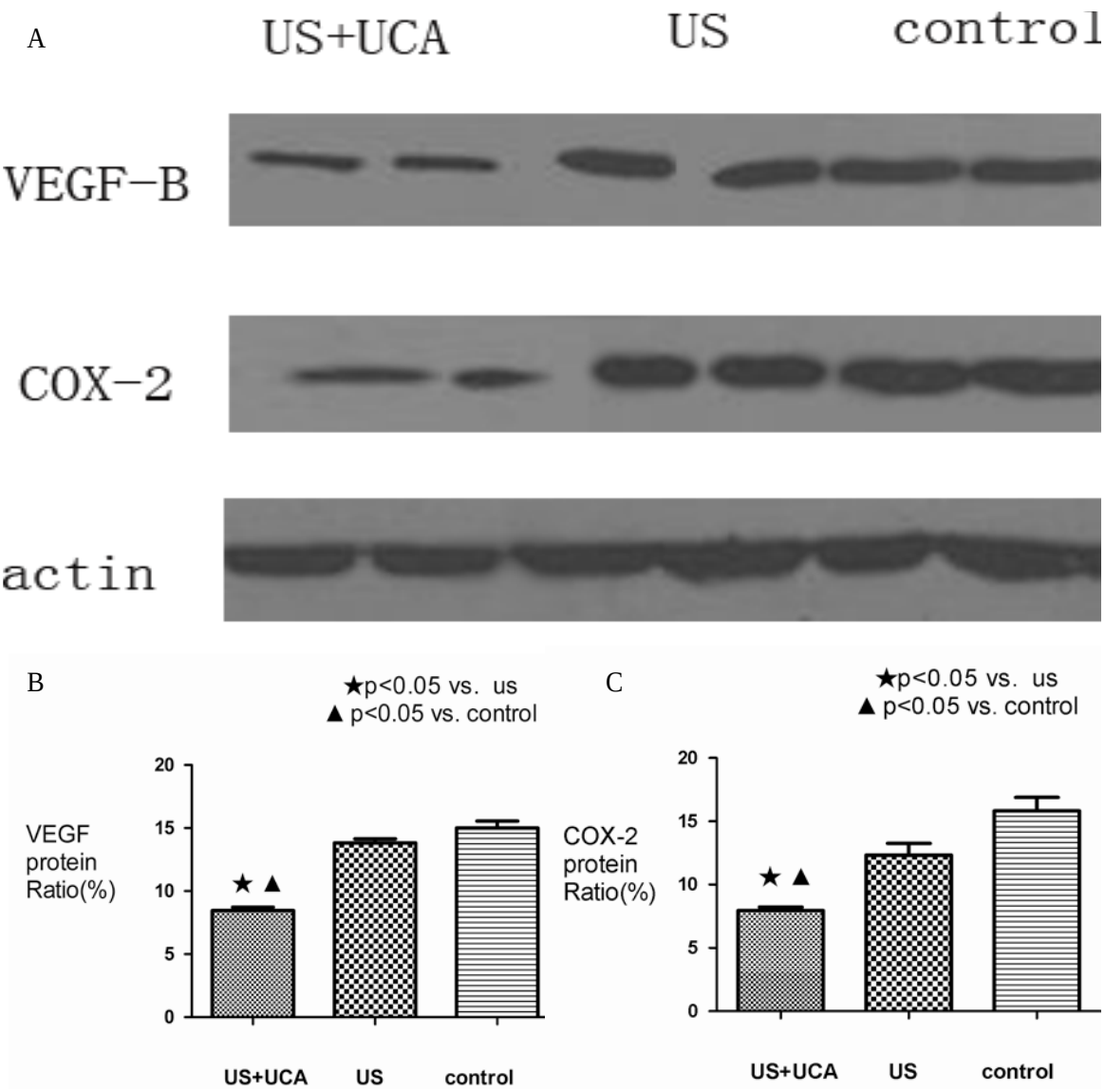

Figure 5. The protein expressions of VEGF and COX-2 in the US + UCA group were significantly lower than those in the US group and control group. A, Western blotting result; B, VEGF expression; C, COX-2 expression.

