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Activation of Bak in ultrasound-induced, JNK- and p38-independent apoptosis and its inhibition by Bcl-2

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Abstract

The molecular mechanisms underlying ultrasound-induced apoptosis remain poorly understood. We have demonstrated that in Jurkat cells, the over-expression of the anti-apoptotic protein Bcl-2 inhibited ultrasound-induced apoptosis, but not necrosis. Inhibition of caspase activity also protected the cells from apoptosis, but not from necrosis, showing the involvement of different mechanisms in ultrasound-induced apoptosis and necrosis. Bak, a pro-apoptotic member of the Bcl-2 family proteins, was activated by ultrasound and its activation was completely inhibited by Bcl-2 over-expression, but not by caspase inhibition. Antioxidant N-acetyl cysteine did not protect the cells from ultrasound-induced apoptosis or necrosis, nor did the inhibition of either c-Jun N-terminal kinase or p38, key factors in the radical oxygen species (ROS)-mediated cell stress response, suggesting that ROS do not play a crucial role in ultrasound-induced apoptosis. Our results confirm that ultrasound induces apoptosis via a pathway that involves Bak, Bcl-2, and caspases, but not ROS.

Introduction

Because ultrasound has found diagnostic and therapeutic applications, its bioeffects are of great interest in the biological and medical area. Therapeutic applications include thermal coagulation of tissues [1,2], vascular occlusions, and thrombolysis under MRI or diagnostic ultrasound image-guidance. The development of microbubble-based ultrasound contrast agents facilitates blood brain barrier disruption [3-5] and ultrasound-induced sonoporation, a transient change in cell-membrane permeability, making it possible to introduce exogenous substances into the cell *in vitro* and *in vivo* [6-8]. This method represents a powerful alternative to conventional techniques used for the transfection of cells with DNAs or siRNAs [7,8].

It has also become evident that ultrasound can cause cell death by an apoptotic mechanism as well as cell lysis or necrotic cell death both *in vitro* [9-18] and *in vivo* [5,19]. The triggers and the molecular pathway to lead each cell death mechanism are not well elucidated, and should be clarified for overall understanding of the ultrasound-induced cell death.

Apoptosis of other systems is known to be finely controlled by endogenous key regulators that determine whether the cell lives or dies [20]. As apoptotic cell death triggers a lower level of

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inflammatory response than necrotic cell death [20], its induction by ultrasound is of interest in patients with malignancies in a closed organ, e.g. the brain, for reducing the potential for complications caused by tissue swelling. As induction of apoptosis by ultrasound is an appealing technique, detailed mechanism of ultrasound induced apoptosis should be elucidated to achieve better control.

Bcl-2 family proteins play an important role during apoptosis induction. They consist of two major groups; one includes anti-apoptotic members such as Bcl-2 and Bcl-x_L, the other, pro-apoptotic members such as Bak and Bax. Many apoptotic stimuli induce the activation of Bak and/or Bax that accompanies conformational changes at their N-terminal regions; the anti-apoptotic activity of Bcl-2 prevents their activation [21-25]. Activated Bak and/or Bax induce mitochondrial events, including the loss of the mitochondrial membrane potential and the release of the apoptogenic factors, such as cytochrome *c* [22-26], which activates caspases, cysteine proteases specifically involved in apoptosis [26]. As ultrasound has been suggested to generate reactive oxygen species (ROS) [27], ROS are thought to be one of the key factors for the initiation of apoptosis by ultrasound [10,15]. In other apoptotic systems, ROS reportedly activate ASK-1, which subsequently activates c-Jun N-terminal kinase (JNK) and p38, key molecules in the ROS-mediated cellular response [28]. The activation of JNK and p38 can result in Bak/Bax activation [29].

In the current study we specifically attempted to elucidate the molecular mechanisms involved in ultrasound-induced apoptosis. We focused on Bcl-2, Bak, and caspases, key factors in the regulation of apoptotic cell death, and also investigated whether ROS, JNK, and p38 are involved in ultrasound-induced apoptosis.

Materials and Methods

Chemicals

The p38 inhibitor SB203580, and anti-Bak (Ab-1) antibody (TC-100) that recognizes the exposed N-terminus of the active Bak protein were from Calbiochem (Merck KGaA, Darmstadt, Germany). Alexa-488-conjugated anti-mouse IgG secondary antibody and the annexin V/propidium iodide apoptosis assay kit (Vybrant™ Apoptosis Assay Kit #2) were from Molecular Probes/Invitrogen (Carlsbad, CA). N-acetyl-cysteine (NAC), cell membrane permeable pan-caspase inhibitor z-VAD-fmk, and JNK inhibitor SP600125 were from Sigma-Aldrich (St. Louis, MO). The CellTiter-Blue (CTB) cell viability assay system was from Promega (Madison, WI).

