Published in final edited form as:

Ultrasound Med Biol. 2010 April ; 36(4): 677–692. doi:10.1016/j.ultrasmedbio.2010.01.010.

Changes in cell morphology due to plasma membrane wounding by acoustic cavitation

Robyn K. Schlicher^{*,†}, Joshua D. Hutcheson[†], Harish Radhakrishna[‡], Robert P. Apkarian^{§,§}, and Mark R. Prausnitz^{*,†,¶}

^{*}The Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory University, Atlanta, Georgia 30322

[†]School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, Georgia 30322

[‡]School of Biology, Georgia Institute of Technology, Atlanta, Georgia 30332

[§]Integrated Microscopy & Microanalytical Facility, Emory University, Atlanta, Georgia 30322

Abstract

Acoustic cavitation-mediated wounding (i.e., sonoporation) has great potential to improve medical and laboratory applications requiring intracellular uptake of exogenous molecules; however, the field lacks detailed understanding of cavitation-induced morphological changes in cells and their relative importance. Here, we present an in-depth study of the effects of acoustic cavitation on cells using electron and confocal microscopy coupled with quantitative flow cytometry. High resolution images of treated cells show that morphologically different types of blebs can occur after wounding conditions caused by ultrasound exposure as well as by mechanical shear and strong laser ablation. In addition, these treatments caused wound-induced non-lytic necrotic death resulting in cell bodies we call wound-derived perikarya (WD-P). However, only cells exposed to acoustic cavitation experienced ejection of intact nuclei and nearly instant lytic necrosis. Quantitative analysis by flow cytometry indicates that wound-derived perikarya are the dominant morphology of nonviable cells, except at the strongest wounding conditions, where nuclear ejection accounts for a significant portion of cell death after ultrasound exposure.

Keywords

ultrasound; drug delivery; microscopy; plasma membrane wounding; cell death; cavitation

Introduction

Acoustic cavitation from low-frequency ultrasound has been used to induce intracellular uptake of drugs by either direct transport from the extracellular environment to the cytosol (Mitragotri

© 2010 World Federation for Ultrasound in Medicine and Biology. Published by Elsevier Inc. All rights reserved.

[¶]To whom correspondence should be addressed. Address editorial correspondence to: Mark R. Prausnitz School of Chemical and Biomolecular Engineering Georgia Institute of Technology 311 Ferst Drive Atlanta, GA 30332-0100 Tel: (404) 894-5135 Fax: (404) 894-2291 prausnitz@gatech.edu.

[§]Deceased February 28, 2006.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2005, Pitt and Hussein 2008, Postema and Gilja 2007, Rapoport et al. 2004), or by inducing release of agents from carriers such as liposomes or micelles (Feril et al. 2005, Gao et al. 2005). Many studies have also shown cavitation-mediated transfection by internalized DNA for gene therapy ((Miller et al. 2002, Newman and Bettinger 2007), (Larina et al. 2005, Mitragotri 2005, Yuh et al. 2005).

Forces created during acoustic cavitation (Apfel 1982, Lauterborn et al. 2007) can transiently disrupt the plasma membrane, to allow intracellular uptake (Brayman et al. 1999, Miller et al. 2002, Schlicher et al. 2006). Ultrasound exposure can also result in cellular and tissue damage (Alter et al. 1998, Bailey et al. 1983) and cell death via routes including both necrosis and apoptosis (Cochran and Prausnitz 2001, Feril and Kondo 2004, Miller and Dou 2009, Miller and Qudus 2002).

Previously, plasma membrane effects including pores and loss of cytoplasm have been imaged by scanning electron microscopy in cells sonicated in the presence of a photosensitive drug (Tachibana et al. 1999) or contrast agent (Mehier-Humbert et al. 2005). However, it was our goal to see native effects of ultrasound alone, without the addition of drugs or contrast agents. We also wished to elucidate reasons for the previously observed heterogeneity of sonication outcomes (Guzman et al. 2001), and thus performed detailed microscopic analysis using multiple techniques for the study of cells exposed to ultrasound.

Cells exposed to mechanical stresses, such as those created by acoustic cavitation, may experience transient breaches to their plasma membranes that allow transport of materials into cells (McNeil and Kirchhausen 2005, Mehier-Humbert et al. 2005, Mitragotri 2005). We have observed in this study that acoustic cavitation can also lead to multiple types of membrane blebbing, formation of perikarya, nuclear ejection and instant cell lysis, depending on the degree of wounding incurred. We propose that these outcomes of ultrasound exposure are based upon mechanical wounding due to acoustic cavitation and are also conserved across different wounding techniques including acoustic cavitation, mechanical shear, and laser ablation. Physiologically, cell wounding and repair routinely occurs throughout the body: mucosal cells of the digestive system can be harmed by passage of food (Miyake et al. 2006), contractile fibers of cardiac and skeletal muscles are sensitive to breakdown due to overexertion (Warren et al. 1995) and alveolar cells of the lungs experience shear damage during ventilator-induced breathing (Gajic et al. 2003).

Due to the seemingly routine nature of wounding, there exist endogenous repair mechanisms that enable cells to recover from such damage. A breach in the plasma membrane causes an influx of Ca^{++} to the cytosol, which has been shown to activate a vesicle-based repair response hypothesized to create a patch of lipids to block flow both into and out of the cell (McNeil and Terasaki 2001, Togo et al. 1999) or to cause rapid multiple exocytosis events to the plasma membrane, increasing the surface area until the wound edges are closed (Meldolesi 2003). This membrane repair after influx of external material to the cytosol allows cells to maintain viability and also entrap any transported extracellular material, such as drugs, siRNA and/or DNA, in the cytosol as “uptake.”

The wounds created by non-invasive, low-frequency ultrasound exposure may be especially promising for uptake of many different types of molecules, as shown by drug and gene delivery in cells, animals and humans (Mehier-Humbert et al. 2005, Mitragotri 2005, Pitt and Hussein 2008). For this reason, as well as the variety of wounds that occur from the heterogeneous effects of ultrasound exposure (Bailey et al. 1983, Keyhani et al. 2001), we feel this method is a good model for producing a large spectrum of wound types, leading to multiple death modes that can be analyzed using high resolution microscopy and flow cytometry. We also utilized more direct methods for creating wounds, i.e., mechanical shear, and laser ablation, which may

mimic some effects of ultrasound on cells (Dunn 1985) while creating more homogenous outcomes than ultrasound exposure, but these methods are likely to be less useful for future clinical applications.

This work provides a detailed morphological analysis of cellular bioeffects after exposure to ultrasound and alternate forms of wounding by two techniques, microscopic and cytometric, which can facilitate development of laboratory applications and medical treatments that seek to control intracellular delivery, cell death and other cellular processes associated with wounding. The study of ultrasound-based wounding may also serve to improve understanding of normal physiological and pathophysiological processes in the body in which cell wounding occurs (Dewey et al. 1981, McNeil and Khakee 1992, Miyake et al. 2006), which may suggest treatments for traumatic injury and chronic diseases involving cell membrane damage.

Materials and Methods

Cell culture

DU145 prostate-cancer cells (American Type Culture Collection, Manassas, VA) were grown to 80% confluence on T-150 flasks (BD Falcon, Franklin Lakes, NJ) in growth media (RPMI-1640, Cellgro, Herndon, VA) supplemented with 10% fetal bovine serum (FBS, Cellgro or Atlanta Biologicals, Atlanta, GA) at 37°C, 5% CO₂, and 90% relative humidity prior to harvest during exponential growth by trypsinization. Primary human astrocyte cells (HA, Sciencell Research Laboratories, Carlsbad CA) were grown to 90% confluence on T-150 flasks (BD Falcon, Franklin Lakes, NJ) coated with 2 mg poly-L-lysine (Sciencell) in DDI water in astrocyte media supplemented with fetal bovine serum, antibiotics (P/S) and astrocyte growth supplement (AGS, all provided by Sciencell) at 37°C, 5% CO₂, and 90% relative humidity prior to harvest during exponential growth by trypsinization. Studies with human cells were carried out according to the guidelines of a Georgia Tech-approved Chemical Hygiene Plan to assure safe and appropriate handling. Although these cell types are typically adherent, we studied them here in suspension due to experimental constraints, including the nature of cavitation and the requirement for quick preparation for microscopy immediately after sonication ended. Had the experiments been performed on cells adhered to substrates, asymmetry of the cavitation field would have occurred. Furthermore, removing the cells from the substrate would take too long (up to minutes) and the use of trypsin could introduce artifacts observable in high resolution electron microscopy.

