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Abstract: Breast cancer is the common malignant tumor, the incidence increases with age. Photodynamic therapy (PDT) is a new technique applied in tumors, which involves the administration of a tumor localizing photosensitizer and it is followed by the activation of a specific wavelength. Pyropheophorbide-a methyl ester (MPPa), a derivative of chlorophyll, is a novel potent photosensitizer. We are exploring the photodynamic effect caused by MPPa-PDT on breast cancer. The in vitro and in vivo experiments indicate that MPPa is a comparatively ideal photosensitizer which can induce apoptosis in breast cancer.

Investigation of photodynamic effect caused by MPPa-PDT on breast cancer

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1. Introduction

Breast cancer is one of the most common malignancies that affect women. The pathogenesis of breast cancer remains poorly understood [1,2]. Photodynamic therapy (PDT) combines a drug which is called a photosensitizer or photosensitizing agent with a specific wavelength of light to kill cancer cells [3–5]. A photosensitizer is taken up by malignant or dysplastic tissues with some selectivity. The energy of the activated photosensitizer is subsequently transferred to molecular oxygen with a series of energy transfers, and the highly reactive oxygen species are produced, resulting in cell death [6–8]. Skin photosensitivity is the most common side effect of PDT and in clinical situations needs to be avoided.

The majority of the photosensitizers studied at present have a heterocyclic ring structure similar to that of chlorophyll or hemoglobin. Chlorophylls and their derivatives have shown themselves to be good potent photosensitizers. Pyropheophorbide-a methyl ester (MPPa) is a semisynthetic photosensitizer derived from chlorophyll a which was evidenced to be an efficient photosensitizer in our previous work in prostate carcinoma [9,10]. The study of effects of MPPa-PDT on breast cancer is performed to expand the research area.

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2. Materials and methods

2.1. Photosensitizer, chemicals, and cell line

MPPa (C_{34}H_{36}N_{4}O_{3}, MW 548.7, 95% of purity) purchased from Sigma-Aldrich was used without any further purification. A stock solution (2 mM) was made in dimethylformamide (DMF), filtered with 0.2 μm polytetrafluoroethylene syringe filter (Alltech Association, Inc., Deereld, IL), and then stored in dark at −20°C. Antibiotics penicillin-streptomycin, fetal calf serum (FCS), DMEM, and trypsin-EDTA were from Gibco-BRL. The human breast cancer cell lines MDA-MB-231 and MDA-MB-435 were obtained from Chinese Academy of Medical Science. They were cultured in Leibovitz L-15 medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 1.1 g/l NaHCO3, and 20000 kIE/l trasylol. MCF-7 cells were cultured in DMEM supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). Cells were incubated at 37 °C in a humidified 5% CO2 incubator.

2.2. Photodynamic therapy

1 × 10^6 cells were inoculated into cell culture plate. The plate was placed into an incubator and grown overnight. The steps below were conducted in dark condition. Drug solution was added and then mixed by gentle shaking. The plate was placed back to the incubator and the cells were incubated for 24 h. It was washed twice with phosphate buffer solution (PBS) and exposed to red light at 670±11 nm (with 4 mW/cm²) for different periods of time to get different light dosages. Cells without MPPa-PDT serve as uninduced controls.

2.3. MTT assay

After PDT, cell viability was determined by MTT reduction assay. Medium was removed and cells were washed twice with PBS. MTT dilution (stock solution diluted with culture medium) was added to each well and then the plate was gently mixed and placed back to the incubator for 4 h. To dissolve the insoluble purple formazan crystal formed, the solution was replaced by dimethyl sulfoxide and incubated for 10 min before reading the absorbance using an ELISA reader.

2.4. Apoptosis measured by flow cytometer

Cells after PDT were fixed in 70% ethanol for 30 min at 4°C. After washing twice with PBS the cells were rinsed with PBS containing 0.1% Triton X-100. The cells were stained with a solution containing 25 μg/ml propidium iodide (PI) and 10 μg/ml ribonuclease for 15 min at room temperature. Analysis was carried out at activating wavelength 488 nm and monitoring fluorescence emission at 623 nm by FACScan (Becton-Dickinson).

2.5. Animal model

Athymic nude mice were immunocompromised allowing the successful implantation of a variety of neoplasms. MDA-MB-231 cells suspended in 300 μl sterile physiological saline solution) were subcutaneously implanted into the right axillary region of BALB/c nude mice (male, 6–8 weeks, 16–20 g). The mice were housed in laminar-air-flow cabinet and allowed free access to food and water throughout the course of the experiment. Ten days after cell injection when the diameter of tumor was about 0.5 cm, nude mice were divided into experiment and control groups randomly. MPPa was injected into tumor topically prior to laser irradiation 120 J/cm² at 675 nm 3 h later in experiment group (2 sub-group, 10 mice in the first sub-group for tumor measurement, and 10 in the second sub-group for transmission electron microscope observation). Tumor ablation and tumor measurement were performed two weeks after PDT in the first sub-group and control groups. Specimens for observation under transmission electron microscope were fixed 4 and 12 h after PDT.

