HYPOTONIA-INDUCED CELL SWELLING ENHANCES ULTRASOUND-INDUCED CELL KILLING

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Abstract

To determine the effects of hypotonia on ultrasound (US)-induced cell killing, we used 146 mOsm medium to induce nonlethal osmotic swelling of U937 cells before exposure to 1-MHz US at 0.5 or 1.0 W/cm^2 for 10 min, or 5 min before exposure to 2.0 W/cm^2 for 1 min. Instant cell killing was determined by Trypan blue dye exclusion test immediately after treatment, while apoptosis and necrosis were measured by flow cytometry after 6 hr. Ion imaging were also done. Enhancement of cell lysis was observed in all intensities, and most prominently at 2.0 W/cm^2, while apoptosis was enhanced at intensities 0.5 and 1.0 W/cm^2 but not at 2.0 W/cm^2. The enhancement is attributed to the increased susceptibility of the cells to mechanical damage, while the effect of hypotonia on membrane damage-and-repair mechanism of the cells is also considered. These findings suggest the potential value of hypotonia in therapy with US.

Introduction

The biological effects of US are being investigated in many different medical fields. This is whether to evaluate the safety of the widely used diagnostic US or to investigate its potential in therapy [1-3]. Low intensity US has been shown to cause cell lysis, loss of cell viability, necrosis, and apoptosis in vitro [3-5]. In vivo, regression of tumors [2], ablation of cancer tissue [6], coagulation of bleeding tissue [7], revascularization of occluded blood vessels [8], and enhanced gene delivery [9-10] were observed. In these studies, US is being used either alone or in combination with other modalities. The limited effect of US in therapy has been improved by the combined methods to enhance all these desired effects, which are also aimed at minimizing the side effects of the therapy as a whole [2].

The mechanisms of these effects by US are being investigated. The thermal effect of US is now recognized to play a key role in these biological effects. However, being a mechanical form of energy, US undoubtedly also exert some degree of mechanical damage to the cell or tissue [11]. This is being indicated by studies showing that US can induce cell death even without lethal increase in temperature [3,12]. This mechanical effect can be produced by shear stress and more prominently by the generation of cavitation. Previous studies have clearly shown such effects by showing good correlation between cell killing and the occurrence of cavitation [13]. The use of agents such as saturation with air or other gases, addition of microbubbles or echo-contrast agents [14], and other similar methods, has successfully enhanced the formation of US-induced cavitation in those studies. Other factors considered in this biological effect, includes the chemical effect of US such as generation of free radicals [13,15] at the event of inertial cavitation, and probably potentiation of some agents by US through other mechanisms [2]. On the other hand, studies have also shown that the factors related to the cells and tissue being exposed to US need to be considered. In vitro, these factors include, type of cell, cell density, age of the cell culture, nutritional condition of the cells, and oxygenation of the cell or medium used [2,16]. However, to our knowledge, no study was done on the effect of osmotic cell swelling on US-induced cell killing. In this study, we intend to modify cell size by exposing the cells to a hypotonic solution, at a certain degree of hypotonia not harmful to the cells by itself. We then hypothesized that physiologic swelling of the cells by this osmotic stress, will make the cells more susceptible to mechanical damage by US.

Methods

To determine the effect of hypotonia on US-induced cell killing, we exposed human myelomonocytic leukemia cells (U937) to a hypotonic RPMI medium (146 mOsm vs 288 mOsm) just before exposure to 0.5 and 1.0 W/cm^2 for 10 min and 5 min before exposure to 2.0 W/cm^2 for 1min. The ultrasonic apparatus (ES-1, OG Giken Co. Ltd, Okayama, Japan), described in previous papers [12,17], with a resonant frequency of 1 MHz continuous wave was used. The samples were exposed while thermally regulated at 37℃. Effective output was determined and indicated that the intensities 0.5, 1.0 and 2.0 W/cm^2 on the reading meter of the US device have measured spatial-average-temporal-average intensity (I_{SATA}) values of 0.312, 0.692 and 1.43 W/cm^2 respectively as measured 20 cm away from the transducer without the inverted T-tube containing the sample. However, in this paper we used the reading output of the device (0.5, 1.0 and 2.0 W/cm^2) to refer to these measured values.

The hypotonic cell swelling was monitored by direct microscopy. After treatment, 200 □l from each of the samples were assayed for cell viability using Trypan blue dye exclusion test. The remaining cells were then incubated for 6 hr at 37℃ before doing

flow cytometry with Annexin V-FITC and PI staining to determine early apoptosis and secondary necrosis. The cells were also analyzed for intracellular calcium ion using Fura 2/AM for fluorescence imaging while to include other cellular ions scanning by secondary ion mass spectrometry (SIMS) or ion microscopy was done. Ultrasound-induced free radical production was detected using EPR spin trapping with DMPO.

