mTHPC-photodynamic therapy induced apoptosis in nasopharyngeal carcinoma cells

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ABSTRACT

In this study, the early apoptotic events of mTHPC-medicated photodynamic therapy (PDT) was explored in two human nasopharyngeal carcinoma (NPC) cell lines - NPC/HK1 cells and NPC/CNE2 cells. Cells (5×10^3) were incubated with mTHPC (0.8 μg/ml) in chamber slides for 20 h and subjected to light irradiation at 2 J/cm^2 (LD_{80}). Morphologic changes of treated cells were examined at 0- 4 h after the light irradiation by a light microscopy. The early stage of apoptosis was detected by fluorescein-conjugated Annexin V (Annexin V-FITC) assay. Mitochondrial membrane damage and cytochrome c release were determined by flowcytometric analysis. The Bcl-2 expression was measured by Western blot analysis. One hour after mTHPC-mediated PDT, membrane blebbing and cell shrinkage appeared in both HK1 and CNE2 cells. Annexin V-FITC assay showed that a considerable number of HK1 and CNE2 cells became apoptotic at 1 h after PDT. Flowcytometric analysis showed that the cytochrome c was released at 1 h after PDT. The Bcl-2 expression also declined significantly in both cell lines compared to the control groups. mTHPC-mediated PDT can effectively induce apoptotic responses in NPC cells which might be modulated by mitochondrial damage and Bcl-2 inhibition.

Key words: mTHPC, photodynamic therapy PDT, apoptosis, nasopharyngeal carcinoma

1. Introduction

In some areas of China the morbidity and the mortality of nasopharyngeal carcinoma (NPC) are still high. There is a need to develop less invasive and more effective modalities for the treatment of NPC. Photodynamic therapy (PDT) is a relatively new treatment modality which involves the administration of a photosensitizer followed by local illumination with visible light of specific wavelength(s). In the presence of oxygen molecules, the light illumination of photosensitizer can lead to a series of photochemical reactions and consequently the generation of cytotoxic species, which can destroy cancer directly and/or indirectly. PDT is a promising modality for the treatment of NPC. Several Chinese groups started to explore the feasibility of PDT for the treatment of NPC since the early 1980's. Those NPC PDT procedures involve multiple sessions of treatment using domestic or imported photosensitizers and patient responses are encouraging.[1-4] The expression of pro-apoptotic genes have been detected in post-PDT examination in NPC patients.[5] Several *in vivo* studies suggest that PDT mediated by various photosensitizers can trigger apoptosis in human NPC cells.[6-10]

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Recently, the 2nd generation photosensitizer drug Foscan containing active ingredient temoporfin or mTHPC (mesotetra hydroxyphenyl chlorin) has been approved in Europe for patients with advanced head and neck cancer who have failed prior therapies and are unsuitable for radiotherapy or chemotherapy. The same protocol has also been investigated in USA and shown significant clinical benefits.[11] Our previous *in vitro* studies demonstrate that mTHPC-mediated PDT possesses a strong phototoxicity toward human NPC cells.[12-13] Other groups have demonstrated that mTHPC-medicated PDT can induce various degrees of apoptosis in other cancer cells.[14,15] In this study, the mechanism action of mTHPC-medicated PDT was explored in two different NPC cell lines generated from local NPC patients - NPC/HK1 cell line (a well-differentiated squamous cell carcinoma) and NPC/CNE2 cell line (a poorly differentiated squamous cell carcinoma).[16-17] Chronological cellular events from early apoptotic events to the downstream cascades were studied by microscopic and flowcytometric analyses.

2. Materials and Methods

2.1 Microscopic examination of morphologic features

In order to determine if apoptosis was involved in the mTHPC PDT-mediated photokilling of NPC cells, morphological changes of PDT-treated cells were examined. Cells (5×10^3) were incubated with mTHPC (0.8 μg/ml, Biolitec, Germany) in chamber slides for 20 h and subjected to light irradiation at 2 J/cm^2 using a 400 W quartz–halogen lamp with heat isolation filter and a 600 nm long-pass filter. The spectral intensity was basically flat from 600 to 800 nm and the total intensity was 14 mW/cm^2. MTT assay confirmed that this dose would induce 80% of cell killings (LD_{80}). Cells were examined under a phase-contrast microscope (CK-2; Nikon, Japan) for morphological changes of necrotic and apoptotic features at 0 - 4 h post mTHPC PDT. Microscopic images were acquired using a digital camera.

