Apoptosis Induction of Human Myeloid Leukemic Cells by Ultrasound Exposure

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ABSTRACT

Therapeutic ultrasound (ULS) and the resulting cavitation process has been shown to induce irreversible cell damage. In this study, we wanted to further investigate the mechanism of ULS-induced cell death and to determine whether apoptosis is involved. High intensity focused pulsed USL sonication at a frequency of 750 KHz was delivered to HL-60, K562, U937, and M1/2 leukemia cell line cultures. ULS exposure used with induction of transient cavitation in the focal area was delivered with an intensity level of 103.7 W/cm² and 54.6 W/cm² spatial-peak temporal-average intensity. As a control, ULS of lower intensity was delivered at 22.4 W/cm² spatial-peak temporal-average intensity, presumably without generation of cavitation. Our results indicated that DNA damage induced by ULS cavitation did not involve generation of free radicals in the culture media. Morphological alterations observed in cells after exposure to ULS included: cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, and apoptotic body formation. Apoptotic cells were evaluated by fluorescence microscopy and detected using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay, which identifies DNA breaks, and by the leakage of phosphatidylserine from the inner to the outer side of the membrane layer of treated cells. Some bioeffects induced on sonicated HL-60 cells, such as inhibition of cell proliferation, DNA repair, and cell-dependent apoptosis, were found to be similar to those produced by γ-irradiation. Thus, much of the cell damage induced by therapeutic ULS in leukemia cells surviving ULS exposure appears to occur through an apoptotic mechanism.

INTRODUCTION

The effect of ULS on lysis of cell suspensions has been correlated with the generation of acoustic cavitation (1–3). Ultrasound cavitation, defined as generation and oscillation of gas bubbles in the experimental medium, may cause irreversible cell damage and modify the membrane structure and functional properties of the cell (4–6).

Attempts to evaluate the sonoelastic effectiveness of a given ultrasound exposure on a heterogeneous blood cell population support the observation that larger cells such as lymphocytes are more susceptible to mechanically induced forces than are smaller cells such as erythrocytes (7). In fact, morphological changes on cell surface membranes were detected after the cells were exposed to diagnostic ULS (8). Because the lipid bilayer of the membrane is a self-assembled structure, it can be easily disordered, resulting in increased bilayer permeability. Shock waves generated by the collapse of cavitation bubbles may also contribute toward the disruption of the cell machinery (5, 9).

Malignant cells were found to be sensitive to ULS treatment, resulting in a transient decrease in cell proliferation (10). In a suspension of carcinoma cells exposed to 1 MHz ultrasound, cell killing was induced, accompanied by DNA strand breaks. This was attributable, mainly, to free radical formation and to pyrolytic processes (11). Ultrasonically induced single-strand DNA breaks accompanied by stimulation of DNA repair synthesis were described in tumor cells (12, 13). Depending on the ultrasound intensity used, the exposure of tumor cells to therapeutic levels of ULS may result in inhibition or stimulation of DNA synthesis (13).

Ablation of adult T-cell leukemia cells and lysis of HL-60 cells by ULS is enhanced in the presence of a photosensitizing drug, indicating that the photosensitizing drug potentiates the cytotoxicity of ULS (14, 15).

We have reported previously that therapeutic ultrasound causes structural changes in the cytoskeleton. These are expressed by altered morphology and functional changes in the sonicated cells (16). Subsequent to these findings, we investigate here whether in a human leukemic cell population sonication may cause an apoptotic cell death process. The human myeloid leukemia cell line, HL-60, was chosen for our experiments because of its ability to undergo apoptosis in response to multiple stimuli (17–19).

Apoptosis or programmed cell death is a mechanism of cellular self-destruction having uniquely defined morphological and molecular characteristics and plays an important role in a variety of biological events, including surveillance against tumors or other malfunctioning cells (20, 21). The morphological criteria that characterize apoptosis include nuclear chromatin condensation, nuclear fragmentation, and ultimately, the formation of apoptotic bodies that are phagocytosed by other cells (22, 23). Apoptotic cell death occurs in response to a large variety of stress signals (24–30).

In the present study, we determined the fate of viable leukemic cells after therapeutic ULS treatment in culture. We show here that ULS of high power and low frequency may induce time-dependent apoptosis in cultured myeloid leukemic cells.