2.6. Transmission electron microscope observation

To be observed under electron microscope, the prostate cancer pieces were fixed with 3% glutaraldehyde and followed by 1% osmium tetroxide fixation, dehydrated in graded ethanol, and embedded in Epon 812. Ultrathin sections were cut with glass knives and mounted on copper grids, stained with uranium acetate and lead citrate, and finally examined with transmission electron microscope (Jeol Ltd., Japan).

2.7. Statistical analysis

All experiments were carried out in strict accordance and the values were the average of three experiments expressed as mean ±SD. Differences between data from two groups were assessed by t test and P < 0.05 was considered to be significant.

3. Results and discussion

3.1. Photo and dark cytotoxicity of MPPa in MDA-MB-231, MDA-MB-435, and MCF-7

The photocytotoxicity of MPPa in the three cell lines showed light- and drugdose dependent manners (Fig. 1).
The results in Fig. 1 show that cytotoxicity was induced in all the three cell lines. It was found that an especially low photodynamic dose (drug concentration $\times$ light dose) was required in MDA-MB-231, which may be useful in reducing skin photosensitivity. Dark cytotoxicity of cells with various concentrations of MPPa was evaluated by MTT reduction assay. No significant dark cytotoxicity was observed at the dose range of 0.25 – 8 $\mu$M (data not shown).

3.2. Apoptosis caused by MPPa-PDT on the three lines

Apoptosis is a Greek word; Apo meaning “from”, and -ptosis meaning “fall”. Apoptosis is recognized as an important cellular event during both normal development and specific disease progression. Apoptosis possesses unique morphologic and biochemical features which distinguish themselves from necrosis. Kerr first lodged this concept in 1972 to describe cell-programmed death during the normal developmental course. During apoptosis, the intracellular content is wrapped in a small bubble of the membrane and swallowed by phagocyte with no harm coming to the surrounding cells. The induction of apoptosis has been identified – intrinsic and extrinsic, caused by a caspase cascade. PDT can induce tumor cell apoptosis or necrosis [11–14].

Apoptosis caused by MPPa-PDT (2 $\mu$M + 50 kJ/m$^2$) was determined by the method of flow cytometry analysis of propidium iodide-labeled cells 24 h after PDT. Apoptosis peak was found in the three cell lines. The same PDT dose may lead about one third of $5 \times 10^5$ MCF-7, $1 \times 10^6$ MDA-MB-435, and $1.5 \times 10^6$ MDA-MB-231 to undergo apoptosis (Fig. 2). It showed that MDA-MB-231 is more...
3.3. Inhibition rate of tumor

Because MDA-MB-231 is proved to be more sensitive in vitro and so we set animal model to explore the in vivo effects. The effectiveness of MPPa-PDT was evaluated by measurement of tumor inhibition rate. Similar in vivo effect was seen, there was obvious difference between experiment and control groups in tumor size throughout the observation time. The growth of the implanted tumors was significantly inhibited and the inhibition rate of tumor weight was about 30% under the condition of 10 mg/kg and 120 J/cm², and 43% under the condition of 20 mg/kg and 120 J/cm², respectively (P < 0.05). These findings indicated that the cytotoxic effect of PDT observed in vitro was also present in vivo. Nude mice in treatment group...
were in better life conditions, more active than the control ones. Exudation and scab could be seen after PDT and finally the skin was still integrated and slick after scab disengaged. The definite therapeutic action suggested that MPPa-PDT could be used as an adjuvant treatment [15–17].

3.4. Observation under transmission electron microscope

Moreover, the fine structure of the cells was examined by electron microscope [18]. Apoptotic and necrotic cells can be studied and distinguished by electron microscope [19,20]. Tumor cells usually have one or more nucleoluses (Fig. 3a and Fig. 3c) and abundant endoplasmic reticulum (Fig. 3d). In the treatment group, apoptosis did not occur as early as that occurred in PC-3M, there is no obvious apoptosis when it is 4 h after PDT. 12 h after PDT, extensive apoptotic MDA-MB-231 cells with intact karyotheca and condensation of nuclear chromatin could be seen. The nuclear chromatin condensed to block and attached to the nuclear membrane (Fig. 3b, Fig. 3c, and Fig. 3d). Swollen mitochondria and their disappeared cristae were also features of apoptosis (Fig. 3d). There are still some apoptotic cell of late stage with wide perinuclear spaces, cellular shrinkage and apoptotic bodies could be seen (Fig. 3e). There are little necrotic cells and they could be seen somewhere.

4. Conclusion

All of the above indicates the obvious photodynamic effect of MPPa-PDT on MDA-MB-231 cells. The results in vitro and in vivo indicate the death way of cells induced by MPPa-PDT is mainly via mild apoptotic way and there is a good cure effect. All demonstrate it to be a good photosensitizer candidate.

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