Sonodynamic therapy inhibits angiogenesis and tumor growth in a xenograft mouse model

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Abstract

Studies of sonodynamic therapy (SDT) have mainly focused on its direct cytotoxic effect on tumor cells. Its effects on the tumor microenvironment, especially angiogenesis, remain unknown. In this study, we found that SDT significantly inhibited endothelial cell proliferation, migration, invasion, and tube formation. Furthermore, in a tumor xenograft mouse model, SDT was found to remarkably suppress tumor growth, intratumoral vascularity, and expression of vascular endothelial growth factor in tumor cells. An ultrastructural study showed damage and disruption of tumor microvasculature after STD. Our results indicate that SDT inhibits neovascularization in tumor, which is partially responsible for the anti-tumor effect of SDT.

1. Introduction

Angiogenesis is a complex process including endothelial cell proliferation, migration, basement membrane degeneration, and new tube formation. It is required for a variety of physiologic processes, such as development and reproduction. Angiogenesis also plays important roles in some disease states, typically cancer [1,2]. The new blood vessels grow and infiltrate into the tumor, providing it with essential nutrients and oxygen, and a route for tumor metastasis [3,4]. Thus, anti-angiogenesis has been thought to be one of the most important anti-cancer therapies.

Sonodynamic therapy (SDT), based on the synergistic effects of ultrasound and sonosensitizers [5,6], is a promising approach for cancer treatment, first proposed by Umemura et al. in 1990 [7]. Ultrasound has the ability to penetrate deep into biological tissues noninvasively, due to its relatively low tissue attenuation coefficient. Furthermore, the energy of the ultrasound wave can be focused on sites of malignancy and, thus, locally activate sonosensitizers that have preferentially accumulated in tumor tissues, causing injuries to desired cells with minimal damage to peripheral healthy tissue. Currently, studies of SDT have mainly focused on its direct killing effects on tumor cells [8–12]. Many mechanisms had been proposed by different research groups, including apoptosis [13–17] and autophagy [18,19]. But until now, SDT effects on the tumor microenvironment, especially angiogenesis, remain unknown.

Protoporphyrin IX (PpIX), as a hematoporphyrin derivative, is one of the most commonly used sonosensitizers [8,11,13,16]. In addition to an exogenous supply of PpIX itself, the accumulation of PpIX in tissues or cells may be produced by administration of its precursors, such as 5-aminolevulinic acid (ALA) or ALA derivatives [20–24]. The main advantage of using ALA is that ALA itself is not a sonosensitizer, and metabolizes quickly. This can reduce or avoid the risk of skin phototoxicity.

The purpose of the present study is to examine the anti-angiogenic potency of ALA-mediated SDT in vitro and in vivo.
instructions. The ECGM consists of endothelial cell medium supplemented with 5% fetal bovine serum, endothelial cell growth supplement, and penicillin/streptomycin solution. HUVECs from the 2nd to 5th passage were subcutaneously injected into the flanks of BALB/c mice. When tumors reached 100 mm³ in size (about 10 days after inoculation), mice in the ALA and US + ALA groups were exposed to ultrasound (1.1 MHz, 1 W/cm²) for 60 s duration in the dark. Instead of ALA, an equivalent quantity of medium was used for the Control and US groups. After 4 h incubation, the cells in the US and US + ALA groups were exposed to ultrasound again (1.1 MHz, 1 W/cm², 50% duty cycle) for 60 s duration in the dark. After the treatments, cells were harvested and prepared for subsequent analyses.

2.5. Treatment protocols

HUVECs were divided into four groups: control (Control), ALA alone (ALA), ultrasound alone (US), and ultrasound plus ALA (US + ALA). For the ALA and US + ALA groups, the cells were incubated with 1 mM ALA in the dark. Instead of ALA, an equivalent quantity of medium was used for the Control and US groups. After 4 h incubation, the cells in the US and US + ALA groups were exposed to ultrasound (1.1 MHz, 1 W/cm², 50% duty cycle) for 60 s duration in the dark. After the treatments, cells were harvested and prepared for subsequent analyses.