Cell lines and culture

Jurkat human T-cell leukemia cells transfected with pCAGGS-hBcl-2 to over-express the human *bcl-2* gene (HB10), and those transfected with an empty vector pCAGGS (V2) were described previously [30]. The cells were maintained in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen) with 5% CO₂ at 37°C.

Sonication device setup

Details of the experimental setup are shown in Fig. 1A. A thin plastic membrane was placed on the top of the polystyrene 48-well plates (catalog number 353230, BD Falcon, Bedford, MA) filled with cell suspension and all of the air bubbles were eliminated out of the well. For sonication of cells, we used a 1.653 MHz frequency in-house manufactured spherical focused ultrasound transducer, which has a diameter of 10 cm and a radius of curvature of 8 cm. The transducer parameters are close to those used for clinical MRI guided focused ultrasound surgery trials [31]. The radiofrequency (RF) signals to drive the ultrasound transducers were generated by a pulsed-output ultrasound driving system (model UDS 04PF-CSA, Advanced

Surgical Systems, Inc., Tucson, AZ). 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. In this setting, standing wave is almost completely eliminated. A rubber plate, serving as an absorber material was placed in the water 5 cm above the cell suspension. The water in both the upper and lower chamber was degassed and heated to 37 °C. Pulse wave sonication with an acoustic power of 0 to 19.25 W was used depending on the experiments. With this power range, 40 ~ 80 % cell viability was obtained right after sonication. The sonication parameters were 2 Hz repetition frequency, 10% duty cycle with 100 seconds exposure.

Induction of cell death with microbubble enhanced ultrasound

Cells (1×10^6 /ml) were suspended in fresh FBS-supplemented medium. OPTISON®, a microbubble ultrasound contrast agent (Amersham Health Inc. Princeton, NJ) was added to the cell suspensions at a final concentration of 0.1%. Sonicated cells were cultured for the time indicated (2~6 hr) for further analysis. In experiments using inhibitors (SP600125, SB203580, NAC, and z-VAD-fmk), the cells were incubated for 1 hr with the inhibitors prior to sonication and through out the experiments.

Cell death assay

To measure the viability of sonicated cells, we used CellTiter-Blue assay or the annexin V/propidium iodide (PI) assay. Both assays were carried out according to the supplier's instructions. In the CellTiter-Blue assay, cell viability was estimated by measuring the amount of reduced resorufin by its fluorescent intensity (ex. 560 nm, em. 590 nm) using a fluorescent microplate reader (SPECTRAMax GEMINI XS, Sunnyvale, CA). In the annexin V/PI assay, cells were recovered at 6 hr post-sonication, washed with phosphate-buffered saline (PBS), and were incubated with an Alexa-488-conjugated annexin V and PI mixture for 15 min.

Detection of N-terminus exposed active Bak

Sonicated cells were recovered 3 hr post-sonication, fixed for 5 min at room temperature (RT) with 0.25% paraformaldehyde in PBS, washed twice with PBS, and then incubated with a solution comprised of anti-Bak antibody (dilution 1:50)/digitonin (100 µg/ml)/PBS at RT for 30 min. They were washed twice with PBS, incubated with Alexa-488-conjugated goat anti-mouse IgG antibody (5 µg/ml in PBS) at RT for 30 min, washed with PBS, and then analyzed on a FACScan flow cytometer (Becton Dickinson). Positive cells were detected on the FL-1 channel.

Results and Discussions

Since the first discovery of ultrasound being capable of inducing apoptosis [5,9-19], the exact mechanisms of apoptosis caused by ultrasound are unknown and only a few documentations can be found challenging this issue. The involvement of Ca^{2+} , free radicals [15], cell membrane damage [12,13] and DNA damage with p53 activation [10] have been proposed for the mechanisms of apoptosis induced by ultrasound. Some have speculated that direct destruction or disturbance of the mitochondrial membrane upon ultrasound exposure can result in apoptotic cell death [10,15].

We first investigated whether the over-expression of Bcl-2, an anti-apoptotic protein, can inhibit ultrasound-induced cell death. When cells are exposed to ultrasound, some portion of the cells will instantly be killed and lysed (cell lysis) while the rest of the cells will survive this initial traumatic event (Fig. 1B). Cell viability right after sonication assessed by CellTiter-Blue assay decreased as the ultrasound energy increased regardless of the existence of Bcl-2 (Fig.

1C). Microscopic analysis showed that the number of cells stained by trypan-blue suggestive of cell lysis right after sonication was almost the same as the amount of non-viable cells determined by CellTiter-Blue assay (data not shown). These results suggest that instant cell lysis was not inhibited by Bcl-2 over-expression (Fig. 1C). After 6 hr-incubation, cell numbers counted by the flow cytometer also corresponded to those of cells that survived after the initial cell lysis (data not shown).