MATERIALS AND METHODS

Leukemia Cell Line Cultures

All cultures of human leukemic cells HL-60, K562, and U937 were performed in RPMI 1640 containing 10% heat-inactivated FCS and incubated in a humidified incubator at 37°C containing 10% CO2 in air. The M1/2 mouse myeloid leukemia with reconstitution of wild-type p53 expression was grown in the presence of conditioned medium (31).

Apparatus

Experimental high energy therapeutic ULS equipment was used to sonicate cells. The exposure to ULS was performed in a rectangular (15 × 21 × 29-cm) Plexiglas water bath fixture, filled 10 mm above the transducer, with degassed, deionized water as the coupling medium. The instrument for inducing cavitation contained an air-backed ultrasound transducer, fixed on the width of the water bath wall.

The therapeutic transducer, which resonates at a frequency of 750 KHz and built as an acoustic lens, consists of one to three composite materials and was designed as a semispherical ring (diameter, 90 mm; radius of curvature, 75 mm) with focal area 50 mm from the transducer surface (Angiosonics, Ltd., Tel Aviv, Israel). The shape of the focal area (−6 dB), measured below the cavitation threshold with a calibrated hydrophone (sprh-s-100; SEA, Soquel, CA), was accepted 12/15/99. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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4 The abbreviations used are: ULS, ultrasound; DC, duty cycle; PD, pulse duration; SPTA, spatial-peak temporal-average intensity; HU, hydroxyurea; MITT, 3-[4,5-diimethylthiazol-2-yl]-2,5-diphenyltetrazolium; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PS, phosphatidylserine; PI, propidium iodide.
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CA), was aspheroid with 12-mm diameter on the symmetry axis and a maximum diameter of 1.5 mm perpendicular to the symmetry axis. An ULS imaging transducer (12 MHz mechanical annular array) was integrated into the center of the therapeutic transducer in a concentric configuration. Monitoring of the cavitation by on-line ultrasound imaging activity allowed for control of the cavitation phenomenon by changing the wave parameters (Ultrasound Technologies, Mahwah, NJ; Ref. 32).

The cell culture flask was fixed in a frame, immersed vertically in the water, perpendicular to the central axis of the ultrasound beam, positioning the specimen at a distance of 60 mm at the acoustic focal zone of the transducer. The frame was connected to a motorized X-Y-Z positioning device, which moved across the ULS focal area at a constant velocity under continuous ULS imaging control.

High intensity focused pulsed ULS at a 750-KHz carrier frequency was delivered to a vertically suspended 50-ml polystyrene cell culture flask containing 15 ml of the cell suspension.

Measurement of Acoustic Emission

During the disruption of a bubble in the transient cavitation process, an acoustic signal is generated (33). To determine the intensity of the cavitation effect, a microphone was attached to the flask containing cell culture medium, and the signal from the microphone was amplified and filtered to the 10–14-KHz frequency band.

Protocol for Therapeutic ULS Treatment

Leukemic cells were suspended 10^6/ml RPMI 1640–10% FCS in a total volume of 15 ml in a 50-ml volume polystyrene culture flask (Nunc). The culture flask was positioned in the sonicator as described above.

During sonication, the cell suspension was moved across the ULS focal area under continuous ULS imaging control at a constant velocity of 4.15 mm/s, covering the cell suspension area during a sonication time of 30 s.

The ULS signal was in the form of a tone pulse wave at a DC of 1.25 and PD of 50 μs, and two different ultrasonic exposures in the focal area were used, 103.7 W/cm² SPTA and 22.4 W/cm² SPTA. The lower intensity pulse presumably did not induce observable cavitation. ULS signal at PD 100 μs, DC 1:50 with transient cavitation effect, and intensity level of 54.6 W/cm² SPTA was tested.

All experiments were performed at room temperature. The temperature in the culture flask during and after sonication was measured by thermocouple at the power and time range used. No significant variation of temperature was detected (<1–2°C).

The viability of sonicated cells were evaluated by the trypan blue exclusion assay. After sonication, the number of viable cells were evaluated; cells were washed, resuspended and transferred into six-well culture plates at a concentration of 1×10^5 viable cells per 3-ml culture medium. The cells were incubated for different time periods (3, 6, 24, or 48 h), the number of vital cells was evaluated by the trypan blue exclusion assay. After sonication, the number of viable cells were evaluated; cells were washed twice with PBS and resuspended (1×10^6 cells) in binding buffer.

