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●Original Contribution

PHYSICAL FACTORS INVOLVED IN STRESS-WAVE-INDUCED CELL INJURY: THE EFFECT OF STRESS GRADIENT

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Abstract—We have studied the biological effects of ablation-induced stress waves *in vitro*. Mouse breast sarcoma cells (EMT-6) were exposed to stress waves that differed only in rise time. Two assays were used to determine cell injury: incorporation of tritiated thymidine (viability assay), and transmission electron microscopy (morphology assay). We present evidence that the rise time of stress waves can significantly modify cell viability and that cell injury correlates better with the stress gradient than peak stress.

Key Words: Ablation, Photoacoustics, Shock waves, Stress gradient, Cell viability, Membrane permeability.

INTRODUCTION

The effects of stress (pressure) waves on tissues, cells, organelles and macromolecules have been extensively investigated. This area of research encompasses such diverse fields as ultrasonics, extracorporeal shock-wave lithotripsy and photoacoustics. The biological response of tissues and cell cultures to stress waves is complex because it can be mediated by many different mechanisms such as heat, cavitation, plasma and free radicals (*e.g.*, Suslick 1988). It was recognized quite early, however, that in many instances the observed cell damage was not caused solely by temperature rise in tissue (Fry et al. 1950) or cavitation and its concomitants (Fry et al. 1951) but also induced by other physical factors (*e.g.*, mechanical stresses). The term *shock wave* has been used often to describe stress transients generated by extracorporeal lithotripters or induced by pulsed lasers. The salient feature of a shock wave is a discontinuity in stress (pressure), density, particle velocity and internal energy (*e.g.*, Duvall and Fowles 1963). We use the general term *stress wave*, to indicate a compressive wave with a fast rise time which is not necessarily a shock wave in the strict sense.

Although the response of tissues and cells exposed to stress waves has been the subject of considerable study (Brümmer et al. 1989; Doukas et al. 1993; Hart-

man et al. 1990; Kaver et al. 1992; Smits et al. 1991a, 1993; Watanabe et al. 1988), the influence of the stress-wave parameters to cell functions, to the best of our knowledge, have not been investigated in any detail. Red blood cell (RBC) lysis is, perhaps, the best studied system. Everett et al. (1972) summarized the available data on RBC lysis under a variety of conditions of shear stress. They concluded that the required magnitude of shear stress to produce lysis was dependent on the exposure time. Smith et al. (1992) compared cell damage induced by piezoelectric and electrohydraulic lithotripters. They found that the mechanism of *in vitro*-induced damage depended on the source of stress waves. They hypothesized that the stress-wave characteristics, such as rise time duration, repetition rate and other parameters, could have biological significance.

In the last few years the capability of stress waves to retard tumor growth alone (Randazzo et al. 1988; Russo et al. 1987; Weiss et al. 1990) or in combination with chemotherapeutic drugs (Flotte et al. 1993; Holmes et al. 1990; Gambihler and Delius 1992) has brought into focus the therapeutic potential of stress waves. Our interest in understanding the biological effects of stress waves was motivated by observations in our laboratory that the stress-wave-induced enhancement of drug cytotoxicity was dependent on the characteristics of stress waves applied to cell cultures (Flotte et al. 1993, unpublished data).

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In a previous study (Doukas et al. 1993), we used an experimental arrangement to investigate the stress-wave-induced cell injury *in vitro* as a function of peak stress with minimal interference from ancillary effects, such as plasma, heat and UV radiation. In this report, we present evidence that the rise time of the stress wave is an important parameter in determining cellular response.

