Detection and photodynamic therapy of inflamed atherosclerotic plaques in the carotid artery of rabbits

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Abstract
Photodynamic therapy (PDT) has been applied in the treatment of artery restenosis following balloon injury. This study aimed to detect the accumulation of 5-aminolevulinic acid (ALA)-derived protoporphyrin IX (PpIX) in inflamed atherosclerotic plaque in rabbit model and evaluate the efficacy of PDT. The inflamed atherosclerotic plaque in the common carotid artery was produced by combination of balloon denudation injury and high cholesterol diet. After intravenous administration of ALA, the fluorescence of PpIX in plaque was detected. At the peak time, the correlation between the fluorescence intensity of PpIX and the macrophage infiltration extent in plaque was analyzed. Subsequently, PDT (635 nm at 50 J/cm2) on the atherosclerotic plaques (n = 48) was performed and its effect was evaluated by histopathology and immunohistochemistry. The fluorescence intensity of PpIX in the plaque reached the peak 2 h after injection and was 12 times stronger than that of adjacent normal vessel segment, and has a positive correlation with the macrophage content (r = 0.794, P < 0.001). Compared with the control group, the plaque area was reduced by 59% (P < 0.001) at 4 week after PDT, the plaque macrophage content decreased by 56% at 1 week and 64% at 4 week respectively, the smooth muscle cells (SMCs) was depleted by 24% at 1 week (P < 0.05) and collagen content increased by 44% at 4 week (P < 0.05). It should be pointed out that the SMC content increased by 18% after PDT at 4 week compared with that at 1 week (P < 0.05). Our study demonstrated that the ALA-derived PpIX can be detected to reflect the macrophage content in the plaque. ALA mediated PDT could reduce macrophage content and inhibit plaque progression, indicating a promising approach to treat inflamed atherosclerotic plaques.

Keywords:
Atherosclerosis
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Fluorescence intensity
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1. Introduction
Vulnerable atherosclerotic plaques are responsible for most cardiovascular events, including myocardial infarction and stroke [1,2]. Local inflammatory cells, particularly macrophages play a crucial role in plaque instability [3–5]. Matrix metalloproteinases (MMPs) secreted by macrophages can digest collagen, thus weakening the protective fibrous cap covering the atheromatous core [5]. Targeting macrophages, there is a growing interest in identification and treatment of vulnerable plaques.

In recent years, several strategies have been proposed for detecting vulnerable plaques [6]. One promising method for the assessment of plaque composition and stability is laser induced fluorescence spectroscopy [7]. Some mechanical interventions, such as endarterectomy, balloon angioplasty and stenting, have significantly decreased the mortality of atherosclerotic diseases. However, the above techniques are correlated with restenosis resulting from intimal hyperplasia or constrictive remodeling [8–11]. Better methods to identify and pacify vulnerable plaques are still needed. Photodynamic therapy (PDT) is a promising treatment modality for tumor and some non-malignant diseases [12,13]. During PDT, inert photosensitizer is activated by low energy light to produce reactive oxygen species (ROS), resulting in cytotoxic effect [14]. PDT has been shown to prevent and inhibit restenosis after balloon injury or stent placement [15–19]. Protoporphyrin IX (PpIX), the metabolic prod-
uct of 5-aminolevulinic acid (ALA), can be activated by red light (635 nm) to produce cytotoxic effect. ALA has been proven to preferentially accumulate in rapidly proliferating tissue and plaque [20].

Thereby, we hypothesized that macrophages could phagocytize ALA, the fluorescence intensity of ALA-derived PpIX reflect macrophage content in plaque, and ALA mediated PDT reduce macrophage content and inhibit plaque progression. In the present study, we examined the accumulation of PpIX in atherosclerotic plaque by fluorescence spectroscopy, analyzed the correlation between the fluorescence intensity and the macrophage content in it, and evaluated the efficacy of ALA mediated PDT on atherosclerotic plaque stability.

2. Materials and methods

2.1. Photosensitizer prepared

The 99% pure ALA powder was obtained as a hydrochloride from Xi’an Natural Field Bio-technique Co., Ltd. It was dissolved in phosphate-buffered saline for intravascular administration. The concentration of ALA was 100 g/L.

