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Research Paper

Intracellular Delivery of Bak BH3 Peptide by Microbubble-Enhanced Ultrasound

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Purpose. To investigate the possibility of intracellular delivery of Bak BH3 peptide using sonoporation effect by microbubble-enhanced ultrasound.
Methods. HeLa and BJAB cells were exposed to 1.696-Mhz focused ultrasound with 2% microbubble contrast agents (OPTISON®). Cell-impermeable calcein was used as an indicator for successful sonoporation, and propidium iodide staining was used for cell viability assessment. Peptides were also exposed to ultrasound with OPTISON® and analyzed with mass spectrometry for evaluation of stability under ultrasound exposure. The effect of transduced Bak BH3 peptide was evaluated by the cell viability of successfully sonoporated cells.
Results. Bak BH3 peptides did not undergo mechanical degradation with microbubble-enhanced ultrasound exposure. With the increase of acoustic energy exposure, the sonoporation efficiency saturated both in BJAB and HeLa cells, while direct cell death rate by ultrasound exposure tended to increase. When BJAB cells were treated with 100 μM Bak BH3 peptides, and ultrasound exposure with ultrasound contrast agents (OPTISON®), an increased 35% cell death was confirmed. On the other hand, although HeLa cells had a similar trend, they failed to exhibit statistical significance.
Conclusions. Our results suggest that microbubble-enhanced focused ultrasound peptide transduction is possible. Further optimization of ultrasound exposure conditions may be necessary.

KEY WORDS: BH3 peptide; focused ultrasound; microbubble ultrasound contrast agent; OPTISON®; peptide transduction; sonoporation.

INTRODUCTION

Emerging knowledge suggests that bioactive peptides such as BH3 peptides, the death domain of the Bcl-2 family of proteins, may be promising candidates as anticancer agents because of their pro-apoptotic activities (1–5). As these peptides function in the intracellular component of the cell, they should be delivered into the cells to exert their function. In previous reports, technologies such as microinjection (3) or synthesis of cell-permeable BH3 peptides using protein-transduction domain (PTD) (2,4,5) have been used for intracellular delivery of peptides, and it has been shown that BH3 peptides can exert their cell-killing activity once delivered into the intracellular component of the cell. However, for successful treatment using these bioactive peptides, a means for delivering them into cells in a site-specific fashion is necessary, which neither of the previously mentioned technologies can achieve. Although luteinizing hormone releasing hormone (LHRH) receptor–targeted BH3 peptide transduction is one promising technology (6,7), using this technique is limited only to LHRH receptor expressing tumor cells. On the other hand, with the advancement of focused ultrasound technology and microbubble ultrasound contrast agent, it is now possible to use image guidance to site-specifically sonoporate and deliver macromolecules such as plasmid DNA into the intracellular component of the cell (8–10). In this investigation, we will demonstrate that intracellular delivery of Bak BH3 peptide is also possible using microbubble-enhanced focused ultrasound and that these peptides can exert their cell-killing function inside the cell, suggesting that microbubble-enhanced focused ultrasound peptide transduction may be a useful technology for future cancer treatment.

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ABBREVIATIONS: ATCC, American Type Culture Collection; BH3/4, Bcl-2 homology 3/4; CW, continuous wave; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HIV, human immunodeficiency virus; HPLC, high-pressure liquid chromatography; LHRH, luteinizing hormone releasing hormone; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; PI, propidium iodide; PTD, protein-transduction domain; PW, pulse wave; RF signals, radiofrequency signals.

MATERIALS AND METHODS

Cell Lines and Culture

HeLa cells were obtained from the American Type Culture Collection (ATCC). BJAB cells, a human B-cell lymphoma cell line, were kindly provided by Dr. Fred Wang of Harvard Medical School. HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA), BJAB cells in RMPI-1640 (Invitrogen) at

37°C with 5% CO_2. Both media were supplemented with 10% fetal bovine serum (FBS; Invitrogen).