MATERIALS AND METHODS

Figure 1 shows the experimental arrangement. Stress waves were generated by ablation of polyimide film (300 HN Kapton, Dupont, DE) and launched into the medium containing the cells. The laser source was an excimer laser (Lambda Physik Model EMG 103, Acton, MA) operating at a 1-Hz repetition rate. Two wavelengths were used, 193 nm (ArF) and 248 nm (KrF). The laser beam was focused on the polyimide target to a rectangular spot 6×3 mm in size. The pulse energy was varied from 25 to 240 mJ using quartz attenuators. In the present study, the apparatus was modified to reduce secondary cell injury caused by deformation of the polyimide film. The ablation was not confined and a thicker polyimide film (75 μ m) was used. This geometry generated stress waves of lower peak value than the confined geometry previously used. It eliminated, however, the deformation of the polyimide film that might cause cell injury. In

Fig. 1. Experimental arrangement. Target cells were placed in capillary tubes sealed at one end with a polyimide film. A pulse for the excimer laser (~ 20 ns FWHM) was moderately focused on the polyimide film. Ablation of the film induced a stress wave which was launched into the medium containing the cells.

separate experiments we measured the deformation of the polyimide film under the highest fluence used in the experiments. A He-Ne laser beam was directed parallel to the polyimide surface and focused to a silicon photodiode. There was no change in the He-Ne laser intensity indicating that the deformation of the polyimide was much less than the size of the beam, ~ 25 μ m.

Stress waves were measured at the target site with a PVDF (polyvinylidene fluoride) transducer (Zweig et al. 1993). A 75- μ m polyimide film was placed on the transducer using silicone grease for acoustic contact. An aperture was positioned over the central part of the laser beam with the transducer mounted directly behind it. The transducer signal was recorded by a storage oscilloscope (Tektronix 7934, Tektronix Inc., Beaverton, OR) or a programmable digitizer (Tektronix 7912AD) using 1 M Ω termination. The transducer was calibrated by measuring the signal generated by a known momentum transfer. A light stainless-steel ball bearing was dropped on the transducer. The impact force was calculated from the conservation of momentum, the mass of the ball bearing, and the time between impacts (Bur and Roth 1985; Venugopalan 1994). The temporal resolution of the transducer was measured using a 40-ps pulse from a Nd:YAG mode-locked laser to generate a stress wave on aluminum foil in contact with the transducer. Leung et al. (1985) have shown that laser-induced stress waves generated under similar conditions had a rise time of less than 3 ns. Therefore, the measured rise time in our experiments, 5 ns, represented the upper limit of the temporal resolution of the measuring system. The measured stress was first corrected for the acoustic impedance difference between the polyimide and the transducer to determine the stress amplitude in the polyimide. The peak stress applied to the cell cultures in the gel was estimated from the acoustic impedances of the polyimide [3.1 MPa/(m/s)] and gel [1.6 MPa/(m/s)].

Figure 2 shows the rise time of the stress waves generated during the ablation of polyimide by the ArF and KrF lasers. The stress waves had substantially different rise times: ArF ablation of polyimide generated a stress wave with a rise time of ~ 10 ns (7–12 ns), while KrF ablation generated a stress wave with a rise time of ~ 25 ns (22–30 ns). Both stress waves had the same duration, ~ 150 ns (Fig. 2 insert). The rise time of the stress waves remained constant over the range of stress amplitudes employed in the experiments. Occasionally, ablation of the polyimide by the ArF laser produced stress waves of ~ 25 -ns rise time, probably caused by changes in the structure of the laser pulse. The biological effects of ArF, on those occasions, were indistinguishable from the effects of

Fig. 2. The leading edge (0–100%) of the stress waves generated during the ablation of polyimide by ArF (a) and KrF (b). The calibration constant (e_{33}/c) of the transducer was 2 mV/N, the beam diameter 2.3 mm and the combined temporal resolution of the transducer oscilloscope ~ 5 ns. The insert shows the complete waveform of the stress wave. The second peaks shown in the figure are the reflected waves at the polyimide–transducer interface.

the KrF laser. The linear attenuation and the nonlinear properties of the medium can modify the rise time of the stress waves (Lyamshev and Naugol'nykh 1981; Muir and Carstensen 1980). In our experiments, however, these phenomena are negligible because of the thin samples ($< 200 \mu\text{m}$) employed. In separate experiments, the stress waves were measured after propagation through a $150\text{-}\mu\text{m}$ layer of gel without observable change in the rise time. In addition, the stress wave propagating through the cell culture was planar given that the beam diameter was much larger than the thickness of the sample (Kermani and Lubatschowski 1991).

