Apoptosis Induced by Photodynamic Therapy with Benzoporphyrin Derivative Monoacid Ring A and Exploration of its Potential Mechanism in Bladder Cancer Cells

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OBJECTIVE To investigate apoptosis induced by photodynamic therapy with benzoporphyrin derivative monoacid ring A (BPD-MA) and explore its potential mechanism in human bladder cancer cells.
METHODS Photosensitization of BPD-MA was activated with a red light Laser (632.8nm) delivered at 10 mW/cm^2 to give a total dose of 2.4 J/cm^2. Cellular apoptosis was measured with flow cytometry analysis and an in-situ terminal deoxyuridine nick end-labeling (TUNEL) assay. Changes in mitochondrial membrane potential ($\Delta\psi m$) were monitored by a flow cytometric method with Rhodamine 123 staining and the expression of bcl-2 in BIU-87 cells was detected with immunocytochemical staining.
RESULTS At 8 h following photodynamic treatment, the degree of apoptosis was significantly increased when analyzed with flow cytometry and TUNEL assay. Treatment of the BIU-87 cells by PDT with BPD-MA resulted in the collapse of the $\Delta\psi m$ and a decrease of bcl-2 expression.
CONCLUSION BPD-MA-mediated PDT can effectively induce apoptosis in BIU-87 cells. The mechanism probably is through a mitochondrial-initiated pathway.

KEYWORDS: photodynamic therapy, BPD-MA, human bladder cancer cell, apoptosis, mitochondrial membrane potential, bcl-2.

Photodynamic therapy (PDT) shows considerable promise as a new treatment for malignant tumors.[1,2] PDT with use of a hematoporphyrin derivative (HpD) has been approved as an alternative method for bladder cancer.[3-6] However, some shortcomings of HpD have limited its clinical applications. Benzoporphyrin derivative monoacid ring A (BPD-MA), a new photosensitizer, has been put forward in view of its advantageous physicochemical properties. BPD-MA-based photodynamic therapy has shown important promising results in treating patients with many malignant tumors.[7,8] Our recent studies have shown that BPD-MA can effectively photokill the bladder cancer cells and induce apoptosis. The objective of this study was to further investigate apoptosis induced by BPD-MA-mediated photodynamic therapy in bladder cancer cells and to explore its potential mechanism.

MATERIALS AND METHODS

Photosensitizer

Liposomally formulated BPD-MA (QLT PhotoTherapeutics Co., Van-

couver. BC. Canada), provided by Dr. Aiqin Liu from the General Hospital of Beijing Military Region PLA, was kept in the dark at -20℃. BPD-MA was diluted in the culture medium without fetal calf serum just before use and added to exponentially growing cells.

Cell culture

The bladder cancer cell line BIU-87 used in this study was obtained from the Research Center of Urologic Surgery, Beijing University, China. Cells were grown in Roswell Park Memorials Institute RPMI-1640 medium supplemented with 10% fetal calf serum (FCS, Gibco-BRL, UK) and antibiotics (100 U/ml penicillin, 100 U/ml streptomycin). The cells were incubated at 37℃ in a humidified CO_2 (5%) incubator.

Photodynamic treatment

For photodynamic studies, the cells were incubated for a total of 90 min at 37℃ with BPD-MA (625 ng/ml) in RPMI 1640 medium without serum. After incubation with BPD-MA, the cells were exposed to red light (632.8 nm) delivered at 10 mW/cm^2 to give a total dose of 2.4 J/cm^2. After photodynamic treatment, the cells were washed twice with 2 ml of D-Hanks buffered saline without phenol red and cultured for 8 h in RPMI 1640 medium with 10% heat-inactivated fetal calf serum.

Analysis of DNA status

The propidium iodide fluorescence analysis procedure was used to detect changes in the status of cellular DNA. The cells (1×10^6) were scraped off the plates, washed twice with ice-cold PBS, permeabilized and fixed in 70% ethanol at 4 ℃ for 1 h. The cells were then washed twice in ice-cold PBS and treated with RNase A (5 μ/ml, DNase-free) and stained with propidium iodide (50 μg/ml) in PBS. Samples were analyzed by flow cytometry. The percentage of cells containing subdiploid levels of DNA (sub-G_1) (apoptotic index) was calculated from single-parameter flow cytometry for propidium iodide fluorescence using a flow cytometer (Coulter Electronics Enc, Hialeah, FL) with the fluorescence emission at 530 nm (FL1). At least 10,000 cells per sample were acquired in histograms and data analyzed by CellQuest software.

