Enhancement of Photodynamic Antitumor Effect With Pro-Oxidant Ascorbate

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Background and Objective: Photodynamic therapy (PDT) is a treatment modality that utilizes photosensitizers activated by light to induce cell death via the formation of singlet oxygen and other free radicals. Although this method has its advantages for tumor treatment, it cannot be well performed for involving so many therapeutic parameters during use. Tumor recurrence is common due to insufficient treatment. Therefore, a supplemental or complementary treatment is necessary for PDT.

Study Design/Materials and Methods: L-ascorbate, commonly known as vitamin C, is an essential nutrient for humans. It is also a well-established pro-oxidant in the presence of certain transition metal ions. In our experiments, ascorbate was administered to tumor-bearing mice by intraperitoneal injection (i.p.) for 10 days after they were treated with PDT. We hypothesize that this supplement may improve the therapeutic outcome by as a result of the reactions between ascorbate and the metal ions induced by PDT.

Results: The results demonstrate that PDT can cause Fe and Cu ions to be released from their protein complexes. The reactions between the ions and ascorbate resulted in a post-PDT surge in reactive oxygen species (ROS) as demonstrated in vitro with chemiluminescence detection. This ultimately leads to enhanced tumor cell death and, thus, an improved treatment outcome.

Conclusion: Based on the results that PDT induces metal ion release and ascorbate reacts with the metal ions producing subsequent ROS, an internal related, complementary and strengthened tumor treatment is established by combination of both PDT and ascorbate, as a low-toxicity and effective method. Lasers Surg. Med. 44:69–75, 2012. © 2012 Wiley Periodicals, Inc.

Key words: photodynamic therapy; reactive oxygen species; transition metal ion; tumor

INTRODUCTION

As a treatment modality, photodynamic therapy (PDT) involves the use of a photosensitizing drug excited with a specific wavelength light to kill target cells via the generation of reactive oxygen species (ROS) [1–3]. Several photosensitizers have been used in clinical PDT [4–7]. Advantages of PDT over other therapies include low toxicity, tumor selectivity, and noninvasive treatment [8]. However, the efficacy of topical PDT is limited by several factors, such as local oxygen (O2) concentration, light transmission, and photosensitizer uptake [9]. These limitations often result in tumor recurrence [10,11]. In clinical practice, PDT is often combined with other modalities such as surgery and chemotherapy to improve the outcome.

It has been reported that PDT works via ROS generation and proteins damage, causing transition metal ions, such as cuprous (Cu+) and ferrous (Fe2+) to be released from the storage protein in biological system [12]. Another mechanism of ferrous ion release during PDT was also reported: ferric iron is present in cells in the form of ferritin, and by penetrating into ferritin, superoxide reduces ferric iron to a free ferrous ion [13]. Transition metal ions are involved in many reactions. They can react with oxygen to produce superoxide anion. They are involved in at least three reactions in biological systems, accompanied by the formation of new free radicals: the reactions with hydrogen peroxide, hypochlorite and lipid hydroperoxides. Fenton reaction is the iron-salt-dependent decomposition of dihydrogen peroxide. It results in the formation of hydroxyl radical, which is highly reactive with proteins and nucleic acids, accompanied by protein denaturation, mutagenic and lethal effects. Hydroxyl radical is also produced when transition metal ions interact with hypochlorite and apparently with a greater yield compared to the reaction of transition metal ions with hydrogen peroxide (H2O2) [14].

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Usually the transition metal ions in an organism are complexed with proteins and their toxicity is inhibited. However, when the combination is broken the stored metal ions are released, increasing their oxidative activity greatly. Damages to cell and tissue, such as the post-ischemic reperfusion damage [15], spinal cord injury [16], dithiocarbamates induced apoptosis [17], and PDT [12,18] are then induced.

Ascorbic acid (L-ascorbate) is a sugar acid with antioxidant properties by being available for oxidation. Being a good electron donor in water, ascorbate can initiate and promote free radical reactions in the presence of free metal ions. Ascorbate in an aqueous solution is easily oxidized by molecular oxygen in the presence of metal ions. This makes it a potential pro-oxidative compound in certain metabolic contexts, thus producing ROS and exhibiting cytotoxicity [19].

