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● *Original Contribution*

ULTRASOUND-MEDIATED MICROBUBBLE DESTRUCTION SUPPRESSES MELANOMA TUMOR GROWTH

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Abstract—Melanoma is one of the most aggressive types of cancer, and its incidence has increased rapidly in the past few decades. In this study, we investigated a novel treatment approach, the use of low-intensity ultrasound (2.3 W/cm^2 at 1 MHz)-mediated Optison microbubble (MB) destruction (UMMD) to treat melanoma in a flank tumor model. The effect of UMMD was first evaluated in the melanoma cell line B16 F10 (B16) *in vitro* and then in mice inoculated with B16 cells. MB$^+$B16 cells were exposed to US *in vitro,* resulting in significant cell death proportional to duty cycle ($R^2 = 0.74$): approximately 30%, 50%, 80% and 80% cell death at 10%, 30%, 50% and 100% DC respectively. Direct implantation of tumors with MBs, followed by sonication, resulted in retarded tumor growth and improved survival ($p = 0.0106$). Immunohistochemical analyses confirmed the significant changes in expression of the cell proliferation marker Ki67 ($p = 0.037$) and a microtubule-associated protein 2 ($p = 0.048$) after US + MB treatment. These results suggest that UMMD could be used as a possible treatment approach in isolated melanoma and has the potential to translate to clinical trials. (E-mail: kee.jang@nih.gov)

Key Words: **Low-intensity ultrasound, Microbubbles, Ultrasound-mediated microbubble destruction, Melanoma, Optison microbubbles.**

INTRODUCTION

Cutaneous melanoma is the sixth most common cancer in the United States (Miller et al. 2016; Siegel et al. 2016). The incidence of cutaneous melanoma has increased in past decades and is associated with ultraviolet radiation exposure that may result in genetic mutation in the melanocytes in the basal layer of the epidermis (Lazovich et al. 2010; Parkin et al. 2011). Melanoma can be treated by surgical excision, immunotherapy and chemotherapy; however, potential morbidity and adverse events are associated with these approaches (Lee et al. 1995; Rosenberg et al. 1994; Weber et al. 2012). Although wide surgical excision when possible is currently used to treat isolated melanoma lesions, it would be advantageous to have alternative or additional therapeutic approaches to effectively eradicate or limit tumor progression.

Low-intensity ultrasound (US), typically <5 W/cm^2 (Wood and Sehgal 2015; Xin et al. 2016), has been used as a diagnostic and therapeutic modality and, when coupled with an infusion of US contrast agent-biocompatible gas-filled microbubbles (MBs), can increase image contrast in lesion detection or, when coupled with therapeutic or focused ultrasound, can be used to enhance drug or gene delivery (Blomley et al. 2001; Miwa et al. 2012; Timbie et al. 2015). The acoustic response of MBs is highly dependent on the level of US pressure as well as the size, stability, diffusion and surface tension of MBs (Chen et al. 2013; Emmer et al. 2009). Recent studies have found that the mechanical effects of dynamic interactions between US and MBs can be utilized for tumor treatment (Hernot and Klibanov 2008; Liu et al. 2014; Pu et al. 2014). MBs oscillate linearly by changing their size and shape in a manner that is inversely proportional to the US pressure amplitude when exposed to low peak negative pressure (PNP) (*i.e.,*

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mechanical index [MI] < 0.05). MBs exposed to high PNP undergo inertial oscillating non-linearly that can lead to complete destruction. MB fragmentation occurs secondary to inertial cavitation that produces mechanical shock waves, elevates local microenvironment temperature and can induce free radical formation (Chomas et al. 2000, 2001; de Jong et al. 2009; Hernot and Klibanov 2008). For these reasons, the behavior of non-linear MB oscillation has been extensively investigated for tumor treatment (Carson et al. 2012; Chen and Hwang 2013; Feril et al. 2003; Hassan et al. 2009). However, few studies have investigated the effect on tumor treatment of MBs directly injected into tumors for targeted drug delivery with applied US-mediated MB destruction (UMMD) (Sonoda et al. 2007; Watanabe et al. 2008). In addition, the direct injection of MBs alone into tumors with the intent of increasing cell kill after US exposure has not been thoroughly evaluated.