Synthesis of Bak BH3 Peptides

Peptides were synthesized in the Molecular Biology Core Facility of the Dana-Farber Cancer Institute (Boston, MA, USA) using a peptide synthesizer (model 433A peptide synthesizer; Applied Biosystems, Foster City, CA, USA). The purity of each peptide was determined to be >97% by reverse-phase C18 column high-pressure liquid chromatography (HPLC) (model 218TP1010; Grace Vydac, Columbia, MD, USA). The molecular weight of the peptides was determined by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry (Voyager-DE STR; Applied Biosystems), and the amino acid sequence was analyzed using an amino acid analyzer (model 420A amino acid analyzer; Applied Biosystems). The synthesized peptides were 19-amino-acid Bak BH3 peptides (residues 71-89; MGQVGRQLAIIGDDINRRY); they were resuspended in DMSO (Fisher Scientific, Hampton, NH, USA) at 5 mM and stored at –20°C until use.

Sonication Device Setup

Detail of the experiment setup is shown in Fig. 1. A thin plastic membrane was placed on the top of the 48-well plates filled with cell or peptide suspension, and all of the air bubbles were eliminated out of the well. For sonication of cells or peptides, we used a 1.696-MHz frequency, in-house manufactured, spherical focused ultrasound transducer (designed by K.H.), which has a diameter of 10 cm and a radius of curvature of 8 cm. All through the experiment, the same transducer was used. The transducer's efficiency was equal to approximately 55% and was used to convert the electrical power measured to acoustical power. The radio-frequency (RF) signals to drive the ultrasound transducer was generated by a pulsed output ultrasound driving system (model UDS 04PF-CSA; Advanced Surgical Systems, Inc., Tuscon, AZ, USA). Both pulse wave (PW; 50-ms pulse length, 2-Hz pulse frequency) and continuous wave (CW) sonication with an acoustical power of 2.75 W were used depending on the experiments. Exposure time ranged from 5 to 200 s in pulse wave sonication and 2 to 20 s in CW sonication. The ultrasound beam was focused in the suspension of experimental materials and then propagated through the membrane into the water on the other side of the membrane. A rubber plate as an absorbent material was placed in the water 5 cm above the cell suspension. The water used in both the upper and lower chamber was degassed and heated up to 37°C.

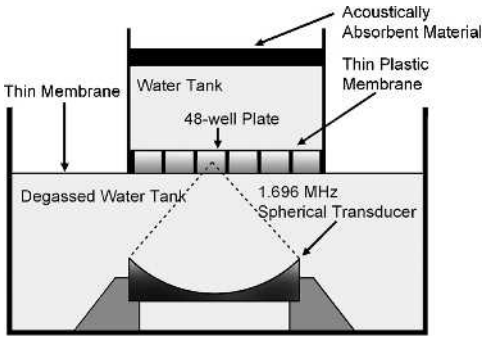

Fig. 1. Experimental setup for cell sonication using focused ultrasound. A 48-well plate was placed in a device designed to minimize ultrasound reflection or scattering. Focused ultrasound (1.696-MHz center frequency, acoustical power of 2.75 W) was delivered vertically to the cells from the bottom through a water tank.

Mass Spectrometry of Peptides Exposed to Ultrasound with Ultrasound Contrast Agent OPTISON

Peptides were suspended in distilled water (final concentration 100 μM) containing 2% OPTISON, which is a microbubble ultrasound contrast agent available from Amersham Health Inc. (Princeton, NJ, USA). Experimental samples were sonicated using the experimental setup described above. The sonication conditions were 10-s CW sonication with an acoustical power of 2.75 W. Peptide degradation was evaluated by measuring the molecular weight of the possibly degraded products using a MALDI-TOF mass spectrometry (Voyager-DE STR; Applied Biosystems).