Some reports have proved that by using both transition metal ion and ascorbate, even millimolar-concentrations of ascorbate in vivo can achieve antitumor activity [20–23]. The antitumor mechanism of ascorbate has been deduced as below [13,14,24,25] (AscH, ascorbic acid; Mn, metal ion; LOOH, peroxidized fatty acid; HCLO, hypochlorite):

\[
\text{AscH}^- + \text{Mn}^{(n+1)+} \rightarrow \text{Asc}^{*^-} + \text{Mn}^{n+} + \text{H}^+ \quad (1)
\]

\[
\text{Mn}^{n+} + \text{O}_2 \rightarrow \text{Mn}^{(n+1)+} + \text{O}_2^- \quad (2)
\]

\[
\text{HO}_2^- + \text{O}_2^- + \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (3)
\]

\[
\text{Mn}^{n+} + \text{H}_2\text{O}_2 \rightarrow \text{HO}^* + \text{HO}^- + \text{Mn}^{(n+1)+} \quad (4)
\]

\[
\text{Mn}^{n+} + \text{LOOH} \rightarrow \text{LO}^* + \text{HO}^- + \text{Mn}^{(n+1)+} \quad (5)
\]

\[
\text{Mn}^{n+} + \text{HCLO} \rightarrow \text{HO}^* + \text{CL}^- + \text{Mn}^{(n+1)+} \quad (6)
\]

The equations suggest that when transition metal ions are present, ascorbate can lead to a series of reactions to produce radical molecules that can damage cell.

Researchers have reported that the biological or chemical reactions occurring in a tumor after PDT treatment are as important as the reactions during the PDT light irradiation [26]. Based on how PDT can induce metal ion release and the ROS-producing reactions between ascorbate and metal ions, ascorbate was investigated as an in vivo pro-oxidant to enhance cell death, thus improving the overall antitumor response. The concept and method are projected as Scheme 1.

**MATERIALS AND METHODS**

**Chemiluminescence Detection of Reactive Oxygen Species Produced by Transition Metal Ions and Ascorbate**

Ascorbic acid (Guangzhou Baiyunshan Pharmaceutical Co. Guangzhou, China) was buffered to pH 7.0 with sodium hydroxide and prepared immediately before use. Luminol (Sigma, Poole, Dorset, UK) and CuCl₂ were dissolved in 1 ml deionized water with pH 11 in a dish (Luminol: 1 mM, CuCl₂: 250 μM). The signal was recorded with a chemiluminescence (CL) detection system. After 1-minute detection 1 μl ascorbate (250 mM) was quickly pushed into the dish with a capillary. CL was detected simultaneously. The CL detection for FeCl₃ was the same as CuCl₂ detection. FeCl₂ (250 μM) was added in Luminol solution (1 mM) and CL was monitored.

**In Vitro Enhancing PDT Effect With Ascorbate**

EMT6 cells (Mouse mammary carcinoma EMT6 cell lines purchased from Fourth Military Medical University Experimental Centre) were cultured (RPMI 1640 with 10% FCS at 37 °C) in a 96-well microplate at a density of 5 × 10³ cells/well for 24 hours. Wells were divided into eleven groups. Cells in each group were administered with different protocols, including Photofrin-only, Photofrin plus ascorbate, ascorbate-only, ascorbate plus light (irradiation time: 240 seconds), PDT-only (including three groups with 60 seconds, 120 seconds and 240 seconds irradiation time, respectively), PDT plus ascorbate (including three groups with the same irradiation time as above) and None. Photofrin was used with 5 μg/ml (24 hours pre-incubation for cells). After transmitting fresh culture medium in all cell-containing wells, cells for PDT were irradiated by laser (10 mW/cm², 635 nm, semiconductor laser: NL-FBA-2.0-635, nLight Photonics Corporation, Vancouver, WA). Ascorbate (1 mM) was then administered to cells according to the scheme. After an additional 24 hours culture, cell viability was assessed with CCK-8 reader (Infinite M200, TECAN, Maennedorf, Switzerland). The absorbance value was at 450 nm and the OD450 is inversely proportional to the degree of cell death in a culture medium (n = 4).