Target cells were placed in $200\text{-}\mu\text{L}$ capillary tubes, 1.5 mm in diameter, sealed at one end with polyimide film $75 \mu\text{m}$ thick. The capillaries were placed at the position of the aperture and irradiated with five pulses. Each experiment covered three to six different stress values, which were determined using the PVDF transducer. Four to six capillary tubes were used at each stress value. Control samples were not irradiated, but were otherwise subjected to identical procedures. In a previous study (Doukas *et al.* 1993), we have shown that this arrangement completely eliminates the effects of plasma, heat and UV radiation. The question of whether cavitation is generated in the gel during irradiation cannot be entirely resolved. The fact that the cells are embedded in gel (Brümmer *et al.* 1989; Smits *et al.* 1991b) and that the stress waves are compressive should certainly reduce the degree

of cavitation. Incidentally, no tensile component was observed in the transducer trace for at least 500 ns. One of the side effects of cavitation is the formation of free radicals. To test if cavitation was induced by stress waves, we applied the Fricke dosimetry test as described by Morgan *et al.* (1988). In separate experiments, the Fricke solution in capillary tubes was exposed to 50 pulses (an order of magnitude higher than the number of pulses used in our experiments) at ~ 650 bar under otherwise identical conditions with those used in our experiments. The fact that the Fricke dosimetry test was done in solution should accentuate the formation of free radicals. The absorbance at 224 nm of the solution exposed to stress waves was measured and compared with that of the control. The absorbance of the exposed solutions did not increase relative to controls. These measurements indicated that if any cavitation was present it was below the sensitivity of our measurements.

Target cells

Mouse breast sarcoma cells (EMT-6) were used as targets. EMT-6 cells were washed three times in HBSS (Gibco, Grand Island, NY) without phenol red. The cells were adjusted to a concentration of $2 \times 10^6/\text{mL}$ in complete medium with 5% Knox gel (denatured collagen) at 37°C . Several capillary tubes sealed with polyimide were loaded with $50 \mu\text{L}$ of cell suspension, corresponding to 10^5 cells. The capillary tubes were placed in 5-mL test tubes filled with water and kept in a water bath at 37°C . The capillary tubes were centrifuged at 1000 rpm for 5 min to get the cells in contact with the polyimide. Subsequently, the cells were spun for 5 min at 500 rpm at 10°C to prevent the separation of the gel from the polyimide during solidification. The capillary tubes were then placed in an ice-water bath to complete the solidification of the gel and slow cell metabolism. They were taken out of the ice bath briefly to expose them to stress waves and returned to the ice bath until processing. In a number of control experiments, we checked the number of cells in the capillaries during the procedure to insure that there was no loss of cells. The question whether the preparations of cell cultures in gels makes them more sensitive to stress waves cannot be completely resolved at present. In recent experiments with preparations of cell cultures in media only, the observed cell injury was in the same range. It should be pointed out, however, that the applied stress was lower, below 350 bar.

After irradiation the capillary tubes were warmed to 37°C to melt the gel. The polyimide was then removed and the contents of each capillary tube flushed with 0.5 mL of complete medium into separate 5-mL test tubes containing 0.5 mL of complete medium.

These test tubes were spun at 2000 rpm for 10 min. After centrifugation, the supernatant in each tube was aspirated and discarded. Cell pellets were then resuspended with 200 μ L of complete medium with 0.25 μ Ci of tritiated thymidine (specific activity 6.7 Ci/mM; New England Nuclear, Boston, MA) and plated in a 96-well flat-bottom microliter plate. The plate was incubated for 18 h. The cells were then disrupted, the contents were collected on glass fiber filter strips and washed freely using an automated harvester (MASH II, Microbiological Associates, Walkersville, MD). The dried filter papers were suspended in scintillation fluid and the radioactivity measured in a liquid scintillation spectrometer (Beckman LS 3801). The mean of the radioactive counts per minute (cpm) of the samples for each condition was calculated, and the means for stress-exposed cells were then expressed as a percentage of the cpm of the control. The results from many experiments (an average of five experiments or 20 to 30 capillaries per point) have been combined to produce the graphs presented here. The error bars shown in the figures are standard errors of the mean.