γ-Irradiation Treatments

γ-Irradiation of HL-60 cells was performed using a 137Cs γ-ray source (Gammacell 1000, Kanata, Ontario) for a total radiation dose of 4 Gy or 10 Gy/cell sample given at a dose rate of 4.1 Gy/min.

DNA Repair Synthesis

HU is a replicative DNA synthesis inhibitor and has a selective action on cells in the S phase. It appears to block cells at the G1-S boundary. HU (10 mM; Sigma) was added to HL-60 cells, 1×10^6/ml in RPMI 1640–10% FCS, and cultured for 30 min in a humidified 37°C incubator in an atmosphere of 10% CO₂ in air. The cell suspension, 15 ml in a 50-ml volume culture flask, was sonicated as described above and was kept on ice. To measure DNA repair synthesis, sonicated viable cells (1×10^6/ml) were transferred to a round-bottomed, 96-microwell plate (Nunc; 0.1 ml/well), pulsed with [3H]thymidine (1 μCi/ml), and incubated for 3, 4, 5, and 6 h. Cells were lysed with 0.5 N NaOH, followed by neutralization with 0.5 N HCl, deposited onto filter paper, and washed with cold trichloroacetic acid. The filters were dried, and radioactivity was evaluated in a liquid scintillation analyzer (1600 TR; Packard, Meriden, CT). DNA repair was defined as the ratio of the mean cpm of sonicated cells to the mean cpm of unsonicated cells, both cultured in the presence of HU. For a positive control, specimens that had been exposed to a 137Cs γ-ray dose of 4 or 10 Gy were used. Total DNA synthesis was evaluated by culturing HL-60 cells in the absence of HU.

Cell Proliferation

The effect of ULS on the rate of cell growth and proliferation of sonicated or γ-irradiated (as positive control) viable cells (5×10^6/ml) was evaluated using a colorimetric assay (34). Cells were seeded in flat-bottomed, 96-microwell plates (0.1 ml/well) in RPMI 1640 supplemented with 10% FCS for 24–96 h of culture. Tetrazolium (MTT), dissolved in DMSO and diluted in PBS to a final concentration of 7 mmol/l, was added (10 μl of MTT/well) to each well for the last 4 h of culture. After incubation, 100 μl of 0.004% HCI in isopropanol was pipetted into each well, and the contents were vigorously mixed by repeated pipetting. After 5 min, the plates were read in an ELISA reader (Kontron SLT-210) at 550 nm. The stimulation index is defined as the ratio of the mean absorbance (A) of sonicated HL-60 to the mean A of untreated cells. γ-irradiated cells served as controls.

Analysis and Evaluation of Apoptosis by Fluorescent Microscopy. Uptake of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml) in PBS (Sigma) excites green and red/orange fluorescence, respectively, when they are intercalated into DNA. Cultured sonicated and control cells were washed and resuspended at 1×10^6/ml in PBS. The cell suspension (25 μl) was mixed with 1 μl of the fluorochrome mixture, and 10 μl of the samples were examined under an Axiolab fluorescence microscope (×200; Zeiss, Germany). Viable cells present an intact, bright green nucleus. Early apoptotic cells contain a bright green nucleus, however, with condensation of chromatin, and the late apoptotic cells contain a red/orange nucleus showing chromatin condensation.

Analysis of Apoptotic Cell Morphology. Morphological changes of the sonicated cells were examined after cytosin preparation and May-Grünwald-Giemsa staining under light microscope (Axiolab, Zeiss). Cells were also studied under a scanning electron microscope (JEOL, SEM 840, Tokyo, Japan) and compared with nontreated cells (36).

Detection of Apoptotic Cells by the TUNEL Assay. Genomic DNA strand breaks characteristic of apoptosis were labeled in situ by the TUNEL method (37) for detection of fragmented DNA, according to the protocol supplied by the manufacturer (Boehringer Mannheim, Mannheim, Germany). In brief, after sonication, air-dried cytosin cell preparations were fixed for 30 min at room temperature in 4% paraformaldehyde solution and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The slides were incubated with the TUNEL reaction mixture (terminal deoxynucleotidyl transferase, FITC-labeled nucleotides) for 1 h and covered with anti-fluorescin antibody conjugated with alkaline phosphatase for 30 min at 37°C. The substrate solution was added to the samples for 10 min and analyzed under light microscope (×480). Unsonicated cells were used as control.

