Cell Loading with Laser-Generated Stress Waves: The Role of the Stress Gradient

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Purpose. To determine the dependence of the permeabilzation of the plasma membrane on the characteristics of laser-generated stress waves.
Methods. Laser pulses can generate stress waves by ablation. Depending on the laser wavelength, fluence, and target material, stress waves of different characteristics (rise time, peak stress) can be generated. Human red blood cells were subjected to stress waves and the permeability changes were measured by uptake of extracellular dye molecules.
Results. A fast rise time (high stress gradient) of the stress wave was required for the permeabilization of the plasma membrane. While the membrane was permeable, the cells could rapidly uptake molecules from the surrounding medium by diffusion.
Conclusions. Stress waves provide a potentially powerful tool for drug delivery.

KEY WORDS: drug delivery; drug carriers; red blood cells; sonophoresis; shock waves; ultrasound; phonophoresis; phonoporation.

INTRODUCTION

The effects of stress waves on cells and tissue have been a recurrent theme of investigation in ultrasonics (1), extracorporeal shock wave lithotripsy (ESW) (2), and laser-tissue interactions (3). Laser-generated stress waves (LSWs) are broadband, unipolar waves which do not produce a measurable tensile component (3) and thus exclude the biological effects induced by cavitation (4). The ability of short laser pulses to generate stress waves was demonstrated shortly after the invention of the Q-switched laser. Laser generated stress waves can be generated by: optical breakdown, ablation, and rapid heating of an absorbing medium (3). The characteristics of the stress waves depend on the laser parameters (wavelength, pulse duration, and fluence) as well as the optical and mechanical properties of the target material. To effectively investigate the biological effects of stress waves it is necessary to eliminate all other sources of cellular injury such as cavitation, thermal denaturation and bulk displacement. The experimental arrangement used eliminates these effects, allowing an examination of the cellular response caused strictly by the stress waves (6,14).

Stress (shock) waves (LSWs and ESWs) as well as ultrasound can render the plasma membrane permeable. Changes in membrane permeability can be detected through the release of cellular components (5–7) or uptake of molecules present in the surrounding medium (8,10–13). The change of the membrane permeability induced by LSWs is transient and the barrier function of the membrane recovers within a minute (9). Furthermore, the cells remain viable (9). This provides an opportunity to load a variety of potentially therapeutic molecules into cells *in vitro*.

In a previous study, we showed that cell injury produced by LSWs to EMT-6 cell line (mouse breast sarcoma) depended on the stress gradient (the peak stress divided by the rise time) (14). Cell cultures, for example, exposed to stress waves of the same peak stress but different rise time sustained substantially different levels of cellular damage (14). In this report we show that the rise time of the propagating compressive wave is vital for efficient membrane permeabilization.

Red blood cells (RBCs) were chosen because they provide a simple model, as they lack the large number of complex membrane enclosed organelles common to other eukaryotic cells. Human RBCs have previously been shown to be capable of uptake of membrane impermeable fluorescent dyes after exposure to LSWs (15). The RBC is also a potent target for a drug delivery vehicle due to its access to most bodily tissues (16). By opening the membrane temporarily we are left with an intact cell that can carry its molecular cargo without generating an immune response or being subjected to interference from blood enzymes.

In this study we used two different lasers (ArF excimer at 193 nm and ruby at 694.3 nm) to generate stress waves in two different target materials (polyimide 76 μm thick and polystyrene 1 mm thick). The various combinations of lasers and targets allow us to generate stress waves with different parameters and thus determine the dependance of plasma membrane permeabilization on the characteristics (rise time and peak stress) of laser-generated stress waves.

MATERIALS AND METHODS

Cell Preparation and Handling

Fresh human blood was used in this study to insure that the cellular membranes were intact and unaltered. Any extracellular material will interact with and affect membrane first, so minimizing the manipulation of the blood was of paramount importance. The cells were not separated. Because RBCs constitute 99% of the cell volume of blood, the contribution of the other cell types could be effectively ignored. Additionally, the use of flow cytometry allowed other cell types to be excluded from the analysis.

