5-Aminolevulinic Acid Enhances Ultrasound-mediated Antitumor Activity via Mitochondrial Oxidative Damage in Breast Cancer

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Abstract. Background/Aim: 5-Aminolevulinic acid (5-ALA), a precursor of protoporphyrin IX (PpIX), is now used for photodynamic therapy (PDT) of pre-cancers of the skin and photodynamic diagnosis (PDD) of brain tumors. Sonodynamic therapy (SDT) of cancers with ultrasound has been studied using 5-ALA as a sonosensitizer. In this article, we evaluated the sonosensitizing activity and mode of action of 5-ALA/PpIX by using mouse mammary tumor EMT6 cells. Results: 5-ALA-SDT showed significant antitumor effects toward EMT6 cells in vitro and in vivo. The fluorescence of MitoSOX Red, an indicator specific for mitochondrial superoxide, was significantly increased by 5-ALA-SDT. Moreover, the fluorescence derived from JC-1, an indicator of mitochondrial membrane potential, was also significantly increased by 5-ALA-SDT. These findings suggest that mitochondria are one of the target organelles of 5-ALA-SDT. PpIX enhanced reactive oxygen species (ROS) production from tert-butyl hydroperoxide (tBHP), suggesting that PpIX might stabilize or promote ROS generation from tBHP. Conclusion: 5-ALA-SDT showed an antitumor effect in mouse mammary tumor EMT6 cells through oxidation of the mitochondrial membrane via ROS production.

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Materials and Methods

Chemicals. 5-ALA hydrochloride was obtained from SBI Pharmaceuticals Co. Ltd. (Tokyo, Japan). Doxorubicin hydrochloride was from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan); aminophenyl fluorescein (APF) from Sekisui medical Co. Ltd. (Tokyo, Japan);...
Cell culture. Mouse mammary EMT6 tumor cells (supplied by Dr. Shin-ichiro Masunaga, Kyoto University, Kyoto, Japan) were maintained in Eagle’s minimum essential medium (Sigma-Aldrich, Shin-ichiro Masunaga, Kyoto University, Kyoto, Japan) were humidified atmosphere of 5% CO2 at 37˚C. Cells were cultured in a humidified atmosphere of 5% CO2 at 37˚C.

Cytotoxicity and intracellular kinetics of 5-ALA. To evaluate the cytotoxicity of 5-ALA, the cells (2×10^5 cells/well) were seeded into a 96-well plate and incubated with various concentrations (0-10 mM) of 5-ALA for 5 h, washed with PBS and, then, incubated for 72 h. The survival rate of the cells was determined using the WST-1 assay. To evaluate the intracellular kinetics of PpIX synthesized from 5-ALA, 3×10^5 cells/dish were incubated for 24 h and, then, 100 μM of 5-ALA was added. The cells were washed with PBS and collected using 2% Triton X-100 solution at every measurement time point. Fluorescence intensity of PpIX (excitation: 410 nm, emission: 630 nm) was measured using a microplate reader, Infinite M200 (Tecan Group Ltd. Männedorf, Switzerland).

ROS detection. ROS production, by the reaction between PpIX and ultrasound (1 MHz, 20% duty cycle, 2.15 W/cm^2, 2 min) or between PpIX and tBHP (20 mM), was determined by measuring the absorbance of DPBF at 410 nm for singlet oxygen or fluorescence of APF (490 nm excitation and 515 nm emission wavelengths) and DCFH for hydroxyl radicals (488 nm excitation and 522 nm emission wavelengths). Intracellular ROS content was determined by measuring the fluorescence of DCFH-DA for cytoplasmic ROS and MitoSOX Red for mitochondrial ROS. The cells (4×10^4 cells/well for DCFH-DA or 2×10^4 cells/well for MitoSOX Red) were seeded in a 24-well plate and 1 mM 5-ALA was added for incubation for 5 h. DCFH-DA (50 μM) or MitoSOX Red (5 μM) was added and then exposed to ultrasound (1 MHz, 20% duty cycle, 2.15 W/cm^2, 2 min). The fluorescence intensity of DCFH was measured at 488 nm excitation and 530 nm emission wavelengths by using a microplate reader at every measurement time point. After 3 h, the fluorescence intensity of MitoSOX Red was measured at 550 nm excitation and 600 nm emission wavelengths using a fluorescence microscope BX-700 (KEYENCE Co. Ltd., Osaka, Japan).

Antitumor activity of 5-ALA-SDT on EMT6 cells in vitro and in vivo. Figure 2A shows the cytotoxicity of 5-ALA. 5-ALA showed strong cytotoxicity at a final concentration of equal or more than 3 mM after treatment for 72 h. The intracellular concentration of 5-ALA increased in a time-dependent manner and the maximum concentration was reached after incubation for 5 h (Figure 1B).