Ultrasound apparatus

Samples were exposed to ultrasound in a device consisting of a cylindrical piezoelectric transducer (Channel Industries, Santa Barbara, CA) sealed between two PVC pipes (5.1 cm inner diameter) to create a watertight sonication chamber, as described previously (Cochran and Prausnitz 2001). This chamber was filled with deionized water that had been degassed for at least 2 h in a bell jar (Nalgene, Rochester, NY) using a vacuum pump (KNF Neuberger, Trenton, NJ) to remove bubble nucleation sites and thereby reduce cavitation in the water bath that could alter the pressure field in the sample chamber.

Ultrasound was generated at 24 kHz by a function generator (DS354 SRI, Stanford Research Systems, Sunnyvale, CA) and amplifier (Macro-Tech 2410, Crown Audio, Elkhart, IN) that controlled the transducer via a matching transformer (MT-56R, Krohn-Hite, Avon, MA). Using this system, the frequency, duty cycle, incident pressure, and pulse length were set and controlled. The exposure time was controlled manually. The incident pressure, a measure of the ultrasound power, was determined using a calibrated hydrophone (Reson model TC4040, Goleta, California). Radial variability in pressure across the width of the cell sample chamber

(9 mm wide) was less than 10%, and axial variability up and down the cell sample chamber height (21 mm tall) was less than 20% (Cochran and Prausnitz 2001).

Ultrasound exposure

Well-mixed cell suspensions at concentrations of either 10^6 cells/ml (for flow cytometry analysis) or up to 10^7 cells/ml (for microscopy analysis) in RPMI-1640 with or without serum (as noted) were stored on ice and gently introduced into either: 1.2 ml sample chambers (No. 241 SEDI-PET SAMCO, San Fernando, CA transfer pipets with stems cut to 2 cm in length) or 0.5 ml sample chambers (No. 293 PADL-PET SAMCO, San Fernando, CA transfer pipets with stems cut to 2 cm in length and end plugged with silicon gel) using a 22-gauge needle and 3-ml syringe (Becton-Dickinson, Franklin Lakes, NJ), ensuring that no bubbles were introduced into the sample chamber. Prior to introduction into the sample chambers, the cell suspension was open to the environment and thus contained a gas content at equilibrium with the surround environment, i.e. the media was saturated with oxygen and nitrogen. After completely filling the chamber, either a stainless steel rod (1.6 mm diameter) for 1.2 ml samples or a plastic rod (polypropylene, 4 mm diameter) for 0.5 ml samples was carefully inserted into the pipet stem to seal the chamber and provide a means to suspend the chamber in the water bath. Sample chambers were then positioned in the exposure chamber water bath by fixing the end of the suspending rod into the chamber cover at a location that positioned the sample chamber in the axial and radial center of the transducer. Samples were exposed at room temperature to 20 acoustic pulses each at 24 kHz, with 0.1 s pulse length at 10% duty cycle at different pressures reported as 0.36, 0.54 and 0.71 atm after conversion from the peak-to-peak voltage. Sham samples (non-sonicated controls) were prepared identically, but no ultrasound was applied.

Wounding device by microchannel fabrication

A microchannel flow device developed by Hallow et al. (Hallow et al. 2008) was used to image cells wounded by shear. To create the device, sheets of 100 μ m thick polyethylene terephthalate (Mylar, DuPont, Wilmington, DE) were first cut into 15 mm discs using a CO₂ laser (LS500XL, Gravograph-New Hermes, Duluth, GA). Cylindrical microchannels were created by drilling holes through the center of the discs using an ultraviolet excimer laser (Resonetics Micromaster, Nashua, NH). The laser was typically operated at 60 Hz with a 248 nm wavelength and energy of 200 mJ. Suspensions of 5×10^5 DU145 cells/mL in RPMI 1640 plus FBS were flowed through 100- μ m long, 60- μ m diameter channels at 100 mL/h using 3 mL syringes connected to a syringe pump to create controlled shear at a designated flow rate. These conditions were selected because they were previously found to cause significant bioeffects, e.g. uptake of molecules and loss of viability (Hallow, Seeger 2008), allowing us to readily observe wounds created by the device.

Laser ablation of cells

Individual cells in suspension being observed using the LSM 510 microscope were ablated using a focused Ar 488nm laser for 10^3 iterations of 1 s duration pulses with a spot radius of 1 μ m². Images were captured as movies using images recorded approximately every 1.5 s.

Fixation and post-staining of DU145 cells

Samples were sonicated in RPMI-1640 (plus FBS), then added in a 1:1 ratio to ~4% glutaraldehyde (making the final concentration of fixation roughly 2%) at a specific time after sonication ended, either immediately (1-2 s), 15 s, 30 s or 1 min. Some samples were divided into portions and fixed at different time points to indicate changes in the cells as a function of time after exposure ended. Samples remained in fixative for approximately 10 min at room temperature, after which they were washed by centrifugation (6 min, 4 °C, $1000 \times g$) and then

washed 1-2 times with staining buffer: PBS with azide (for sample stability) and FBS (to block non-specific binding and reduce background fluorescence upon viewing by microscopy). In some cases, samples were then incubated with 5 μ M BODIPY phalloidin in staining buffer with 0.2% saponin (Sigma-Aldrich), which allows gentle permeabilization of plasma membranes, thus allowing the phalloidin to stain intracellular actin, for either 2 h at room temperature while rotating on a nutator orbital shaker (Fisher Scientific, Waltham, MA) or overnight (up to 12 h) at 4 °C while stationary (Urs et al. 2005).

Fluorescent preparation of cells

Plasma membranes were fluorescently labeled prior to sonication with either 2 mM TRITC- or FITC-labeled wheat germ agglutinin (WGA) (Sigma-Aldrich, St. Louis MO) for 20 min at 2 °C (Sharon and Lis 1989) or 5 μ M DiO (Invitrogen, Carlsbad CA) at 37 °C for 10 min (flow cytometry) or 10 μ M DiO at 37 °C for 8 min (LSCM). Excess label was removed by ≥ 1 wash step in PBS before sonication in fresh RPMI-1640 (Cellgro, MediaTech, Herndon VA) supplemented with fetal bovine serum (FBS, Atlanta Biologicals, Atlanta, GA). Actin cytoskeleton was labeled using 5 μ M BODIPY phalloidin (Invitrogen) by either delivering the compound during wounding or by staining after fixation (Barak et al. 1980) using 0.2% saponin in PBS with 0.5% sodium azide and 10% FBS as blocking agent for nonspecific binding. Endoplasmic reticulum was stained by Organelle Lights (Invitrogen) baculovirus constructs following the company's recommended protocol (Fliegel et al. 1989) with cells cultured in T-25 flasks (Falcon, BD Biosciences). Intracellular lipid was labeled using incubation of adherent cells in RPMI-1640 (with FBS) containing 10 μ M FM1-43 for 12–16 h at 37 °C (Togo et al. 1999). Labeled cells were harvested by trypsinization.

To study uptake, cells were sonicated in RPMI-1640 (with FBS) with plasma membrane impermeant markers: 10 μ M (flow cytometry) or 100 μ M (LSCM) calcein (Invitrogen) or 10 μ M sulforhodamine101 (LSCM, Invitrogen). After exposure, nonfixed samples recovered for 10 min at 22 °C. Excess label was removed by ≥ 2 wash steps in PBS and samples were assayed in 7.5 μ M (flow cytometry) or 15 μ M (LSCM) PI (Molecular Probes) to determine cell viability. Some samples were fixed (as noted) using 2% paraformaldehyde (Sigma-Aldrich) for 10 min at 22 °C within 5 s - 2 min after sonication to compare to samples prepared for EM by rapid fixation (within seconds after exposure).

