Hematoporphyrin monomethyl ether-mediated photodynamic effects on THP-1 cell-derived macrophages

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**Abstract**

Photodynamic therapy (PDT) has been shown to attenuate atherosclerotic plaque progression and decrease macrophage-infiltration. The effectiveness of PDT depends strongly on the type of photosensitizers. Hematoporphyrin monomethyl ether (HMME) is a promising second-generation porphyrin-related photosensitizer for PDT. This study is designed to characterize effects of HMME-based PDT on THP-1 cell-derived macrophages and define the cell-death pathway. HMME was identified to accumulate in the macrophages by fluorescence microscopy and confocal scanning laser microscope. Our data demonstrated that the intensity of laser-induced HMME fluorescence in macrophages steadily increased with the increasing incubation concentration of HMME. The survival rate of macrophages determined by MTT assay decreased with the increasing HMME concentration and irradiation time. HMME-based PDT induced macrophage apoptosis via caspase-9 and caspase-3 activation pathway detected by caspase fluorometric assay decreased with the increasing HMME concentration and irradiation time. HMME-based PDT increased the number of apoptotic macrophages by 14-fold at 12 h post irradiation by 9 J/cm² 635 nm diode laser. These results imply that photodynamic therapy with HMME may therefore be a useful clinical treatment for unstable atherosclerotic plaques.

**Keywords:**
- Photodynamic therapy (PDT)
- Hematoporphyrin monomethyl ether (HMME)
- Macrophage
- Apoptosis

**1. Introduction**

Atherosclerosis is the leading cause of morbidity and mortality in developed and some developing countries, largely due to events caused by the sudden rupture of atherosclerotic plaques [1]. Such rupture-prone plaques are characterized by large necrotic lipid cores, thin fibrous caps, and dense macrophage-infiltration. The pathology of vulnerable plaques has been investigated extensively, and ample evidences suggest that macrophages play a crucial role in the instability of such plaques [2–5]. Therefore, the therapeutic targets for these plaques should be the local inflammatory cells, particularly activated macrophages [6,7].

Recently, photodynamic therapy (PDT) has evolved as a promising treatment for cardiovascular pathologies, including atherosclerosis and restenosis [8]. Photoangioplasty using a photosensitizer, such as ALA [9], motexafin lutetium [10] or MV0611 [6], has been used in a hyperlipidemic rabbit model of atherosclerosis. It was found that PDT attenuated the progression of plaque, and promoted the stabilization of plaque, vessel healing and repair [11]. The number of macrophages and foam cells also decreased after PDT. One possible reason for the dropout of vascular cells without the promotion of inflammatory responses during PDT is apoptosis triggered by the redox-sensitive pathway and the activation of caspases [12].

Hematoporphyrin monomethyl ether (HMME) is a second-generation, porphyrin-related photosensitizer that has recently been developed [13]. HMME consists of two monomeric porphyrins, i.e., 3-(1-methyloxyethyl)-8-(1-hydroxyethyl) deuteroporphyrin IX and 8-(1-methyloxyethyl)-3-(1-hydroxyethyl) deuteroporphyrin IX (Fig. 1). Experimental studies and clinical trials have demonstrated that HMME which can be selectively taken by tumor tissues has a stronger photodynamic effect, lower toxicity and shorter-term skin photosensitizations. Moreover, HMME is less costly compared with other drugs [14–18].

It is therefore of interest to know whether HMME is capable of inducing photodynamic cytotoxic effects on macrophages. In this work, THP-1 cell-derived macrophages were used to examine the possible effects and mechanisms of HMME-mediated PDT in vitro.
2. Materials and methods

2.1. Chemicals

HMME was provided by the Pharmacology Laboratory of the Second Military Medical University (Shanghai, China). A stock solution was made in ethanol at a concentration of 10 mg/ml and kept in the dark at −20 °C. Fetal bovine serum (FBS) and RPMI 1640 were bought from Hyclone Laboratories, Inc. (HyClone, Logan, UT, USA). Phorbol-12-myristate-13-acetate (PMA) was purchased from EMD Biosciences, Inc. (La Jolla, USA). The ApoAlert Annexin V-FITC kit and the ApoAlert fluorescent assay kits of caspase-9 and caspase-3 were purchased from BD Bioscience. All other drugs and chemicals used for this study were purchased from Sigma Chemical Co., Ltd.

