HYPOCRELLIN B ENHANCES ULTRASOUND-INDUCED CELL DEATH OF NASOPHARYNGEAL CARCINOMA CELLS

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Abstract—Hypocrellin B, a natural pigment from a traditional Chinese herb, has been attracting extensive attention. The present study aims to investigate whether hypocrellin B can enhance cell death induced by ultrasound sonification on nasopharyngeal carcinoma cells in vitro. The sonodynamic action of hypocrellin B was investigated on nasopharyngeal carcinoma cell line CNE2 cells as tumor model cells. In the experiments, the hypocrellin B concentration was kept constant at 2.5 μM and the cells were subject to ultrasound exposure for 15 s at an intensity of 0.65 W/cm². Cytotoxicity was investigated 24 h after ultrasound sonification. Apoptosis was evaluated using flow cytometry with annexin V-FITC and propidium iodine staining and nuclear staining with Hoechst 33258. Cell ultrastructure morphology was observed using transmission electron microscopy (TEM). No significant dark cytotoxicity of hypocrellin B in the CNE2 cells was observed at the concentration of 2.5 μM. The cell death rate induced by ultrasound sonification was significantly higher in the presence of hypocrellin B than in the absence of hypocrellin B. Flow cytometry showed that ultrasound exposure in the presence of hypocrellin B significantly increased the early and late apoptotic rate, 18.64% and 22.57%, respectively, compared with the controls. Nuclear condensation was observed in the nuclear staining and swollen mitochondria and more vacuolar and broken cell membrane were found in TEM after the treatment of hypocrellin B and ultrasound. Our findings demonstrated that the presence of hypocrellin B significantly enhanced the cytotoxicity of ultrasound radiation in CNE2 cells, suggesting that hypocrellin B is a novel sonosensitizer and hypocrellin B-mediated sonodynamic therapy is a potential therapeutic modality in the management of malignant tumors. (E-mail: xcshan@163.com or awnleung@cuhk.edu.hk) © 2010 World Federation for Ultrasound in Medicine & Biology.

Key Words: Hypocrellin, Sonodynamic therapy, Apoptosis, Necrosis, Nasopharyngeal carcinoma.

INTRODUCTION

Malignant tumor remains to be the main cause of death worldwide. The therapeutic modalities for malignancy, such as surgery, radiotherapy chemotherapy and biologic therapy, are initially successful in most of the cases. However, their long-term efficacy needs to be improved. Therefore, novel therapeutic strategies including photodynamic therapy (PDT) and sonodynamic therapy (SDT) have been spawned.

Photodynamic therapy is a relatively new therapeutic modality in the management of malignant tumors. PDT destroys tumor cells dependent on the photochemical reaction of photosensitizers induced by visible light of specific wavelength (Ali et al. 2001; Yow et al. 2007). However, the poor penetration of visible light in the biologic tissues is a main limitation to clinical application of PDT. Recent studies showed that ultrasound, as a mechanical wave, could efficiently activate some sensitizers to produce reactive oxygen species (ROS), which can deactivate cancer cells directly or indirectly (Hiraoka et al. 2006; Rosenthal et al. 2004; Kuroki et al. 2007). The new therapeutic treatment is named sonodynamic therapy (SDT). The great penetration of ultrasound in biologic tissues will overcome the limitation for the clinical application of light as an activating source (Hiraoka et al. 2006; Rosenthal et al. 2004; Kuroki et al. 2007). Therefore, SDT might be developed as a novel alternative modality for treating malignant tumors.

Many sensitizers used in current SDT research are primarily photosensitizers for example porphyrin and its
derivatives (Hiraoka et al. 2006; Rosenthal et al. 2004; Kuroki et al. 2007; Wang et al. 2008). Exploring new and efficient sonosensitizers is the subject of intensive investigation for our SDT research team. In our previous studies, we have investigated the combined effect of ultrasound and several sensitizers on tumor cells. Our findings showed that hematoporphyrin monomethyl ether (HMME) and 5-aminolaevulinic acid (5-ALA) could enhance the “sonokilling” action of ultrasound in tumor cells. Hypocrellin B, a natural pigment of perylquinone derivatives, was mainly isolated from the traditional Chinese herb *Hypocrella bambuase* (Ali et al. 2001; Yu et al. 2002; Ma et al. 2004; Xu et al. 2001, 2004). In China, hypocrellin B was extensively used to treat rheumatoid arthritis, gastric diseases and skin diseases (Ma et al. 2004). Recently, it has been confirmed that hypocrellin B is an efficient singlet oxygen generator during light-activation. In anti-tumor and antivirus research, hypocrellin B was found to have a strong photodynamic effect on malignant tumor, human immunodeficiency virus type I (HIV-I) and vesicular stomatitis (Ali et al. 2001; Yu et al. 2002; Ma et al. 2004; Xu et al. 2004). However, its absorption wavelength is located in the range of 450 nm to 550 nm. The low absorption in the photodynamic window (600~900 nm) limits the clinical application of hypocrellin B-induced PDT (Xu et al. 2001; Liu et al. 2009). In the present study, we have explored whether hypocrellin B (HB) (Fig. 1), the natural photosensitizer from the traditional Chinese herb, could enhance the ultrasound-induced cell death of nasopharyngeal carcinoma cells as tumor model cells in vitro.