Microscopy: Scanning Electron Microscopy (SEM), Cryo-SEM and Laser Scanning Confocal Microscopy (LSCM)

Cells sonicated in RPMI 1640 (without FBS) were fixed in 2.5% EM-grade glutaraldehyde (Sigma-Aldrich) 2 s or 30 s after sonication and prepared using standard techniques (Castejon et al. 2001) for viewing by SEM (DS-130F Schottky Field Emission SEM/STEM, Topcon, Tokyo Japan). For cryo-SEM, cells were flash frozen in solid gold planchets (Balzers), cracked with a cold blade while under liquid nitrogen, loaded into a cold stage and sputter coated (Gatan CT-3500, Pleasanton CA) following the methods developed by Apkarian (Apkarian et al. 1999). Fluorescently labeled cells in suspension were imaged both chemically fixed and live in real time by LSCM (LSM 510 Carl Zeiss MicroImaging, Thornwood NY) in an Attofluor cell chamber (Invitrogen) using 40X, 63X or 100X oil immersion objectives. Movies were created on the LSCM by setting a time series to capture images every 5 s, 10 s, or 1 min. The images are viewed in a sequential series, creating a time lapse movie.

Flow Cytometric Analysis

To determine the relative importance of the wound-induced morphological changes identified by microscopy, we quantified the frequency of occurrence for specific morphologies at several levels of ultrasound exposure. Morphological changes in cells observed by microscopy can be classified among seven populations: (Pop1A) cells with normal morphology; (Pop1B) cells

with normal morphology and uptake of extracellular marker compound, calcein; (Pop2C) necrotic cells; (Pop2D) WD-P; (Pop2E) isolated nuclei; (Pop3F) nuclear debris and (Pop3G) cytosolic and plasma membrane debris from lysed cells. Blebbing cells and cells ejecting nuclei are interpreted as transient states not easily captured by flow cytometry that ultimately lead cells to one of these seven categories.

Specific populations identified during the microscopy analysis are quantifiable by flow cytometry using (i) forward light scatter as a measure of event size, (ii) propidium iodide (PI) fluorescence as a measure of plasma membrane integrity, (iii) calcein fluorescence as a measure of reversible plasma membrane wounding and (iv) DiO fluorescence as a plasma membrane marker, as summarized in Table 1. Although flow cytometry cannot identify all features evident by microscopy, we believe that many hallmark features can be identified to correlate flow cytometry with microscopy findings.

Samples were run on a BD-LSR flow cytometer using FACS DiVa software (BD Biosciences, Franklin Lakes, NJ). Relative cellular size was obtained through measurements of the light scattering properties of the cells as detected by forward scatter (FSC) measurements in the flow cytometer. The fluorescence of green marker compounds (including calcein and DiO) was measured with a 488-nm argon laser excitation and a 530/30 bandpass filter for emission. Red-fluorescent propidium iodide was also excited at 488 nm and measured using a 675/20 nm bandpass filter for emission. For each sample, 3×10^4 events were collected from a gated region known from previous work (Canatella, Karr 2001) to contain DU145 cells.

Data were analyzed using FCS Express (De Novo Software, Thornhill, Ontario) flow cytometry analysis software. The flow cytometric events were plotted on a 2-dimensional dot plot with FSC on the abscissa and propidium iodide fluorescence on the ordinate. The data could be separated into characteristic populations based upon size (FSC) and membrane integrity (PI). The uptake and membrane stains described above give the plot a third dimensionality to analyze specific characteristics of interest and divide the main populations into subpopulations (as shown in Table 1 of the Results section).

The populations were quantified as a percentage of the total cells present in the control samples. Since many cells are lysed by sonication—and thus undetected as whole cells by the flow cytometer—the gated total cells of all of the sonicated samples were normalized to the gated total cells in the control, sham-exposure samples. The control samples were assumed to have no cellular debris. This assumption ensures that all of the quantified cellular lysis in sonicated samples resulted from the actual sonication. The quantification of debris was done by recording the amount of time required to collect 3×10^4 cells in each sample. Since the cells lost due to lysis resulted in a longer required collection time for sonicated samples (i.e., the sonicated samples have a lower cell concentration), the number of lost cells was calculated as the balance of cells that would be required for the sonicated samples to have the same flow cytometry collection time as the control samples.

Results

High resolution microscopy shows cellular features

We first prepared and imaged sham-exposed DU145 cells by electron microscopy and laser scanning confocal microscopy (LSCM) to establish their typical morphology. Cells viewed using scanning electron microscopy (SEM) had plasma membranes covered by microvilli, microruffles and normal blebs (arrow, Fig 1a), which consisted of transparent membranes containing grainy material, possibly cytosol. LSCM of optically sectioned cells showed solid rings of intact plasma membrane (Fig 1b, TRITC-labeled WGA, red) and actin cytoskeleton

(Fig 1c, BODIPY-phalloidin, green) surrounding multi-lobed large nuclei (stained blue with Hoechst 33342).¹

Plasma membrane wounds lead to uptake of molecules

We next imaged cells wounded by acoustic cavitation during ultrasound exposure (sonication) and fixed <1 min after treatment. Consistent with our previous work (Schlicher, Radhakrishna 2006), cells imaged by SEM had patches of missing plasma membrane, as indicated in Fig 1d (arrow) by a smooth area surrounded by normal cell surface covered with microvilli. LSCM imaging of a cell whose plasma membrane was labeled with TRITC-WGA also had evidence of plasma membrane loss, as shown in Fig 1e by a discontinuity, or break, in the red ring (arrow) through which transport of a green fluorescent compound, calcein, occurred, as compared to non-wounded controls with no internalization of calcein (Fig 1b). These disruptions to the cell surface may also penetrate through the actin cytoskeleton, causing loss of actin near the plasma membrane (where it is normally concentrated in suspension cells), and redistribution of this cytoskeletal component into the cytosol (Fig 1f).

Intracellular lipids create multiple forms of blebs at wound sites

To study the development of blebs after wounding, we used a dual lipid labeling method in conjunction with LSCM to differentiate internal lipid (labeled green) from plasma membrane lipid (labeled red) at the time of wounding. This allowed us to observe both breaks in the plasma membrane and lipid response cells during repair. We found that wounded cells produced blebs at sites of plasma membrane disruption as shown in Fig 2. We observed wound-associated blebs to be different from blebs on cells in sham exposed samples, assumed to be normal blebs, in terms of transparency, internal composition, size and shape.

In Fig 2a, a spherical bleb protrudes from the plasma membrane (arrows at disruption site). The bleb color (green) indicates it was formed by stained internalized lipids and not plasma membrane lipids, which were labeled with TRITC-WGA just prior (~15 min) to sonication, in order to avoid internalization of the red labeled lipid. This bleb likely derived from lipid stored in a compartment near the plasma membrane disruption site. Another wounded cell, similarly labeled with FM1-43, but not TRITC-WGA, also produced a large bleb connected via a long neck to an inner part of the cell. We describe spherical protruding blebs as “balloons,” that pinch off into the extracellular environment, a phenomenon we have observed hundreds of times after wounding cells by several different means. We do not observe blebs of this nature in sham exposed, fixed and processed samples, which we always prepare and image for comparison to wounded samples in every experiment. Furthermore, many examples of these blebs, including our initial observations of the phenomena, were made in unfixed samples, and in unfixed cells damaged by mechanical shear and laser ablation, supporting our contention that these effects are not artifacts of the fixation process, but are a result of wounding and cell damage.

Furthermore, balloon blebs may contain small round lipid vesicles deriving from endoplasmic reticulum (Fig 2c), indicating that blebs access the cytosol and ‘fill in’ disruptions through which some internal materials may exit and become entrapped in the bleb. Similar blebs were seen to break through the cytoskeleton in cells stained for actin by BODIPY phalloidin, (Fig 2d1, yellow arrows), suggesting that actin is lost or disrupted at the sites of bleb formation (Fig 2d1 red arrows, Fig 2d2 close-up), supporting our observation that some types of shear wounding can disrupt and re-distribute actin normally located at the plasma membrane (Fig 1c).