Sonication of Cells

HeLa- and BJAB cells (10^6 cells/ml), suspended in their respective culture medium containing 10% FBS and 2% OPTISON, were transferred to 48-well plates, and each well was filled to the top of the wells. The total amount in each well was approximately 1.5 ml. Cell-impermeable calcein (Sigma-Aldrich, St. Louis, MO, USA) was added to the cell suspension; the final concentration was 100 μM for HeLa and 37.5 μM for BJAB cells. In the peptide transduction experiments, we added Bak BH3 peptides (final concentration 100 μM for both cell lines). For the control, equivalent amount of DMSO was added. After sonication, the cells were kept for 15 min at 37°C with 5% CO_2 to allow recovery and then washed 3 times with Hanks buffer (Mediatech, Inc., Herndon, VA, USA). They were then either resuspened in DMEM or RPMI-1640 with 10% FBS and 50 U/ml penicillin (Invitrogen) or stained with propidium iodide (PI; Sigma-Aldrich) for examination of cell viability.

Cell Viability Assay

To determine cell "sonoporation" and viability, we used calcein and PI staining. As cells were sonicated in the presence of cell-impermeable calcein, upon excitation with blue light, successfully sonoporated viable cells contained green calcein-derived fluorescence, whereas nonviable cells contained red PI-derived fluorescence. Using a fluorescent microscope (model IM; Olympus, Tokyo, Japan), we recorded calcein-positive and PI-negative cells as viable cells that had taken up the peptides. At the indicated times, the ratio of calcein-positive and PI-negative cells in the whole cell population was determined by counting at least 82 randomly selected cells (average 239 cells).

Statistical Evaluation

Statistical evaluation was performed by the Student's *t* test using Microsoft Excel analytical tools, and a p value of less than 0.05 was considered statistically significant.

RESULTS

Bak BH3 Peptides Are Stable Under Microbubble-Enhanced Ultrasound Exposure

As there was a possibility of peptides degradation upon microbubble-enhanced ultrasound expose, we first evaluated the stability of Bak BH3 peptides under ultrasound exposure. As shown in Fig. 2B, even when the Bak BH3 peptides were exposed to ultrasound with microbubble contrast agent at the extreme settings for cell sonoporation (2.75 W acoustical power, 10-s CW exposure), no degraded product was observed compared to control (Fig. 2A) by mass spectrometric analysis, and the Bak BH3 peptides were still intact (Figs. 2A and 2B, arrows).

Sonoporation Efficiency and Bioeffects of Microbubble-Enhanced Ultrasound Exposure Are Both Energy and Cell-Type Dependent

Next we looked for the optimal parameters for sonoporation. We used cell membrane–impermeable calcein and PI-staining to evaluate sonoporation efficiency and cell viability immediately after microbubble-enhanced ultrasound exposure. In BJAB cells, sonoporation efficiency was saturated at around 20% at 50-s PW exposure; the ratio of nonviable cells continued to increase with increasing exposure time. We found that 50-s PW exposure had an effect on sonoporation efficiency and cell-killing that was similar to the effect of the equivalent energy-dose applied in 5-s CW exposure (Fig. 3A). In HeLa cells, the saturation of sonoporation efficiency was as low as 10%, and the ratio of nonviable cells was much higher than in BJAB cells (Fig. 3B). At 3 h postsonication, successfully sonoporated viable HeLa cells attached to the culture dish and contained green calcein fluorescence. The presence of some nonviable cells containing green fluorescence suggests delayed cell death due to ultrasound exposure (Figs. 3C and 3D). Based on these results, we postulate that the sonoporation efficiency and bioeffects of microbubble-enhanced ultrasound exposure depend both on the cell type and the total energy applied to the cells.