The protocol was approved by the MGH subcommittee on human studies and informed consent was obtained from the volunteers. Human blood was drawn in heparinized syringes. The blood was immediately diluted into 40 ml of phosphate buffered saline (PBS) without calcium or magnesium. The blood solution was then spun briefly at 1200 RPM (200 g for 5 minutes) to pellet the blood and remove the plasma. The pellet containing the mixture of blood cells was then re-suspended to an adjusted concentration of 40×10^6 cells/ml in PBS. The cell solution (100 μl/well) was then added to the wells of the 96-well black polystyrene plates (Dynatech Laboratories,

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ABBREVIATIONS: ESWs, extracorporeal shock wave; FITC, fluorescein isothiocyanate; LSW, laser-generated stress wave; PBS, phosphate buffered saline; PI, polyimide; PS, polystyrene; PVDF, polyvinyledene fluoride; RBC, red blood cell.

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Chantilly, VA). An equal volume of 200 μM 4.4 kDa dextran-fluorescein isothiocynate (FITC-dextran, Sigma Chemicals, St. Louis, MO) in PBS was added to each well for a final dye concentration of 100 μM. In some experiments a home-built plate was used with polyimide film (300 HN kapton, Dupont, DE) as the laser target material (11). In this case, 50 μl/well of the cell solution was used and an equal volume of the FITC-dextran solution was added. The wells in the polystyrene plate had a diameter of 6 mm while the wells in the polyimide plate were 3 mm in diameter. The plates were then placed in a refrigerator at 4°C for 30 minutes to allow the cells to settle to the bottom of the plates. The plates were then carefully removed, brought to room temperature and subjected to 1, 5, or 20 stress waves. In some experiments the FITC-dextran solution was added to the well 15 min after exposure of RBCs to stress waves in order to determine whether the barrier function of the plasma membrane had recovered.

After the application of stress waves, the cells in solution were removed from each well and diluted in an excess of PBS. The resulting solution was then spun (1200 RPM for 10 minutes), the solution decanted, and re-suspended in fresh PBS. The cells were washed a total of 3 times to remove any extracellular FITC-dextran from the solution. After the final wash the cells were suspended in 2 ml of PBS and analyzed by flow cytometry.

Stress Wave Generation

The experimental setup for the stress wave generation is shown in Fig. 1. The laser beam was directed onto the bottom of the plates, black polystyrene (PS) 1 mm thick, or polyimide (PI) 76 μm thick, which acted as the target material. It was focused to a spot size equal to the diameter of the well, namely 6 or 3 mm. The stress waves were generated by laser ablation of the target and launched into the aqueous medium containing the cells. The laser pulse was totally absorbed by the target so that the RBCs were not exposed to the laser radiation.

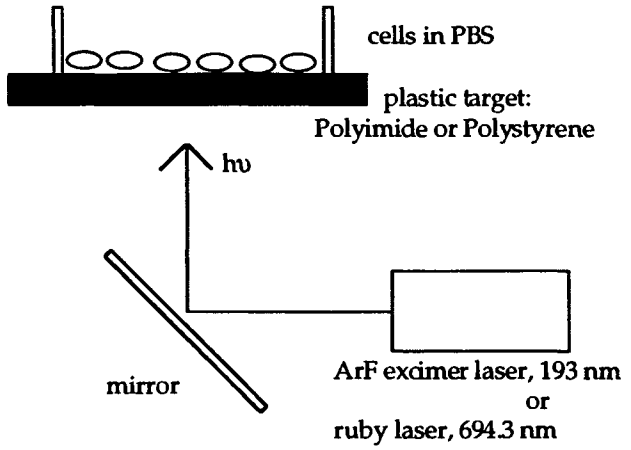

Fig. 1. Diagram of the setup used to generate stress waves *in vitro*. In each case the laser beam was focused off a single mirror in order to strike the plastic target from beneath. For the Ruby laser the beam was focused to a final diameter of 6 mm. For the ArF excimer laser the laser spot size was adjusted to either 3 or 6 mm by adjusting the sample stage. In both cases the laser pulse was totally absorbed by the target so that the RBCs were not exposed to the laser radiation.