2.2 Measurement of phosphatidylserine by Annexin V assay

Phosphatidylserine redistribution in the early stages of apoptosis was detected by Fluorescein-conjugated Annexin V (Annexin V-FITC; Roche, Germany) to quantitatively determine the percentage of cells undergoing apoptosis. Propidium iodide (PI; Sigma, USA) was used to distinguish viable cells from nonviable cells. Cells were treated with mTHPC PDT as described before. At 1 and 2 h post PDT treatment, 5 μL of Annexin V-FITC and 2 μL of PI were co-incubated for 15 min at room temperature in the dark. After the addition of 400 μL binding buffer to each slide, cells were examined by a fluorescent microscope (Eclipse E600; Nikon, Japan) equipped with a digital camera for image capturing. The green channel with band-pass filter (505 – 550 nm) recorded Annexin V-FITC fluorescence signals (excitation 488 nm, emission 510 nm) and the red channel with 590 nm long-pass filter recorded PI red fluorescence.

2.3 Flowcytometry measurement of cytochrome c release

In most of the experimental models, PDT-induced mitochondria damage could cause the release of cytochrome c. In this study, cytochrome c release was detected with a FITC-conjugated monoclonal anti-cytochrome c antibody (BD Pharmingen, USA) and aflow cytometry assay. Cells were treated with mTHPC PDT as described before. At 1 and 2 h post treatment, 1×10^6 cells were incubated with 1 μg of anti-cytochrome c antibody for 2 h. The proportion of cells with positive cytochrome c staining after PDT treatment was quantified by a flow cytometry (Coulter Epics Elite ESP, Beckman Coulter, USA).

2.4 Detection of Bcl-2 expression

In order to assess the expression of Bcl-2, an apoptotic inhibitor, on cell susceptibility to mTHPC PDT, HK1 and CNE2 cells were treated at 2 and 4 J/cm^2 as described before. Treated cells were lysed at 1 h post PDT. Cell protein extracts were prepared by lysing 1×10^7 of control and treated cells in 200 μL of lysis buffer (1× phosphate buffered saline, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 μg/mL aprotinin, and 100 μg/mL phenyl-methy-sulphonyl-fluoride) at 4°C for 30 min. Lysed cells were further disrupted and homogenized by passing through 21 gauge needles. After removal of insoluble

material by centrifugation, the supernatants containing cell extracts were recovered. The concentration of protein was determined by the BioRad Protein reagent kit

(BioRad, USA). One hundred μg of protein from each sample was loaded on to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, and then transferred onto a nitrocelluloase membrane (BioRad). The blot was blocked by 5% nonfat milk and probed with anti-Bcl-2 antibody (1:100 dilution; Dako, Denmark). A secondary probe constituted with horseradish peroxidase-conjugated anti-mouse secondary antibody (Dako) at 1:1000 dilution was incubated for 1 h. Antigen-antibody protein complexes were detected using an ECL chemiluminescence reagent kit (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. Rainbow markers (Amersham, UK) served as standard molecular weights. Relative densitometry analysis of autoradiographs on control and treated samples bands with the Bcl-2 expression were qualitatively measured by the Lumi-Imager imaging system (Roche, Germany).

3. Results

3.1 Morphological changes of apoptotic features

NPC cells were examined under a phase-contrast microscope for morphological changes at 0 - 4 hours post mTHPC PDT. After mTHPC PDT at LD_{80} dose level, both HK1 and CNE2 cells revealed a sequence of apoptotic morphological alternations including cell shrinkage, membrane blebbing and DNA fragmentation as early as 1 h post PDT (Fig. 1&2).