Transmission electron microscopy

After irradiation the polyimide was removed under a dissecting microscope and the gel samples were gently extruded into vials containing 4% glutaraldehyde fixative. After initial fixation (overnight), the gels were rinsed in 0.1 M cacodylate buffer and postfixed in 2% OsO_4 for 2 h. The samples were then dehydrated in a graded ethanol series and flat embedded with Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). One-micrometer-thick cross-sections were cut through the samples for light microscopy on an ultramicrotome (Reichert-Jung Ultracut, Vienna, Austria) and stained with 1% toluidine blue. Ultrathin sections were cut, stained with uranyl acetate and lead citrate examined with a transmission electron microscope (CM-10, Philips, Eindhoven, The Netherlands).

RESULTS

Figure 3 shows the incorporation of tritiated thymidine by EMT-6 cells exposed to five stress waves as a function of peak stress in the gel. Thymidine incorporation is given as a percent of thymidine incorporation of the control group and is a measure of cell viability (Cleaver 1967). In a previous study (Doukas et al. 1993), we compared thymidine incorporation with ethidium bromide/fluorescein diacetate (EB/FDA) exclusion assay under the same conditions. The two assays gave similar results; thymidine incorporation, however, was more sensitive to cell injury. The salient feature in Fig. 3 is the difference in the response of cells exposed to

Fig. 3. Incorporation of tritiated thymidine by EMT-6 cells exposed to five pulses as a function of peak stress (σ) generated in the gel by ArF and KrF lasers, respectively. Thymidine incorporation is given as a percent of thymidine incorporation of the control group. Solid lines indicate the best linear fit. Error bars are the standard error of the mean.

stress waves of different rise times. At the highest stress, ~ 650 bar, thymidine incorporation is more than two times lower for the cultures exposed to 10-ns than to 25-ns rise-time waves. A one-dimension analysis of variance (1D ANOVA, LabView 3, National Instruments, Austin, TX) was applied to the two sets of measurements. The analysis showed that the effect of stress was statistically significant for the ArF-induced but not for KrF-induced stress waves. The level of significance was 2×10^{-6} and 3×10^{-1} , respectively. A two-dimensional analysis (2D ANOVA) where both the rise time and the peak stress were considered gave a level of significance for the rise time of 3×10^{-4} . The difference in the level of cell damage can, therefore, be attributed to the rise time, since the stress waves were nearly identical in every other respect. The lines represent the best fit of the data to a linear regression with slopes of -0.13 ($R = 0.96$) and -0.07 ($R = 0.93$), respectively. The two slopes differ by a factor of two, roughly equal to the ratio of the two rise times.

Transmission electron microscopy was performed on EMT-6 cells exposed to stress waves using ArF and KrF lasers (10- and 25-ns rise times, respectively) with the same peak stress (410 and 390 bar, respectively). Both samples were exposed to five pulses. Fig. 4 shows the sham-treated EMT-6 control cells. Figure 4b shows the cells exposed to stress waves generated by the ArF laser. As compared to controls, there are changes ranging from cells showing disruption of the cell wall to dilated endoplasmic reticulum. Figure 4c is the sham control for the cells exposed to stress waves generated by the KrF laser. Figure 4d shows cells exposed to stress waves generated by the KrF laser. These

Fig. 4. Transmission electron photomicrographs of EMT-6 cells in gels. The experimental arrangement is discussed in the text. The polyimide was located at the bottom of the figure for all specimens. The horizontal black bars represent discontinuities in the representation of the specimens related to the grid bars which supported the specimen in the electron microscope. (a) Sham control for the cells exposed to stress waves generated by ArF. (b) Cells exposed to stress waves generated by ArF (10-ns rise time, 410-bar peak stress in gel, five pulses). Note the cells showing disruption (arrows) and dilated endoplasmic reticulum (arrowheads). (c) Sham control for the cells exposed to stress waves generated by KrF. (d) Cells exposed to stress waves generated by KrF (25-ns rise time, 390-bar peak stress in gel, five pulses). Note lack of differences between the control and exposed cells. Bar scale equals 10 μm .

cells show no significant differences in morphological features as compared to sham-treated controls. These observations indicate that morphological alterations of cells may be sensitive to stress gradient. They are also consistent with *in vivo* observations previously described that have been attributed to stress injury (Watanabe *et al.* 1988).