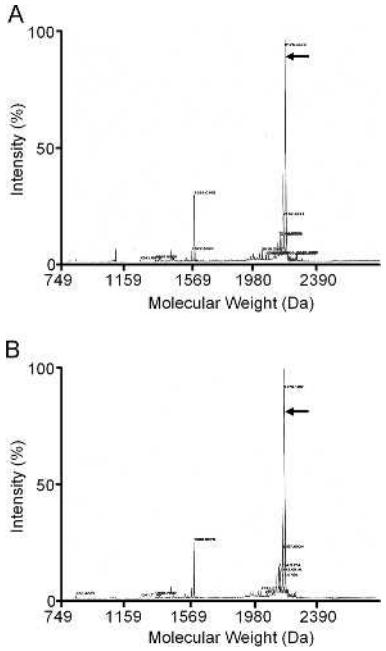

Fig. 2. Bak BH3 peptides are stable under microbubble-enhanced focused ultrasound exposure. Bak BH3 peptides treated with (B) and without (A) focused ultrasound (continuous wave, 10 s) exposure and 2% OPTISON were analyzed by mass spectrometry. No degraded products were detected. The peak at a molecular weight of 2175 is the intact Bak BH3 peptide (arrows).

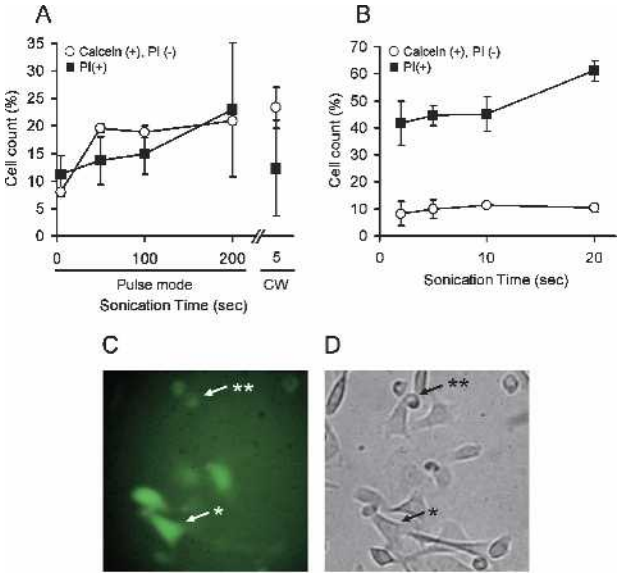

Fig. 3. Sonoporation and cell-killing effect of focused ultrasound with 2% OPTISON. (A) Sonoporation efficiency and cell-killing rate of BJAB cells by microbubble-enhanced focused ultrasound exposure. BJAB cells were sonicated with 2% OPTISON and calcein. Sonications were performed both in pulse wave (50-ms pulse length, 2-Hz pulse frequency) and continuous wave (CW) for the indicated time. Sonoporation efficiency and cell viability were assayed under a fluorescent microscope using PI staining. Open circles, sonoporated viable cells; filled squares, nonviable cells. Data are expressed as the mean ± SD of 3 independent experiments. (B) Sonoporation efficiency and cell-killing rate of HeLa cells by microbubble-enhanced focused ultrasound exposure. HeLa cells were sonicated in the presence of 2% OPTISON and calcein. Sonications were performed with continuous wave for the indicated time. Sonoporation efficiency and cell viability were assayed under a fluorescent microscope using PI staining. Open circles, sonoporated viable cells; filled squares, nonviable cells. Data are expressed as the mean ± SD of 3 independent experiments. (C and D) Successful sonoporation of HeLa cells. After sonication of HeLa cells in the presence of calcein, cells were washed, cultured for 3 h in DMEM containing 10% FBS, and inspected under an inverted fluorescent microscope. Both fluorescent (C) and phase-contrast (D) images are presented. Upon excitation with blue light, successfully sonoporated cells emit green fluorescence deriving from calcein (asterisk). Sonoporated, nonviable cells are indicated by double asterisks.