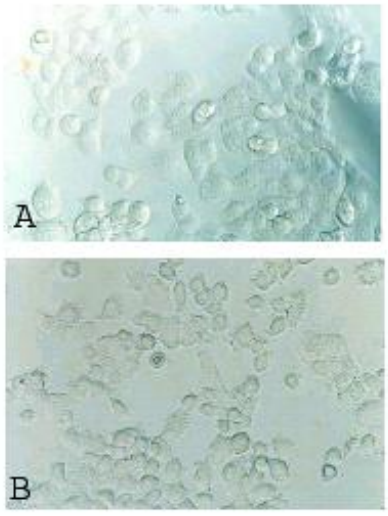

Fig. 1. HK1 cells. One hour post PDT.
A: Control. B: treated cells.

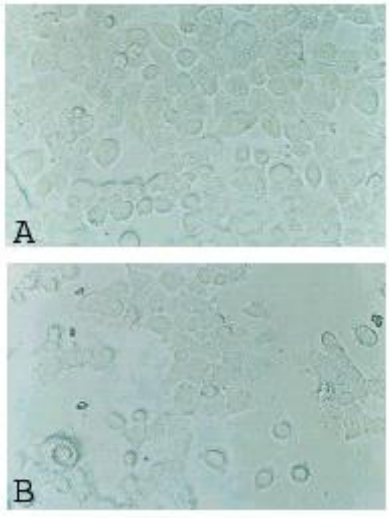

Fig. 2. CHE2 cells. One hour post PDT.
A: Control. B: treated cells.

3.2 Fluorescent staining of apoptotic cells

To confirm the early apoptosis, Annexin V-FITC was used to stain the phosphatidylserine, apoptotic cells in early phase then can be visualized by a confocal fluorescence microscopy. When co-incubated with PI, viable cells with intact membranes excluded PI and therefore were negative for both Annexin V-FITC and PI staining (Ann V-/PI-), whereas membranes of dead or damaged cells were permeable to PI (Ann V-/PI+). Cells that stained positive for Annexin V-FITC and negative for PI (Ann V+/PI-) were in the early stages of apoptosis. Cells that stained positive for both Annexin V-FITC and PI (Ann V+/PI+) were either in the later stages of apoptosis or were already dead.

Confocal microscopic image sets of dark control and treated HK1 and CNE2 cells showed that a considerable number of cells (both HK1 and CNE2) progressively showed up of green stain (i.e. Ann V+/PI-) 1 to 2 h after the onset of PDT treatment, confirming that apoptosis had incurred. Meanwhile, there were a few cells showing both green and red (i.e. Ann V+/PI +), reflecting necrosis might happen but only in a very minute fraction (< 5%) in both NPC cell lines.

3.3 Release of cytochrome c

The release of cytochrome c due to the mTHPC PDT-mediated mitichondria damage was examined by a flow cytometry. The proportion of cells with positive cytochrome c staining after PDT treatment was quantified. In the dark control groups, about 2% of HK1 and CNE2 cells were of cytochrome c positive. One hour after PDT treatment at LD_{80}, the percentage of cytochrome c positive HK1 and CNE2 cells were 42% and 26%, respectively. The additional release of cytochrome c was detected in both cell lines at 2 h post PDT at levels of 77% and 73%, respectively. This verified that mTHPC PDT indeed induced apoptosis in NPC cells through mitichondria pathways.

3.4 Attenuation of Bcl-2 expression

Figure 3 a & b are the results obtained from Western blot densitometry analysis by the Lumi-Imager imaging system. Results showed that there was no difference in Bcl-2 expression for the cell control and dark control (mTHPC only) in both HK1 and CNE2. However, 1 h after mTHPC PDT, the Bcl-2 expression was decreased in both cell lines at light dose levels of 2 and 4 J/cm^2. Approximately 15% attenuation in HK1 cells and 10% in CNE2 cells were observed at 2 J/cm^2 when compared to the control groups. Stronger inhibition was obtained at a higher dose, e.g. 52% in HK1 cells and 44% in CNE2 cells at 4 J/cm^2. A decrease in Bcl-2 expression would promote the initiation of apoptosis, thereby enhancing an apoptotic response in NPC cells.

