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Apoptosis of vascular smooth muscle cells induced by photodynamic therapy with protoporphyrin IX

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ABSTRACT

Photodynamic therapy (PDT) had been shown effective in the treatment of intimal hyperplasia, which contributes to restenosis, by eradicating cells in the vessel wall. This study is designed to evaluate the effects of PDT with protoporphyrin IX (PpIX) on the viability of vascular smooth muscle cells (SMCs) and to define the cell-death pathway. Fluorescence microscopy and laser-induced fluorescence spectroscopic detection showed that SMCs selectively uptake PpIX, and the intracellular PpIX concentration increases with the amount of PpIX in the incubation solution. PDT with PpIX impaired cellular viability from 93 ± 3.4% to 36 ± 3.9% when the light intensity increases from 2 to 9 J/cm^2 and intracellular PpIX concentration increases from 0.5 to 20 μg/ml. Although PDT induced both apoptosis and necrosis, the ratio of apoptotic cells increased with light dosage or intracellular PpIX concentration. The loss of mitochondrial membrane potential coincided with the apoptotic ratio. Our results indicated that the induction of apoptosis of SMCs may be one of the mechanisms by which PDT inhibits restenosis *in vivo*.

Introduction

In recent years, significant advances have been made in the prevention and treatment of coronary artery disease, resulting a noticeable reduction in the related incidences of morbidity and mortality [1,2]. Most frequently used treatment approaches include atherectomy, bypass surgery, endarterectomy, balloon angioplasty and implantation of medical stents. Especially, drug eluting stents have successfully reduced restenosis cases after percutaneous interventions. However, a critical reassessment of evidences has been recently published regarding the hypothesis of over-estimation of benefits, safety and costeffectiveness for the drug eluting stents [3]. There are still 5–10% patients develop restenosis even with long term drug treatment after the procedure.

Photodynamic therapy (PDT) involves the combined use of a photosensitizing agent that accumulates in the target tissues and endovascular illumination on the target to produce cytotoxic singlet oxygen [4,5]. Investigations showed that PDT induced damage to SMCs mainly through cellular depletion due to apoptosis [6], promoted early reendothelization, control of IH and matrix production [7,8]. Initial clinical applications showed that PDT safely and effectively prevented restenosis after angioplasty up to a 6 month follow-up [9].

During the last few years a large number of suitable dyes were synthesized and tested [10]. At present, the predominantly used photosensitizers are represented by porphyrins and their analogs [11]. The selective build-up of PpIX in the atheromatous plaque was 10 times higher than in normal vessel walls, providing an atherosclerosis-selective treatment agent [12].

This study aimed to observe the intracellular accumulation of PpIX, quantify the effect of PpIX mediated PDT on SMCs, and measure the apoptotic or necrotic ratio of SMCs under PDT. Mitochondria membrane potential changes were also detected.

Materials and methods

Chemicals. Protoporphyrin IX was from Sigma–Aldrich reconstituted in 100% DMSO (Sigma–Aldrich) to 0.1 g/ml and stored at room temperature. JC-1 probe was provided by Beyotime Institute of Biotechnology (Haimen, China). Hoechst 33258, propidium iodide (PI) and thiazolyl blue tetrazolium bromide (MTT) were from Sigma–Aldrich. Fetal bovine serum and DMEM were from Hyclone

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Laboratories, Inc. (HyClone, Logan, UT, USA). All other reagents were obtained from Sigma Chemical Co. Ltd.

Cellular uptake of PpIX and PDT procedure. SMCs cell line (ATCC), which was derived from murine thoracic vascular aorta, was maintained in DMEM supplemented with 10% FBS, penicillin, and streptomycin. Cells were grown at 37 °C in a humidified 5% CO_2/95% air atmosphere.

In the PDT procedure, SMCs were first put into serum-free medium, then incubated with PpIX (from 0.5 to 50 μg/ml) in 5% CO_2 atmosphere at 37 °C for 3 h. Afterward, PpIX-containing or vehicle-containing media were removed, and the cells were washed with sterile PBS and then illuminated in a darkroom using a 635 nm diode laser at a total power of 2–9 J/cm^2. After illumination, PBS was removed, and serum-containing medium was added. The viability and apoptosis of SMCs were assessed after 6 h more incubation. All data are presented as means ± SD. Groups were compared by the Dunnett-*t* test using the SPSS Software system. A value of $P < 0.05$ was considered significant.

