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Effect of platinum nanoparticles on cell death induced by ultrasound in human lymphoma U937 cells

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ABSTRACT

In this study, we report on the potential use of platinum nanoparticles (Pt-NPs), a superoxide dismutase (SOD)/catalase mimetic antioxidant, in combination with 1 MHz ultrasound (US) at an intensity of 0.4 W/cm^2, 10% duty factor, 100 Hz PRF, for 2 min. Apoptosis induction was assessed by DNA fragmentation assay, cell cycle analysis and Annexin V-FITC/PI staining. Cell killing was confirmed by cell counting and microscopic examination. The mitochondrial and Ca^{2+}-dependent pathways were investigated. Caspase-8 expression and autophagy-related proteins were detected by spectrophotometry and western blot analysis, respectively. Intracellular reactive oxygen species (ROS) elevation was detected by flow cytometry, while extracellular free radical formation was assessed by electron paramagnetic resonance spin trapping spectrometry. The results showed that Pt-NPs exerted differential effects depending on their internalization. Pt-NPs functioned as potent free radical scavengers when added immediately before sonication while pre-treatment with Pt-NPs suppressed the induction of apoptosis as well as autophagy (AP), and resulted in enhanced cell killing. Dead cells displayed the features of pyknosis. The exact mode of cell death is still unclear. In conclusion, the results indicate that US-induced AP may contribute to cell survival post sonication. To our knowledge this is the first study to discuss autophagy as a pro-survival pathway in the context of US. The combination of Pt-NPs and US might be effective in cancer eradication.

1. Introduction

Over the last decade, ultrasound (US) has gained credibility as a tool in cancer therapy seen in the spreading use of High Intensity Focused Ultrasound (HIFU) over the world. However, the performance of low intensity US as a therapeutic modality is still awaiting optimization through parametric modulation or combination with other chemical agents [1,2]. What imparts superiority to therapeutic US is its being inexpensive, non-invasive, deeply penetrating mechanical waves with a high degree of targeting precision. With correct tuning and adjustment, US can affect through different mechanisms: cavitational, thermal and non-thermal/non-cavitational mechanisms. Cavitation the oscillation and/or collapse of gaseous microbubbles in a sound field-mediates various mechanical stresses on nearby cells such as shear, shock wave and high pressure. The chemical stress occurs due to sonolysis of water and the consequent formation of reactive oxygen species (ROS). By creating unfavorable conditions, US manifests its potential in altering cell signaling and gene expression ending in cell killing or cell survival [3–6]. The sway of US outcomes between extremes highlights the resilience of living structures. Cell death can be perceived as loss of battle to survive [7]. There is always an inflection point beyond which damaged cells lose control and enter a vicious cycle of deterioration [8]. The identification of this "no return" point in sonicated cells can achieve a breakthrough in

Abbreviations: Pt-NPs, platinum nanoparticles; Au-NPs, gold nanoparticles; US, ultrasound; Nec-1, necrostatin-1; ROS, reactive oxygen species; O_2^-, superoxide; H_2O_2, hydrogen peroxide; ·OH, hydroxyl radical; MMP, mitochondrial membrane potential; SOD, superoxide dismutase; AP, autophagy; DF, duty factor; PRF, pulse repetition frequency; CCA, cell cycle analysis.

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the applications of US and help tune it for the purpose; cell survival in wound healing and macromolecular delivery, or cell killing in cancer eradication [9,10].

Macroautophagy, here autophagy (AP), has been recently reported to be upregulated following US exposure [11]. AP is a degradative pathway in which excess/damaged organelles are sequestered into double-membraned vesicles known as autophagosomes and degraded by hydrolytic enzymes into their basic building molecules for recycling. As such, AP can be considered as cellular housekeeping system operating in atonement for stress and/or damage to maintain homeostasis and provide energy to the *whole* living system. It might be speculated that such mechanism should operate initially as pro-survival pathway for the individual cell and later as pro-survival pathway for the rescue of the *whole* through intercellular signaling [12]. This means that AP can promote individual cell death in circumstances of severe irreversible damage where non-functioning cytoplasmic content is processed and used to start a new life [13]. In the context of US, AP has been studied in sonodynamic therapy in presence of inert chemical agents which are only activated upon combination with US [14,15]. Also, Wang et al., have observed autophagosomes in cells treated with US alone after 18 h from exposure [16]. Because these combination treatments were intended for cancer cell killing, the induction of AP has been interpreted as a pathway for cell death. However, there is no evidence to negate a pro-survival role of AP in sonicated cells up to date.