A Q-switched ruby laser (Laser Applications, Inc., Orlando, FL) and an ArF excimer laser (LPX-305i, Lambda Physik, Ft. Lauderdale, FL) were used to generate stress waves. The operating parameters of the ruby laser were 694.3 nm, 2.1 J, and 23 ns pulse duration. The excimer laser operated at 193 nm, 350 mJ, and 29 ns pulse duration. The excimer laser was used with both PS and PI targets, while the ruby laser was used exclusively with the PS target. Polyimide absorbs poorly at 694.3 nm and cannot be used as a target for the ruby laser. The targets were selected because they absorb light very well at the wavelengths used, the polyimide has a penetration depth of 24 nm at 193 nm (19). The pentration depth of the polystyrene is not known, but the material is totally opaque. In each case this resulted in all the laser radiation being absorbed by the target material and none penetrating to the cell sample.

The stress waves were measured using a calibrated polyvinyledene fluoride (PVDF) transducer placed at the target site and recorded by a digital oscilloscope (LeCroy 9360, LeCroy, Chestnut Ridge, NY). The temporal resolution of the transducer-oscilloscope was ~5 ns. The energy of the laser pulse was varied to achieve the desired peak stress by placing neutral density filters in the beam path. In previous publications (6,14), we described the methodology for generating the stress waves, as well as the experimental conditions that insure that the observed phenomena are being generated solely by the compressive stress waves and not by heat, light or cavitation.

The oscilloscope traces of the stress waves generated by the ArF and ruby laser on PI and PS targets are shown in Fig. 2. The stress waves generated by the two lasers have different rise times. The stress wave generated by the ArF laser has a rise time of 8 ns which is practically limited by the resolution of the transducer. On the other hand, the ruby laser generates a stress wave with a 40 ns rise time, five times that of the ArF laser. Thus, the stress waves generated by the two lasers produce different stress gradients across the cells. The stress wave generated by the ArF laser on polyimide (PI) has also a rise time of

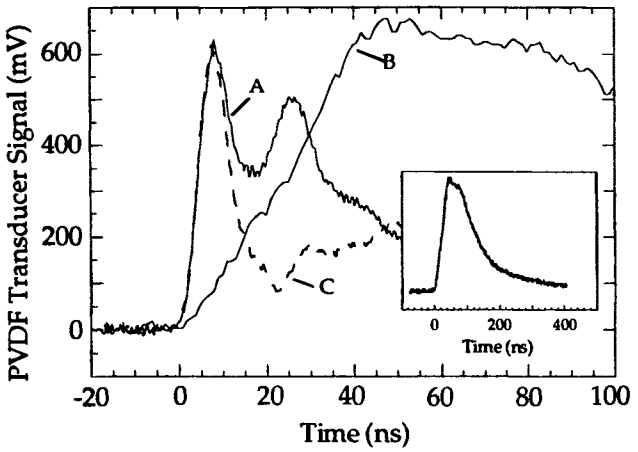

Fig. 2. The oscilloscope trace of the leading edge of the stress waves generated by the ablation of the plastic target. Trace A corresponds to the ArF excimer laser on a polyimide target, trace B to the ruby laser on a polystyrene target, and trace C to the ArF excimer laser on a polystyrene target. The second peak shown in trace A is the reflected wave at the target-transducer interface and corresponds to the round trip time inside the target. The insert shows the complete waveform of the stress wave generate by the ruby laser. The measured signal intensity (in mV) was used to calculate the peak stress.

8 ns. The difference in the rise time of the stress waves generated by the ArF and ruby lasers is probably due to the different mechanisms involved in the generation of the stress waves. Ultraviolet ablation with the ArF laser causes a decomposition of the polymer at the target surface. (20) The microscopic fragments are ejected from the target surface at high velocity. A stress wave with a high peak stress is generated within the intact target material by the imparted recoil momentum. This process generates stress waves with a fast rise time. On the other hand, an infrared laser source creates a plasma at the air-target interface. There is a considerable impulse delivered to the target even after the termination of the laser pulse as the pressure of the plasma relaxes to the ambient value (21). Thus, the formation of plasma results in increasing the rise time of the stress wave.