Intracellular accumulation of PpIX. SMCs (3×10^4 cells per well) were seeded in 24-well tissue culture plates, PpIX (from 1 to 50 μg/ml) containing medium was added to the cells, then incubated for 3 h. Intracellular accumulation of PpIX was confirmed by using fluorescence microscopy imaging. In order to quantify the PpIX uptake, laser-induced fluorescence (LIF) spectra was measured under the excitation of a 405 nm diode laser.

SMCs viability after PDT. The cultured PpIX containing SMCs were illuminated to perform the PDT process as previously described. The survival rate of SMCs was measured using a colorimetric assay based on the uptake of MTT by viable cells. Because uptake and cleavage of MTT occurs only in living cells, the color reaction is a function of the number of viable cells, and can indicate the percentage of surviving cells after the treatment. The percentage of survival cells was calculated in PDT treated SMCs and compared to controls incubated with PpIX but without illumination from the same plate.

Apoptosis and necrosis assessment. After PDT, cells were washed in PBS and stained with Hoechst 33258 and PI, according to the manufacturer's protocol. SMCs were first incubated with 10 μg/ml PI for 10 min at 37 °C in the dark, then incubated with 5 μg/ml Hoechst 33258 for 5 min, After that, PBS was removed, and serum-containing medium was added. A fluorescence microscope equipped with a digital camera was used for image capturing. Necrotic cells will be stained with red fluorescence while apoptotic cells will be stained with blue fluorescence. The fraction of apoptotic or necrotic cells was determined in five random microscopic images with at least 1000 cells/group.

Mitochondrial transmembrane potential assessment. Mitochondrial transmembrane potential was assessed using the probe JC-1, a sensitive fluorescent dye. After PDT, SMCs were incubated with 10 mg/ml JC-1 for 10 min at 37 °C in the dark and monitored by using a fluorescence microscope. Red-orange fluorescence is attributable to a potential-dependent aggregation in the mitochondria. Green fluorescence, reflecting the monomeric form of JC-1, appeared in the cytosol after mitochondrial membrane depolarization.

Results

Cellular accumulation of PpIX

The accumulation of PpIX observed by fluorescence microscopy is shown in Fig. 1A. Intracellular PpIX fluorescence increased when the cells were given more PpIX (from 1 to 50 μg/ml). LIF spectra were measured for different PpIX concentration (from 0.5 to 50 μg/ml). As shown in Fig. 1B, PpIX has two fluorescence emission peaks centered at 635 and 701 nm. The area beneath the curve could be used as a measure for the dosage of PpIX within the SMCs. Intracellular PpIX concentration was proportional to the PpIX concentration before 10 μg/ml, but only 1.5 times more in 50 μg/ml compared with 10 μg/ml, which shows saturation effect.

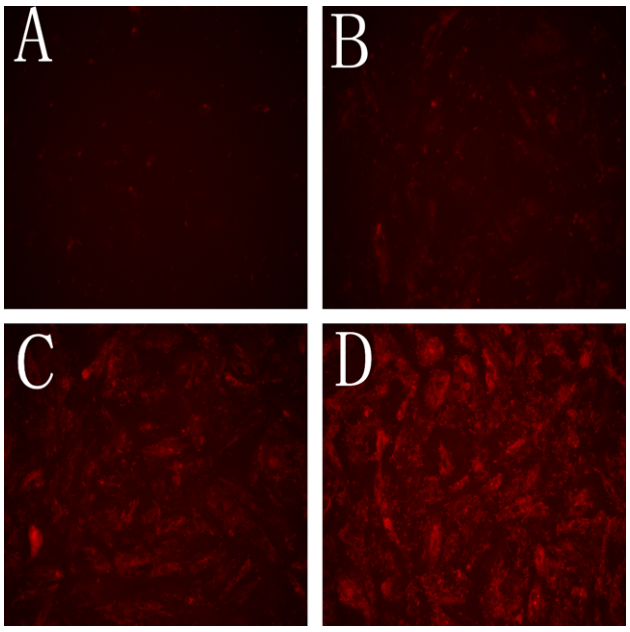

Fig. 1A. Fluorescent photomicrograph of SMCs after 3 h incubation with different concentration of PpIX, original magnification 400×, PpIX concentration: 1 μg/ml (A), 5 μg/ml (B), 20 μg/ml (C), and 50 μg/ml (D).