Results and discussion

No instant cell killing was observed at 0.5 W/cm^2 for 10 min. At 1.0 W/cm^2 for 10 min, 15.8±3.7% (mean ± SD) cell lysis was observed, while at 2.0 W/cm^2 for 1 min, 25.5±7.3%. Exposure to hypotonia did not produce significant loss of cell viability. When hypotonia was combined with 0.5 W/cm^2 US, minimal instant cell killing was observed, while with 1.0 W/cm^2 for 10 min and 2.0 W/cm^2 for 1 min, significant synergistic cell killing (28.2±5.8 and 48.5±6.7% (mean±SD), respectively) were observed. These data showed that hypotonia synergistically augmented the ability of US to produce instant cell lysis by making the cells more susceptible, even if the US is subthreshold for free radical production (0.5 and 1.0 W/cm^2).

The US-induced early apoptosis 6-hr post-incubation, increased with increasing US intensity. Exposure to hypotonic solution did not induce significant apoptosis or secondary necrosis. While under hypotonia, a synergistic apoptosis induction was induced with ultrasound 0.5 W/cm^2 (2.7±0.8% vs. US only 0.9±0.6%) and 1.0 W/cm^2 (7.6±0.9% vs. US only 2.5±0.3%) as measured by flow cytometry after 6-hr incubation. However at 2.0 W/cm^2, no enhancement of apoptosis was observed. Also, no significant enhancement of secondary necrosis was observed in all intensities used.

From this result, we could say that hypotonia can enhance the susceptibility of the cells to the US-induced cell damage even at intensity 0.5 W/cm^2 where relatively ineffective in causing instant cell lysis in itself and is considered far below the threshold for free radical production. The enhanced cell lysis, but not apoptosis at 2.0 W/cm^2, shows that at condition above threshold for inertial cavitation, the cells (if osmotically swollen) are likely to die by breaking-up mechanically than by apoptosis.

The enhancement could be attributed by both the effect of hypotonia on the ultrasound waves (as low density medium promotes cavitation formation) and another is on the effect of ultrasound on the swollen cells. The first effect was investigated using free radical detection as indicator of cavitation. No significant changes in ultrasound-induced free radical production were noted, thus down playing the role of enhanced cavitation as part of the low viscosity effect of hypotonia. Cell swelling is more likely playing a major role in the enhancement. The swollen cells are made a bigger target for the mechanical effect of ultrasound. In addition, membranes of the swollen cells are apparently considerably stressed to make it more susceptible to ultrasound. The mechanical effect on a swollen cell can supported by a mathematical equation [18] that describes the force $\boldsymbol{F}$ towards the direction x, acting on a particle of volume **v** in a liquid medium where radiation pressure (as in acoustic streaming and micro-streaming) is applied, $\boldsymbol{F}$ is given by

$$\boldsymbol{F}=\mathbf{v}\,(1-\Phi)\partial \boldsymbol{T}(\partial x)^{-1}$$

where $\boldsymbol{T}$ is the time-averaged volume density of kinetic energy, while $\Phi=\rho_o/\rho$, is the density ratio. Here, ρ_o is the density of the medium, and ρ is the density of the particle (cell).

The addition of hypotonic medium into the cells will result to a decrease in ρ value, thus increasing the value $\boldsymbol{F}$. This is followed by cell volume increase which will result to a directly proportional increase of the value $\boldsymbol{F}$. This would mean that a 3 x increase in cell volume (at about 5 min of hypotonia), would result to more than 3 x increase in magnitude for $\boldsymbol{F}$ when combined with the density effect.

The data on increase intracellular calcium ion further points to membrane damage as main point of the bio-effects. Ion micrographs by SIMS showed decrease in number of cells undergoing cell membrane repair (patch formation with high density Ca ions) [19] in the combine treatment, suggesting an impaired cellular repair mechanism due to hypotonia.

In summary, we could say that US-induced cell killing can be enhanced when combined with non-lethal hypotonia. The mechanical damage induced by US on the cell membrane is likely the main mechanism involved, while associated membrane damage-and-repair impairment is also suggested. This finding might be helpful in elucidating the mechanical nature of the US-induced biological effects, and the cellular response to these effects. Eventually, this may also be useful clinically when infusion of hypotonic fluid to the target tissue is applied in conjunction with US therapy, especially in cancer therapy.

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