**MATERIALS AND METHODS**

**Sensitizer**

Hypocrellin B was supplied by the Institute of Chemistry, Chinese Academy of Sciences. A stock solution was made in dimethyl sulfoxide (DMSO) at a concentration of 100 mM and kept in the dark at −20°C.

**Cell culture**

Nasopharyngeal carcinoma cell line CNE2 cells were provided by the Shanghai Biology Institute under the approval from the ethics committee of Chongqing Medical University. The cells were grown in Roswell Park Memorials Institute (RPMI)-1640 medium supplemented with 10% fetal calf serum (FCS, Gibco, New York, NY), 50 μg/mL penicillin, 50 μg/mL streptomycin and 10 μg/mL neomycin. The cells were incubated at 37°C in a humidified CO2 (5%) incubator.

**Ultrasound treatment**

The CNE2 cells were exposed to ultrasound 5 h after the incubation with hypocrellin B (2.5 μM). A 1 cm-diameter transducer with a central frequency of 1.7 MHz and intensity 0.65 W/cm² was employed to generate continuous ultrasound energy at various exposure times. The plane transducer was placed in a water tank filled with degassed water and the temperature was kept at 37°C during ultrasonic exposure (see Fig. 2). All experiments were randomly divided into four groups: hypocrellin B-ultrasound treatment, ultrasound sonification alone and the controls including hypocrellin B treatment alone and sham sonification. For the hypocrellin B-ultrasound treatment, the cells were pretreated by hypocrellin B incubation before the ultrasound exposure. The cells in the ultrasound sonification alone group were only exposed to ultrasound but not incubated by hypocrellin B. Those in the hypocrellin B treatment alone group were only pretreated by hypocrellin B incubation with no ultrasound sonification and the cells in the sham sonification group were not treated by both ultrasound and hypocrellin B.
Cytotoxicity

The cytotoxicity of ultrasound and hypocrellin B on the CNE2 cells was assessed using Cell Counting Kit-8 (Beyotime, Jiangshu, China). Briefly, the CNE2 cells (5 × 10³ cells/well) were incubated in a 96-well microplate at 37°C with hypocrellin B (2.5 μM) in the dark for 5 h. Then the unbound drugs were washed away and the cells were exposed (except the dark controls) to ultrasound. The cells were incubated at 37°C for 24 h. Cytotoxicity was then determined using a cell counting kit-8. The optical density (OD) was measured using an iEMS Analyzer (Type 1401; Labsystems, Finland) at the wavelength of 650 nm. The percentage of cytotoxicity was calculated using the following equation:

\[
\text{Cytotoxicity(\%) = \left(\frac{\text{OD control group} - \text{OD treatment group}}{\text{OD control group}}\right) \times 100%}
\]

Cell apoptosis detection

After ultrasound treatment, the CNE2 cells were incubated in culture flasks at 37°C for 18 h. Then, cell apoptosis was analyzed by flow cytometry with annexin V-FITC apoptosis detection kit (Beyotime, Jiangshu, China). Briefly, the cells from each sample were suspended in the 195 μL of 1× annexin V-FITC binding buffer and 5 μL annexin V-FITC. The cells were incubated at room temperature for 10 min. Then each sample was centrifuged at 1000 g for 5 min, suspended again in 190 μL of binding buffer and 10 μL of propidium iodide (PI) working solution was added. Then the samples were analyzed by flow cytometric method (FCM). The population was separated into three groups: live cells with a low level of fluorescence, apoptotic cells in the earlier period with green fluorescence and necrotic and advanced stage apoptotic cells with both red and green fluorescence.

Nuclear staining

The CNE2 cells (1 × 10⁵ cells/well) were incubated in a 24-well microplate at 37°C for 18 h after ultrasonically induced cell damage in the presence and absence of hypocrellin B. The cells were washed with PBS and nuclear staining was performed. Briefly, the treated and the control cells were stained with Hoechst 33258 (5 μg/mL) (Stock solution 1 mg/mL in sterile water) for 5 min at 37°C. Those stained cells were washed two times with PBS and then observed immediately under a fluorescence microscope. A filter set of Ex/Em of BP330–380/LP420 nm was used and the images were recorded by a colorful charge-coupled device camera.