In the in vivo experiment, tumor-bearing mice were randomly divided into four groups in the same manner as the cells. ALA was intraperitoneally injected into mice in the ALA and US + ALA groups at a dose of 250 μg/kg body weight. After 4 h of administration of ALA, the mice in the US and US + ALA groups were anesthetized with 1.2% avertin and were placed on a plexiglass plate with the tumor immersed into the degassed water. Tumors were irradiated by ultrasound (1.1 MHz, 2 W/cm², 50% duty cycle) for 5 min in the dark. The treatment above was carried out twice a week for two weeks on a Monday/Thursday schedule. Tumor growth was monitored every other day by measuring the tumor volume, calculated as volume = \( (\pi/6) \times A \times B^2 \) (A and B are the long and short diameters of the tumor, respectively). At the end of experiment, mice were sacrificed. Solid tumors were removed and processed for immunohistochemical and ultrastructural analyses.

2.6. Cell proliferation assay

HUVECs were seeded into 96-well plates (2 \times 10⁴ cells/well). After 48 h incubation, cell proliferation was determined with an MTT assay. Briefly, 50 μL MTT solution (0.5 mg/mL) was added to each well and incubated at 37 °C for 4 h to allow the formation of blue formazan crystals. Residual MTT was carefully removed, and crystals were dissolved by incubation with dimethyl sulfoxide (150 μL). Plates were shaken for 10 min; then, absorbance at 490 nm was measured using a microplate reader.

2.7. Transwell assay

Cell invasion assay was performed using Transwell chamber with a 6.5-mm-diameter polycarbonate membrane (8-μm-sized pores). The upper surfaces of Transwell inserts were coated with Matrigel. The bottom chamber of the apparatus contained 600 μL ECGM. HUVECs (100 μL) were added to the upper chamber (2 \times 10⁴ cells/well) and incubated in endothelial cell medium. After 16 h incubation at 37 °C, non-invasive cells on the upper membrane surfaces were removed by wiping with cotton swabs. Invading cells were fixed with methanol and stained with Giemsa. Cell invasion was quantified by counting cells on the lower surface using phase contrast microscopy. Cell migration assay was carried out as described for the invasion assay, with no coating of Matrigel.

2.8. Tube formation assay

The effect of SDT on HUVEC differentiation was examined by in vitro tube formation on Matrigel. Matrigel was thawed on ice overnight, spread evenly over each well of 48-well plates and polymerized for 30 min at 37 °C. HUVECs (2 \times 10⁴ cells/well) were plated onto the Matrigel layer and cultured in ECGM. After 8 h of incubation at 37 °C, cell morphological changes were observed and captured with a phase contrast inverted microscope.

2.9. Immunohistochemistry

Four micrometer sections of formalin-fixed, paraffin-embedded tumor tissues were consecutively cut onto silanized glass slides, and were routinely dewaxed and rehydrated. Antigens were retrieved in 10 mM citrate buffer (pH 6.0) for 15 min in a pressure cooker. After rinsing with phosphate-buffered saline (PBS), the sections were immersed in 3% hydrogen peroxide solution for 10 min to block endogenous peroxidases. Non-specific binding was prevented by incubation in 5% normal goat serum for 20 min in a humidified chamber. The sections were then incubated with anti-CD31 antibody (1:50 dilution) or anti-VEGF antibody (1:200 dilution) overnight at 4 °C. After washing with PBS, antibody binding was detected
with horseradish peroxidase-conjugated secondary antibody (Zhongshan Goldenbridge Biotechnology Co. Ltd.) for 1 h at 37 °C. The sections were visualized with diaminobenzidine (DAB) solution, and then lightly counterstained with hematoxylin.

2.10. Transmission electron microscopy

Xenografts were dissected and fixed in 2.5% glutaraldehyde for 1 h at 4 °C, followed by post-fixation in 1% osmium tetroxide for 2 h at 4 °C. After washing with PBS, the samples were dehydrated by graded alcohol, and embedded with Epon812 for 72 h at 60 °C. Ultra-thin sections were cut, stained with uranium acetate and lead citrate, and then observed under a transmission electron microscope (Hitachi, Tokyo, Japan).