We have also reproducibly seen “blister” blebs created by lipids integrated with the plasma membrane and filled with clear fluid. This type of bleb is eventually re-integrated with the

normal plasma membrane of the cell. An example of both bleb types, blister and balloon, is shown in an ultrasound-wounded cell imaged by cryo-SEM (Figure 2d). The upper bleb (Fig 2d, yellow arrow) is connected to an inner region of the cell by a neck area and protrudes like an inflating balloon. The lower bleb (Fig 2d, red arrow) is integrated to the plasma membrane surface. Furthermore, using LSCM, live cells showing blister blebs in real time after exposure to wounding modes appeared to enable cells to repair after damage (Fig 2f1-f3, 2h-i), as shown by collecting images over extended periods of time and described below. Cells in Fig 2a, b, d, and e were fixed seconds to minutes after wounding, thus stopping full development of blebs. We therefore viewed live cells with bright field microscopy (BFM) sometimes overlaid with LSCM (as indicated) within minutes after wounding to study bleb kinetics.

In a field of unfixed cells imaged by BFM overlaid with LSCM, most were stained with propidium iodide, indicating viability loss; however an unstained, live cell in the middle (Fig 2f1, circle) had a blister bleb (Fig 2f2, arrow) that we followed using time lapse imaging. Over time (~15 min) the cell redistributed its cytosolic components while the bleb expanded along the cell periphery, but remained integrated with plasma membrane (Fig 2f3, arrow, Supporting Information (SI) Fig S1, Movie M1). Despite the overall distortion of the cell's surface and inner contents, this cell never became permeant to PI, unlike neighboring cells (Fig 2f1), thus indicating it maintained viability. Therefore, we surmise that this cell recovered from wounding by a mechanism related to bleb formation.

Similar observations in cells wounded by two other mechanisms suggest that formation of blebs is a conserved response to plasma membrane wounding that appears associated with its repair, as shown in Fig 2g-2i. Cells wounded by flow-induced mechanical shear through a microfluidic channel also produced both balloon (Fig 2g) and blister (Fig 2h) blebs similar to those exposed to ultrasound. Cells ablated using a high-intensity laser beam and viewed by BFM in real time also created a blister bleb at the wound site (Fig 2i) which then expanded to create a clear compartment (SI Fig S2, Movie M2) like that shown in Fig 2f1-2f3.

Cytosol loss through plasma membrane wounds creates wound-derived perikarya

During observations of cell repair, we also saw evidence of cell death due to non-recoverable injuries from mechanical wounding. One type of non-repairable wounding caused the creation of small cell bodies we term wound-derived perikarya (WD-P), based on their morphological similarities, e.g. intact nuclei affiliated with some cytosol and plasma membrane, to bodies formed during autschizis, a programmed cell death (PCD) mode previously observed in DU145 cells overdosed with vitamin K (Gilloteaux, Jamison 2003). However, we do not believe WD-P seen in this study form by this type of PCD, but instead represent a rapid necrosis that differs from traditional cell lysis after injury.

Our studies show that some wounds resulted in rapid loss of cytosol and organelles (in cells fixed within seconds after ultrasound exposure) as observed by SEM (Fig 3a, arrow); we propose that this loss of material through a non-repairable wound is the mechanism by which WD-P form. As shown in Fig 3b, a cell body captured using SEM of sonicated cells fixed within seconds after exposure consists of an exposed intact nucleus (Fig 3b, **) affiliated with a small amount of plasma membrane and cytosol (Fig 3b, arrow). The nucleus is identified by its smooth surface and distinct shape, while spheres of cytosol and internal lipids, as well as plasma membrane with microvilli are also seen. Unlike perikarya reported in literature, which are believed to be surrounded by plasma membrane, we show that the plasma membranes of WD-P are partially missing and often extremely damaged. Using LSCM (Fig 3c), we can specifically stain for organelles, supporting our analysis of images captured with EM and proving that WD-P structures do contain PI-stained nuclei (Fig 3c, large red body) with some DiO-labeled plasma membrane (Fig 3c, arrow, green). Furthermore, WD-P are extremely important because they are the primary outcome of cells killed by acoustic cavitation, except

at the highest ultrasound pressure used in this study, where another unique form of cell death is also significant, as described below.

WD-P were also seen in cell suspensions exposed to microfluidic wounding and observed by BFM (Fig 3d, arrow indicates plasma membrane/cytosol and ** indicates nucleus). We were able to capture formation of WD-P by laser ablating cells during BFM time-lapse imaging (Fig 3e). We observed that, immediately after laser treatment, the ablated region of membrane grew a blister bleb similar to those described above (Fig 3e1, arrow) that ruptured after expanding for ~15 min, thereby causing extensive cytosol loss (SI Fig S3, Movie M3) through the resulting large plasma membrane disruption. The body that remained consisted of an intact nucleus with affiliated plasma membrane (Fig 3e2, arrow indicates lost cytosol, ** indicates nucleus), similar to the WD-P observed after sonication and microfluidic shear.

Increased wounding due to high sonication pressure causes nuclear ejection

We observed that some cells exposed to high acoustic cavitation pressure and rapidly fixed afterward were in the process of ejecting morphologically intact nuclei, but did not appear to have other wound responses (e.g. repair blebs). As shown in Fig 4, a cell imaged by SEM has a nucleus that is largely present on the outside of the plasma membrane, apparently in the process of extrusion (Fig 4a). A rapidly fixed optically sectioned cell stained for plasma membrane and nuclear material imaged by LSCM (Fig 4b), shows its nucleus starting to protrude through a break in plasma membrane. Complete nuclear extrusion occurs over time as shown in Fig 4c, where a PI-stained (red, arrow) unfixed intact nucleus with no affiliated cell components is shown. This nucleus is next to a damaged cell whose plasma membrane is labeled with DiO (green), indicating that nuclei ejected by these means do not have any affiliated plasma membrane. We have also observed that these ejected nuclei have no apparent cytosol (indicated by calcein-AM) or actin cytoskeleton (indicated by BODIPY phalloidin) within proximity.

However, in cell suspensions fixed <1 min after sonication, we never saw isolated nuclei fully ejected from cells; while in cell suspensions imaged in real time minutes after sonication, we often found isolated ejected nuclei, but never saw nuclei undergoing the process of ejection. This suggests that nuclear ejection occurred over a timescale on the order of 1 min. We do not think this observation is an artifact of the fixation method because (i) nuclear ejection was only seen repeatedly in significant numbers at high ultrasound pressure (≥ 0.72 MPa) and (ii) nuclear ejection was seen in both fixed and unfixed samples, just at different phases of the process. Fully ejected nuclei with no associated plasma membrane were also identified by flow cytometry (see below).

Extreme wounding causes nearly instant cell lysis

We have repeatedly observed that cells with extreme damage can be nearly instantly lysed. Using SEM (Fig 4d), remnants of mechanically fragmented cells were found <5 s post sonication. Using cryo-SEM, rounded lipid-type cell remains were seen (Fig 4e). Debris observed by LSCM (Fig. 4f) consists of nuclear fragments (stained blue using Hoechst 33342), disrupted plasma membrane (stained yellow using FITC-WGA) and released cytosol (stained red using sulforhodamine 101). Unlike traditional, or osmotic, lysis, which typically occurs over minutes to hours, these cells were completely ruptured within seconds of treatment, indicating that extreme wounding induced nearly instantaneous lysis and uncontrolled cell death.

Wounding outcomes are also observed in a primary cell line

We found that primary human astrocyte (HA) cells exhibited many of the same characteristic outcomes as DU145 cells after ultrasound treatment using sham-exposed HA cells (Fig 5a) as

a comparison for cell effects of wounding. Ultrasonically wounded HA cells were able to internalize calcein (Fig 5b) in a manner similar to DU145 cells. We also observed large repair blebs (Fig 5c), where a balloon bleb is indicated by an arrow and the nucleus remains blue (i.e., has not been PI-stained), an indication of viability. HA cells were also seen to form WD-P (Fig 5d), in which remnants of calcein-stained cytosol are in proximity to a PI-stained intact nucleus. The appearance of calcein in this cell body supports our hypothesis that WD-P cells are wounded prior to forming the perikaryon, thus allowing transport into the cytosol; however, the cell was unable to repair and become an intact uptake cell.