**Metal Ions Release Induced by PDT and Ascorbate**

Mouse tumor cells were cultured for several days before use. EMT6 cells (1 × 10⁵ cells/ml, 150 μL) were inoculated subcutaneously to BALB/c female mice (average weight 20 g, Center of Experimental Animal Sun Yat-sen University, Guangzhou, China). Animal procedures were...
in agreement with the guidelines of the Institutional Animal Care and Use Committee. When tumors grew up to 15 mm in diameter, 3 mice were used as controls and 18 mice were injected with the photosensitizer Photofrin (5 mg/kg, Axcan Pharma Ltd, Ireland) in vein. Twelve hours later, control mice and mice other than three mice were sacrificed directly. The remaining mice (three/group) were all irradiated with laser (135 mW/cm², 635 nm) for 500 seconds and sacrificed at different survival time: 0, 0.25, 0.5, 3, 12, and 48 hours. Each tumor mass was harvested and 500 mg was transferred to a tube. The mass was cut into small pieces with a surgical scissors. After adding 1.5 ml cell Lysis Buffer (50 mmol/L Tris–HCl pH8.0, 150 mmol/L NaCl, 1% Triton X-100, 100 mg/ml PMSF), the tumor tissue pieces were disrupted for 10 minutes in ice-bath condition with an Ultrasonic Cell Disrupter System (JY92-1, SCIENTZ, Shanghai, China). The supernatant liquid of the mixture was separated by a centrifuge (5415R, Eppendorf, Hamburg, Germany) at 10,000 rpm at 4°C for 10 minutes. Bathophenanthroline disulfonic acid disodium salt (BPSA) hydrate (C24H14N2Na2O6S2·3H2O, Toronto Research Chemicals Inc., North York, ON, Canada) dissolved at the primary concentration 50 mM was used to detect Fe ion [27]. The tissue supernatant liquid (239 μl) was mixed with 10 μl BPSA and 1 μl ascorbate (250 mM). Fifteen minutes later, the mixed solution was transferred into three wells of a 96-well plate (50 (250 mM). Fifteen minutes later, the mixed solution was transferred into three wells of a 96-well plate (50 μl/cell). All solutions were analyzed by the Tecan Infinite® M200 multimode microplate reader. Absorbance at 535 and 600 nm were detected.

Mice (n = 15) were administered with PDT as above but injected with ascorbate by i.p. (1.25 g/kg) at once after PDT. In this protocol ascorbate was employed twice a day, considering its pharmacokinetics in vivo [28]. 0.25, 0.5, 3, 12, and 48 hours following PDT, mice (three/group) were sacrificed and free Fe ions of tumors were detected.

In Vivo Enhancing PDT Effect With Ascorbate

EMT6 tumor cells were cultured and transplanted into mice subcutaneously. Once the volume of tumor approached 150 mm³, mice in different groups (n = 4) were subjected to PDT-only, ascorbate-only, PDT plus ascorbate and none protocols respectively. Photofrin (PDT group) was (5 mg/kg) injected in vein. Twelve hours later, tumors were irradiated with laser for 500 seconds (635 nm, 135 mW/cm²). Ascorbate was injected by i.p. (1.25 g/kg, twice a day). Two days later mice were sacrificed and tumor masses were cut into small pieces with surgical scissors in glass dishes. Cell dissociation buffer (3 ml) was added to these dishes. Five minutes later, the masses were dissociated into single cell suspension. With a pipette the suspension was transferred into a 10 ml centrifuge tube to which 1 ml cell culture medium had been pre-added. The same operation was repeated three times and the cell suspension was spun down at 10,000 rpm for 10 minutes to separate the cells. Cells were collected and blew into single cells in PBS medium (pH 7.4). After filtering with a cell strainer, cells were incubated with FITC (fluorescein isothiocyanate)-annexin V for 15 minutes in the dark at room temperature according to the manufacturer’s instructions. Apoptosis was detected analytically using the BD FACSCanto II flow cytometer (Becton Dickinson Biosciences, Le Pont-de-Clai, France).

With the same treatment protocols as described above, four mice were employed for EMT6 staining to observe histological changes: mice were sacrificed after treatment. Tumors were harvested and fixed in Bouin’s solution for 12 hours. The tumors were then embedded in paraffin and sectioned to 4 μm thickness. The sections were stained by hematoxylin and eosin, and viewed with a microscope (ECLIPSE 80i, Nikon, Tokyo, Japan).