Measurement of Dextran Uptake

The permeabilization of the plasma membrane caused by the application of stress waves was measured by the uptake of the FITC-dextran probe using flow cytometry. Flow cytometry measures the fluorescence intensity of individual cells. This allows for the rapid fluorescence measurement of a large number of cells while simultaneously measuring the size and shape of the cells passing through the instrument. The flow cytometer (Becton Dickinson, FACScan, Bedford, MA) was set to collect data on 10,000 cells using an excitation wavelength of 488 nm and detecting fluorescence at 525 nm. The same number of cells were observed from each sample, correcting for any difference in cell density. The following conditions were measured: (A) Controls, RBCs in PBS; (B) RBCs exposed to stress waves only; (C) RBCs in the solution of FITC-dextran but not exposed to stress waves; and (D) RBCs exposed to stress waves in the presence of FITC-dextran.

RESULTS

Figure 3 shows the flow cytometry measurements of RBCs exposed to 5 stress waves from the ArF excimer laser (8 ns rise time, 600 bar peak stress). In the absence of the FITC-dextran, the cells had a very low background fluorescence. Exposure of these cells to stress waves had no effect on this background level of fluorescence. Addition of dextran in the absence of stress waves slightly increased the fluorescence background of the cells. Application of stress waves in the presence of the FITC-dextran, resulted in an increase in the cellular fluorescence corresponding to the uptake of the probe molecules. Figure 4 shows the flow cytometry measurements of RBCs exposed to 5 stress waves from the ruby laser (40 ns rise time, 615 bar peak stress). There is little difference between samples exposed to stress waves in the presence of FITC-dextran and samples in FITC-dextran solution but not exposed to stress waves.

The forward scatter and side scatter of the laser light did not change by the addition of FITC-dextran or the application of stress waves, suggesting that neither the fluorescent probe nor the stress waves caused any gross alterations to the morphology of the cells (data not shown).

Figure 5 shows the change in molecular uptake as the peak stress of the stress wave was increased (5 stress waves applied at each stress). The dextran uptake by RBCs exposed to 1, 5, and 20 stress waves at 500 bar is shown in Fig. 6. The molecular uptake as indicated by the integrated fluorescence was calculated from the flow cytometry data of 10,000 cells. The total number of pulses does not effect the net uptake of 4.4 kDa FITC-dextran. A single pulse was sufficient for maximum delivery of the 4.4 kDa dextran into the RBCs.

DISCUSSION

It has been shown previously that LSWs caused injury to cells which was dependent on the (rise time) stress gradient (14). The present measurements show that the permeabilization of the plasma membrane is also dependent on the rise time. Ablation of the PS target with the ruby laser resulted in a stress wave with a rise time of over 40 ns which did not cause any measurable increase in the uptake of FITC-dextran. Ablation

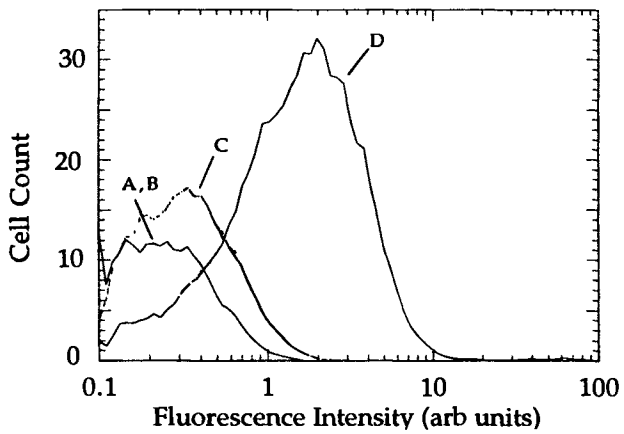

Fig. 3. Flow cytometry measurements showing the uptake of 4.4 kDa FITC-dextran following the application of 5 LSWs with the ArF excimer laser (8 ns rise time, 600 bar peak stress). The following conditions are shown: (A) Controls, RBCs in PBS; (B) RBCs exposed to stress waves only; (C) RBCs in the solution of FITC-dextran but not exposed to stress waves; and (D) RBCs exposed to stress waves in the presence of FITC-dextran.