Cellular viability and PDT

As shown in Fig. 2, PDT impaired cellular viability from 93 ± 3.4% (light dose = 2 J/cm^2, PpIX = 0.5 μg/ml) to 36 ± 3.9% (light dose = 9 J/cm^2, PpIX = 20 μg/ml). The cellular viability descends with higher light intensity and/or higher intracellular PpIX concentration. Moreover, induction of cell death requires the combination of PpIX and light illumination, neither drug nor light alone had significant effects on cellular viability.

The apoptosis and necrosis of SMCs induced by PDT

The condensed and fragmented nuclei with blue fluorescence were apoptotic cells and they were stained more brightly than nor-

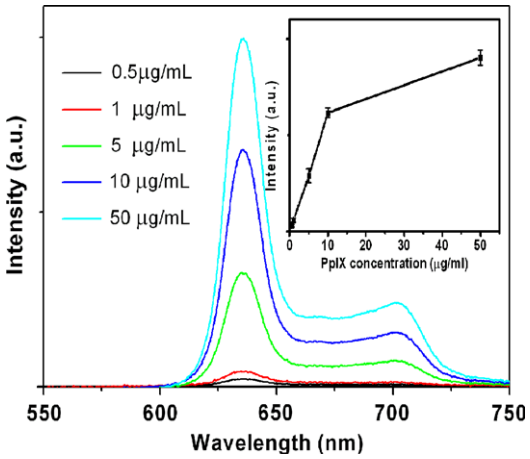

Fig. 1B. The LIF spectra detection of SMCs incubated with different concentration of PpIX. After stimulated by laser at $\lambda = 405$ nm, PpIX emission wavelength fluorescence was at both $\lambda = 635$ nm and $\lambda = 701$ nm. The amount of intracellular PpIX steadily increased with the increase of photosensitizer concentration (inset).

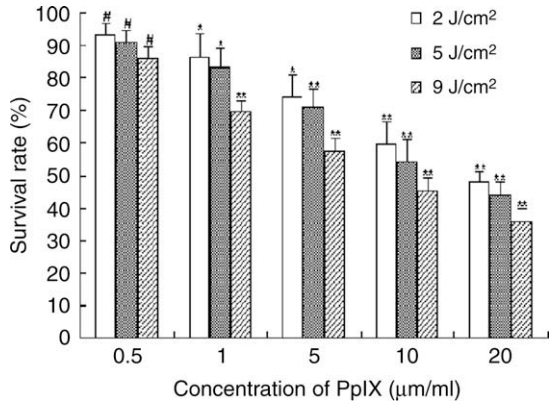

Fig. 2. Cellular viability after PDT. The effect of PDT with PpIX (from 0.5 to 20 μg/ml) on SMC viability was assessed by using a MTT assay. Data are representative of three independent experiments. $^{\#}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.005$ vs controls.

mal cells, while the necrotic cells were labeled by red fluorescence. As shown in Fig. 3, PDT induced both apoptosis and necrosis of SMCs, and apoptotic ratio was higher than necrotic ratio with appropriate conditions. At the same time, necrotic cells kept increasing with higher light dosage or/and higher intracellular PpIX concentration. The blue line represents the apoptosis/necrosis ratio (Fig. 3A and B).