To detect tumor growth and mice survival, the same protocols described in this section, including PDT-only, ascorbate-only, PDT plus ascorbate and none (n = 8) were employed. Ascorbate was injected by i.p. for 10 days (1.25 g/kg, twice a day). Tumor dimensions were measured every day for 30 days and the volumes were calculated with a formula (volume = length × (width)²/2) [29]. The time for a tumor volume to reach 1,000 mm³ after treatment was regarded as the end of a mouse.

Statistical Analysis

Data were presented as mean ± SD and were processed with Origin6.0 program (OriginLab, Hampton, MA). The in vitro, in vivo results were all tested with the Student’s t-test for any statistical difference. A value of P < 0.05 was considered statistically significant.

RESULTS

Luminol was used to detect ROS production. The CL probe can react with ROS to emit 425 nm photons [30]. Figure 1 shows CL was enhanced after injection of ascorbate in metal ion solution. The results indicate that ascorbate can react with Cu²⁺ or Fe³⁺ to produce ROS. The reaction rate of Cu²⁺ is faster than that of Fe³⁺++. Fe³⁺ alone can also induce ROS production to yield the CL signal (Fig. 1C).

After irradiation by laser for PDT, cells were incubated with ascorbate. The results of cell viability (Fig. 2) show that ascorbate significantly improved cell death of PDT, especially in the low light dose group. But when PDT light dose was high (up to 240 seconds), the enhancement of cell death was limited. The result also indicates that ascorbate alone could kill cells at the investigated dose level. The combination of ascorbate and a 240 seconds laser irradiation for cells was also investigated, and the cytotoxicity was not improved comparing with the ascorbate-only group. As a control, the blank and Photofrin groups showed a low death rate.

The release of transition metal ions from their protein complexes was studied. Figure 3 shows the in vivo result of Fe ion release. The concentration gradually increased and reached its maximum value at about 3 hours after PDT, then followed by a drop. The results also indicate that the combination with ascorbate further enhanced the in vivo ion release.

After tumor treatment, tumor masses were disrupted to single cells and the apoptotic cells were detected by a flow...
cytometry based on FITC/PIS staining (Fig. 4). The flow cytometry result and H&E staining result both show that the PDT plus ascorbate group had the highest cell death rate. The combination with ascorbate can improve tumor treatment outcome. The results of tumor growth rate and survival rate (Figs. 5 and 6) further demonstrated the consequences: the tumor growth rate of PDT plus ascorbate group is significantly low (Fig. 5), and by contrast the other three treatment protocols the survival time of the mice were prolonged obviously (Fig. 6). Clearly, although

Fig. 2. Enhancement of PDT effect with ascorbate in vitro. PDT groups were irradiated with time 60, 120, and 240 seconds, respectively (Photofrin: 5 μg/ml, laser: 10 mW/cm²). Ascorbate concentration was 1 mM. Ascorbate plus light group was irradiated with time 240 seconds. Cell viability was assessed with OD value after CCK-8 staining. Data are presented as mean ± SD and asterisk means significant Student’s t-test between the two in same column (Asc, ascorbate).

Fig. 3. The release of transition metal ions induced by PDT. Bathophenanthrolinedisulfonic acid disodium salt hydrate (BPSA, 50 mM) was used to detect Fe ions. Each of the tissue supernatant liquors 239 μl was mixed with 10 μl BPSA and 1 μl ascorbate. The absorbance at 535 and 600 nm were detected. The difference between 535 and 600 nm was as the relative absorption. The data are present as mean ± SD and asterisk means significant Student’s t-test.
both the PDT-only and ascorbate-only group could induce tumor cell death and prolong the mice life, the PDT plus ascorbate presented a remarkable enhancement, indicating the combination improved tumor treatment effectively.

**DISCUSSION**

As reviewed in the Introduction Section, ascorbate at millimolar concentration is toxic to tumor cells. However, the tumor-restraining rate with ascorbate alone is limited. The CL results in solution indicate that the metal ions Cu$^{2+}$, Fe$^{3+}$ can react with ascorbate to produce ROS, as described in Equations (1)–(4). The ROS generation suggests a potential cell toxicity of ascorbate in the presence of transition metal ions. If the quantity of transition metal ions is large, its cytotoxicity will increase remarkably.