TUNEL assay

TUNEL assays were performed by using TUNEL label mix and tunnel enzyme according to the procedure described by the manufacturer (Wuhan Boster Biotechnology Co., Ltd. Wuhan, China). Briefly, after photodynamic treatment, the cells were cultured in RPMI-1640 medium supplemented with 10% FCS for 8 h prior to fixation. The cells were then washed twice in PBS, fixed in paraformaldehyde (4%) and permeabilized in 70% ethanol. The cells were digested with proteinase K (2 mg/L) for 3 min. After 2 washes in PBS, the cells were kept in labeling buffer at room temperature and then labeled with Dig-dUTP in the presence of terminal deoxynucleotidyl transferase and incubated for 2 h at 37 ℃ in a humidified atmosphere in the dark. After 3 washes in PBS, the cells were treated with Stop/Wash buffer for 30 min at 37℃, and then incubated for 30 min at 37 ℃ in anti-Dig-Biotin antibody and SABC. The cells were analyzed by the DAB displayed color reaction.

Mitochondrial membrane potential

Changes in mitochondrial membrane potential ($\triangle\psi m$) were monitored using the flow cytometric method. Briefly, BIU-87 cells were sensitized with BPD-MA for 90 min. Cells were then irradiated at a light dose of 2 J/cm^2, and incubated further for 8 h. Rhodamine 123 was supplied by Molecular Probes and dissolved in DMSO to produce a 1 mg/ml stock solution and 5 μg/ml was added 30 min before cell harvesting. Washed cells were resuspended in PBS and then were analyzed using a flow cytometry with the excitation setting at 488 nm, acquiring signals in the FL-2 channel. At least 10,000 cells per sample were collected in the histograms and data analyzed by CellQuest software.

Bcl-2 immunocytochemical staining

The BIU-87-treated cells were rinsed with PBS and fixed with acetone at 4℃ for 10 min. The positive percentage of bcl-2 expression was determined by using the SABC method. Non-specific binding sites were blocked with 10% normal goat serum for 30 min. When the serum was removed, the cells were incubated with monoclonal rabbit anti-rat bcl-2 antibody (4℃, 24 h) and goat antirabbit IgG (25℃, 30 min), and then the cells were treated with avidin DH-biotinylated horseradish peroxidase H complex (ABC) and counterstained with haematoxyin. The samples were analyzed by microscopy. At least 100 cells were counted and the percent of bcl-2-positive cells determined.

Statistical analysis

All data were expressed as the mean±standard deviation ($\bar{x}\pm SD$) and were analyzed by one-way analysis of variance. Groups with adducts were compared with the control. A *P*-value of less than 0.05 was considered

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significant.

RESULTS

Apoptosis

Fig.1 shows that BPD-MA-mediated photosensitization significantly increased the number of TUNEL-positive cells as compared to the controls. The results of flow cytometry showed that with BPD-MA the sub-G_1 peak and the apoptotic index were significantly increased up to 26.11±2.59%. Treatment with laser irradiation alone or BPD-MA alone or sham radiation did not significantly affect the apoptotic index of the BIU-87 cells (Data no shown).

Collapse of the Mitochondrial membrane potential (△ψm)

Experiments were performed to measure the change of mean fluorescent intensity in the △ψm. The BIU-87 cells were sensitized with BPD-MA and irradiated at a light dose of 2 J/cm². Table 1 and Fig.2 show significant changes of mean fluorescent intensity in the △ψm in the BIU-87 cells 8 h after PDT with BPD-MA. The collapse of the △ψm , as judged from a leftward shift of the fluorescence curve, was observed in the BIU-87 cells treated by PDT with BPD-MA.

Table 1. The Changes in the △ψm in BIU-87 cells after PDT with BPD-MA (%).