Interestingly, we were able to capture a HA cell in the process of nuclear extrusion, as shown in Fig 5e. This image shows the nucleus (red) is exposed to the outer environment, but still connected to the main cell body by a thin line of cytosol (arrow) with some actin (green). Organelle-specific labeling indicates that the large cell body contains no nucleus and that the actin of the cell body is damaged or depolymerized (indicated by the small amount of green stain in the image). This image is consistent with our hypothesis that cytoskeleton disruption occurs due to or during exposure to mechanical shear, due to the similarity of this image to those described in the literature after cytochalasin treatment (Mori et al. 1984). Finally, an intact ejected nucleus from a HA cell is shown in Fig 5f. We also observed rapid cell lysis (data not shown) after ultrasound exposure of these cells, indicating that all characteristic death modes observed in suspensions of DU145 cells are also present in HA cells after exposure to similar forms of wounding.

Wounding outcomes can be quantified by flow cytometry analysis

As shown in Fig 6, we were able to use flow cytometry analysis to find seven populations, similar to those observed in microscopy analysis, by plotting PI fluorescence vs. cell size (i.e., forward scatter) with a “third axis” of either calcein fluorescence indicated by events colored green (Fig 6a-b) or DiO fluorescence indicated by events colored red (Fig 6c). Non-sonicated cells are shown in Fig 6a, where almost all events have normal cell size and are negative for both PI and calcein staining.

Representative samples of sonicated cells are shown in Fig 6b-c. The population of events in the lower right quadrant has normal cell size and negative PI staining (Pop1), which indicates viable cells. Within this population, some cells lack calcein uptake (Pop1A normal cells) and others have calcein uptake (Pop1B uptake cells), which identifies cells with reversible plasma membrane wounding.

A few events have normal cell size and are positive for PI, calcein and DiO staining (Pop2C necrotic cells). This population has cells with irreparably damaged plasma membranes. Although necrotic, they have not undergone cell swelling or lysis at time of analysis. This population has few cells, probably because they are in a transient state en route to lysis.

Previous studies have not addressed cell size changes after wounding. We found that most nonviable cell events are smaller than control cells, i.e., Pop2D WD-P and Pop2E ejected nuclei. Strong PI fluorescence of these cells indicates that they contain intact nuclei; thus, diminished size must be due to cytosol loss. Many events in this population have calcein and DiO staining (Pop2D WD-P), indicating presence of plasma membrane consistent with WD-P. The other events mostly lack calcein and DiO staining (Pop2E ejected nuclei), indicating lack of plasma membrane consistent with isolated nuclei.

Finally, we were able to identify debris as very small events that were either PI positive and DiO negative (Pop3F nuclear debris) or PI negative and DiO positive (Pop3G cytosolic debris containing lipid components). For the purposes of quantification these two populations were reported together as total cellular debris.

Mechanical sorting confirms interpretation of flow cytometry results

To further validate our interpretation of flow cytometry data, cells were mechanically sorted into Pop1, Pop2 and Pop3 and viewed by LSCM. Sorted Pop1 cells were viable (PI⁻) either without calcein uptake (Pop1A normal cells, Fig 6e) or with green-fluorescent calcein uptake (Pop1B uptake cells, Fig 6f). Cells acquired by gating Pop2 included: non-viable (PI⁺) calcein-positive cells of similar size to Pop1 cells (necrotic cells, Pop2C necrotic cells, Fig 6g); relatively small, non-viable (PI⁺) green-fluorescent DiO-positive cell bodies comprised of intact nuclei with affiliated plasma membrane and cytosol (WD-P, Pop2D WD-P, Fig 6h); and intact PI⁺ nuclei with no affiliated plasma membrane or cytosol (Pop2E ejected nuclei, Fig 6i). Pop3 gating yielded fragmented PI⁺ nuclei (Pop3F nuclear debris) and cytosolic components (Pop3G cytosolic debris).

Quantification of wounding outcomes by flow cytometry analysis

We next quantified the number of cells in each population as a function of wounding intensity. Non-sonicated control samples contained >90% of events appearing as normal cells (Pop1A normal cells), confirming that most cells were intact and viable prior to ultrasound exposure (Fig 6d). After moderate wounding (0.36 MPa ultrasound pressure) half the cells were viable (PI⁻) and half were non-viable (PI⁺) (Fig 6d). Of viable cells, 34% were “unaffected” Pop1A normal cells and 16% were Pop1B uptake cells, indicating reversible membrane wounding. Of nonviable populations, most were in Pop2D WD-P (45%) with the rest approximately evenly divided between Pop2C necrotic cells, Pop2E ejected nuclei, Pop3F nuclear debris and Pop3G cytosolic debris with 1-2% in each.

At stronger wounding conditions (0.54 MPa), viable cells dropped to 27%, with 15% in Pop1A normal cells and 12% with uptake in Pop1B uptake cells. Of nonviable populations (73%), the majority (57%) were in Pop2D WD-P, while debris increased to 10% (Pop3), isolated nuclei from Pop2E ejected nuclei increased to 5% and intact nonviable cells in Pop2C necrotic cells remained at 1%.

At strongest wounding (0.72 MPa) Pop1A normal cells increased somewhat (17%) but Pop1B uptake cells (7%) were halved. Percent Pop2 nonviable cells (76%) was similar to weaker conditions, but distribution among populations changed; Pop2D WD-P decreased (27%) and Pop2E ejected nuclei increased correspondingly (32%). Lysed cells in Pop3 also increased (16%), while Pop2C necrotic cells remained unchanged (1%). Decrease in WD-P and increase in isolated nuclei suggests that strongest wounding conditions either affect cytoskeleton to make cells unable to contain the nucleus or cause wound repair that increases plasma membrane surface tension so that it extrudes the nucleus.

Discussion

This study provides a detailed examination of morphological changes in combination with quantification of these effects in cells after exposure to acoustic cavitation. We used multiple forms of electron and confocal microscopy to identify outcomes we were then able to assess with quantitative flow cytometry. We were able to identify specific characteristics of wounds in cells exposed to sonication, and then observed similar morphological changes in cells wounded by microfluidic shear and laser ablation, suggesting that these findings may be broadly relevant to cell wounding. Furthermore, the data are supported by two cell types, a continuous line and a primary line, indicating that these outcomes may be conserved across different types of cells.

At moderate ultrasound wounding conditions, ~1/6 of cells had reversible membrane wounding shown by calcein uptake, ~1/3 were apparently unaffected, and ~1/2 were nonviable. Notably,

most nonviable cells became WD-P, which has not been reported before and is named for its similarity to programmed cell death by autschizis (Gilloteaux et al. 2003). We propose that this outcome represents a new form of controlled necrosis that results from either plasma membrane resealing that occurs too slowly and permits excessive cytosol leakage, or due to wounds too extensive for the cell to repair.

A distribution of viable cells with and without uptake and nonviable cells after wounding is consistent with literature (Hallow et al. 2008, McNeil and Ito 1990). Other studies have better optimized wounding conditions and achieved higher levels of intracellular uptake and viability, which is important to some applications, but such optimization was beyond the scope of this work.

At higher levels of sonication, uptake and viability were both reduced; nonviable cells still included large numbers of WD-P and the number of lysed cells increased, which is consistent with harsher mechanical treatment. Notably, isolated nuclei became significant after strongest wounding. This has not been reported before and may represent either a new form of cell death by non-chemical-induced nuclear extrusion or an extreme form of WD-P, in which cytosol and plasma membrane are completely lost due to excessive wounding. Morphologically similar nuclear ejection has been reported in the literature to be caused by cytochalasin, an agent used in chemotherapy due to its ability to disrupt the cytoskeleton (Carter 1967). This suggests that some wounding modes cause a similar breakdown of the cytoskeleton, which could occur due to intense mechanical wounding, a finding supported by the work of Alter et al (Alter et al. 1998), where ultrasound exposure was found to affect cytoskeletal components of cells, including effects hypothesized to be similar to those caused by cytochalasins. Although we report two types of nuclei-based cell bodies as outcomes of wounding, our observations suggest nuclear ejection and formation of WD-P occur by different mechanisms; one via loss of cytosol through an irreparable wound (WD-P) and the other by some yet unknown destabilization of cytoskeleton (ejected nuclei).