2.2. Cell line and cell culture

Human THP-1 cells, a monocytic cell line (ATCC), were seeded at a density of 0.5 × 10⁶ cells per milliliter in RPMI 1640 medium containing 10% FBS, 200 mg/ml penicillin and 200 mg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ [19]. THP-1 cells were stimulated with PMA (100 ng/ml) for 72 h to induce a macrophage phenotype in 96-well plates or 35-mm Petri dishes.

2.3. Cell uptake of hematoporphyrin monomethyl ether

To investigate the intracellular kinetics of HMME, the cells were put into serum-free medium and incubated with HMME (0–40 µg/ml) in a dark, 5% CO₂ atmosphere at 37 °C for 3 h. Then the medium was removed. The incubated cells were rinsed three times with cold phosphate-buffered saline (PBS) and illuminated with a 405 nm violet light to trigger the fluorescence of HMME inside the cells. The 5 cm × 5 cm square light source was composed of one hundred violet light-emitting diodes (Sunlight Shenzhen Opto-Electronic Technology Co. Ltd.) with a nominal operating wavelength of 405 nm and a linewidth of 20 nm. A quartz lens with a focal length of 80 mm was used to focus the light onto the surface of the cells, creating an irradiated light spot with a diameter of 0.2 cm. The resulting fluorescence was detected by a multimode optical fiber (Ocean Optics Inc. OFLV-200-1100), which was coupled to an 8 nm-resolution spectrometer composed of a monochromator and a 2048-element CCD-array detector (Ocean Optics Inc. USB2000). All spectral measurements were performed at room temperature.

The intracellular localization of the fluorescence was determined by an OLYMPUS IX81 fluorescence microscope, and the images were captured by an UPLSAPO objective and a CCD camera, and subsequently processed using Image-Pro software (Media Cybernetics, USA). To determine the intracellular localization of HMME in the cells further, a sterile quartz coverslip (0.5 mm in diameter, 0.2 mm thick) was placed onto the bottom of a 35 mm Petri dish. During the treatment with HMME, the medium was aspirated and replaced with medium containing HMME (30 µg/ml). After 3 h incubation, the cells were rinsed three times with PBS. The cells were then incubated with Hoechst 33342 dye (10 µg/ml) for 5 min and examined immediately by OLYMPUS FLUOVIEW 500 confocal scanning laser microscope (OLYMPUS, Japan). Fluoview (version 4.3) was used to encode and process the fluorescence images.

2.4. Phototoxicity assay

The survival rate of the cells after PDT was measured by MTT assay. During the experiments, 1 × 10⁶/ml cells were incubated with different concentrations of HMME (0–40 µg/ml) in a 96-well culture plate for 3 h. Then the drug-containing medium was aspirated and the cells were rinsed with PBS. The medium was replaced with 200 µl RPMI 1640 before illumination. The laser source was a diode laser device (High Power Devices) with a maximal output of 500 mW. The irradiation was carried out for 0.5–3 min by 635 nm light with an output power of 100 mW/cm² (a diode laser device from High Power Devices, Inc., NJ, USA). Following PDT, the medium was replaced with 10% FCS RPMI 1640 and the cells were able to proliferate. At 12 h post irradiation, 20 ml of MTT (final concentration: 0.5 mg/ml) was added to each well and the cells were incubated for 4 h at 37 °C. Afterwards, the culture medium was replaced with 200 ml DMSO. The optical density (OD) of the 96-well culture plate was examined immediately at 490 nm with a micrometer reader (BioTek ELX800). The cell survival rate was calculated as the ratio of the absorbance of the treated cells over untreated cells.

2.5. Determination of cell death

After illumination at a fluence of 9 J/cm² (HMME = 30 µg/ml), the amount of cell apoptosis was assessed by the Annexin V-FITC apoptosis kit according to the manufacturer’s instructions. Approximately 0.5 × 10⁶ treated or untreated cells were gently scraped off and washed twice with cold PBS. The cells were resuspended in 480 µl of binding buffer and incubated with 5 µl of Annexin V and 10 µl of propidium iodide (PI) for 20 min at room temperature in the dark. Cells from each sample were then analyzed by FacsCalibur flow cytometer (Becton–Dickinson, USA). The data were analyzed using the CELLQuest software (Becton–Dickinson, USA). The results were interpreted in the following fashion: cells in the lower-left quadrant (Annexin-V⁻/PI⁻) represent living cells; those in the lower-right quadrant (Annexin-V⁻/PI⁺) represent early apoptotic cells; those in the upper-right quadrant (Annexin-V⁺/PI⁺) represent late apoptotic cells and those in the upper-left quadrant (Annexin-V⁻/PI⁺) represent necrotic cells. The total apoptotic rate is calculated as the ratio of the cells in the lower-right quadrant and in the upper-right quadrant to those the other two quadrants.