Photochemical reactions can kill cells and induce metal ion release from its complex with proteins; but PDT alone often kills local tumor cells incompletely, which results in tumor recurrence. By combining ascorbate with the metal ions induced by PDT, the tumor cure rate in vivo is enhanced.

The *in vitro* results show that cells can be killed by photodynamic reaction alone, as well as millimolar-concentration ascorbate. By combining PDT with ascorbate the cell viability decreased greatly, suggesting a more severe cell-killing effect. The mechanism may be described as below: The transition metal ions in normal cells exist as complexes with proteins, such as metallothionein, ceruloplasmin, ferritin, transferrin and so on, to restrain their toxicity and storage for use. PDT damages the metal-binding proteins which results in the release of the stored metal ions. The released ions are toxic to cells mainly due to production of ROS and other free radicals. But the toxicity is limited due to the oxidation depletion. When ascorbate is present, the reaction deoxidizes the high valent metal ions and recycles the producing ROS process (as Equations 1–6 described). This yields more ROS to damage cells.

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*Fig. 4. In vivo tumor cell death analysis by a flow cytometry based on FITC/PIS staining and H&E staining. Ascorbate, PDT-only, PDT plus ascorbate and control were compared (A, flow cytometry; B, H&E-staining, the bar is 50 μm). Quantitative analysis of the flow cytometry data were shown in C. Data are presented as mean ± SD (n = 4, *P* < 0.05).*
The result in Figure 2 indicates that ascorbate is not phototoxic. As a reducer, ascorbate in cells usually acts as an antioxidant to prevent damage. It can effectively quench singlet oxygen which is the main component for tumor cell killing in PDT. The quenching produces hydrogen peroxide and further is eliminated by catalase in the cells [31]. It is likely that administering ascorbate prior or even during a PDT irradiation may adversely affect the treatment outcome. As shown in Figure 3 the maximum transition metal ion accumulation occurred at 3 hours post-PDT. Based on the results and considering the pharmacokinetics of ascorbate [20,32], ascorbate was administered only upon completion of the laser irradiation. The result in Figure 3 also suggests that the administered ascorbate not only continued cell damage by acting as a pro-oxidant to produce more ROS, but also induced more metal ion release. The positive feedback is likely to further promote tumor cell killing and improve the treatment effect.

The results including Figures 4–6 demonstrate the enhancement of tumor cell killing by administration of ascorbate in vivo. The result in Figure 4 indicates that, by administering ascorbate after PDT, cytotoxicity was enhanced. The cell apoptosis increases 30% compared to the control. The data also suggest that by further extending the associated administration period, it may further improve post-PDT cell killing. The results of tumor growth and mice survival show a significantly enhanced treatment effect with a 10-day administration of ascorbate.

Reports have shown that the toxicity of ascorbate to tumor cells is dose-dependent [20,32,33]. Clearly, using higher dosage of ascorbate, as well as PDT, will enhance the treatment effect. In the research, ascorbate was administered with concentrations at 1 mM in vitro and 1.25 g/kg in vivo. These concentrations are relatively low compared with that used in clinical applications [34]. Yet, at the investigated dosage levels, the effectiveness of the modality is clearly demonstrated. The main objective of this research is to study how the therapeutic effect could be improved by the combination of PDT and ascorbate. With higher dose administration, the enhancement was less clear as it approached a therapeutic cure (Fig. 2). For the purpose of the study, subtractive dosages were then used to demonstrate the effectiveness of the combined therapies. The results indicate that at the dosage level, the treatment outcome was improved. It can thus be deduced that ascorbate administration will help to compensate for the insufficient cell killing of PDT, especially in the low-dose region of a therapeutic application.

In conclusion, with transition metal ions induced by PDT acting as catalyst, the administration of ascorbate enhanced ROS production post-PDT. The internal related, complementary and strengthened combination improved tumor treatment effect. Ascorbate, as a medically used drug the combination with PDT for tumor treatment is prospective for clinical application. Of course, it would be useful and interesting to carry out further work to clearly distinguish how to administer ascorbate and PDT to achieve the best treatment efficacy in clinic.

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