For each sample, a control of TUNEL mix excluding the enzyme terminal deoxynucleotidyl transferase was included. The typical alkaline phosphatase staining in apoptotic cells was observed.

Annexin V/PI Staining. The test was performed to discriminate between intact and apoptotic cells. Double staining for Annexin V-FITC binding to membrane PS and for cellular DNA using PI was performed according to the protocol provided by the manufacturer (Genzyme Diagnostics). In brief, cultured leukemic cells were treated according to the sonication protocol. Cells were washed twice with PBS and resuspended (1×10^6 cells) in binding buffer with FITC-conjugated Annexin V and PI at final concentration of 0.5 and 5 μg, respectively.

Samples were analyzed by the FACSort using CELL Quest software (Becton Dickinson). Different labeling patterns in this essay enabled us to identify different cell populations: vital cells (PI−/Annexin V−); early apoptotic cells (PI+/Annexin V−); and cells undergoing apoptosis/necrosis (PI+/Annexin V+).
Nitrite Determination

The concentration of NO$_2^-$ secretion into the culture medium was measured with Griess reagent as described (38). Briefly, 50-μl aliquots were removed from the supernatants of the sonicated and control cells and incubated in flat-bottomed microtiter plates (Nunc) with an equal volume of Griess reagent (Sigma Chemical Co., St. Louis, MO) at room temperature for 10 min. The colored product of the diazotization reaction was spectrophotometrically quantified at 550 nm in a microplate ELISA reader. Sodium nitrite dissolved in the same medium was used as a standard curve.

Hydrogen Peroxide Measurement

Determination of free radicals in the cell suspension of the sonicated cells was done as described previously (39). Briefly, 50-μl aliquots of a sonicated cell suspension were added to 950 μl of freshly prepared FOX1 solution (25 mM H$_2$SO$_4$, 250 mM ammonium ferrous sulfate, 100 mM xylenol orange, and 100 mM sorbitol) in 1-ml microcentrifuge tubes; the suspension was vortexed and incubated at room temperature for at least 30 min. The absorbance was read at 560 nm by a spectrophotometer (Biochrom, Cambridge, England). Peroxidation was determined using a standard curve of 0–5 μM H$_2$O$_2$.

Statistical Analysis

All values are expressed as mean ± SE. Differences between sonicated cells and control were assessed with the Student t test. Statistical significance was established at a value of P < 0.05.
RESULTS

Ultrasound Dose, Cell Viability, and Free Radicals. We first established the parameters for our ULS system and chose conditions to deliver a high power and low frequency focused and pulsed ULS to HL-60 cells to result in cavitation and cell damage to LD50. The parameters chosen to generate cavitation were a sonication time of 30 s, a frequency of 750 KHz, PD 50 µs, DC 1:25, and intensity level of 103.7 W/cm² SPTA. These parameters resulted in LD50 tested by the trypan blue exclusion assay after ULS treatment, and transient cavitation effect was observed. The number of viable cells surviving ULS was ~50% under these conditions, and an increase in apoptotic cells occurred during cell incubation in comparison to untreated cells and cells sonicated at lower intensity, without generation of cavitation (22.4 W/cm² SPTA; Fig. 1, A and B). For leukemia cell lines K562, U937, and M1/2, the parameters chosen to generate cavitation were: PD 100 us, DC 1:50, and intensity level of 54.6 W/cm² SPTA at focal area that resulted in apoptotic cell death (Fig. 1C). No significant temperature changes (1–2°C) and no free hydrogen peroxide or nitric oxide radicals were found to be generated in response to the cavitation (data not shown).

DNA Repair. DNA repair and DNA replication were distinguished by the hydroxyurea method. Single-strand breaks were detected in HL-60 cells, which remained vital after ULS. Stimulation of DNA synthesis was demonstrated in these cells, and this was detected by [3H]thymidine incorporation.

Exposure of cells to 103.7 W/cm² (SPTA) showed a significant increase of strand breaks 5–6 h after ULS, which was comparable in magnitude with that induced by 10 Gy of 137Cs γ-rays (Fig. 2).