2.6. Measurement of caspase-9 and caspase-3 activities

The activities of caspase-9 and caspase-3 were measured using the fluorescent assay kit at various times after illumination at a fluence of 9 J/cm² (HMME = 30 µg/ml). The treated or untreated cells were collected by centrifugation at various times post-PDT and resuspended in 50 µl cell lysis buffer for 10 min at 0 °C. Then, the
cell lysates were centrifuged to precipitate cellular debris. After centrifugation, 50 μl of reaction buffer and 5 μl of caspase substrate were added to the supernatants. After incubating for 1 h at 37 °C, the fluorescence was determined by fluorescence spectrophotometer (Shimadzu UVmini-1240, Japan). The caspase-9 Fluorescence Assay was detected a shift in the fluorescence of 7-amino-4-methoxy coumarin (AMC). On cleavage, the fluorescence of AMC can be measured by a 380-nm excitation filter and a 460-nm emission filter. Meanwhile, the caspase-3 Fluorescence Assay was detected a shift in the fluorescence emission of 7-amino-4-trifluoromethyl coumarin (AFC). After the substrate is cleaved by the protease, AFC emits a yellow–green fluorescence at 505 nm when excited at 400 nm. The caspase activities were calculated as the ratio of the emitted fluorescence of treated cells over that of untreated cells.

2.7. Transmission microscopy examination

At predetermined times after 9 J-PDT, the treated and untreated cells were trypsinized, harvested by centrifugation, fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and post-fixed with 1% OsO4. The cells were dehydrated in a graded series of ethanol solutions, finishing with isoamyl acetate. The cells were microsectioned into ultra-thin sections, stained with uranyl acetate and lead citrate and then examined under a transmission electron microscope (JEM-1220, Japan).

2.8. Statistical analyses

The statistical evaluation was based on the Dunnet-t test using SPSS Software (version 13.0; SPSS, Chicago, IL, USA). The data are presented as the mean values ± SD. A value of \( p < 0.05 \) was regarded as statistically significant.

3. Results

3.1. Cellular accumulation

Fluorescence microscopy and confocal scanning laser microscope were used to examine the intracellular distribution of HMME in the cells. Untreated cells showed no fluorescence. After incubating with 30 μg/ml of HMME, \(^{1}\) red fluorescence was identified in the cells (Fig. 2A). The dye in the cells was distributed a punctate pattern throughout the cytoplasm. The nuclei were stained blue using Hoechst 33342, as shown in Fig. 2B. The fluorescence emission profiles of the cells incubated with different concentrations (10–40 μg/ml) of HMME for 3 h are shown in Fig. 2C. The fluorescence intensity of HMME within the macrophages is dose-dependent.

3.2. Cellular viability post-PDT

The survival rate of the macrophages after PDT was determined by MTT assay. If the cells were not exposed to light, lower concentrations (≤40 μg/ml) of HMME did not influence cell survival rate. The survival rate of the PDT group was significantly lower than that of the untreated group, although the use of low power light irradiation alone did not inhibit cell growth (data not shown). The survival rate of the macrophages decreased as the increase of HMME concentration and the prolongation of irradiation time. The survival rate of the cells incubated in medium containing 30 μg/ml HMME (irradiated for 1.5 min, fluence 9 J/cm²) was 50.73 ± 4.45% (p < 0.005) (Fig. 3). Our results indicated that HMME-mediated PDT was capable of inducing cell death significantly.

3.3. Studies of cell apoptosis

Apoptosis of the cells was assessed with Annexin V/PI staining by flow cytometer at 12 h post 9 J-PDT (HMME concentration was 30 μg/ml). The apoptotic rate was calculated as the percentage

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\(^{1}\) For interpretation of color in Fig. 2, the reader is referred to the web version of this article.
of cells in the lower-right (Annexin-V+/PI-) and upper-right (Annexin-V+/PI+) quadrants. Using 9 J-PDT increased the number of apoptotic macrophages 14.02 ± 1.43-fold at 12 h post irradiation (mean ± SD, n = 3, p < 0.0005; Fig. 4C), whereas the percentage of necrotic cells did not increase from the baseline after PDT. The apoptotic rate of the group administered HMME without light radiation was 2.79 ± 1.75% at 12 h (Fig. 4B), almost the same as the untreated group (1.99 ± 1.43%) (Fig. 4A) (p > 0.05, HMME only vs. untreated group). The apoptosis rate of each group was shown in the histogram graph (Fig. 4D).