2.11. Statistical analysis

Data are presented as the mean ± SD. Statistical differences were evaluated by one-way ANOVA, with P < 0.05 considered significant.

3. Results

3.1. Effect of SDT on proliferation of HUVECs

To assess the anti-angiogenic potency of SDT in vitro, its inhibitory effect on endothelial cell proliferation was first evaluated by MTT assay. The cell proliferation rate in the ALA group was 96.4%, and ALA alone showed no apparent cytotoxic effect on HUVECs compared with control. The cell proliferation rate was 81.6% in the US group, and declined to 70.6% in the US + ALA group. Both ultrasound alone and in combination with ALA significantly inhibited HUVEC proliferation (Fig. 2A).

3.2. Effect of SDT on migration of HUVECs

Migration of endothelial cells is a critical initiating event in the formation of new blood vessels and the repair of injured vessels. We carried out Transwell migration assays to investigate the effect of SDT on endothelial cell migration. Compared with control, endothelial cells treated with ALA alone did not show an obvious decrease in migration, while HUVEC migration in the US group was clearly reduced, and the reduction became more significant in the presence of ALA (Fig. 2B).

3.3. Effect of SDT on invasion of HUVECs

During angiogenesis, migrating endothelial cells must break and transverse through their basement membranes to form new blood vessels. We thus conducted Transwell invasion assays to observe the effect of SDT on endothelial cell invasion. Fig. 2C shows that ultrasound treatment somewhat suppressed HUVEC invasion compared with the control group, and ultrasound irradiation in the presence of ALA could induce more significant suppression than ultrasound alone. No obvious difference could be seen between the ALA and Control groups.

3.4. Effect of SDT on tube formation of HUVECs

Although angiogenesis is a complex process involving several types of cells, tube formation of endothelial cells is one of the key steps. After seeding on a Matrigel surface, HUVECs spread and aligned with each other to develop hollow, tube-like structures in both the Control and ALA groups. However, treatment with ultrasound alone significantly reduced the capillary network formation of endothelial cells, including decreased areas covered by HUVECs and lengths of the network. Moreover, HUVECs formed fewer capillary-like structures when exposed to ultrasound plus ALA (Fig. 3).

3.5. Effect of SDT on tumor growth in vivo

We used a human tongue cancer SAS xenograft mouse model to investigate the effect of SDT on tumor growth. As shown in Fig. 4A, compared with the control group, mice treated with ALA exhibited no obvious tumor growth suppression, while ultrasound treatment exerted a significant anti-tumor effect. In the US + ALA group, the inhibitory effect was more marked than that in the US group. At the end of the 2-week treatment period, the tumor volume inhibition ratios were 22.38% and 43.77% in the US and US + ALA groups, respectively. There was no statistically significant difference in body weight among different treatment groups (Fig. 4B). Furthermore, no adverse effects, such as skin ulceration or toxic death, were observed in any of the groups.

3.6. Effect of SDT on tumor angiogenesis in vivo

Angiogenesis was evaluated by microvessel density (MVD), which reflects the growth state of the tumor and has become the morphological standard for evaluating new vasculature in tumor tissue. The results in Fig. 5A shows that MVD in the ALA group were similar to that in the Control group. Ultrasound treatment significantly decreased MVD compared with control, and the reduction of MVD was more prominent in the US + ALA group. Accordingly, as a critical pro-angiogenic factor, the expression level of VEGF was reduced in tumors treated with ultrasound irradiation. Ultrasound plus ALA induced more significant decrease in VEGF expression than ultrasound alone (Fig. 5B). Similar to the in vivo results, ultrasound inhibited the secretion of VEGF in SAS cells more significantly in the presence of ALA (Supplementary Fig. 1).