Bak BH3 Peptides Transduced by Microbubble-Enhanced Ultrasound Exposure Can Exert Cell-Killing Function in BJAB Cells

We investigated the effect of Bak BH3 peptides transduced into the cells by microbubble-enhanced ultrasound exposure. The viability of successfully sonoporated BJAB cells was measured 0.5, 4, and 8 h after PW (50-ms pulse length, 2-Hz pulse frequency, 100-s exposure) and CW sonication

(5-s exposure) with and without Bak BH3 peptides. Under both conditions, at 8 h postsonication, a viability loss of approximately 30% was observed in the peptide-untreated control group. This confirms the occurrence of delayed cell death after sonoporation. In the presence of Bak BH3 peptides, the viability loss increased to approximately 65% (Figs. 4A–4D). As Bak BH3 peptides had no effect on BJAB cells when added to the culture medium without any treatment (data not shown), it was suggested that this additive 35% increase in BJAB cell death was attributable to peptide-transduction into the cells. On the other hand, although cell death tended to increase in BH3 peptide–exposed HeLa cells at 6 h postsonication, the increase was not statistically significant (Fig. 4E). This suggests that HeLa cells are more resistant than BJAB cells to transduced Bak BH3 peptides, a result consistent with findings made with the microinjection technique (2).

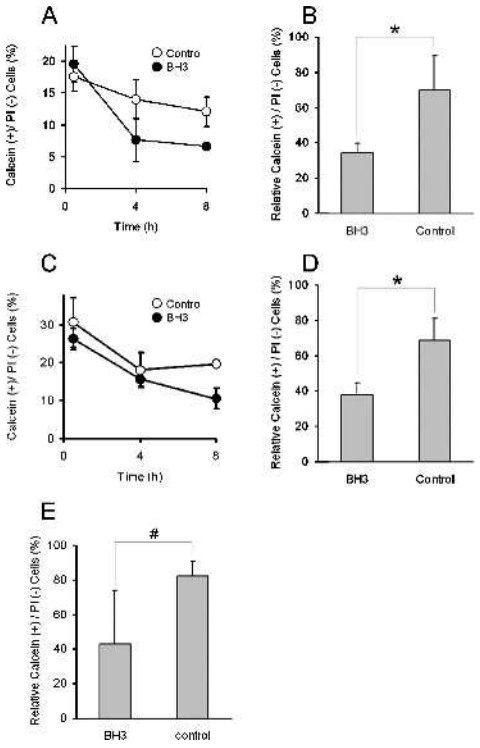

Fig. 4. Bak BH3 peptides transduced by microbubble-enhanced focused ultrasound exposure can exert cell killing function. (A to D) Enhanced cell-killing by Bak BH3 peptides of BJAB cells. The cells were sonoporated in the presence of 100 μM Bak BH3 peptides by 100-s microbubble-enhanced pulse-wave (A and B) or 5-s continuous-wave focused ultrasound exposure (C and D). Viable calcein-positive cells were counted at the indicated times (A and C). The relative ratio of calcein-positive viable cells at 8 h postsonication was calculated by dividing the ratio of calcein-positive viable cells at 8 h by the ratio obtained at 0.5 h postsonication in the same experiment (B and D). Cell death was enhanced by transduced Bak BH3 peptides under pulse-wave ($p = 0.04$) and continuous-wave ($p < 0.01$) sonication conditions. (E) HeLa cells are resistant to Bak BH3 peptides transduced by microbubble-enhanced focused ultrasound exposure. The cells were sonoporated in the presence of 100 μM Bak BH3 peptides by 10-s microbubble-enhanced continuous-wave focused ultrasound exposure. The relative ratio of calcein-positive viable cells at 6 h postsonication was calculated by dividing the ratio of calcein-positive viable cells at 6 h postsonication by the ratio obtained at 0.5 h postsonication in the same experiment. The Bak BH3 peptides failed to exhibit HeLa cell-killing effect ($p = 0.09$). All data are expressed as the mean ± SD of 3 (A, B, and C) or 4 (D and E) independent experiments.