Morphological changes reported here may represent a continuum of effects caused by different degrees of plasma membrane wounding. Cells that avoid wounding or have extremely minor wounding appear to be unaffected (Pop1A normal cells). Cells with repairable wounds retain viability and take up exogenous compounds during repair (Pop1B uptake cells). This repair appears to involve blebbing to patch the wound, where blister blebs may form during repair of smaller wounds and balloon blebs may be associated with larger wounds.

In rare cases, membrane repair occurs without significant loss of cytosol, but stresses associated with wounding nonetheless render cells nonviable (Pop2C necrotic cells). More frequently there is significant loss of cytosol and internal organelles, which yields nonviable WD-P (Pop2D WD-P). Extreme damage to plasma membrane, and possibly cytoskeleton, leads to complete dissociation of nuclei from their cell bodies (Pop2E ejected nuclei). Finally, massive wounding can lead to instant cell lysis leaving debris of nuclear (Pop3F nuclear debris) and cytosolic and membrane (Pop3G cytosolic debris) origin.

This study emphasized analysis of effects due to cavitation generated using low-frequency ultrasound. Cavitation under these conditions is known to include stable and transient bubble collapse that can lead to bubble oscillation, mechanical impact on neighboring cells by acoustic streaming, shock waves, fluid jets and other effects (Leighton 1994). We found that mechanical impact generated by direct fluid mechanical shear and laser-induced ablation had similar cellular effects, which is consistent with a mechanism based on fluid mechanical impact on the cell. Due to the low frequency of the ultrasound used in this study, contrast agents were not required to induce cavitation effects; however, we hypothesize that similar effects will be seen in cells sonicated at higher frequencies in the presence of such agents, since effects such

as cell and tissue damage, as well as drug and gene delivery at these frequencies have been reported (McPherson and Holland 2003, Miller et al. 2008, Unger et al. 2001).

These observations may help develop laboratory and medical applications of ultrasound, as well as mechanical cell wounding involving other methods. They may also help in understanding physiological and pathophysiological processes in the body in which cell wounding occurs. Typically, cell death due to wounding is an undesirable outcome that occurs as a side effect of opening plasma membranes for intracellular delivery of drugs, proteins or genes. Analysis of this study suggests that wounding intensity should be carefully controlled to avoid excessive wounding leading to WD-P or, under extreme conditions, nuclear ejection or cell lysis. Because controlling intensity of wounding in multicellular populations or in tissues is difficult, methods to promote rapid plasma membrane resealing or prevent excessive cytosolic leakage should be beneficial.

In conclusion, this study supports the hypothesis that exposure to acoustic cavitation, as well as other mechanical methods can cause cell wounding effects leading to intracellular uptake of molecules, multiple forms of membrane blebbing, and formation of WD-P. Ultrasound alone was seen to be responsible for a new observation, nuclear ejection after exposure to mechanical shear, and instant cell lysis. Finally, we propose that the outcome of wounding, i.e., repair or death by one of the identified modes, is dependent on the degree of wounding experienced by the cell.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Tim Tolentino, Daniel Hallow, Richard Seeger, Kevin Caran, Elizabeth Wright and Jeannette Taylor for technical discussions and assistance. This work was supported in part by the National Institutes of Health.

References

- Alter A, Rozenszajn LA, Miller HI, Rosenschein U. Ultrasound inhibits the adhesion and migration of smooth muscle cells in vitro. *Ultrasound Med Biol* 1998;24:711–21. [PubMed: 9695275]
- Apfel RE. Acoustic cavitation: a possible consequence of biomedical uses of ultrasound. *Br J Cancer Suppl* 1982;5:140–6. [PubMed: 6950749]
- Apkarian RP, Caran KL, Robinson KA. Topographic Imaging of Chromium-Coated Frozen-Hydrated Cell and Macromolecular Complexes by In-Lens Field Emission Scanning Electron Microscopy. *Microsc Microanal* 1999;5:197–207. [PubMed: 10383992]
- Bailey KI, O'Brien WD Jr, Dunn F. Ultrasonically induced morphological damage to mouse ovaries. *Ultrasound Med Biol* 1983;9:25–31. [PubMed: 6308869]
- Barak LS, Yocum RR, Nothnagel EA, Webb WW. Fluorescence staining of the actin cytoskeleton in living cells with 7-nitrobenz-2-oxa-1,3-diazole-phalloidin. *Proc Natl Acad Sci U S A* 1980;77:980–4. [PubMed: 6928695]
- Brayman AA, Coppage ML, Vaidya S, Miller MW. Transient poration and cell surface receptor removal from human lymphocytes in vitro by 1 MHz ultrasound. *Ultrasound Med Biol* 1999;25:999–1008. [PubMed: 10461730]
- Canatella PJ, Karr JF, Petros JA, Prausnitz MR. Quantitative study of electroporation-mediated molecular uptake and cell viability. *Biophys. J* 2001;80:755–64. [PubMed: 11159443]
- Carter SB. Effects of cytochalasins on mammalian cells. *Nature* 1967;213:261–4. [PubMed: 6067685]
- Castejon OJ, Castejon HV, Apkarian RP. Confocal laser scanning, conventional scanning and transmission electron microscopy of vertebrate cerebellar granule cells. *Biocell* 2001;25:235–55. [PubMed: 11813540]