3.4. Caspase-9 and caspase-3 activation after PDT

To understand the mechanism of cellular photoinactivation, the activity of caspase-9 and caspase-3 was determined after PDT with a fluence of 9 J/cm² (30 μg/ml HMME). The activations of caspase-9 and caspase-3 in the macrophages at 6 and 12 h after photosensitization were examined (Fig. 5A and B). The activities of caspase-9 and caspase-3 were clearly stimulated by HMME-based PDT, and the maximal activities were measured at 6 h (30 ± 4.5-fold and 36 ± 2.4-fold vs. the untreated group, respectively) after the phototreatment (n = 3).

3.5. Electron microscopy studies

The apoptosis of the cells was also studied by electron microscopy. Cells in the untreated group presented the integral cellular membrane with abundant microvillia, dense cytoplasm and complete nuclear structure (Fig. 6A). Cells incubated in the dark with HMME (Fig. 6B) displayed no significant morphological changes compared with untreated cells, indicating HMME was noncytotoxic. The cells following PDT showed typical apoptotic morphological changes, such as parts of the cell microvilli vanishing, a decrease in the volume of the cell nucleus and condensation of the chromatin on the nuclear envelope (Fig. 6C and D).

4. Discussion

Waksman et al. [6] successfully demonstrated that PDT simultaneously reduced the inflammation of plaque and attenuated the progression of the disease. Similarly, the use of intra-arterial, trans-catheter PDT has favorably influenced the response to balloon injury both in coronary and peripheral arteries [9,10,20,21]. The effectiveness of PDT is determined by the combination of photosensitizer and light illumination [22,23]. HMME is a second-generation photosensitizer and has already been used clinically for PDT of cancers. Our study has shown that HMME-mediated PDT is very effective in promoting the apoptosis of macrophages. So it might develop a promising treatment for atherosclerosis.

Here, we confirm that HMME can accumulate in THP-1-derived macrophages and distribute a punctate pattern throughout the cytoplasm. It has been proposed that the main mechanism for its selectivity is the covalent conjugation of a photosensitizer to a ligand that specifically recognizes and internalizes cell-surface receptors. The class A, Type-I scavenger receptor of macrophages is a good candidate for conjugation by the photosensitizers, as they recognize serum albumin and have a higher capacity than other ligands. Therefore, scavenger receptor-targeted PDT gives a high degree of specificity toward macrophages [24,25]. In addition, ox-LDL functions as a specific delivery system for photosensitizers to the scavenger receptors are expressed on the macrophages in atherosclerotic lesions, increasing the beneficial effects of PDT for cardiovascular diseases [26]. The present findings clearly indicate that the intensity of the fluorescence increases along with the HMME concentrating in cells.

Disease-associated cells expressing molecules, including proteases, receptors, and adhesion molecules, are different with their normal counterparts [27]. Therefore one intention in targeting therapy is to develop chemically derived drugs or drug vectors targeting defined cells via specific recognition mechanisms and overcoming biological barriers [28]. It is demonstrated that local
inflammatory cells particularly activated macrophages play a crucial role in plaque instability. Different charged colloidal particles, when mixed in an aqueous solvent with oppositely charged linear polyelectrolytes, have been recently shown to be able to self-assemble, forming long-lived, finite-size mesoscopic aggregates [29,30]. So there are many possible strategies to make HMME delivery efficiently. This aspect is particularly appealing in developing new strategies in PDT. Therefore, synthesizing specific HMME derived drugs or drug vectors to increase the concentration of HMME in macrophages need further studies.

Macrophages play a crucial role in the progression of atherosclerosis. Cytokines released by macrophages promote the progression of atherogenesis and the continuous infiltration of macrophages. These, over time, result in the accumulation of lipoproteins and the aggregation of free cholesterol that contribute to the formation of a necrotic lipid core, leading to plaque instability. Moreover, the release of macrophage metalloproteinases and other proteolytic enzymes can weaken the fibrous cap and promote plaque rupture [6]. Therefore, an increased resistance of macrophages to apoptosis in early atherosclerotic lesions has been associated with an increased plaque burden [31]. Apoptosis may be central to the proposed clinical use of PDT to prevent and treat restenosis [32], or as a primary atherosclerotic plaque ablating therapy, which would allow for cell dropout without promoting inflammatory responses. The sustained removal of macrophages is a pivotal component of PDT in promoting plaque stabilization. The results from our studies indicate that HMME-based PDT decreases the survival rate of macrophages and induces the apoptosis of macrophages via the caspase-9 and caspase-3 activation pathways.