Ultrastructural morphologic changes

Transmission electron microscopy (TEM) was performed to identify ultrastructural morphologic changes of the CNE2 cells 18 h after the ultrasound exposure. Fixed cells were postfixed in 2% OsO₄, dehydrated in graded alcohol and flat embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA, USA). Ultra-thin sections (100 nm) were prepared, stained with uranyl acetate and lead citrate and examined under an electron microscopy (H-600; Hitachi, Tokyo, Japan).

Results

Cytotoxicity of hypocrellin B and ultrasound on the CNE2 cells

To assess the cytotoxicity of hypocrellin B and ultrasound in nasopharyngeal carcinoma cell line CNE2 cells, the ultrasound-treated cells were incubated for 24 h in the presence and absence of 2.5 μM hypocrellin B. The cytotoxicity of hypocrellin B and ultrasound on the CNE2 cells was shown in Figure 3. No significant dark cytotoxicity of hypocrellin B in the CNE2 cells was observed at the concentration of 2.5 μM in this study. The death rate of CNE2 cells exposed to ultrasound (1.7 MHz) at 0.65 W/cm² was increased along with ultrasound exposure time. With the same ultrasound sonification time, the death rate of CNE2 cells induced by ultrasound was significant higher in the presence of hypocrellin B than in the absence of it \((p \leq 0.05)\). These showed that the presence of hypocrellin B obviously enhanced the sonokilling action.

Apoptosis induction

Flow cytometer with annexin V (annexin V-FITC) and PI staining can identify early and late apoptotic cells by annexin V-FITC binding to PS exposed on the outer membrane of the cell.

Statistical analysis

All data were processed using a one-way analysis of variance (ANOVA). A p value of less than 0.05 was considered a significant difference.
leaflet in early apoptotic cells. They can also distinguish early apoptosis from late apoptosis or necrosis because PI is impermeant to live cells and early apoptotic cells (Jaruga et al. 1998). The results from our studies showed that ultrasound exposure in the presence of hypocrellin B increased the early and late apoptotic rates of CEN2 cells. Figure 4a showed that the early and late apoptotic rates of the CNE2 cells 18 h after the treatment of sham sonification were 0.79% and 0.75%, respectively. Figure 4b showed that the early and late apoptotic rates of the CNE2 cells after the treatment of hypocrellin B alone were 0.65% and 0.48%, respectively. Figure 4c showed that the early and late apoptotic rates of the CNE2 cells after the treatment of ultrasound sonification in the absence of hypocrellin B were 2.83% and 2.37%, respectively.

Figure 4d showed the early and late apoptotic rates after ultrasound sonification in the presence of hypocrellin B (2.5 µM) significantly increased to 18.64% and 22.57% respectively, under the intensity of 0.65 W/cm² for 15 s. These findings suggested that ultrasound sonification in the presence of hypocrellin B could obviously enhance the early and late apoptosis in the CNE2 cells. To further verify hypocrellin B-SDT induced apoptosis of the CNE2 cells, the cells were stained using Hoechst 33258 after SDT with hypocrellin B (2.5 µM). Normal cells displayed a weak fluorescence and apoptotic cells showed an increased bright fluorescence and typical apoptotic body. As shown in Figure 5, typical apoptotic characteristics such as nuclear condensation were observed 18 h after ultrasound sonification in the presence of hypocrellin B.

Fig. 4. Apoptosis of CNE2 cells was analyzed 18 h after ultrasound radiation in the presence of hypocrellin B (2.5 µM) under ultrasound irradiation (1.7 MHz) with the intensity of 0.65 W/cm² for 15 s using flow cytometry with annexin V-FITC and PI staining. Annexin V-FITC in conjunction with PI staining could distinguish early apoptotic (annexin V-FITC positive, PI negative; bottom right quadrant of each panel) from late apoptotic or necrotic (annexin V positive, PI positive; top right quadrant of each panel) cells. Fluorescence intensity for annexin V-FITC is plotted on the x-axis, and PI is plotted on the y-axis. (a) Sham radiation. (b) Hypocrellin B alone. (c) Ultrasound radiation alone. (d) Ultrasound radiation with hypocrellin B.
Ultrastructural morphologic changes

Transmission electron microscopy (TEM) showed integrity of cell membrane, many normal mitochondria and a variety of cell nuclei of the CNE2 cells 18 h after the treatment of sham sonification, hypocrellin B alone and ultrasound sonification in the absence of hypocrellin B. (Fig. 6A, B, C and E). After ultrasound sonification in the presence of hypocrellin B, swollen mitochondria and some vacuoles were found in the treated cells. Even then some cells showed typical characteristics of apoptotic and necrotic cells such as nuclear condensation and broken cell membranes with nuclear lysis (Fig. 6D and F). These demonstrated ultrasound sonification in the presence of hypocrellin B significantly resulted in the damage of the ultrastructure of CNE2 cells.