Figure 5 shows the incorporation of tritiated thymidine as a function of the stress rate of change ($d\sigma/dt$) and the spatial stress gradient ($d\sigma/dr$). The spatial stress gradient can be calculated from the rise time of the stress wave and the speed of sound. The response of the cells exposed to stress waves of 10- and 25-ns rise times, is similar. The experiments described here show that the characteristics of the stress transients, specifically the rise time, can significantly modify the response of cells exposed to stress waves. Furthermore, our results show that the stress rate of change is a better determinant of cell damage than the peak stress. Cell cultures exposed to stress waves below 15 bar/ns (10 bar/ μm) showed

Fig. 5. Incorporation of tritiated thymidine by EMT-6 cells exposed to five pulses as a function of the stress rate of change ($d\sigma/dt$) generated by ArF and KrF lasers, respectively (the stress gradient, $d\sigma/dr$, is shown on the top of the abscissa). Thymidine incorporation is given as a percent of thymidine incorporation of the control group.

increased thymidine incorporation relative to control samples. This kind of stimulation induced by stress waves has been reported before for human lymphocytes (Doukas et al. 1993). Stimulation of cells has also been observed in other types of insult, *e.g.*, stimulation of human keratinocytes exposed to elevated temperature (Maytin et al. 1990).

DISCUSSION

The role of the stress gradient in determining cell injury may be responsible to some extent for the difference in the number of pulses required to produce cell injury between our measurements and those reported in the literature. In our experiments, five pulses are sufficient to produce substantial decrease in cell viability. Cell damage induced by lithotripters, on the other hand, require 100 to 2000 pulses. Lithotripters generate stress waves of the same order of magnitude as those generated in our experiments. The rise time of the stress wave, however, can vary from 30 to 450 ns depending on the type of the lithotripter (Coleman and Saunders 1989). Thus, the stress gradient generated by lithotripters is much lower than the stress gradient produced in our experiments. The close correlation between the rise time and cell damage also indicates that the contribution from cavitation if any is minimal. Otherwise, cavitation should be proportional to the rise time of stress waves. This is unlikely given the statistical nature of the generation of cavitation.

The exact cell sites and functions that are affected by the stress waves are not known. It is likely that cell injury, to some extent, is mediated by changes of the cell plasma membrane. In fact, a number of experiments have shown increased permeability of the cell membrane following exposure to stress waves. Holmes et al. (1992) observed that influx and efflux of extra/intracellular components occurred in human neutrophil cultures following the application of stress waves. Gambihler et al. (1992) and Flotte et al. (1993) have shown that intracellular dye concentration increased after cell cultures were exposed to stress waves. It is also possible that other structures inside the cell can be damaged by stress waves. Damage to the endoplasmic reticulum, for example, has been observed in the transmission electron photomicrographs. In addition, other possible targets of injury include DNA and other macromolecules. Stress waves, for example, are known to cause DNA breaks in viruses (Hamrick and Cleary 1969).

In this work we have shown that the rise time plays a significant role in the biological effects of stress waves *in vitro*. It is probable that other characteristics of stress waves, such as stress duration and decay may

have biological significance. The limiting factor in the present study was our inability to generate stress waves with independently adjustable parameters. The current techniques of generation and measurement of stress waves do not provide the necessary sensitivity and accuracy. Thus, only gross biological responses can be investigated. Lamb et al. (1994) have demonstrated the generation of stress waves, using a free electron laser, with independently adjustable parameters, namely rise time, duration and peak stress. Tailor-made stress waves can facilitate the study of cellular response to stress waves.

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