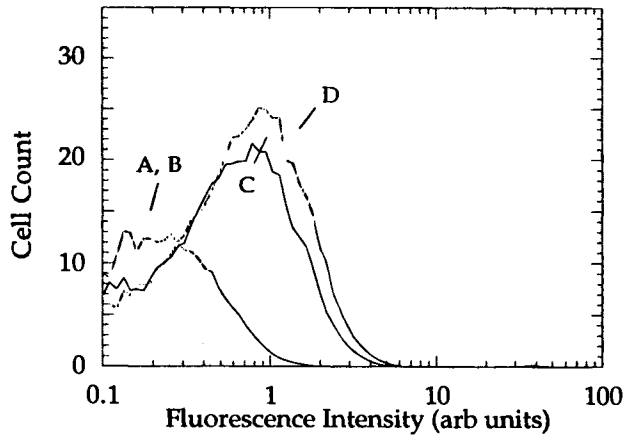

Fig. 4. Flow cytometry measurements showing the uptake of 4.4 kDa FITC-dextran following the application of 5 LSWs with the ruby laser (40 ns rise time, 615 bar peak stress). The following conditions are shown: (A) Controls, RBCs in PBS; (B) RBCs exposed to stress waves only; (C) RBCs in the solution of FITC-dextran but not exposed to stress waves; and (D) RBCs exposed to stress waves in the presence of FITC-dextran.

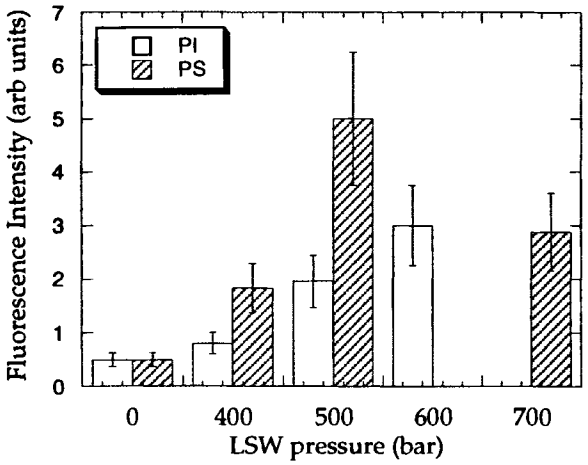

Fig. 5. The integrated cellular fluorescence intenstity following 5 stress waves with different peak stress. The integrated fluorescence was calculated from the flow cytometry data on 10,000 cells. An ArF excimer laser was used to generate LSWs on PI (clear bars) and PS (shaded bars) targets.

of the PS and PI targets by the ArF excimer laser resulted in slightly different waveforms and different stress of maximal uptake. The maximal uptake of FITC-dextran for the PS target was observed at 500 bar with the total uptake declining above and below that peak stress. On the other hand, the maximal uptake of the FITC-dextran for the PI target was observed at 600 bar with the total uptake declining at lower stress. Peak stress of greater than 600 bar could not be generated using the current laser system. These differences help to demonstrate the important role the stress gradient has in membrane permeabilization. Neither peak stress nor rise time alone control the interaction between the stress wave and the membrane. The peak stress of the ruby was varied over a wide range (up to 2 kbar), and yet was never able to increase the uptake of FITC-dextran. Conversely, the stress waves generated by the ArF excimer laser on the two different plastic targets had very similar rise times, yet each had a different peak stress for maximal uptake. The stress gradient, the combination of rise time and peak stress in the material, appears to govern the effectiveness of membrane permeabilization.

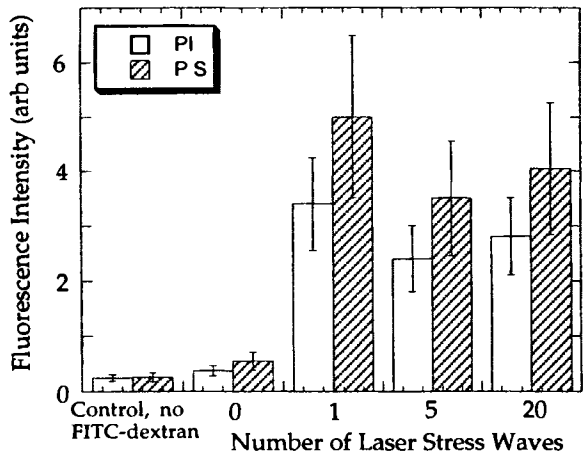

Fig. 6. The integrated cellular fluorescence intensity following 1, 5, or 20 pulses. The integrated fluorescence was calculated from the flow cytometry data of 10,000 cells. An ArF excimer laser was used to generate the LSW using PI (clear bars) and PS (shaded bars) targets. The peak stress was 440 and 550 bar, respectively. One LSW was sufficient to achieve maximal cell loading.