The purpose of this study was to test the hypothesis that UMMD in melanoma flank tumors would suppress their growth. We evaluated the effects of duty cycle (DC)-modulated US on MB destruction and on inducing tumor cell death *in vitro* and evaluated the efficacy of UMMD in the treatment of melanoma in a mouse model.

METHODS

Low-intensity ultrasound system

A versatile sweep function generator (BK Precision, Yorba Linda, CA, USA) was used to generate 1-MHz sinusoidal continuous or pulsed (pulse repetition frequency [PRF] fixed at 1 KHz) waveforms. Input pulses (pulse length = 1 μs) with the modulation of DC at 0%, 1%, 10%, 30%, 50% and 100% (total on time was 0, 10, 100, 300, 500 and 1000 ms/s, respectively) were generated by adjusting the number of cycles per US burst (total number of cycles: 0, 1×10^4, 1×10^5, 3×10^5, 5×10^5 and 1×10^6 cycles, respectively). DCs of 0% and 100% are equivalent to no US (control) and continuous US, respectively. The pulses were amplified with a radiofrequency power amplifier (Electronic Navigation Industries, Rochester, NY, USA) and transferred to an ultrasonic transducer (unfocused linear transducer 3 cm in outer diameter, center frequency at 1 MHz; Ultrasonic S-Lab, Concord, CA, USA). The output power of US was determined by radiation force balance (RFB) technique and was regarded as spatially averaged and temporally averaged (SATA) (Preston 1986a, 1986b; Zeqiri and Bickley 2000). US intensity at 0%, 10%, 50% and 100% DC was determined as 0, 0.03, 0.15 and 0.27 W/cm^2, respectively.

In vitro MB destruction by US

Two hundred microliters of culture medium containing 10% (v/v) biologically safe MBs (Optison, GE Healthcare, Princeton, NJ, USA) was prepared and exposed to DC-modulated US at 0, 1%, 10%, 30%, 50% and 100% for 10 s. The entire volume of cells/MB suspension was exposed to US that traveled through degassed water. Immediately after US exposure, the MBs remaining in suspension were counted using a hemocytometer and light microscope.

In vitro cytotoxicity by UMMD

The melanoma cell line B16 F10 (B16) was purchased from ATCC (Manassas, VA, USA) and cultured in 90% Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES, Gibco), 50 μg/mL gentamycin (Cellgro, Manassas, VA, USA), 1 mM GlutaMAX (Gibco) and 1 mM sodium pyruvate (Gibco) at 37°C and 5% CO_2 (Overwijk and Restifo 2001). For *in vitro* cytotoxic evaluation of UMMD, intermediate cells/MB suspension (0.2×10^6 cells/mL containing 10% v/v Optison MBs) was prepared by mixing Optison MBs with B16 cells by gently pipetting up and down several times. The 200 μL of cells/MB suspension was then transferred into a polymerase chain reaction (PCR) tube (BR781326, Sigma), and the tube was placed at the bottom of a sterile specimen container (Medsupply Partners, Atlanta, GA, USA) filled with degassed water. When moving the PCR tube from an upright to a horizontal position, there was no cell/MB suspension movement as a result of the surface tension, suggesting that there was no potential interference of ultrasound propagation between air bubbles and cells/MB suspension. A 1-MHz unfocused water-immersible transducer with 30-mm outer diameter was placed at a 1-cm distance from the near-field zone; the effective area of the transducer was large enough to cover the entire volume of cells/MBs. The cells/MBs were exposed to DC-modulated US for 10 s using (n = 3–9 per group). This procedure was performed as quickly as possible before the cells were separated from MBs, which tend to float. After sonication, cells in suspension were labeled with 1 μM Calcein-AM, a live cell indicator (Invitrogen Life Technologies, Waltham, MA, USA), and 1 μM ethidium homodimer-2 (EthD-2), a dead cell indicator (Invitrogen Life Technologies), and were counted with a hemocytometer using a fluorescence microscope (BX60, Olympus, Center Valley, PA, USA). Cell viability was determined as the percentage of live cells to the total cells.

In vivo *UMMD treatment*

All animal experimental procedures were approved and performed according to the guidelines established by the Institutional Animal Care and Use Committee (IACUC) at the University of Iowa. Eight-week-old female C57BL/6J mice (n = 30) were purchased from Harlan Laboratories