2.2. Animal model produced

All animal procedures were approved by the Ethics Committee for Animal Experiments, Harbin Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

New Zealand white adult male rabbits, weighing 2.5–3.0 kg, were continuously housed at the hospital’s animal care facilities (The Second Affiliated Hospital of Harbin Medical University). Rabbits were anesthetized with ketamine (5 mg/kg), xylazine (5 mg/kg) and acepromazine (0.75 mg/kg) via intramuscular injection. Anesthesia was maintained with 1% isoflurane. A midline neck incision was made to expose the right common carotid artery (CCA) and its bifurcation. A 2F sheathing canal balloon catheter (CCA) was introduced via the lingual artery of the right CCA. Then, a balloon (2.5 mm × 10 mm balloon dilatation catheter (Medtronic, Inc., USA)) was guided into the right CCA with guidewire assistance. The balloon catheter was inflated to 4–6 atmospheres, and withdrawn to the carotid bifurcation with a rotating motion to produce endothelial denudation [21]. After three passages, the catheter was removed, and the lingual artery was ligated. The rabbits were fed an atherogenic diet (1.5% cholesterol, 7% lard and 15% yolk powder, Shanghai Solarbio Bioscience & Technology Co., Ltd.) for 8 weeks, producing atherosclerotic lesions in the right CCA.

2.3. Fluorescence detection of PpIX in plaque

ALA (60 mg/kg) was administered intravenously into 12 randomized atherosclerotic rabbits, which were euthanized at 1–6 h after injection (n = 2). Another rabbit was given phosphate-buffered saline as a blank control. Then, the carotid plaques and adjacent normal vessel segments were removed. The arteries were cut longitudinally to expose the intimal surface and washed thoroughly with normal saline. Fluorescence spectra of the intimal surface were excited by illumination of 405 nm violet light with a linewidth of 20 nm. A quartz lens was used to focus the light on target tissue, creating an irradiated light spot of about 2 mm in diameter. The fluorescence spectra were recorded by a spectrometer (USB2000, Ocean Optics Inc., USA). All spectral measurements were performed at room temperature with the same experimental configuration. Emission spectra from 350 to 900 nm were collected every 3 mm across the entire area of the exposed intimal surface. Data were analyzed by the OriginLab Origin-Pro 7.0 software.

2.4. Evaluation of the correlation between fluorescence intensity of PpIX and macrophage infiltration extent

Based on the optimized time post ALA injection, another 12 rabbits were euthanized and their atherosclerotic vessels were removed. Fluorescence measurement of the intimal surface was performed as described above. Each specimen was marked at three sites for detection and stored in 4% paraformaldehyde. At each marked site, three serial transverse sections (5 μm) were acquired for immunohistochemical analysis of macrophages. Rabbit macrophages were identified with a monoclonal antibody (anti-RAM-11, 1:1200, Dako North America, Inc., Carpinteria, California, USA). The presence of smooth muscle cells (SMCs) was assessed using monoclonal anti-a smooth muscle actin antibody (1:1200, Sigma–Aldrich, St. Louis, Missouri, USA).

2.5. PDT on balloon-injured rabbit arteries

In all rabbits (n = 48), vascular ultrasound was performed before PDT to confirm the formation of plaques. However, ultrasound (7.5–10 MHz) did not have enough resolution to distinguish the change of plaque on the same rabbit before and after PDT. Therefore, in the current work, the treatment effect was evaluated in comparison with the control group.

Forty-eight rabbits were randomly divided into four groups: PDT (n = 12), light only (n = 12), ALA only (n = 12) and control (n = 12). In PDT group, ALA (60 mg/kg) was administered to each rabbit via the marginal vein of its ear 2 h before light illumination. After the rabbit was anesthetized, a midline neck incision was made and the right common carotid artery and its bifurcation were exposed. The light diffuser connected to a diode laser (635 nm) was placed at the point determined by the vascular ultrasound and the light (50 J/cm², 210 mW/cm²) was delivered to activate PpIX within the plaque. The time for light delivery was from 8 to 15 min based on the area of the irradiated artery. The neck structures were shielded so that only the targeting artery section for PDT was exposed to the laser beam. Meanwhile, rabbits of other groups were treated correspondingly. The rabbits were fed on a diet containing 0.075% cholesterol until euthanasia at 1 and 4 week respectively after PDT.