Platinum nanoparticles (Pt-NPs) is an advancement of nanomedicine intended to increase the innate activity of platinum as an antitumor agent [17]. Platinum (Pt) as a noble element is known to be toxic and DNA damaging. On the contrary, studies conducted on Pt-NPs showed that they can act as SOD/catalase mimetics scavenging free radicals owing to their large surface area. Combined treatment with physical stressors, such as ultraviolet radiation, X-irradiation and hyperthermia, known to mediate cell killing through oxidative stress showed that Pt-NPs can protect (cancer) cells [18–20]. When AP is questioned with Pt-NPs, the difficulty of predicting the outcomes mounts to the highest levels since antioxidants would suppress ROS-induced AP but nano-sized formulations would act as AP inducers [21–23]. In this study, the combination of Pt-NPs and low-intensity US at 1 MHz has been investigated using human lymphoma U937 cells. Insights to the underlying molecular mechanisms have been presented as well. The role of AP has been explored and correlated to the results. To our knowledge, this is the first report on the combination of Pt-NPs and US and the first to address the role of AP as pro-survival pathway in US-induced bio-effects.

2. Materials and methods

2.1. Cell culture

Human myelomonocytic lymphoma U937 cells were obtained from Human Sciences Research Resource Bank (Japan Human Sciences Foundation, Tokyo, Japan). The cells were grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37 °C in humidified air with 5% CO_2.

2.2. Preparation of nanoparticles

Pt-NPs were prepared by citrate reduction of H_2PtCl_6, according to a previous report with minor modifications [24]. Required concentrations of Pt-NPs were prepared in complete RPMI 1640 medium immediately before experiments. Preliminary experiments to choose the best Pt-NPs dose were performed before conducting the study.

Gold nanoparticles (Au-NPs) used in this study are 2 nm in size which were obtained from BBI Solutions (British Biocell International Limited, United Kingdom). Au-NPs at a dose of 20% were prepared in complete RPMI 1640 medium immediately before experiments.

2.3. Ultrasound apparatus

Sonicmaster ES-2 (OG Giken Co., Ltd., Okayama, Japan) with a resonant frequency of 1 MHz, previously used to study and optimize US-induced bio-effects, was employed in this study. This device is equipped with a built-in digital timer, intensity regulator, and duty factor controller. The intensity ranges from 0.1 to 2.0 W/cm^2 at an increment of 0.1 W/cm^2, while the duty factor (DF) is variable at 5%, 10%, 20%, 30%, 50% and 100% with fixed pulse repetition frequency (PRF) of 100 Hz. For the sonication procedure, a planar transducer with a diameter of 5.0 cm was fixed so that the transducer was facing upward. The culture dishes were placed directly above the transducer surface with partially degassed water as a coupling agent. This setup allowed for standing wave formation due to the reflection of US waves at water/air interface. The intensity of 0.4 W/cm^2 (device-indicated) at 10% DF was used for 2 min sonication time. The spatial-average temporal average intensity (I_{SATA}) was found to be 0.092 W/cm^2 as measured by an US power meter (UPM-DT-10E, Ohmic Instrument Co., Easton, MD). All experiments were carried out at room temperature.

2.4. Ultrasound treatment

Cells were collected by centrifugation following 20 h pretreatment with 300 μM Pt-NPs and then re-suspended in fresh, air-saturated medium at a cell density of 1×10^6 cells/ml. Two milliliters were transferred into 3.5 cm polystyrene culture dishes (Corning Inc., Corning, NY) and sonicated. After sonication, cells were incubated for designated times before conducting further analyses. Each data point is representative of three separately sonicated dishes.

2.5. DNA fragmentation

For the detection of apoptosis, the percentage of DNA fragmentation was assessed at 3 and 6 h post treatment using the method of Sellins and Cohen with minor modifications [25]. Briefly, approximately 2.5×10^6 cells were lysed using 200 μl of lysis buffer (10 mM Tris, 1 mM EDTA and 0.2% Triton X-100, pH 7.5) and centrifuged at 13,000*g* for 10 min. Subsequently, each DNA sample in the supernatant and the resulting pellet was precipitated in 25% trichloroacetic acid (TCA) at 4 °C overnight and then quantified using diphenylamine reagent after hydrolysis in 5% TCA at 90 °C for 20 min. The percentage of fragmented DNA in each sample was calculated as the amount of DNA in the supernatant divided by total DNA for that sample (supernatant and pellet).

2.6. Detection of histone H2AX phosphorylation

US have been shown to result in immediate DNA damage through inducing DNA double strand breaks (DSBs) [26,27]. To determine the effect of Pt-NPs on the extent of US-induced DNA damage, Pt-NPs-pretreated cells were collected immediately after sonication and washed with phosphate buffer saline (PBS) containing 0.5% Tween. Samples were reacted sequentially with specific antibodies according the manufacturer's instructions of H2AX Phosphorylation Assay Kit (Upstate Inc., Syracuse, NY). Finally, samples were analyzed using a flow cytometer (Epics XL, Beckman-Coulter, Miami, FL).