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Photodynamic action of methylene blue in osteosarcoma cells *in vitro*

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Osteosarcoma cells;
Cell death;
Apoptosis;
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Summary
Background: Osteosarcoma is a common malignant bone tumor which threatens the life of young people worldwide. To explore alternative strategy for combating osteosarcoma, a light-emitting diode (LED) that activates methylene blue (MB) was used in the present study to investigate cell death of osteosarcoma-derived UMR106 cells.
Materials and methods: Photocytotoxicity in UMR106 cells was investigated 24 h after photodynamic activation of MB using sulforhodamine B (SRB) assay and light microscopy. Apoptosis induction was observed 24 h after photodynamic treatment using a confocal laser scanning microscopy (CLSM) with Hoechst 33342 staining. The change in mitochondrial membrane potential (MMP) was analyzed using a flow cytometry with rhodamine 123 staining.
Results: MB under red light irradiation caused a drug-concentration (0—100 μM) and light-dose (0—32 J/cm^2) dependent cytotoxicity in UMR106 cells. The SRB assay and light microscopy observed a significant decrease in the number of UMR106 cells attached to the bottom of culture well after LED light-activated MB (100 μM, 32 J/cm^2). Nuclear shrinkage, chromatin condensation and fragmentation were found in the treated cells by nuclear staining. In addition, flow cytometry showed that the MMP in UMR106 cells was rapidly reduced by photo-activated MB (100 μM, 32 J/cm^2).
Conclusion: Photodynamic action of MB under LED irradiation could remarkably kill osteosarcoma cells and induce cell apoptosis as well as MMP collapse.

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Introduction

Osteosarcoma is one of the commonest malignant bone tumors threatening the life of young people worldwide [1]. The survival of patients with osteosarcoma has been enhanced by advanced modern remedies [2,3], however, the long-term efficacy of the current therapeutic modalities is very limited due to drug-resistance and cancer recurrence [4,5]. Therefore, novel and more effective strategies are in urgent need for eradicating osteosarcoma cells.

Photodynamic therapy (PDT) is an alternative for combating malignant tumors [6—8] *via* photochemical action-induced cytotoxic reactive oxygen species (ROS) from photosensitizers retaining in over-growing tumor cells activated by appropriate light [9]. In PDT photosensitizer and light source are two key components. In order to explore photodynamic therapy as a new therapeutic technique, we have successfully set up new light sources using light-emitting diodes (LED). Our previous study showed that red light from this LED light source could effectively activate pyropheophorbide-α methyl ester (MPPa) and pheophorbide a (Pa) to kill various tumor cells *in vitro* [10,11]. Blue light from this LED light source could remarkably activate hypocrellin B to deactivate ovarian and breast cancer cells [12,13]. Methylene blue (MB) is a second-generation photosensitizer from phenothiazine dyes with remarkable photocytotoxicity of tumor cells. Emerging studies have shown that MB was retained in the malignant tissues [14]. Recently, Matsubara et al. reported that MB under the irradiation of light from a 500 W xenon lamp source had a strong photocytotoxic effect on mouse osteosarcoma (LM8) cells through affecting cytoplasmic ballooning, and inducing necrosis and apoptosis [15]. In this present study, we used red light from a new LED light source to activate MB for observing the killing efficacy of LED-activated MB on osteosarcoma-derived UMR106 cells *in vitro* and explore apoptotic cell death and mitochondrial membrane potential (MMP) of UMR106 cells induced by photodynamic action of MB.

Materials and methods

Photosensitizer and light source

Methylene blue (MB) ($C_{16}H_{18}ClN_3S$, M.W. 319.90, $\geq$82%) was purchased from Sigma—Aldrich Co. (St. Louis, MO, USA). A stock solution (3 M) was made in phosphate buffer saline (PBS) and stored in the dark at −20 °C until used.

The light source was from a red LED with the wavelength of 630 nm. Its highest power density is 106.5 mW/cm^2 with a uniform distribution over an area of 78.5 cm^2. The samples were exposed to the LED light at the energy density of 0, 2, 4, 8, 16 and 32 J/cm^2, respectively.

Cell culture

Rat osteosarcoma-derived UMR106 cells (ATCC No. CRL-1661), obtained from American Type Culture Collection (Manassas, VA, USA), was presented by Miss Winnie Lee and stored at School of Chinese Medicine, The Chinese University of Hong Kong. The cells were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% PSN (5 mg/ml penicillin, 5 mg/ml streptomycin and 10 mg/ml neomycin; Invitrogen), and cultured at 37 °C in a 5% (v/v) CO_2 humidified incubator.

Sulforhodamine B (SRB) assay

UMR106 cells (1×10^4 cells per well) were incubated with different concentrations of MB (0—100 μM) in 96-well plates. After incubation in the dark for 1 h, the cells were washed with fresh medium and then exposed (except for sham control and MB treatment alone) to red light from the LED light source with the power density of 106.5 mW/cm^2. The cells were then further incubated at 37 °C for 24 h in a humidified incubator. 50 μl of 50% trichloroacetic acid (TCA) was then added to the 200 μl medium already in each well. After incubation for 1 h at 4 °C, the cells were washed 5 times using tap water, and then dried in air. Subsequently, 50 μl of 0.4% SRB dissolved in 1% acetic acid was added to each well at room temperature. After fixing for 30 min, the plates were washed 4 times using 1% acetic acid to remove the unbound dye, and then dried in air again. After that, 150 μl of 10 mM Tris-base was added to each well and then shaken for 10 min on a gyro-rocker (Stuart, Dynalab Corp., USA). The optical density (OD) was measured using a microplate reader at the wavelength of 570 nm. The percentage of photocytotoxicity was calculated using the following equation:

$$\text{Photocytotoxicity}\,(\%) = 1 - \frac{(\text{OD}_{\text{treatment}} - \text{OD}_{\text{blank}})}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}} \times 100\%.$$

Light microscopy

UMR106 cells (1×10^5 cells per dish) were incubated using MB (100 μM) for 1 h in 35 mm culture dishes at 37 °C in the dark. Then unbound drugs were washed away and the cells were exposed to red light from the LED source at the energy density of 32 J/cm^2. The cells were then incubated at 37 °C for 24 h and the changes of cell morphological features were observed under a phase-contrast light microscopy.

Nuclear staining

UMR106 cells (1×10^5 cells/ml) were incubated with MB (100 μM) for 1 h in 35 mm culture dishes. After incubation, the cells were washed to remove unbound MB and medium was replaced with fresh culture medium for LED light irradiation. After incubation for 24 h after LED-activated MB, the cells were stained with Hoechst 33342 (10 μg/l) for 10 min at 37 °C in the dark. The stained cells were washed twice and then observed immediately under a confocal laser scanning microscopy (CLSM) (Eclipse C1, NIKON, Japan). The filter set of $\lambda_{ex}/\lambda_{em}$ (excitation wavelength/emission wavelength) of BP395-380/LP450 nm was used, and images were recorded by a colorful color charge-coupled device camera.