Groups	Mean fluorescent intensity
PDT with BPD-MA	78.47±15.66*
Laser irradiation control	140.96±25.94
BPD-MA alone control	149.08±28.38
Sham radiation control	155.82±22.87

*: P<0.05 *vs.* the controls. The controls including light radiation alone, BPD-MA treatment alone, sham radiation.

Translational expression of bcl-2

To analyze the translational expression of bcl-2 in BIU-87 cells, immunocytochemical staining was used in this study. The cells were positive for bcl-2 staining with the brown-yellow particle-substance distributing over the cytoplasm. The degree of bcl-2 expression in BIU-87 cells was as follows: laser irradiation alone, BPD-MA alone and sham radiation was 54.32±1.98%, 53.78±2.72% and 53.46±1.91% respectively; but the expression was significantly decreased down to 13.32±1.81% after BPD-MA-mediated photodynamic therapy. (Table 2, Fig.3).

Table 2. The degree of bcl-2 expression in BIU-87 cells after PDT with BPD-MA(%).

Groups	The degree of bcl-2 expression
PDT with BPD-MA	13.32 ± 1.81*
Laser irradiation control	54.32 ± 1.98
BPD-MA alone control	53.78 ± 2.72
Sham radiation control	53.46 ± 1.91

*: P<0.05 *vs.* the controls. The controls including light radiation alone, BPD-MA treatment alone, sham radiation.

DISCUSSION

Apoptosis is a carefully regulated process of cell death by characteristic morphological and biochemical changes, including compaction and fragmentation of chromatin, shrinkage of the cytoplasm and loss of membrane asymmetry.[9,10] Recent studies have reported that PDT induces apoptosis similar to other external stimuli that trigger oxidative stress.[11] To confirm that PDT with BPD-MA can induce BIU-87 cellular apoptosis, we performed flow cytometry with PI staining and TUNEL assays. The TUNEL assays showed that BPD-MA-mediated photosensitization significantly increased the number of TUNEL-positive cells. The results of flow cytometry showed that with BPD-MA the sub-G1 peak and the apoptotic index were significantly increased up to 26.11± 2.59% (Data no shown). These findings indicate that apoptosis might be an important mode of cell death induced by PDT with BPD-MA in the BIU-87 cells.

Recent studies have shown that mitochondria play a central role in apoptotic control.[11-13] Members of the bcl-2 family are important regulators of the mitochondrial apoptotic pathway. The bcl-2 protein mainly localizes on the cytoplasmic side of the mitochondrial outer membrane as an anti-apoptotic factor.[14-16] In the present study, the expression of bcl-2 in the BIU-87 cells treated by BPD-MA-mediated photodynamic therapy significantly decreased from 53.46±1.91% in the sham radiated control to 13.32 ±1.81% . The down-regulation of bcl-2 following photodynamic therapy with BPD-MA might enhance apoptosis of the BIU-87 cells induced by reactive oxygen species produced by photochemical reactions.

The △ψm is reduced very early in apoptosis. This decline of △ψm probably initiates the opening of permeability transition pores resulting in mitochondrial swelling and rupture of the outer membrane with cytochrome c released into the cytoplasm.[1] Therefore,

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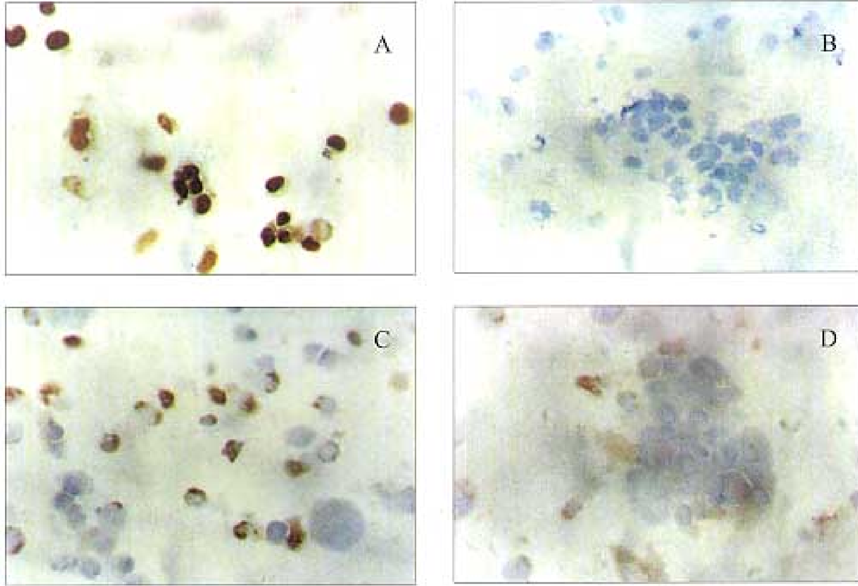