- Cochran SA, Prausnitz MR. Sonoluminescence as an indicator of cell membrane disruption by acoustic cavitation. *Ultrasound Med Biol* 2001;27:841–50. [PubMed: 11516544]
- Dewey CF Jr, Bussolari SR, Gimbrone MA Jr, Davies PF. The dynamic response of vascular endothelial cells to fluid shear stress. *J Biomech Eng* 1981;103:177–85. [PubMed: 7278196]
- Dunn F. Cellular inactivation by heat and shear. *Radiat Environ Biophys* 1985;24:131–9. [PubMed: 4011848]
- Feril LB Jr, Kondo T. Biological effects of low intensity ultrasound: the mechanism involved, and its implications on therapy and on biosafety of ultrasound. *J Radiat Res (Tokyo)* 2004;45:479–89. [PubMed: 15635256]
- Feril LB Jr, Ogawa R, Kobayashi H, Kikuchi H, Kondo T. Ultrasound enhances liposome-mediated gene transfection. *Ultrason Sonochem* 2005;12:489–93. [PubMed: 15848113]
- Fliegel L, Burns K, MacLennan DH, Reithmeier RA, Michalak M. Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. *J Biol Chem* 1989;264:21522–8. [PubMed: 2600080]
- Gajic O, Lee J, Doerr CH, Berrios JC, Myers JL, Hubmayr RD. Ventilator-induced cell wounding and repair in the intact lung. *Am J Respir Crit Care Med* 2003;167:1057–63. [PubMed: 12480613]
- Gao ZG, Fain HD, Rapoport N. Controlled and targeted tumor chemotherapy by micellar-encapsulated drug and ultrasound. *J Control Release* 2005;102:203–22. [PubMed: 15653146]
- Gilloteaux J, Jamison JM, Arnold D, Taper HS, Von Gruenigen VE, Summers JL. Microscopic aspects of autotoxic cell death in human ovarian carcinoma (2774) cells following vitamin C, vitamin K3 or vitamin C:K3 treatment. *Microsc Microanal* 2003;9:311–29. [PubMed: 12901765]
- Guzman HR, Nguyen DX, Khan S, Prausnitz MR. Ultrasound-mediated disruption of cell membranes. II. Heterogeneous effects on cells. *J Acoust Soc Am* 2001;110:597–606. [PubMed: 11508985]
- Hallow DM, Seeger RA, Kamaev PP, Prado GR, LaPlaca MC, Prausnitz MR. Shear-induced intracellular loading of cells with molecules by controlled microfluidics. *Biotechnol Bioeng* 2008;99:846–54. [PubMed: 17879304]
- Keyhani K, Guzman HR, Parsons A, Lewis TN, Prausnitz MR. Intracellular drug delivery using low-frequency ultrasound: quantification of molecular uptake and cell viability. *Pharm Res* 2001;18:1514–20. [PubMed: 11758757]
- Larina IV, Evers BM, Esenaliev RO. Optimal drug and gene delivery in cancer cells by ultrasound-induced cavitation. *Anticancer Res* 2005;25:149–56. [PubMed: 15816532]
- Lauterborn W, Kurz T, Geisler R, Schanz D, Lindau O. Acoustic cavitation, bubble dynamics and sonoluminescence. *Ultrason Sonochem* 2007;14:484–91. [PubMed: 17254826]
- Leighton, TG. *The Acoustic Bubble*. Academic Press; San Diego: 1994.
- McNeil PL, Ito S. Molecular traffic through plasma membrane disruptions of cells in vivo. *J Cell Sci* 1990;96(Pt 3):549–56. [PubMed: 1699955]
- McNeil PL, Khakee R. Disruptions of muscle fiber plasma membranes. Role in exercise-induced damage. *Am J Pathol* 1992;140:1097–109. [PubMed: 1374591]
- McNeil PL, Kirchhausen T. An emergency response team for membrane repair. *Nat Rev Mol Cell Biol* 2005;6:499–505. [PubMed: 15928713]
- McNeil PL, Terasaki M. Coping with the inevitable: how cells repair a torn surface membrane. *Nat Cell Biol* 2001;3:E124–9. [PubMed: 11331898]
- McPherson DD, Holland CK. Seizing the science of ultrasound: beyond imaging and into physiology and therapeutics. *J Am Coll Cardiol* 2003;41:1628–30. [PubMed: 12742307]
- Mehier-Humbert S, Bettinger T, Yan F, Guy RH. Plasma membrane poration induced by ultrasound exposure: implication for drug delivery. *J Control Release* 2005;104:213–22. [PubMed: 15866347]
- Meldolesi J. Surface wound healing: a new, general function of eukaryotic cells. *J Cell Mol Med* 2003;7:197–203. [PubMed: 14594544]
- Miller DL, Averkiou MA, Brayman AA, Everbach EC, Holland CK, Wible JH Jr, Wu J. Bioeffects considerations for diagnostic ultrasound contrast agents. *J Ultrasound Med* 2008;27:611–32. quiz 33-6. [PubMed: 18359911]
- Miller DL, Dou C. Induction of apoptosis in sonoporation and ultrasonic gene transfer. *Ultrasound Med Biol* 2009;35:144–54. [PubMed: 18723272]

- Miller DL, Pislaru SV, Greenleaf JE. Sonoporation: mechanical DNA delivery by ultrasonic cavitation. *Somat Cell Mol Genet* 2002;27:115–34. [PubMed: 12774945]
- Miller DL, Quddus J. Diagnostic ultrasound-induced membrane damage in phagocytic cells loaded with contrast agent and its relation to Doppler-mode images. *IEEE Trans Ultrason Ferroelectr Freq Control* 2002;49:1094–102. [PubMed: 12201456]
- Mitragotri S. Healing sound: the use of ultrasound in drug delivery and other therapeutic applications. *Nat Rev Drug Discov* 2005;4:255–60. [PubMed: 15738980]
- Miyake K, Tanaka T, McNeil PL. Disruption-induced mucus secretion: repair and protection. *PLoS Biol* 2006;4:e276. [PubMed: 16933971]
- Mori Y, Akedo H, Matsuhisa T, Tanigaki Y, Okada M. Extrusion of nuclei of murine suspension culture cells with microtubule poisons. *Exp Cell Res* 1984;153:574–80. [PubMed: 6734763]
- Newman CM, Bettinger T. Gene therapy progress and prospects: ultrasound for gene transfer. *Gene Ther* 2007;14:465–75. [PubMed: 17339881]
- Pitt WG, Hussein GA. Ultrasound in drug and gene delivery. *Adv Drug Deliv Rev* 2008;60:1095–6. [PubMed: 18471928]
- Postema M, Gilja OH. Ultrasound-directed drug delivery. *Curr Pharm Biotechnol* 2007;8:355–61. [PubMed: 18289044]
- Rapoport NY, Christensen DA, Fain HD, Barrows L, Gao Z. Ultrasound-triggered drug targeting of tumors in vitro and in vivo. *Ultrasonics* 2004;42:943–50. [PubMed: 15047411]
- Schlicher RK, Radhakrishna H, Tolentino TP, Apkarian RP, Zarnitsyn V, Prausnitz MR. Mechanism of intracellular delivery by acoustic cavitation. *Ultrasound Med Biol* 2006;32:915–24. [PubMed: 16785013]
- Sharon N, Lis H. Lectins as cell recognition molecules. *Science* 1989;246:227–34. [PubMed: 2552581]
- Tachibana K, Uchida T, Ogawa K, Yamashita N, Tamura K. Induction of cell-membrane porosity by ultrasound. *Lancet* 1999;353:1409. [PubMed: 10227224]
- Togo T, Alderton JM, Bi GQ, Steinhardt RA. The mechanism of facilitated cell membrane resealing. *J Cell Sci* 1999;112(Pt 5):719–31. [PubMed: 9973606]
- Unger EC, Hersh E, Vannan M, Matsunaga TO, McCreery T. Local drug and gene delivery through microbubbles. *Prog Cardiovasc Dis* 2001;44:45–54. [PubMed: 11533926]
- Urs NM, Jones KT, Salo PD, Severin JE, Trejo J, Radhakrishna H. A requirement for membrane cholesterol in the beta-arrestin- and clathrin-dependent endocytosis of LPA1 lysophosphatidic acid receptors. *J Cell Sci* 2005;118:5291–304. [PubMed: 16263766]
- Warren GL, Lowe DA, Hayes DA, Farmer MA, Armstrong RB. Redistribution of cell membrane probes following contraction-induced injury of mouse soleus muscle. *Cell Tissue Res* 1995;282:311–20. [PubMed: 8565058]
- Yuh EL, Shulman SG, Mehta SA, Xie J, Chen L, Frenkel V, Bednarski MD, Li KC. Delivery of systemic chemotherapeutic agent to tumors by using focused ultrasound: study in a murine model. *Radiology* 2005;234:431–7. [PubMed: 15671000]

Fig. 1.

Non-wounded DU145 control cells (a-c) and sonication-wounded cells (d-f). (a) SEM, arrow indicates normal bleb, (b) LSCM, plasma membrane (PM) labeled with TRITC-WGA (red) and nucleus labeled with Hoechst 33342 (blue), (c) LSCM, actin cytoskeleton labeled with BODIPY phalloidin (green) and nucleus labeled with Hoechst (blue), (d) SEM, sonicated at 0.54 MPa, fixed 5 s after, arrow indicates PM wound, (e) LSCM, sonicated at 0.72 MPa, fixed 1-2 s after, arrow indicates hole in PM through which the uptake marker, calcein (green), probably diffused into the cell, (f) LSCM, sonicated at 0.54 MPa, fixed 1-2 s after, actin cytoskeleton labeled with BODIPY phalloidin (green), arrow indicates a break in cytoskeleton, suggesting that ultrasound-mediated wounds go deeper than PM. Scale bars are 1 μm .

Fig. 2.