2.6. Histopathology and immunohistochemistry after PDT

The rabbits were euthanized. Atherosclerotic segments were pressurized at 100 mmHg with 4% paraformaldehyde for 15 min, then harvested, fixed and stored for 24–48 h. Each atherosclerotic artery was cut into three parts (proximal, middle and distal), and embedded in paraffin. Four serial artery transverse sections (5 μm) were cut at each part for histopathology and immunohistochemistry analysis. Hematoxylin and eosin (H&E) and Masson’s stain were performed to determine the internal elastic membrane (IEL), external elastic membrane (EEL) and collagen. Collagen content was quantified by calculating the proportion of positive area within the plaque.

Immunohistochemical analysis of macrophages and SMCs was conducted. Images of stained sections were recorded by an Olympus IX70 microscope (Olympus, Tokyo, Japan). Histopathology was assessed by a pathologist blinded to the groups with computer-assisted color image analysis (Image-Pro Plus, version 6.0, Media Cybernetics Inc., Silver Spring, MD, USA). Immunohistochemistry staining was quantified by setting hue thresholds to include the maximum amount of staining in every slide [22]. The percent of positive staining area per region of interest was collected.
2.7. Statistical methods

Data are expressed as means ± SD. All statistical analyses were performed using SPSS v13.0 (SPSS Inc., Chicago, IL, USA). Values were normally distributed and thus one-way analysis of variance was employed for comparison. *P* < 0.05 was considered statistically significant in our analyses.

3. Results

3.1. Fluorescence intensity of PpIX in plaque

Histopathology showed that luminal plaques occurred in the right CCA of rabbit without evidence of IEL rupture.

Under 405 nm light excitation, an intense red fluorescence was observed in the CCA atheromatous plaque in rabbit after administration of ALA (Fig. 1A). Two fluorescence bands, one centered at 636 nm and another at 706 nm, were in accordance with the emission spectrum of PpIX under the excitation of 405 nm light. Typical fluorescence spectra were measured from segments containing atheromatous plaques at 1–6 h post ALA administration, which showed that the peak fluorescence intensity in the plaque occurred at 2 h. Quantification of the fluorescence at different times was accomplished by calculating the area under the curve (AUC). Fluorescence intensity of the plaque was about 12 times stronger than that of the adjacent normal vessel. After 6 h, the fluorescence of PpIX almost diminished from plaques (Fig. 1B).

3.2. The correlation between fluorescence intensity of PpIX and macrophage content

Fluorescence and immunohistochemical analysis showed that the fluorescence intensity of PpIX was positively correlated with plaque macrophage content (*r* = 0.794, *P* < 0.001, Fig. 2A) and negatively correlated with plaque SMC content (*r* = -0.531, *P* < 0.05, Fig. 2B).

3.3. The effect of PDT on inflamed plaque

There was no statistical significance in any of the parameters measured among control, light only and ALA only groups. In control group, the plaque area increased by 68% at 4 week compared with that at 1 week (0.69 ± 0.08 1 week vs. 0.41 ± 0.07 mm² 4 week, *P* < 0.001). Compared with control group, the plaque area of PDT group decreased by 39% at 1 week (0.25 ± 0.05 mm² PDT vs. 0.41 ± 0.07 mm² control, *P* < 0.001) and 59% at 4 week (0.28 ± 0.03 mm² PDT vs. 0.69 ± 0.08 mm² control, *P* < 0.001) respectively (Fig. 3A, B, and E), and there was no significant difference between 4 week and 1 week (*P* = 0.356). The lumen area in PDT group was found to be larger than that in control group at 1 week (0.81 ± 0.08 mm² PDT vs. 0.59 ± 0.10 mm² control, *P* < 0.001) and 4 week (0.73 ± 0.10 mm² PDT vs. 0.34 ± 0.03 mm² control, *P* = 0.001, Fig. 3A, B, and F). Collagen content was significantly higher in PDT group than in control at 4 week (23.48 ± 3.63% PDT vs. 13.23 ± 6.74% control, *P* < 0.001, Fig. 3C, D).
The EEL area was not significantly different between PDT group and control group at 1 week (1.80 ± 0.18 mm² PDT vs. 1.88 ± 0.15 mm² control, P = 0.523) and 4 week (1.85 ± 0.12 mm² PDT vs. 2.03 ± 0.20 mm² control, P = 0.196).