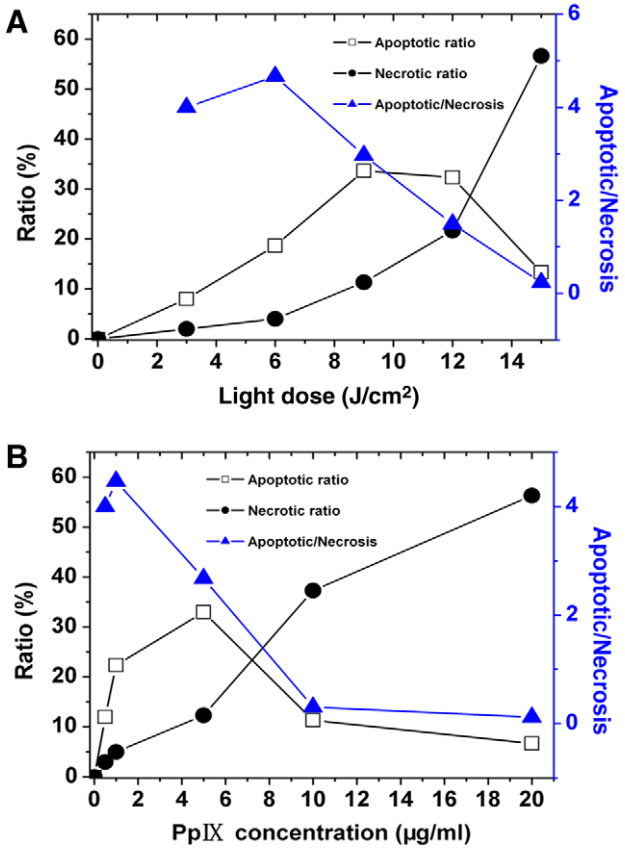

Fig. 3. PDT with PpIX induced both apoptosis and necrosis of SMCs. (A) PDT with identical intracellular PpIX concentrations (5 μg/ml) but the applied light power was from 0 to 12 J/cm^2. (B) PDT with identical light power (9 J/cm^2), and the applied PpIX concentrations was from 0 to 20 μg/ml. The blue line means the apoptosis/necrosis ratio. Results are representative of three independent experiments. (For interpretation of color mentioned in this figure legend, the reader is referred to the web version of the article.)

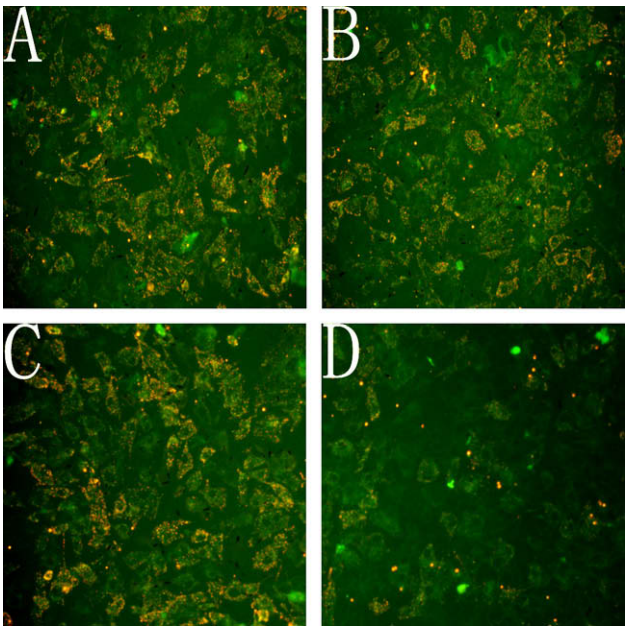

Fig. 4. PDT with PpIX reduced the mitochondrial membrane potential of SMCs. Mitochondrial membrane potential was determined using the potential-sensitive fluorescent probe JC-1. Normal cultured SMCs contained red-orange fluorescent mitochondria in the cytoplasm (A), the light alone (B) or PpIX alone (C) behaved the same as normal cultured SMCs. SMCs subjected to PDT (light dose = 9 J/cm^2, PpIX = 5 μg/ml) showed green fluorescence (D), indicating the loss of mitochondrial membrane potential. Original magnification 400×. (For interpretation of color mentioned in this figure legend, the reader is referred to the web version of the article.)

Mitochondrial membrane depolarization after PDT

As shown in Fig. 4, normal SMCs exhibited red-orange fluorescence (Fig. 4A), and neither light alone (Fig. 4B) nor PpIX alone (Fig. 4C) showed mitochondrial membrane potential change. SMCs after PDT developed a diffuse green staining pattern (Fig. 4D), which is representative of reduced mitochondrial membrane potential. The mitochondrial membrane potential decreased with higher light power or/and higher PpIX concentration.

