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Photosensitization of phycocyanin extracted from *Microcystis* in human hepatocellular carcinoma cells: Implication of mitochondria-dependent apoptosis ☆

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ABSTRACT

The aim of this study was to explore the possibility that *Microcystis* phycocyanin (MC-PC) functions as a photosensitizer and to investigate the mechanism for the apoptosis induced by *Microcystis* phycocyanin-mediated photodynamic therapy (MC-PC-PDT) in human hepatocellular carcinoma cells (HepG2). After incubation with MC-PC, HepG2 cells were exposed to a He–Ne Laser beam and the cell survival rate was detected by MTT and Colony forming assay. The mechanism of apoptosis was determined by ultrastructural observation, reactive oxygen species (ROS) and mitochondrial membrane potential ($\Delta\psi m$) assay, activity detection of caspase-3 and cytosol cytochrome c and flow cytometry (FCM) for cell cycle analysis. Our results demonstrated that MC-PC-PDT effectively inhibits HepG2 proliferation with a half-lethal dose of 100 μg/mL and induces apoptosis at 24 h with a dose of 200 μg/mL MC-PC. MC-PC was found to localized in mitochondria, it could induce a high level of ROS accumulation at 16 h after PDT treatment, cause mitochondrial damage and the release of mitochondrial cytochrome c into the cytosol. These cellular changes are accompanied by a reduction of the $\Delta\psi m$, activation of caspase-3 and G2/M phase arrest, finally leading to apoptosis through a mitochondria-dependent pathway after 24 h. Meanwhile, necrosis was also contributed to cell death in MC-PC PDT process. The present study also identified a new source of phycocyanin from *Microcystis* as a safe and effective photosensitizer.

1. Introduction

Hepatocellular carcinoma (HCC) is the leading cause of liver cancer. Although chemotherapeutic drugs can be quite effective in treating HCC, these agents do not differentiate normal, healthy cells from cancer cells. Hence, there is a need for the development of therapeutic agents against HCC that can overcome drug resistance and reduce the toxin.

Photodynamic therapy (PDT) is a useful and promising therapeutic method for the treatment of neoplastic and non-neoplastic diseases, which involves the activation of a photosensitive substance with preferential uptake by the targeted tissue. Its dominant mechanism of action is the local generation of cytotoxic singlet oxygen and other reactive oxygen species (ROS), which produce considerable cancer cell damage. Such damage can lead to cell death and destruction, which can be caused either by apoptosis or necrosis [1].

C-phycocyanin is a blue, light-harvesting pigment in cyanobacteria that is made up of α and β subunits with molecular weights of 16,500 (α) and 18,500 (β); it has an absorption peak located at 618 nm [2]. This water-soluble biliprotein has several biological activities, including antioxidant [3], anti-arthritic, anti-inflammatory and hepatoprotection properties [4,5]. Compared to Hematoporphyrin derivative (HPD), C-phycocyanin has been reported to exert a much stronger photodynamic action on tumor cells, and no side effects were observed; thus, it could potentially be used as a new type of photodynamic therapeutic agent [6,7].

Microcystis is the most abundant blue–green algae found in lake blooms, This large amount of cyanobacteria is a good source of phycocyanin. There have been approximately 60 different microcystins identified to date. Microcystins consist of a seven-membered peptide ring that is made up of five non-protein amino acids and two protein amino acids, the most common and toxic of which is microcystin-LR. These microcystins restrict the use of *Microcystis* phycocyanin (MC-PC). The toxin can be freed from high-molecular-weight impurities through dialysis [8,9]. *Microcystis* phycocyanin was not reported as a photosensitizer in an anticancer study.

Cell death following the application of PDT is dependent on the type and dose of photosensitizer and the energy and wavelength of the light [10,11]. Apoptosis is the dominant form of cell death in

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PDT because most of the photosensitizer accumulates in the mitochondrial membrane [12]. Apoptosis can be induced by mitochondrial-dependent mechanisms or by the plasma membrane receptors of intracellular signaling pathways involving calcium ions, ceramides, c-AMP, protein kinases, transcription factors, lipid peroxides, etc. [1].

Usually, the phycocyanin used in PDT is isolated from *Spirulina platensis*. Our goal was to study the possibility that *Microcystis* phycocyanin can function as a photosensitizer and to explore the mechanisms of the apoptosis induced by *Microcystis* phycocyanin-mediated PDT in HepG2 cells. We showed that ROS play an important role in the apoptosis induced by MC-PC-PDT through a mitochondria-dependent pathway.

2. Materials and methods

2.1. Materials

MC-PC was extracted from *Microcystis aeruginosa* in Chaohu Lake (Anhui Province, China) and was purified as previously reported [13]. Microcystin-LR was purchased from Alexis Biochemicals (San Diego, CA, USA). DMEM medium was purchased from Gibco (Invitrogen Corporation, USA). FBS (Fetal bovine serum), MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], NAC (N-Acetyl-L-Cysteine), Rh123 [2-(6-Amino-3-imino-3H-xanthen-9-yl) benzoic acid methyl ester] and PI (Propidium Iodide) were purchased from BoMei Biotechnology Co. Ltd. (Hefei, China). DCFH-DA (2′, 7′-Dichlorodihydrofluorescein diacetate) was obtained from Sigma (USA). The primary antibodies against cytochrome c, caspase-3 were purchased from Santa Cruz Biotechnology. Z-VAD-FMK (Santa Cruz biotechnology, Inc.).