mechanisms. Although, therapeutic ultrasound has been found to produce local hyperthermia for thermal ablation of tumors, the exact mechanism underlying the ultrasound-assisted tumor treatment is proposed to be the non-thermal effect of ultrasound (Wu, 1998; Husseini et al., 2002; Dijkmans et al., 2004). Recently, therapeutic application of ultrasound contrast agents emerges. This application is in part, motivated by the requirement of targeted treatment of tumors.Microbubbles have special acoustic properties which meet this requirement. As microbubbles are cavitated by the ultrasound, the local shock waves increase the capillary permeability. In the *in vivo* studies, low-frequency ultrasound has been applied in nude mice receiving injection of microbubbles through tail vein. Subcutaneous cancer of nude mice was exposed to 20 kHz low-frequency and low-energy ultrasound with low duty cycle and fixed pulse parameters in the presence and absence of microbubble injection. Our results demonstrated that low-frequency ultrasound in the presence of microbubbles significantly reduced the tumor size (Figure 2).

The fragile, poorly functioning tumor vessels, whose formation is activated by the tumor's "angiogenic switch," are likely to be more sensitive to insonation. COX-2 and VEGF play critical roles in the cancer angiogenesis. Over expression of COX-2 has been demonstrated to contribute to the carcinogenesis by stimulating cell proliferation, inhibiting apoptosis and enhancing angiogenesis; all of which are thought to be mediated via prostaglandin E2 (PGE2) (Chan, 2002; Dai and Wang,

2006; Scartozzi et al., 2004). VEGF, a highly specific mitogen of vessel endothelial cells, is the most potent tumor-angiogenic factor and is capable of promoting the proliferation and migration of endothelial cells as well as increasing the vascular permeability (Ferrara et al., 2003). In the present study, results showed low-frequency ultrasound significantly down-regulated the expressions of COX-2 and VEGF, which attributes to the promotion of apoptosis cancer cells leasing to suppression of tumorigenesis.

In the present study, intact vascular lumen and normal erythrocytes were observed in the blood vessels following treatment with US alone. However, following ultrasound treatment in the presence of microbubbles, degeneration of endothelial cells, mitochondrial vacuolation and lumen occlusion were observed in these vessels, which indicates the effect of US + UCA is different from that of US alone. US in combination with contrast agent result in apparent damage to the blood vessels in the cancer of nude mice.Some researchers have demonstrated that platelets can aggregate around oscillating bubbles *in vitro* (Brayman and Miller, 1997).

The presence of inertial cavitation, in which a microbubble expands and then collapses in the presence of insonation exerting the bioeffect, this may explain, at least in part, the anti-vascular action in the nude mice. It is hypothesized that the gas released following the rupture of bubbles may further form secondary small bubbles which interact with ultrasound resulting in the cellular bioeffects (Brayman and Miller, 1997; Dalecki et al., 1997; Kamaev et al., 2004). Gas in the bubbles as ultrasound contrast agent also provides nuclei for inertial cavitation (Miller, 2007). Normally, the living organisms lack suitable cavitation nuclei. SonoVue®, BR1 is a preparation of stabilized microbubbles containing sulfur hexafluoride gas and developed as an artificial ultrasound contrast agent (Schneider et al., 1995; Schneider, 1999). The encapsulated microbubbles can persist long enough to act as nuclei when they are exposed to ultrasound. The destabilization of bubbles can serve to nucleate the inertial cavitation. Ultrasound contrast agent-induced endothelial damage such as degeneration of endothelial cells, mitochondrial vacuolation and lumen occlusion were noted in our experiment which leads to the occlusion of blood vessels. Other researchers revealed that, micro bubbles under the low-frequency and low power ultrasound mediated the vascular damage and thrombosis, thereby, limiting the blood flow and blocking the blood supply to cancers (Hwang et al., 2005). Our results showed tumor ischemia and growth inhibition were two main mechanisms underlying the anti-tumor therapy.

Conclusion

Our findings reveal low-frequency and low-energy ultrasound in combination with microbubbles can be a strategy to effectively treat cancers, which may be closely related to the microbubble induced increase of cavitation under ultrasound exposure. We speculate that this technique will be a promising non-invasive approach for the anti-tumor treatment. However, the application of microbubble in combination with low-frequency ultrasound in the cancer therapy is currently in its infancy. The role of ultrasound/microbubble induced cavitation in the therapeutic effect of ultrasound is required to be further studied. The interactions between bubbles, sonication and surrounding tissues such as micro-vessels and endothelial cells might be also important mechanisms.

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