A single stress wave was sufficient to permeabilize the cell plasma membrane and allow the FITC-dextran to diffuse into the cytoplasm. The use of additional stress waves did not increase the uptake of the dextran. Addition of the FITC-dextran 15 minutes after the stress wave resulted in no uptake, indicating that the plasma membrane had recovered its barrier function. This observation is consistent with previous experiments (9) which have shown that a EMT-6 cell line (mouse breast sarcoma) recovers its barrier function approximately 80 seconds after the application of a single stress wave.

The mechanisms of membrane permeabilization by LSWs are not entirely known. A number of experiments suggest that the permeabilization of the plasma membrane is facilitated by membrane proteins (15). The osmotic fragility of the plasma membrane, on the other hand, was not altered when RBCs were exposed to stress waves (22). The mechanisms of action of LSWs appear to be different from those of ultrasound and ESWs. Permeabilization of the plasma membrane by the latter is mediated mostly by cavitation. Delius (23) showed that the application of hydrostatic pressure concurrently with ESWs decreased substantially the uptake of the molecular probes, presumably because of the reduction in cavitation. It should be also pointed out that stress waves are different from the hydrodynamic shear stresses that are used to disrupt the RBC plasma membrane (24). For example, decreasing the magnitude of the shear stress increases the exposure time required to produce the same degree of cell lysis (26). Conversely, decreasing the peak stress requires a shorter rise time to produce the same stress gradient and thus the same degree of permeabilization.

Because LSW's can cause cell damage as well as cell loading, understanding the relationship between these two phenomena is important. We have demonstrated previously (6) that the peak stress necessary for cell loading is lower than that required for cell injury in the cell lines that have been studied so far, human peripheral blood mononuclear cells (6,11) and EMT-6 cell lines (9,14). We have also shown that the degree of cell injury also depends on the rise time (14). RBC cell damage as measured by lysis of intact red blood cells occurs over a range of stresss, and increases with increasing peak stress (6). At 700 bar of stress approximately 95% of the cells remained intact, while at 1.8 kbar only 75% were intact after 5 stress waves (3% of cells lysed in the control sample from the stresses of the cell handling). By choosing the parameters of peak stress and rise time it is possible to effectively load cells while minimizing cell injury.

The capacity of a LSW to transiently permeabilize a cell membrane has potent implications for loading drugs and other active compounds into living blood cells. By loading exogenous compounds and chemicals into a cell they are sequestered from the immune system and blood enzymes. RBCs may be loaded using LSWs with a minimum of cellular injury. The manipulation of the cells is kept to a minimum, and the loading is completed in minutes with the application of a single stress wave. All that is then required is to wash the cells to remove the remaining exogenous material and

any cellular proteins that escaped while the membrane was permeable (11).

The permeabilization of the cell membrane is only one of the many interesting effects facilitated by LSWs that have been observed to this date. We have recently shown that LSWs can permeabilize the stratum corneum *in vivo* (26). The increased permeability is transient and the barrier function of the stratum corneum recovers within minutes. Interestingly, the permeabilization of the stratum corneum and the cell plasma membrane require stress waves of different characteristics. For example, a single stress wave generated by the ruby laser on the PS target can permeabilize the stratum corneum. Yet the same stress wave is not effective on the plasma membrane. It appears, therefore, that the mechanisms of permeabilization of the two systems are different.

While we used laser ablation of a plastic target to generate stress waves in these experiments, this is only one of many techniques available. Extracorporial shock wave lithotripters (2), shock tubes (27), and explosives (28) can all generate stress waves. The differences in the stress waves generated (peak stress, rise time, repetition rate) give them different physiological effects. Stress waves have already been used to deliver target genes to cells in vitro (10,12,13). Cell loading techniques have also been used to turn cells into drug carriers (16). While intact skin provides a very convenient target for stress wave-induced drug delivery, the use of other generation systems such a thermoelastic expansion of fiber-optic based targets and explosives contained within balloon catheters should provide mechanisms for accurately delivering stress waves to a variety of tissues and organs *in vivo*.

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