Next, we examined the action of over-expressed Bcl-2 on the delayed effect of ultrasound. When the fate of the cells which survived instant cell lysis was examined by annexin V/PI staining, vector control cells showed an early apoptotic or a necrotic change 6 hr after sonication (Figs. 1D, E). With sonication at 16.5 W, apoptosis involved about 20% of the vector cells (Fig. 1D), and necrosis about 15% (Fig. 1E). Over-expressed Bcl-2 reduced ultrasound-induced apoptosis to less than half of that observed in control experiments (Fig. 1D). On the other hand, ultrasound-induced necrosis was not significantly affected by the over-expression of Bcl-2 (Fig. 1E). These results suggest that different mechanisms are involved in ultrasound-induced apoptosis and necrosis.

Several apoptosis-inducing stimuli activate Bak and Bax, exposing their N-terminal domain [32], and Bcl-2 prevents their activation. At 3 hr post-sonication, approximately 30% of non-lysed cells exhibited Bak activation (Fig. 2A). In cells that over-expressed Bcl-2, Bak activation was almost completely inhibited (Fig. 2B). It has become obvious that Bcl-2 inhibits apoptotic cell death by canceling the activation of pro-apoptotic proteins such as Bax, Bak and Bad [22], or by interfering with the opening of the voltage-dependent anion channel (VDAC), the pore channel in the outer mitochondrial membrane [23-25]. As both of these processes are involved in the triggering of mitochondrial events in the course of apoptosis, our data suggest that ultrasound-induced apoptosis is not triggered by physical disruption of the mitochondria but rather by other physiological mechanisms. The activation of p53 upon ultrasound-induced DNA damage is a candidate for the mechanism(s) that underlies the activation of Bak or Bax [33]. However, as Jurkat cells are reported to express a negligible amount of p53 [34,35], it seems unlikely that in our system, p53 played a crucial role in the ultrasound-induced activation of Bak.

To examine whether caspases were involved in ultrasound-induced cell death, we analyzed the effects of the broad-spectrum caspase inhibitor z-VAD-fmk on ultrasound-induced apoptosis and necrosis, and on the exposure of the Bak N-terminus. While apoptosis was significantly inhibited in the presence of z-VAD-fmk, it had no effect on necrosis (Fig. 2C). Furthermore, z-VAD-fmk did not inhibit the exposure of the Bak N-terminus induced by ultrasound treatment (Fig. 2D). These results suggest that ultrasound-induced apoptosis is caspase-dependent, and that caspases function primarily downstream of the Bak activation step (Fig. 4). In addition, the fact that Bcl-2 over-expression (Fig. 1D, E) and z-VAD-fmk (Fig. 2C) inhibited apoptosis but not necrosis, confirms that different mechanisms are involved in the appearance of apoptotic and necrotic cell populations following ultrasound exposure (Fig. 4).

Finally, we examined another potential factor that could be involved in the observed ultrasound-induced cell death. Free radical production is associated with the occurrence of inertial cavitation (collapse of microbubbles) triggered by ultrasound exposure. The violent collapse of the microbubbles caused by inertial cavitation can lead water homolysis and the creation of hydrogen atoms ($\cdot\text{H}$) and hydroxyl radicals ($\cdot\text{OH}$). These primary radicals can recombine to produce hydrogen peroxide (H_2O_2) only by themselves or produce hydroperoxyl radicals ($\text{HO}_2\cdot$) in the presence of air, which can both be categorized as reactive oxygen species (ROS) [27]. It has also been reported that intercellular ROS production is one of the key factors for apoptosis induced by ultrasound [10,15]. The exposure of cells to N-acetyl-cysteine (NAC), a potent antioxidant, however, did not confer protection either from apoptosis or from necrosis

(Fig. 3A), although it completely inhibited cell death induced by hydrogen peroxide (Supplementary figure). This result is partly controversial with the findings by Honda et al [15], where NAC moderately inhibited the production of PI positive cell population and caspase-3 activity with a slight suppression of DNA fragmentation. However, they observed no inhibitory effect of NAC on annexin V(+)/PI(−) cell production, which is similar to our result. As JNK and p38 are known to be in the center of the ROS-mediated stress-response signal cascade [28], we used specific JNK and p38 inhibitors to test the hypothesis that ROS stress is one of the key factors in ultrasound-induced apoptosis regulation. We found that the inhibition of JNK or p38 did not confer protection on ultrasound-exposed cells from apoptotic cell death (Figs. 3B-D). According to Feril et al. [13], apoptosis can be induced at ultrasound-exposure conditions at which free radical ($\cdot\text{OH}$) is not produced. In combination, these findings suggest that free radical- and intracellular ROS production is not crucial for ultrasound-induced apoptosis or necrosis (Fig. 4).