ROS production and MMP loss by 5-ALA-SDT. To investigate which type of ROS contributes to the antitumor action of 5-ALA-SDT, we measured the production of singlet oxygen and hydroxyl radicals after a combined PpIX and ultrasound treatment in a cell-free system. The PpIX and ultrasound combination showed no obvious difference between control and 5-ALA alone and ultrasound-alone treatments. Moreover, 5-ALA-SDT showed a significant suppressive effect on tumor growth in tumor-bearing mouse models and the effect of 5-ALA-SDT was equal to that of doxorubicin (Figure 2B).

Statistical analysis. Data are expressed as mean and standard deviation values. The statistical significance of the differences between the results of the independent experiments was analyzed using the Student’s t-test. A p-value of <0.05 was considered statistically significant.
of hydroxyl radicals compared to that observed with control and ultrasound-alone treatments (Figure 3B). In cell culture systems, 5-ALA-SDT did not affect cytoplasmic ROS production (Figure 4A) but it significantly enhanced the production of superoxides in mitochondria (Figure 4B). Moreover, 5-ALA-SDT caused MMP loss in EMT6 cells (Figure 5). To elucidate the mechanism of ROS production by 5-ALA-SDT, we confirmed the sensitizing effect of PpIX

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Figure 1. Cytotoxicity (A) and intracellular kinetics (B) of 5-ALA. All experiments were performed in triplicate. Each error bar represents the standard deviation value.

Figure 2. Antitumor activity of 5-ALA-SDT in EMT6 cells (A) and solid tumor (B). *Tumors treated with 5-ALA-SDT (closed square) and doxorubicin (open square) were significantly smaller than those subjected to control (open circle) (p<0.05) and ultrasound alone (closed circle) treatments (p<0.05).
Figure 3. Production of ROS from the combination of PpIX and ultrasound. (A) Detection of singlet oxygen by measuring DPBF absorbance at 410 nm; control (white bar), ultrasound alone (dotted bar), PpIX and ultrasound (black bar). (B) Detection of hydroxyl radical by measuring the fluorescence of APF. *The combination of PpIX and ultrasound (closed bar) was significantly higher than the ultrasound alone (open bar) (p<0.05).

Figure 4. ROS production from a combination of PpIX and ultrasound in vitro. (A) Detection of ROS in the cytoplasm by measuring the fluorescence of DCFH-DA. *tBHP (black bar) as a positive control was significantly higher than the control (white bar), ultrasound alone (dotted bar), 5-ALA alone (Horizontal striped bar) and 5-ALA-SDT (gray bar) (p<0.05). (B) Detection of ROS in mitochondria by measuring the fluorescence of MitoSOX Red after a 3-h treatment. *5-ALA-SDT was significantly higher than the control, ultrasound alone and 5-ALA alone (p<0.05).
on hydroxyl radical production using tBHP. PpIX greatly enhanced the production of hydroxyl radicals from tBHP. However, ultrasound had no effect on ROS produced from tBHP (Figure 6).

Discussion

In this study, we evaluated the sonosensitizing activity and mode of action of 5-ALA/PpIX using mouse mammary tumor EMT6 cells and its tumor-bearing mice models. It became obvious that 5-ALA-SDT has an antitumor effect through mitochondrial oxidative damage of the EMT6 cell, albeit ultrasound had no effect on ROS production in the cytoplasm of EMT6 cells irrespective of 5-ALA addition. Several researchers have reported that singlet oxygen is important in sonodynamic induction of apoptosis (11, 12); however, singlet oxygen produced by 5-ALA-SDT was not detected in our study. In PDT mechanisms, it has been proposed that photosensitive compounds can undergo efficient energy transfer under light irradiation to engender active triplet-state oxygen molecules (\(^{3}\text{O}_2\)) that give birth to singlet-state oxygen molecules (\(^{1}\text{O}_2\)). However, it is presumed that this reaction is restrictive in SDT as the ultrasound energy of more than 1 MHz is considerably less than that of light.

We found that PpIX strongly enhanced hydroxyl radical production from tBHP. Furthermore, by using proton nuclear magnetic resonance (\(^{1}\text{H-NMR}\)) and high-performance liquid chromatography (HPLC), we confirmed that PpIX does not structurally change in this reaction (data not shown). Based on our observations, it appears possible that PpIX in 5-ALA-SDT traps the ROS generated by the ultrasound and the complex interacts with lipids of the mitochondrial membrane, thus resulting in mitochondrial damage and a sequential apoptotic cell death.

In conclusion, we demonstrated that 5-ALA-SDT showed a potent antitumor effect on mouse mammary tumor through oxidation of the mitochondrial membrane via stabilized or promoted ROS generation.

References


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