Discussion

It is known that most types of mechanical disruptions of the vascular wall inflict a significant injury to the intima and media layers, thereby stimulating a tissue response which is based on the formation of a neointima, this is largely the consequence of the hyperproliferation of smooth muscle cells and myofibroblasts followed by their diffusion to the subintimal space and deposition of extracellular matrix proteins with eventual reduction of the luminal cross-area [13]. Since SMCs play a key role in the pathogenesis of restenosis, several studies have been focused on the photodynamic inactivation of such cells to dynamic inactivation of such cells.

In the present study, a dosage-dependent increase in intracellular PpIX fluorescence was observed in SMCs (Fig. 1A), and we have detected stronger LIF spectra at higher PpIX concentration (Fig. 1B). Intracellular PpIX concentration was proportional to the PpIX concentration before 10 μg/ml, then began to saturate (Fig. 1B inset). PpIX is heme precursor and therefore a physiologic substance, and the peak absorption wavelength is about 635 nm. For *in vivo* applications of PDT, preferred photosensitizers are those efficiently absorbing red light ($\lambda > 600$ nm) [14]. Such wavelengths exhibit a relatively deep penetration power into most human tissues, ranging from about 1 cm in the 600–650 nm interval up to 3 cm in the

750–800 nm interval [15]. Moreover, red/far-red light is poorly absorbed by endogenous constituents of tissues, which minimizes the risk of generalized photosensitization effects.

After quantifying PpIX induced phototoxicity as a function of applied energy density and photosensitizer concentration, we have analyzed in detail the cellular response to PDT. Apoptosis has been found to be the prominent form of cell death in response to PDT for many treated cells [16]. Clinically, apoptosis is preferred over necrosis since it causes less tissue reactions. As shown in Fig. 3, at low dosage photosensitizer and lower power illumination, apoptosis was the main cell-death pathway. High light dosage or higher PpIX concentration increases the ratio of necrosis and eventually makes necrosis the main cell-death pathway. The apoptosis/necrosis ratio showed that lower dosage and lower power PDT would be more preferred in order to elicit more cell apoptosis. This mode of cell death may be essential to the proposed clinical use of PDT to prevent and treat restenosis or as a primary atherosclerotic plaque-ablating therapy, allowing for vascular cell dropout without promoting an inflammatory response.

There are numerous examples in which the ability of PDT exposed cells initiating apoptotic process differs substantially depending on the cell line [17–20], the photosensitizer type and its subcellular location, the overall dosage as well as other conditions [21–24].

Mitochondria play a central role in programmed cell death [25]. Mitochondrial respiration generates a major physiological source of ROS, and activators of apoptosis (e.g., caspase-2, caspase-9, cytochrome *c*, and apoptosis-inducing factor) reside in mitochondria. Mitochondria-derived ROS may signal apoptosis by modifying membrane proteins, such as a large-conductance channel known as permeability transition pore, to modulate mitochondrial membrane potential or by activating downstream targets such as stress-activated protein kinase cascades [26]. In the present study, we have shown that PDT with PpIX results in the loss of mitochondrial membrane potential. Our findings are consistent with the results in apoptosis assay. The precise mechanism of how PDT with PpIX linked to these mitochondrial events remains to be determined.

PDT has a pleiotropic effect, acting not only on SMCs, but also on other constituents of the vessel wall which play an important role in the development of hyperplasia. PDT has been shown to affect both the proliferation and the invasive migration of fibroblasts and SMCs [27–29], stimulate the proliferation of endothelial cells, generate matrix protein cross-links [28], thus hindering invasive cellular migration.

In conclusion, we have identified PpIX accumulation in SMCs *in vitro*, and verified that PDT could induce both apoptosis and necrosis of SMCs. Different PDT conditions could affect the main cell-death pathway as well as the extent of mitochondrial membrane potential changes. Our results demonstrated that PpIX medicated PDT could be clinically useful in the treatment of atherosclerosis with proper optimization of the treatment parameters.

Acknowledgments

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