3.7. Effect of SDT on vascular ultrastructure in vivo

Ultrastructural changes in tumors were studied by electron microscopy. No evidence of morphological alterations was found in either the Control (Fig. 6A and B) or ALA group (Fig. 6C). As shown in Fig. 6D, sonication caused moderate damage to the endothelial cells, with swelling of mitochondria and dilatation of endoplasmic reticulum in the cytoplasm. Condensation of chromatins was observed in the nuclei. The changes in ultrastructure of tumor tissues in the US + ALA group were more marked. A number of red blood cells or platelets clumped against the vascular endothelium (Fig. 6E). Cytoplasmic organelles further degenerated, and vacuolization was observed in the cytoplasm of endothelial cells (Fig. 6F).

4. Discussion

Angiogenesis is critical to tumor growth, metastasis, and recurrence. In the present study, we found that SDT based on the combination of ultrasound and sonosensitizers significantly inhibited in vitro angiogenic processes, including proliferation, migration, invasion, and tube formation in primary cultured HUVECs. Furthermore, SDT also significantly suppressed in vivo angiogenesis as measured by MVD in tumor-bearing mice. As we know, tumor angiogenesis is a complex process involving extensive interactions among endothelial cells, tumor cells, and other components of the microenvironment, and is often triggered by the release of proangiogenic factors [1–4]. Among the numerous secreted factors that promote angiogenesis, perhaps the most important is VEGF [25,26]. Our data showed that, besides the direct inhibitory effect of SDT on human endothelial cells, the decreased expression level of VEGF in tumors treated with ultrasound plus ALA provided another important explanation for the in vivo anti-angiogenic effect of SDT. In addition, we also found evidence of damage and disruption of tumor microvasculature through ultrastructural analysis.
Taken together, all of the above changes resulted in the significant suppression of tumor growth in the US + ALA group.

The therapeutic effect of SDT is influenced by multiple factors, such as the sonosensitizer and ultrasonic parameters. As a precursor of the sonosensitizer PpIX, ALA was used in this study. Our results indicated that ALA-induced PpIX accumulated in endothelial cells and also inhibited angiogenesis in tumor when exposed to ultrasound irradiation, which is similar to the photodynamic studies of Ota et al. [27] and Rodriguez et al. [28]. There have been other reports demonstrating that due to insufficient accumulation of intracellular endogenous PpIX, neither cellular alterations nor photodynamic efficacy could be observed in HUVECs [29]. These discrepancies may be related to the cellular proliferation rate. Wyld et al. [30] found that endothelial cells could accumulate between 1.5 and 4 times more PpIX when proliferating (as they do during tumor-induced angiogenesis) than when quiescent.

Fig. 2. Effect of SDT on HUVEC proliferation, migration, and invasion. (A) Cells were subject to the indicated treatments. After 48 h, cell proliferation was assessed by MTT assay. (B) Cell migration was evaluated by Transwell assay. Cells were seeded onto the culture insert following the indicated treatments and incubated for 16 h. After fixation and staining, the migrating cells were counted using a phase contrast inverted microscope and (C) Transwell assay was also performed to detect cell invasion. Cells were added into the upper chamber coated with Matrigel following the indicated treatments. After 16 h incubation, the HUVECs that invaded through the Matrigel and membrane were stained and quantified. Data are presented as the mean ± SD (n = 3), *P < 0.05 vs. Control; #P < 0.05 vs. US.

Fig. 3. Effect of SDT on HUVEC tube formation. Cells were cultured on a layer of Matrigel following the indicated treatments. After 8 h, tubular structures were visualized and quantified. Data are presented as the mean ± SD (n = 3), *P < 0.05 vs. Control; #P < 0.05 vs. US.
Application of therapeutic ultrasound (i.e., frequency up to 5 MHz and intensity up to 3 W/cm²) is already well-established in medicine. Low intensity ultrasound (usually 30–500 mW/cm²) has been shown to stimulate physiological responses within injured tissues [31] and to accelerate tissue repair [32], partially through promoting angiogenesis [33,34] and by increasing the production of angiogenic factors [35,36]. Yet, this is in contrast to our studies that 1 W/cm² ultrasound has a weak anti-angiogenic effect, which became more significant in the presence of ALA. This may be due to differences in the ultrasonic intensity and frequency used.