DISCUSSION

The limitation to develop hypocrellin B to become an ideal photosensitizer was driving us to explore novel activation strategies. Recent experimental studies have revealed that ultrasound could activate some photosensitizers to produce ROS which eventually kill the neighboring cells (Hiraoka et al. 2006; Rosenthal et al. 2004; Kuroki et al. 2007; Wang et al. 2008). To investigate the effect of ultrasound in the presence of hypocrellin B on tumor cells, the poorly differentiated CNE2 cell line was chosen as tumor model cells for cytotoxic assay 24 h after ultrasound exposure in the present study. The results showed that sonification time-dependent cytotoxicity in the CNE2 cells was present after ultrasound sonification in the presence and absence of hypocrellin B. With the same ultrasound sonification time, the cell death rate of the CNE2 cells was significantly higher in the presence of hypocrellin B than in the absence of it. These findings above demonstrated that the presence of hypocrellin B could obviously enhance the killing efficacy of ultrasound on tumor cells. And TEM showed the integrity of cell membrane, many normal mitochondria and a variety of cell nuclei in the cells after sham sonification, hypocrellin B treatment alone and ultrasound sonification alone. Swollen mitochondria, more vacuolar and typical characteristics of apoptotic and necrotic cells such as nuclear condensation and broken cell membrane with nuclear lysis were found in the CNE2 cells treated by ultrasound sonification in the presence of hypocrellin B. These indicate that hypocrellin B might be a potential sonosensitizer.

In the sonodynamic therapy, the interaction between the excited sensitizer and molecular oxygen produces ROS to induce cell death (Rosenthal et al. 2004; Kuroki et al. 2007; Wang et al. 2008). To investigate the effect of ultrasound in the presence of hypocrellin B on tumor cells, the poorly differentiated CNE2 cell line was chosen as tumor model cells for cytotoxic assay 24 h after ultrasound exposure in the present study. The results showed that sonification time-dependent cytotoxicity in the CNE2 cells was present after ultrasound sonification in the presence and absence of hypocrellin B. With the same ultrasound sonification time, the cell death rate of the CNE2 cells was significantly higher in the presence of hypocrellin B than in the absence of it. These findings above demonstrated that the presence of hypocrellin B could obviously enhance the killing efficacy of ultrasound on tumor cells. And TEM showed the integrity of cell membrane, many normal mitochondria and a variety of cell nuclei in the cells after sham sonification, hypocrellin B treatment alone and ultrasound sonification alone. Swollen mitochondria, more vacuolar and typical characteristics of apoptotic and necrotic cells such as nuclear condensation and broken cell membrane with nuclear lysis were found in the CNE2 cells treated by ultrasound sonification in the presence of hypocrellin B. These indicate that hypocrellin B might be a potential sonosensitizer.

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et al. 2007). Cell death occurs mainly through two major modes including necrosis and apoptosis. Necrosis is a passive route usually ensuing rigorous inflammation, directly resulting in cell death. Apoptosis, a carefully regulated mode of cell death, is distinguished from necrosis in the changes of morphological and biochemical characteristics (Jackel et al. 1999; LaMuraglia et al. 2000). In this study, the apoptosis of the CNE2 cells induced by ultrasound in the presence and absence of hypocrellin B (2.5 μM) was analyzed 18 h after ultrasound exposure using flow cytometer with annexin V and PI staining. The results showed that the early apoptotic rate of the CNE2 cells after ultrasound exposure in the presence of hypocrellin B significantly increased to 18.64% and the late apoptotic rate (necrosis) increased up to 22.57% after ultrasound sonification in the presence of hypocrellin B. Nuclear staining and TEM reinforced these findings above. Our findings indicate that ultrasound sonification in the presence of hypocrellin B could significantly enhance the early and late apoptosis of CNE2 cells.

The cytotoxicity of sonodynamic therapy is dependent on the production of ROS involving free radicals and singlet oxygen (1O2) from the sonosensitization of sensitizers. The free radicals can react rapidly with molecular oxygen in the tissues, resulting in the production of highly reactive oxygen species (e.g. the superoxide and the peroxide anions). Singlet oxygen (1O2) and highly reactive oxygen from free radicals initiate further oxidative reactions in the proximate environment, such as the lipid membranes, enzymes, or nucleic acids, which destroy the tumor cells (Komori et al. 2009a, 2009b; Wang et al. 2008; Maisch et al. 2007). However, it was unclear for hypocrellin B to increase the biological effects of ultrasound on the CNE2 cells mainly through producing free radicals or singlet oxygen. The exact mechanism should be determined by further investigations.
In summary, the present study manifested that the presence of hypocrellin B could effectively enhance ultrasound-induced CNE2 cell death. Our results indicate that hypocrellin B is a novel sonosensitizer and hypocrellin B-mediated sonodynamic therapy is a potential therapeutic modality in the management of malignant tumors.

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REFERENCES