Fig. 3. Western blot – mTHPC PDT effect on bcl-2 expression. a: HK1 cells, b: CNE2 cells. Lane 1 – cell only, Lane 2 – cell with mTHPC only, Lane 3 – 2 J/cm^2 and Lane 4 – 4 J/cm^2.

4. Discussion

Although limited clinical data indicate that PDT is a promising modality for the treatment of NPC, the mechanism action of mTHPC PDT on NPC has not been fully explored. Our previous studies demonstrate that mTHPC-mediated PDT possesses a strong phototoxicity toward human NPC cells.[12-13] This might be partially attributed to a high degree of cellular uptake of mTHPC by NPC cells and severe damage to the mitochondria during mTHPC photosensitization.

There are considerable studies strongly implicating the mitochondria as the critical mediators of apoptosis by rapid release of AIF (apoptotic inducing factor), cytochrome c, activation of caspase-9 and caspase-3 and possibly other protein factors, which promote caspase activation.[18] On the other hand, both pro-apoptotic (Bax, Bak, Bid) and anti-apoptotic members (Bcl-xL) of the Bcl-2 family are found on the mitochondrial membranes and can either promote or inhibit apoptosis.[19] Bcl-2 family proteins also regulate apoptosis by controlling mitochondrial permeability and the release of cytochrome c, thus preventing the activation of the caspase cascade and apoptosis triggered by PDT.[20]

PDT initiating apoptosis has gained a great attention since the last decade. PDT is a potent modulator of apoptosis in many cell types and apoptosis is more effective than necrosis for cell inactivation. Other

groups have demonstrated that mTHPC PDT can induce various degrees of apoptosis in other tumor cell lines.[14,15] In this study, the mechanism action of mTHPC-medicated PDT was explored in two different NPC cell lines generated from local NPC patients. Chronological cellular events from the early apoptotic event to the downstream cascades were studied.

Apoptosis, or programmed cell death, is a highly regulated cell death process, morphologically characterized by cell shrinkage, membrane blebbing and DNA fragmentation. All those typical apoptotic morphological changes were observed in both NPC cell lines at the dose level of LD_{80} at 1 h post PDT (see Fig. 1&2). Further analysis (i.e. Annexin V-FITC vs propidium iodide) confirmed that the majority of PDT-treated cells underwent apoptosis and only a small portion became necrosis.

Mitochondria is one of the initiating target concerned for apoptosis. It is generally recognized that mitochondria play a critical decision-making role in the apoptotic cascade by controlling the release of key factors involved in the apoptotic process.[21] The major apoptotic factor in the mitochondria-regulated pathway is cytochrome c. A disruption of mitochondria membrane function will cause a rapid loss of the mitochondrial inner transmembrane potential ($\Delta\psi_m$) and promote leakage of cytochrome c from the mitochondria. Cytochrome c in turn triggers caspase activation by binding to the caspase-activating protein, Apaf-1. In the presence of deoxyadenosine triphosphate (dATP), Apaf-1 subsequently activates procaspase-9 resulting in formation of functional apoptosome that will activate procaspase-3, one of the effector caspases. Downstream of these effector caspases (including 3, 6, 7 and 8) will provoke the degradation of genomic DNA into nucleosome-sized fragments.[22] Our data showed that at 2 h post mTHPC PDT over 70% of cells released cytochrome c which confirmed that mTHPC PDT could damage the mitochondria membrane and cause a quick release of cytochrome c.

In addition, the mitochondria photodamage catalyzed by mTHPC also resulted in significant decrease of Bcl-2 expression in both cell lines (see Fig. 3), which will favor the action of bax, an apoptotic gene product. So, the increased apoptotic response of mTHPC PDT in NPC cell lines might therefore be attributed to the elevated bax: bcl-2 ratio after PDT. Our data are consistent with the observations of different cells by other groups.[23,24]

In conclusion, mTHPC-mediated PDT can effectively induce apoptotic responses after mitochondrial photodamage in NPC cells and the efficiency of photo-killing in this cell model is high. mTHPC is a potent photosensitizer in the activation of NPC cells. Further *in vivo* studies are worth investigating.

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