In a recent study, under the same experimental conditions as used in our study, inhibition of tube formation in HUVECs by ultrasound was also demonstrated [37].

Distribution of the sonosensitizer is dependent on the time-interval between drug administration and ultrasound irradiation (drug-ultrasound interval, DUI) and to a great extent determines the effectiveness of the sonodynamic therapy.

**Fig. 4.** Effect of SDT on tumor growth in vivo. (A) Tumor-bearing athymic mice of different treatment groups. During the treatment, tumor volumes were recorded and plotted and (B) body weight of mice was also measured. Data are presented as the mean ± SD (n = 6), *P < 0.05 vs. Control; #P < 0.05 vs. US.

**Fig. 5.** Effect of SDT on tumor angiogenesis in vivo. (A) Paraffin sections of tumor tissue were tested by immunohistochemical analysis with anti-CD31 antibody. Representative tumor vasculatures from mice of different treatment groups are shown. Microvessel density was calculated for each group and (B) immunohistochemical staining for VEGF was also analyzed in tumor samples. Data are presented as the mean ± SD (n = 6), *P < 0.05 vs. Control; #P < 0.05 vs. US.
sonosensitizing targets, therefore, DUI may be another important factor influencing sonodynamic efficacy. In general, initially, after administration, the sonosensitizer is confined within the tumor vasculature, and SDT that employs a short DUI will largely damage tumor vasculature (i.e., vascular-targeting sonosensitization). In contrast, a long DUI allows diffusion of the sonosensitizer into the tissue, to be accumulated in the tumor cellular compartment, so that the subsequent sonication will generate more direct tumor cytotoxicity (i.e., cellular-targeting sonosensitization). SDT is usually thought to maximize its therapeutic effect on tumors and minimize side effects on the surrounding healthy tissues when the sonosensitizer ratio of tumor to normal tissue is maximal[8,11]. However, in recent years, increasing evidence in photodynamic therapy (PDT) accumulated suggests that plasma concentration (which reflects endothelial cell exposure) correlated better with the therapeutic effect than tumor concentration[38–42]. In this study, we chose 4 h as our DUI. Four hours is the usual interval from drug administration to light illumination in clinical PDT using ALA [30], and is neither the time interval for maximal tumor concentration [43] nor for maximal plasma concentration [44,45]. Therefore, further study is required to elucidate the relationship between DUI, the distribution of sonosensitizer, and sonodynamic efficacy.

With extensive researches of SDT in the field of oncology, much attention has been paid to the action mechanism of SDT. Recently, a number of studies have shown that reactive oxygen species (ROS), generated from a sonosensitizer activated by ultrasound, play an important role in tumor apoptosis induced by SDT [14,16,43]. Depending on concentration and sub-cellular localization, ROS have been reported to mediate a variety of cellular functions, including pathogen killing, cell proliferation, migration and differentiation [46]. Thus, we believe that SDT inhibits angiogenesis also through the generation of ROS.

In conclusion, our study demonstrated that SDT significantly inhibits angiogenesis in vitro and in vivo, which is at least partially responsible for the anti-tumor effect of SDT.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.02.006.

References


Fig. 6. Effect of SDT on vascular ultrastructure in vivo. Transmission electron microscopy was used to investigate the ultrastructural changes in tumors of different treatment groups. (A) and (B) Endothelial cells in the control group. Framed area in A (×7000) is shown at high magnification in B (×15,000). (C) in the ALA group, vascular structure was intact, and tight junctions (black arrows) are visible (×15,000). (D) swollen mitochondria (black arrows) in endothelial cell after sonication (×15,000). (E) congestion is visible following SDT (×8000). “RBC” means red blood cell and (F) mitochondria (black arrows) were severely swollen and vacuolated in the cytoplasm of endothelial cell after SDT (×25,000).