Fig.1. The determination of apoptosis in BIU-87 cells after photodynamic treatment was examined with the TUNEL assay. A: Positive control. B: Negative control. C: Photodynamic treatment with BPD-MA. D: The control.

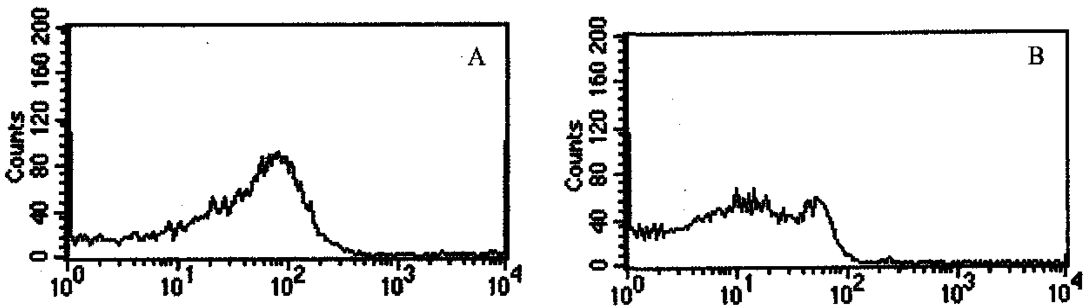

Fig.2. Collapse of the $\Delta\psi m$ in BIU-87 cells after BPD-MA-mediated photodynamic therapy shown by the flow cytometric method. The shift of the fluorescence curve spectrum to the left indicates mitochondrial membrane depolarization. A, the control. B, photodynamic therapy with BPD-MA.

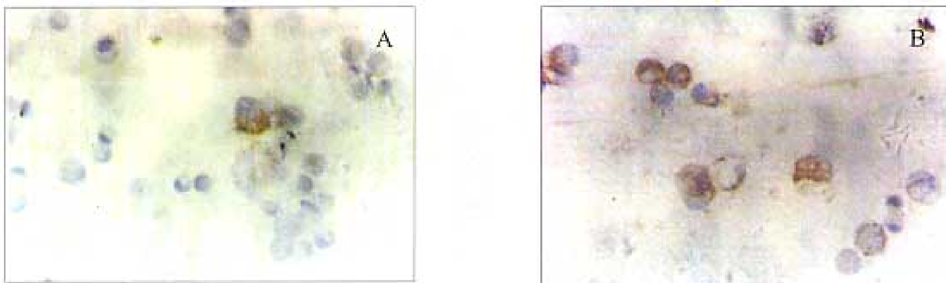

Fig.3. The expression of Bcl-2 protein assayed by immunocytochemical staining. A: sham radiation, B: BPD-MA-mediated photodynamic therapy.

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collapse of the $\Delta\psi m$ has recently been implicated as a key factor initiating a cascade of death-related events.[5] Flow cytometry with Rhodamine 123 (Rh123) staining is widely used as a measure of the $\Delta\psi m$.[17] Following exposure of the BIU-87 cells to PDT with 2 J/cm^2, the cells with low $\Delta\psi m$ (low Rh123 uptake) were observed at 8 h after PDT. The finding that more cells displayed reduced incorporation of $\Delta\psi m$ sensitive dyes (Rh123), indicates that there was a decline in $\Delta\psi m$ after PDT with BPD-MA. These studies have demonstrated that BPD-MA-mediated photodynamic therapy might effectively trigger BIU-87 cells into apoptosis through mitochondrial apoptotic cascades, inducing down-regulation of bcl-2 expression and loss of the $\Delta\psi m$. Whether the precise mechanism of apoptosis induced by BPD-MA-mediated PDT is linked to these mitochondrial events needs to be determined and is the focus of our ongoing studies.

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