Wounding creates cellular blebs. (a) LSCM, sonicated at 0.72 MPa, fixed 30 s after, PM labeled with TRITC-WGA (red) and internal lipids labeled with FM 1-43 (green), arrow indicates a break in PM from which a bleb of internal lipids protrudes, (b) LSCM, sonicated at 0.72 MPa, fixed 30 s after, internal lipids labeled with FM 1-43 (green), arrow indicates internal compartment from which a bleb appears to derive, (c) BFM overlaid with LSCM, sonicated at 0.54 MPa, viewed live within 10 min after, endoplasmic reticulum (ER) labeled using Organelle Lights (green), indicating that small vesicles observed in balloon blebs may be formed from ER-derived lipids, (d1) BFM overlaid with LSCM, sonicated at 0.72 MPa, fixed 1 min after, cytoskeleton labeled with BODIPY phalloidin (green) and nucleus labeled with

propidium iodide (PI, red), yellow arrows indicate balloon blebs that broke through cytoskeleton, red arrows point to holes in cytoskeleton through which blebs protrude, (d2) Close-up view of 2d1, with no BFM exposure, (e) Cryo-SEM, sonicated at 0.72 MPa, fixed ~ 30 s after by freezing in liquefied ethane, yellow arrow indicates a balloon bleb, which protrudes spherically from the cell and is attached by a long neck, red arrow points to a blister bleb, which is contiguous with the PM. (f1) BFM overlaid with LSCM, sonicated at 0.54 MPa, nuclei labeled with PI (red, indicating lost viability), field of live cells imaged after sonication, showing that many cells are dead, but that a cell with a blister bleb (circle) remains viable (excludes PI) after 15 min, (f2) Close-up of circled cell, BFM image showing beginning of image collection following bleb formation within minutes after sonication, (f3) BFM of same cell, ~10 min later, bleb has redistributed as a blister formation (See Fig. S1, Movie M1), (g) BFM, mechanically sheared, arrow indicates balloon bleb, (h) BFM, mechanically sheared, arrow indicates blister bleb, (i) BFM, laser ablated, arrow indicates blister bleb (See Fig. S2, Movie M2). Scale bars are 1 μm , which the exception of f1, where the scale bar is 10 μm .

Fig. 3.

Formation of wound-derived perikarya (WD-P) after PM damage. (a) SEM, sonicated at 0.72 MPa, fixed 5 s after, arrow indicates cytosol released from cell, (b) SEM, sonicated at 0.72 MPa, fixed 5 s after, arrow indicates PM remnant, ** indicates nucleus, (c) BFM overlaid with LSCM, sonicated at 0.54 MPa, arrow indicates PM remnants labeled with DiO (green), ** shows PI stained nucleus (red), (d) BFM, mechanically sheared, arrow indicates PM and ** nucleus, (e1) BFM laser ablated cell captured live producing balloon bleb (arrow), ** indicates nucleus; over time, this bleb grew in size, but burst (e2) causing massive cytosol loss (arrow) and leaving behind nucleus (**) and PM, (See Fig. S3, Movie M3). Scale bars are 1 μm .

Fig. 4.

Ejected nuclei and cell debris. (a) SEM, sonicated at 0.72 MPa, fixed 5 s after; arrow indicates nucleus extruding from cell. (b) LSCM, sonicated at 0.72 MPa, fixed 1 min after, PM labeled with TRITC-WGA (red) and nucleus with Hoechst (blue). Arrows indicate break in the plasma membrane through which nucleus protrudes. (c) LSCM, sonicated at 0.72 MPa, imaged live, PM labeled with DiO (green) and nuclei with PI (red), arrow indicates a PI-stained ejected nucleus with no affiliated PM, part of a different cell appears below to show normal PM (stained green), (d) SEM, sonicated at 0.54 MPa, fixed 5 s after, shows cell debris, e.g. completely fragmented cells that were destroyed either during sonication or within s after, (e) Cryo-SEM, sonicated at 9 atm, fixed ~ 30 after in liquefied ethane shows spheres of cell debris, (f) LSCM, sonicated at 0.72 MPa, fixed 30s after, fragments of PM appear yellow due to overlap of FITC-WGA (green) with attached sulforhodamine uptake marker (red), pieces of nuclei are Hoechst-stained blue, cytosol is red due to sulforhodamine 101 uptake before fragmentation. This image shows that cell debris (d-e) contains all parts of the cell and that rapid death by this means is unlike death that creates WD-P or ejected nuclei. Scale bars are 1 μ m.

Fig. 5.

Human astrocytes after exposure to wounding. Effects are similar to DU145 cells (see main text). (a) BFM overlaid with LSCM, sham exposed control cell, nucleus stained with Hoechst 33342 (blue), (b) LSCM, sonicated at 0.54 MPa, showing uptake of calcein (green) and Hoechst-stained nucleus (blue, **), (c) BFM overlaid with LSCM, sonicated at 0.54 MPa, arrow indicates balloon bleb in cell with uptake of calcein (green) whose nucleus is labeled with Hoechst (blue), (d) LSCM, sonicated at 0.54 MPa, WD-P consisting of a PI stained nucleus (red) and some remaining cytosol with uptake of calcein (green), showing that WD-P cells have wounds capable of intracellular uptake, (e) BFM overlaid with LSCM, sonicated at 0.72 MPa, cell in the presence of Hoechst nuclear stain (blue) sonicated with BODIPY-phalloidin (green, labels actin) as uptake marker whose nucleus has been ejected and shows PI staining (red), but is still connected to the cell by a thread of cytosol (arrow) with some actin (green); the lack of internal cytoskeleton staining indicates that either (i) the cell was not well permeabilized, e.g. no large holes were created by ultrasound exposure or (ii) the cytoskeleton is completely disordered. The large cell body (**) was fully scanned in the z-direction to show that no nucleus is present, (f) BFM overlaid with LSCM, sonicated at 0.72 MPa, PI-stained (red) completely ejected nucleus in a sample stained for actin with BODIPY-phalloidin (green); the lack of green indicates that the nucleus has no cellular actin component, similar samples show that ejected nuclei also have no cytosol or PM components. Scale bars are 1 μm .

Fig. 6.

Separation of cells into characteristic populations using flow cytometry analysis and confocal imaging of mechanically sorted populations. Flow cytometric dot plot shows that (a) cells from sham-exposure control sample fall mainly in Pop1, (b) cells sonicated at 0.54 MPa are more evenly distributed across the populations, where cells with calcein uptake are shown in green and cells without calcein uptake are shown in black, (c) cells PM stained with DiO are shown in red while cells without are shown in black, (d) Bar graph showing the distribution (as percent of total cells) into the identified populations as a function of ultrasound pressure; Pop3F nuclear debris/3G cytosolic debris are combined during quantification; (e-j) BFM overlaid with LSCM, sonicated at 0.54 MPa, imaged live after mechanical sorting. (e) Pop1A normal cells, cell with no uptake; (f) Pop1B uptake cells, cell with uptake of calcein; (g) Pop2C necrotic cells, PI^+ cell similar in size to Pop1; (h) Pop2D WD-P, PI^+ exposed nucleus with plasma membrane (DiO^+). (i) Pop2E ejected nuclei, PI^+ nucleus with no PM; (j) Pop3, debris sorted by gating everything outside of Populations 1, 2, and 3; all scale bars are 10 μm .

Table 1

Analysis of flow cytometry populations

Population	Characteristics	Size ¹	PI ²	calcein ²	DiO ²
(1A)	cells with normal morphology	=	-	-	+
(1B)	normal cells with uptake of calcein	=	-	+	+
(2C)	necrotic cells of normal size	=	+	+	+
(2D)	small necrotic cells with loss of cytosol (WD-P)	<	+	+	+
(2E)	ejected nuclei	<	+	-	-
(3F)	nuclear debris (lysed cells)	<<	+	-	-
(3G)	cytosolic debris (lysed cells)	<<	-	-	+/-

¹ Events can be equal to (=), smaller than (<) or much smaller than (<<) normal cell size.

² Events can be stained (+) or unstained (-) with the various marker compounds.

Table 2

Cell treatments presented in this study.

Treatment	DU145 Prostate Cancer Cells	Human Astrocyte Cells
Ultrasound	EM, LSCM, flow cytometry	LSCM
Mechanical shear	LSCM	---
Laser ablation	LSCM	---
Fluorescent WGA (plasma membrane)	LSCM	---
DiO (plasma membrane)	LSCM, flow cytometry	---
BODIPY phalloidin (actin cytoskeleton)	LSCM	LSCM
Organelle lights™ (endoplasmic reticulum)	LSCM	---
FM 1-43 (internal lipids)	LSCM	---
Calcein (green uptake molecule)	LSCM, flow cytometry	LSCM
Sulforhodamine 101 (red uptake molecule)	LSCM	---