By operating with ULS power that did not affect detectable generation of cavitation (22.4 W/cm²), an insignificant degree of strand breakage was induced in HL-60 cells (Fig. 2).

Cell Proliferation. The tetrazolium assay, in which MTT is converted to the colored formazan derivative by viable cells, served as an indirect measurement of cell number. A significant decrease in metabolic activity in the HL-60 cell cultures occurred 72 h after sonication (103.7 W/cm²) or 48 h after γ-irradiation in comparison with unsonicated cells or cells sonicated presumably without detectable cavitation (22.4 W/cm²; Fig. 3).

Apoptosis: Morphological Changes in Cells Undergoing Apoptosis. We monitored morphological changes in the cells 3, 6, and 24 h after their exposure to ULS. The presence of apoptotic cells was identified by a distinct morphological form characterized by cell shrinkage, membrane blebbing, condensation of nuclear chromatin,
and nuclear fragmentation (apoptotic bodies); features characteristic of apoptosis exhibit bright chromatin staining. The scanning electron microscope view shows apoptotic bodies, membrane collapse, and bleb formation (Fig. 4).

**Fluorescence Microscopy.** The evaluation of viable cells and apoptotic cells using ethidium bromide and acridine orange staining provides a rapid and objective method to study the effect of ULS on a single-cell suspension. Viable cells excluded ethidium bromide and were permeable to acridine orange, which reacts with DNA to yield a green nuclear fluorescence. Nonviable cells show red/orange fluorescence because of entry of ethidium bromide, which reacts with DNA. ULS treatment increased the number of brightly stained apoptotic cells. Progression of chromatin condensation and nuclear fragmentation was visible in comparison with the nontreated cells (Figs. 1, B and C, and 5).

**Detection of Apoptotic Cells by the TUNEL Method.** The TUNEL assay to measure apoptosis is a nick translation assay based on the incorporation of labeled nucleotides into single-strand DNA breaks of apoptotic cells. HL-60 cells 6 h after sonication showed positive staining versus untreated cells. Some cells with TUNEL-positive nuclei also showed TUNEL positive staining in their cytoplasm, suggesting the presence of cytoplasmic DNA fragments in the cells undergoing apoptosis (Fig. 6).

**Measurement of Changes in the Plasma Membrane.** Double staining for Annexin V-FITC binding/PI was performed to study cytoplasmic membrane integrity and the cellular DNA. During early apoptosis, PS residues flip from the inside to the outside of the plasma membrane. This was detected by flow cytometry using Annexin V-FITC, which binds to the PS residue. The percentage of cells at early and late stages of apoptosis after ULS treatment was compared with that of nontreated cells.

Fig. 7 shows the results obtained by a flow cytometry follow-up of HL-60 cells maintained in culture 6 and 24 h after sonication. High

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Fig. 6. Photomicrographs of in situ DNA fragments by TUNEL stain on HL-60 cells in cytospin smear preparations. ULS-treated cells 6 h after culture showed TUNEL-positive brown color, as developed in an alkaline phosphatase substrate system. A, positive staining in sonicated cells. B, untreated control cells, exhibiting only a few stained cells.

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Fig. 7. Contour diagram of Annexin V/PI flow cytometry of HL-60 cells after therapeutic ULS treatment with generation of cavitation (103.7 W/cm²) or without detectable cavitation (22.4 W/cm²) 6 and 24 h after sonication. Nonsonicated cells were used as a control. The cells were incubated with Annexin V-FITC, and PI was added before analysis by fluorescence-activated cell sorter using an argon-ion laser tuned to 488 nm. Lower left quadrants, viable cells (PI⁻/Annexin V⁻/FITC⁻). Lower right quadrants, early apoptotic cells (PI⁻/Annexin V⁺/FITC⁺). The upper right quadrants (PI⁺/Annexin V⁻/FITC⁻) contain nonviable, late apoptotic/necrotic cells. One representative experiment of four is shown.
levels of early and late apoptosis/necrosis was detected 6 h after ULS, and high levels of late apoptosis/necrosis was detected 24 h after high intensity (103.7 W/cm²) ULS. Signaling of early apoptosis was found in HL-60 cells 6 h after low intensity sonication (22.4 W/cm²) with lack of apoptotic cell death.