After PDT, plaque macrophage content decreased by 56% at 1 week (8.67 ± 2.51% PDT vs. 19.54 ± 4.25% control, P < 0.001) and 64% at 4 week (5.81 ± 1.34% PDT vs. 15.97 ± 3.32% control, P < 0.001), and there was no significant difference between 4 week and 1 week (P = 0.148, Fig. 4A–C, and G). In PDT group, the plaque SMC content was less than control group at 1 week (23.18 ± 4.1% PDT vs. 30.55 ± 3.92% control, P < 0.05), but not significantly different with control at 4 week (28.43 ± 4.38% PDT vs. 27.23 ± 5.07% control, P = 0.997). There was a moderate increase in PDT group at 4 week compared with that of 1 week (28.43 ± 4.38% 4 week PDT vs. 23.18 ± 4.1% 1 week PDT, P < 0.05, Fig. 4D–F, and H).

4. Discussion

In the study, the rabbit CCA was used to established the atheromatous plaque, and there was no evidence of thrombosis formation after PDT. Compared with the femoral artery, the wall characteristics and shear stress of carotid artery contribute more to the development of plaque formation [23]. And some current treatments, such as endarterectomy and stenting, are often correlated to thrombosis and rupture, especially in carotid artery. Thus PDT targeting macrophages et al. on carotid artery plaque implied more advantages. In addition, though the diameters of the carotid arteries might vary among individual rabbits, the normality test showed that the data of the plaque area and the lumen area was normally distributed respectively. In the current work, after PDT, histopathology and immunohistochemistry were used to evaluate the inflamed atherosclerotic plaque. Vascular ultrasound (7.5–10 MHz) was not used to distinguish the change of plaque on the same rabbit before and after PDT, because it had no enough resolution.

ALA is a natural porphyrin precursor of PpIX. When abundant exogenous ALA is administrated, it overloads the synthetic pathway and leads to the accumulation of PpIX [24]. It has been reported that PpIX accumulated preferentially in tumor tissue and atherosclerotic plaque [20,24,25]. The mechanism of PpIX uptake and retention in plaque is still not fully understood. As we know, oxidation of LDL and subsequent uptake into macrophages play a
crucial role in atherogenesis [4,26]. De Vries HE et al. demonstrated that oxidized LDL functioned as a delivery system for photosensitizers into macrophages through scavenger receptors [27]. And in our study, it was demonstrated that ALA-derived PpIX preferentially accumulated in plaque, and its fluorescence intensity was positively correlated with plaque macrophage content. As macrophage is responsible for the instability of atherosclerotic plaque, plaque rupture and subsequent life-threatening thrombosis [4,28–30], it provoked an interest in detecting the infiltration of macrophage in order to evaluate the stability of plaque. Although our method for detecting the fluorescence intensity in plaque was not optimized for clinical application, it demonstrated a potential way to identify and quantify the macrophage infiltration extent in inflamed plaque.

After activation of ALA-derived PpIX accumulated in plaque, the positive therapeutic response was showed in inhibiting plaque progression and stabilizing inflamed disease. The macrophage content decreased significantly compared with control at 1 week and 4 week, while the SMC content had a slight decrease at 1 week, and recovered at 4 week. This change suggested that PDT mainly focus on macrophage, which was corresponding to the phagocytosis of the macrophage to PpIX mentioned above. Besides the direct effect, PDT might also cross link collagen and thereby promote accelerated endothelialization [31,32], which could provide a barrier to invading inflamed cells [33]. The SMC content decreased slightly at 1 week suggested that PDT had less influence on SMCs than that on macrophage. This may be attributed to the different affinity of PpIX to macrophages and SMC.

In our study, the EEL area was not significantly different between PDT and control group, indicating that no arterial remodeling happened. Early clinical trials for restenosis in femoral arteries suggested that ALA mediated PDT had positive therapeutic responses, with better tolerance and no adverse events in the uncontrolled experiment [34]. However, Gabeler et al. revealed arterial wall weakening and aneurysmal dilatation in PDT-treated segments 1 year after high energy PDT [35]. In addition, Nitta et al. reported that PDT resulted in a progression of atherosclerosis in experimental rabbit models using fullerene–PEG [36]. It may be associated with the injury of intima by excessive reactive oxygen during therapy. Thus, more studies are needed to assess the safety and effect of this technique prior to the clinical application.

In conclusion, our study demonstrated that the ALA-derived PpIX can be detected to reflect the macrophage content in the plaque. ALA mediated PDT could reduce macrophage content and inhibit plaque progression, indicating a promising approach to treat inflamed atherosclerotic plaque.

**Conflict of interest**

There was no conflict of interest in this study.
Acknowledgments

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