The HPLC was from Agilent (Santa Clara, CA, USA). The chromatogram column was a Dikma Diamonsil C18 (Beijing, China). The He–Ne laser was purchased from Nanjing Latron Laser Company (China), the transmission electronic microscope was a Jem-100sx from Japan electron (Japan), and the fluorescence microscope was from Olympus (Tokyo, Japan). The flow cytometer was a FACSCalibur (Becton Dickinson, USA).

2.2. Methods

2.2.1. Separation of Microcystis phycocyanin

Fresh blue–green algae obtained from Chaohu Lake was washed with distilled water 2–3 times, picked to remove contaminating algae, non-plant material and filtered through cheesecloth. The extract was ultrasonicated and then centrifuged at 10,000*g* for 20 min; subsequently, the supernatant was brought to 15% saturation by adding solid $(NH_4)_2SO_4$ at 4 °C, which was performed to remove any contaminating protein. 55% saturated $(NH_4)_2SO_4$ was added to the last supernatant, and the precipitate was collected by centrifugation at 10,000*g* for 5 min and then dialyzed overnight at 4 °C against a 10 mM PBS buffer (pH 7.2). The protein suspension was subjected to filtration through a Sephedex G-200 chromatography column. The column was immediately developed with a linear NaCl concentration gradient. The purified phycocyanin was collected, desalted, and frozen as a dry powder for storage.

2.2.2. HPLC analysis of Microcystis phycocyanin and microcystin-LR

The purified fraction was characterized according to a HPLC characteristic spectrum compared with the commercial sample (C-PC, from Sigma), which was used as a control. At the same time, HPLC was used to analyze the level of microcystin-LR in the purified phycocyanin.

Sample preparation: The C-PC control and the purified sample were dissolved in PBS at concentrations of 4 mg/mL, respectively. The microcystin-LR standard was dissolved in methanol at a concentration of 0.5 mg/mL. All samples were filtered through a 0.45 μm filter membrane.

Chromatogram conditions for detecting MC-PC included a C18 chromatographic column (46 mm × 10 mm, 5 μm) and a C18 reversed phase chromatographic column (46 mm × 250 mm, 5 μm); the mobile phase, which was a mixture of deionized water and methanol (at a ratio of 40:60); a flow velocity of 1.0 mL/min; a column temperature of 30 °C; an injection volume of 20 μL; and a detection wavelength of 280 nm.

Conditions for detecting microcystin-LR included a C18 chromatogram column (46 mm × 10 mm, 5 μm) and a C18 reversed phase chromatogram column (46 mm × 250 mm, 5 μm); the mobile phase, which was a mixture of deionized water and methanol (at a ratio of 40:60); a flow velocity of 0.7 mL/min; a column temperature of 40 °C; an injection volume of 20 μL; and a detection wavelength of 238 nm.

2.2.3. Cell culture

HepG2 cells and HL7702 cells were cultured in DMEM culture medium supplemented with 10% FBS, 100 units/mL penicillin and 100 units/mL streptomycin, and incubated at 37 °C in the presence of 5% CO_2.

2.2.4. PDT

Cells were inoculated in serum-free DMEM and treated with different concentrations of MC-PC for 4 h before laser irradiation. The PDT irradiation dose by He–Ne laser (632.8 nm, 45 mW/cm^2) was 26 J/cm^2. After irradiation, the cells were cultured in DMEM containing 10% serum for 24 h.

2.2.5. Cytotoxicity assay

The MTT assay was used to evaluate cell viability as previously described [14]. Briefly, The cells were seeded at a concentration of 4×10^3 cells per well in 96-well flat-bottomed plates and incubated overnight at 37 °C. The cells were then treated with various doses of phycocyanin, irradiated by He–Ne laser and cultured for 24 h. To each well, 10 mL of 5 mg/mL MTT was added, and cells were incubated in the presence of MTT for 4 h in the dark at 37 °C. The formazan crystals were dissolved in DMSO. The absorbance was determined with a microplate reader at 490 nm. Absorbance values were normalized to the values obtained for the untreated cells to determine the cell viability.

2.2.6. Colony forming assay

To measure the cell colony formation ability, indicated different concentrations of PC were added to the medium. Briefly, about 50–150 cells per well were exposed to PC for 4 h before laser irradiation and then the cells were allowed to grow in fresh medium for the next 10–14 days. The cells were fixed in 70% ethanol and stained with 10% (v/v) Giemsa (Merck). Colonies that consisted of more than 50 cells were counted.

2.2.7. Analysis of apoptosis by electron microscopy

The treated HepG2 cells were digested with trypsin, harvested by centrifugation (200*g*, 5 min, 4 °C), re-suspended, and fixed in 2.5% glutaraldehyde for 2 h, and then post-fixed with osmium tetroxide. After being dehydrated, embedded and sectioned, the cells were observed under a transmission electron microscope.

2.2.8. Reactive oxygen species (ROS) detection

Intracellular ROS production was measured with DCFH-DA as a fluorescent probe. HepG2 Cells were cultured in 6-well plates at a concentration of 1×10^6 cells/well. Cells were treated with MC-PC-PDT at graded concentrations. After incubation, cells were exposed to DCFH-DA (20 μM) for 30 min at 37 °C away from light,