**DISCUSSION**

Apoptotic cell death occurs in response to a large variety of signals including UV and γ-irradiation. However, none of the modalities currently used to induce apoptosis involve the use of ULS energy. The intensity level of the ULS energy treatment should be high enough to create transient acoustic cavitation at the locus of the target cell. Induction of cavitation was carried out by using an experimental piezoelectric ultrasound transducer, which generated low frequency, high energy, and focused and pulsed ultrasound of 750 KHz. This frequency was chosen to balance the lower cavitation threshold and smaller attenuation in tissue or experimental media obtained at low frequencies with the smaller focal size obtainable at higher frequencies and needed for accurate positioning of the energy. The balance between cell destruction and cell viability achieved depends on the ULS conditions, including pulse duration time, pulse repetition rate, number of pulses accomplished, and the transducer voltage applied.

We have demonstrated previously that high power, low frequency ULS induces structural and functional changes in sonicated cells (16). In the present study, we have investigated DNA strand breaks, cell proliferation, and apoptotic cell death that take place after therapeutic ULS application. This sonication appears to be effective in causing cavitation, having an increased tendency to progress to apoptosis during cell incubation (Fig. 1, B and C).

The morphological cell changes incurred during apoptosis are unique and should be a deciding factor concerning the mechanism of cell death. Cell death by apoptosis is characterized by cell chromatin condensation, together with visible nuclear fragmentation and formation of apoptotic bodies that may be phagocytosed by other cells. These changes may be used as markers for apoptosis (Ref. 40; Fig. 4).

The leukemia cell lines HL-60, K562, and U937 can be induced to undergo apoptosis after therapeutic ULS, despite its being deficient in p53, a tumor suppressor gene required for the induction of programmed cell death initiated by DNA damage. These data indicate that ULS-induced apoptosis is an additional example of the existence of a p53-independent pathway (41, 42). The leukemia cell line M1/2 could initiate apoptosis by a p53-dependent pathway mediated by the restoration of wild-type p53 expression (Ref. 31; Fig. 1C).

The decision to undergo apoptosis induced by X-irradiation appears to be made at the G2 checkpoint (41), whereas apoptosis induced by ULS is unrelated to the functioning of the cell cycle checkpoint, as observed by cell cycle analysis (data not shown). Positive identification of apoptotic cells by specific assays is based on the detection of DNA strand breaks (43). The proliferation capacity and the detectable DNA repair in HL-60 cells after exposure to ULS appears to be comparable in magnitude to that of 4 and 10 Gy γ-irradiation-induced lesions (Ref. 44; Figs. 2 and 3).

Changes in the cell membrane are one of the earliest features of cells undergoing apoptosis. The plasma membrane of apoptotic cells is more permeable than that of normal cells, and a variety of DNA-binding dyes are taken up more rapidly by apoptotic cells (45). When ULS energy was applied without the creation of detectable cavitation, the membrane PS was translocated, as demonstrated by a high number of Annexin V-positive cells (Fig. 7). Lack of apoptosis in experiments below the energy threshold for cavitation suggests an active membrane repair process, avoiding apoptotic cell death. DNA repair was not significantly increased, and no change in cell viability was found by the trypan blue exclusion assay (Figs. 1 and 2).

Several different signal transduction pathways may mediate the induction of apoptosis. The results presented here suggest that induction of apoptosis of the myeloid leukemia cell lines by selected physical parameters of therapeutic ULS may be explained as a response to cell membrane and DNA damage induced by sonication. Having a single unpaired electron, NO is in itself considered to be a free radical and has been implicated as an inducer of apoptosis (46). Negative results obtained by testing nitric oxide and hydrogen peroxide generation may exclude the free radical-dependent mechanism to mediate apoptosis in our system. However, a comprehensive understanding of the initiation step that is responsible for ULS-induced apoptosis is not yet available. This *in vitro* cell system offers the advantage of enabling the study of ULS exposure parameters to evaluate bioeffect information on the potential application of ULS on tissue and large volumes of blood. The ULS mechanism of action in cell suspension under predetermined conditions may differ from those occurring *in vivo* (47). ULS acoustic waves that propagate through a medium find very little fluid in tissue in the spaces between and among cells. This suggests that sonication conditions needed for tissue treatment are likely to be different from those used in cell suspension. The assumption that cancer may be treated by induction of apoptosis suggests ULS as potential modality for successful cancer treatment.

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