DISCUSSION

Bioactive peptides are one of the promising therapeutic agents for cancer and other diseases treatment. During the past, peptides deriving from the BH3 and BH4 domain of the Bcl-2 family of proteins have been spotted for potential therapeutic agents in a variety of diseases including cancer therapy because of their pro- and anti-apoptotic activity (1–5,11–13). These peptides were delivered into cells using the protein-transduction domain (PTD) of *Drosophila* Antennapedia or HIV-1 TAT protein (14,15). By coupling these PTDs at the N-terminus of either BH3 or BH4 peptides, these cell-permeable peptides were transduced into cells where they exerted their pro- or anti-apoptotic activity both *in vitro* and *in vivo* (2,4,5,12,13). In addition, hydrocarbon-stapling of the BH3 helix has recently been shown to render the peptide cell-permeable (16). However, attempts to use cell membrane–permeable BH3 peptides in *in vivo* cancer therapy raise the problem of site specificity because if administered systemically, they are taken up by organs outside the treatment target. Although there are reports that show LHRH receptors can be a target for selective BH3 peptide transduction, this technology is limited to LHRH receptor expressing cells (6,7).

Advances in ultrasound and microbubble ultrasound contrast agent technologies have made it possible to use ultrasound not only for diagnostic but also therapeutic purposes. When a focused ultrasound technique is used, the ultrasound energy is concentrated to within a few millimeters around the focal point of the beam, and surrounding tissue is spared. It is now possible to thermocoagulate a specific area of tissue (17) and even to disrupt the blood-brain barrier locally through the intact skull using microbubble ultrasound contrast agents and focused ultrasound (18,19). Furthermore, it has been shown that microbubble-enhanced ultrasound can change the permeability of the cell membrane for a short time by sonoporation (20–22). It has been reported that intracellular delivery of extracellular molecules achieved thermodynamic equilibrium with the extracellular solution regardless of their molecular size (23). Cavitation energy created by the collapse of the bubbles is believed to be the key mechanism of sonoporation (21), and this technique has been applied for site-specific intracellular delivery of macromolecules such as dextran or naked plasmid DNA both *in vitro* and *in vivo* (8–10,23). It has been reported that this transient permeability change of the cell membrane lasts a few seconds postsonication (22).

We investigated the possibility of using this novel technique for the intracellular delivery of bioactive BH3 peptides. We evaluated whether these peptides, transduced by microbubble-enhanced ultrasound into BJAB and HeLa cells, exert their original function inside these cells. Taking into consideration future use of this technology *in vivo*, we used a "focused" ultrasound transducer to test the feasibility of microbubble-enhanced peptide transduction. As our mass spectrometry results indicated that the Bak BH3 peptide was stable under conditions of microbubble-enhanced ultrasound exposure, we conclude that these peptides can be delivered inside the cell without undergoing mechanical degradation.

Next, when we investigated the sonoporation rate and the direct cell death effect caused by microbubble-enhanced ultrasound both in BJAB and HeLa cells, sonoporation efficiency saturated while direct cell-killing effect tented to increase. These data are quite similar to previous reports (21,24). When BJAB cells were sonicated in the presence of Bak BH3 peptides, the peptides were transduced by microbubble-enhanced ultrasound exposure and exhibited cell-killing effects. However, in HeLa cells the transduced BH3 peptides showed a weaker cell-killing performance, possibly because HeLa cells are more resistant than BJAB cells to injected BH3 peptides. Holinger *et al.* reported that HeLa cells were not affected by microinjected BH3 peptides, although these peptides performed cell-killing functions when coupled to the *Drosophila* Antennapedia PTD (2). Our findings suggest that it may be possible to apply microbubble-enhanced focused ultrasound exposure as a new technique for the localized, site-specific transduction of bioactive peptides. As both the sonoporation efficiency and the bioeffects of microbubble-enhanced focused ultrasound exposure appear to be cell-type dependent, the eventual use of this technology necessitates the optimization of sonication parameters for individual cell and tissue types.

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