Apoptosis induction is an appealing concept from an application perspective. Many non-apoptotic cells, however, are generated by ultrasound-induced apoptosis [11,13-15] and more extensive research on ultrasound-induced cell death is required. Parameters where apoptosis dominates cell death should be determined both *in vitro* and *in vivo* in order to make this technique applicable for clinical use.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

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Abbreviations

JNK, c-Jun N-terminal kinase; NAC, N-acetyl-cysteine; CTB, CellTiter-Blue™; PI, propidium iodide; ROS, radical oxygen species; VDAC, voltage dependent anion channel.

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Fig 1.

Experimental setup for cell sonication using focused ultrasound and cell viability after treatment.

A, A 48-well polystyrene plate was placed in a device designed to minimize ultrasound reflection or scattering. **B**, Schematic presentation of the fate of a cell after ultrasound exposure. Some portion of the cells exposed to ultrasound is instantly lysed and others will stay intact (instant effect of ultrasound). When the cells were able to survive this first traumatic event, they will stay intact or undergo apoptotic or necrotic cell death (delayed effect of ultrasound). **C**, The viability of Jurkat V2 (○) and Jurkat HB10 (●) cells was determined by CellTiter-Blue assay immediately after exposure to ultrasound at the indicated power. Data were expressed

as the mean $\pm$ SD of 3 independent experiments. Apoptosis (**D**) and necrosis (**E**) assays using Jurkat V2 ($\circ$) and Jurkat HB10 ($\bullet$) cells were performed 6 hr after exposure to ultrasound at the indicated power. The ratios of apoptotic (annexin V⁺/PI⁻ **D**) and necrotic (annexin V⁺/PI⁺ **E**) cell populations was plotted. Data were expressed as the mean $\pm$ SD of 3 independent experiments.

Fig 2.

Ultrasound-induced exposure of the Bak N-terminus, its inhibition by Bcl-2 and the effect of the pan-caspase inhibitor z-VAD-fmk on ultrasound-induced apoptosis, necrosis and Bak activation.

Bak activation was assessed by examining exposure of the N-terminus at 3 hr post-sonication. Jurkat V2 (**A**) and Jurkat HB10 (**B**) cells were exposed to ultrasound (16.5W). After 3-hr-incubation, they were stained with an antibody specifically recognizing the N-terminal region of Bak, and analyzed with a flow cytometer. Representative flow cytometric data are shown and the quantitative data are expressed as the mean $\pm$ SD of 3 independent experiments. **C**, Effect of z-VAD-fmk on ultrasound-induced apoptosis and necrosis. Jurkat V2 cells were

pretreated with 200 μ M z-VAD-fmk for 1 hr and then the same amount of OPTISON-mixed medium was added to achieve a final concentration of 0.1% OPTISON. Cells were then exposed to ultrasound (16.5 W), and apoptosis and necrosis assays were performed at 6 hr post-sonication. Data are the mean $\pm$ SD of 3 independent triplicate experiments (n=9, * p <0.001, Student's t -test). **D**, Effect of z-VAD-fmk on ultrasound-induced Bak activation. Jurkat V2 cells were treated as in **C**. Bak activation was assessed at 3 hr after sonication. Representative flow cytometric data are shown and similar results were obtained in 3 independent experiments (data not shown).

Fig 3.

Effect of antioxidant N-acetyl-cysteine (NAC), and JNK (SP600125) and/or p38 (SB203580) inhibitors on ultrasound-induced apoptosis and necrosis.

A, Jurkat V2 cells were pretreated for 1 hr with 10 mM NAC and sonicated (16.5 W) in the presence of 0.1% OPTISON. **B-D**, Jurkat V2 cells were pretreated for 1 hr with 10 μ M SP600125 (**B**), SB203580 (**C**) or with SP600125 and SB203580 (**D**) and sonicated (16.5 W) in the presence of 0.1% OPTISON. In control experiments, an identical amount of DMSO, used as a reagent solvent, was added. After 6-hr-incubation, apoptosis and necrosis were assessed by annexin V/PI assay. Data are the mean $\pm$ SD of 3 independent triplicate experiments (n=9).

Fig 4.

Diagram of the signaling pathways of ultrasound-induced cell lysis and death.

The signaling pathways of ultrasound-induced cell lysis and cell death, proposed by our experimental data are summarized. Ultrasound initiates the apoptotic or necrotic signal cascade by an unknown, JNK-, p38-, and ROS-independent stress source. The apoptotic signal initiates upstream of the Bak and caspase activation steps, which can be inhibited by Bcl-2 and caspase inhibitors, respectively.