Caspase-9 is activated very early in the apoptotic cascade by cytochrome c, which is released from the mitochondria in response to apoptotic stimuli [33]. Activated caspase-9 then initiates the proteolytic activity of other downstream caspases, including caspase-3 and caspase-6. Following these events, the apoptotic machinery leads to DNA fragmentation and cell death. Mitochondrial respiration generates a major physiological source of reactive oxygen species (ROS) and activators of apoptosis reside in the mitochondria [34]. Here, we have shown that HMME-based PDT results in the activation of caspase-9 and caspase-3, suggesting that mitochondria play a central role in this process. The precise mechanism of HMME-based PDT linked to these mitochondrial events remains to be determined.

The combination of photoangioplasty and photosensitizing agent which accumulates in the target tissue and endovascular illumination can produce cytotoxic singlet oxygen for the treatment of primary atherosclerosis as well as the prevention and treatment of restenosis [35]. The relative contributions of apoptosis and necrosis depend on the cell line, photosensitizing agent and/or experimental conditions. In the present study, we have used diode-based lasers, which are compact, portable and relatively inexpensive compared to pulse lasers and dye lasers. The 630 nm light penetrates adequately, allowing it to be delivered directly or via hollow waveguides or fibers terminating in optical configurations, without having to obstruct the blood stream. As is known, obstructing the blood flow may lead to many negative consequences, including myocardium ischemia and arrhythmia. Early clinical trials indicated that HMME has a stronger photodynamic effect, lower toxicity and shorter-term skin photosensitizations, which suggest that HMME-based PDT is well tolerated and could produce positive therapeutic responses in patients [16–18]. No adverse effects were noted in the uncontrolled experiment [36]. However, Gabeler et al. revealed that PDT could cause arterial wall weakening and aneurismal dilation in the treated segment of the common iliac artery [37]. Thus, high-energy PDT might be associated with adverse effects. More studies are needed to estimate the safety of this promising treatment for atherosclerotic plaques before any clinical application.

**Fig. 5.** The activation of caspase-9 and caspase-3 at 6 and 12 h after photosensitization. The cells were incubated for 3 h with 30 µg/ml HMME and irradiated for 1.5 min using 635 nm light at a fluence of 100 mW/cm² (the dose of light was 9 J/cm²). The data shown are the means ± SD (n = 3) and are representative of three independent experiments. *P* < 0.05; **P** < 0.005 vs. the untreated controls.

**Fig. 6.** Representative transmission microscope images. The morphological alterations induced by HMME-PDT of the macrophages. The untreated macrophages after trypsinization (A, 5000×). The macrophages incubated for 3 h with 30 µg/ml HMME and not irradiated (B, 5000×) and irradiated for 1.5 min (C and D, 5000×). After irradiating for 1.5 min, some of the cells showed typical apoptotic morphology. The microvilli can be found around the cells of the control and HMME only group (black arrows in A and B), but vanished after PDT (black arrowheads in C and D). Condensation of the chromatin can be observed on the nuclear envelope in the cells of PDT group (white arrowheads in C and D). Scale bar: 2 µm.
In the present study, apoptotic mechanisms appear to prevail after a short period of low-energy irradiation (9 J/cm²). The parameters used in the present study were chosen to induce the apoptosis, but not the necrosis of the cells, as apoptosis is effective in reducing the plaque burden whereas necrosis can lead to plaque instability. The use of 9 J-PDT increased the number of apoptotic macrophages by 14-fold, whereas the percentage of necrotic cells did not increase after PDT treatment. Therefore, an optimal condition of HMME-mediated PDT must be evaluated to treat atherosclerosis. It is important to note that the vascular effects of PDT are not limited to apoptosis, but also to other important biological processes, including the modification of extracellular matrix proteins, the reduction of vascular cell migration and the modulation of adventitial fibroblast function. PDT has also been shown to modulate the release or activation of cytokines which promote vascular growth. Experiments about these factors are currently underway.

In conclusion, our results show that HMME selectively accumulates in macrophages and HMME-based PDT induces the apoptosis of macrophages via the caspase-9 and caspase-3 activation pathways. This study is preliminary and the treatment effectiveness needs to be verified in vivo in the future, but it strongly suggests that HMME-mediated PDT might be a promising method for atherosclerosis and merits further evaluation in experimental studies and clinical practices.

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