Bio-Medical Materials and Engineering 26 (2015) S2083–S2089
DOI 10.3233/BME-151514
IOS Press

Potential mechanism of apoptosis induced by ultrasound in human hepatocarcinoma cells via comparative proteomic analysis

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Abstract. To analyze the potential molecular mechanism of ultrasound induced apoptosis in cancer cells, comparative proteomic methods were introduced in the study. After ultrasound exposure at the intensity of 1.2 W/cm^2, the human SMMC-7721 hepatocarcinoma cells were stained by trypan blue to detect the morphologic changes, and then the flow cytometry was used to examine the percentage of early apoptosis via double staining of FITC-labelled Annexin V and Propidium iodide. The proteins were separated by two-dimensional (2D) SDS polyacrylamide gel electrophoresis (PAGE). Among them, the differently expressed proteins were identified by MALDI-TOF mass spectrometry to reveal the key proteins response to ultrasound exposure. It's proved early apoptosis of cells were induced by focused ultrasound. After ultrasound exposure, the expressing characteristics of several proteins changed, in which some proteins in HSP family are associated with apoptosis initiation. It is suggested that the focused ultrasound could be applied in the assistant cancer therapy. Moreover, it is proved the comparative proteomic methods could supply information about the protein expression to analyze the metabolic processes related to bio-effects of biomedical ultrasound.

Keywords: Focused ultrasound, apoptosis, comparative proteomic methods, signaling pathway, assistant cancer therapy

1. Introduction

The bio-effects of ultrasound (US) to biological tissues or cells could not be ignored when it is used in the therapy of diseases, as well as the diagnostic imaging introduced with ultrasound contrast agents. Sometimes, it is the bio-effects that are used to get the therapeutic purpose. Plenty of bio-effects induced by biomedical ultrasound have been studied. However, to our knowledge, most of them are used to detect the related phenomena. The biomedical ultrasound can damage the genes [1]; it also can improve the gene transfection and curative effects of gene therapy via enhancing the permeability of cells [2]. As for the proteins macromolecules, biomedical ultrasound can reduce its activity and lead to its denaturalization. It also showed biomedical ultrasound can induce the apoptosis of cells [3].

US induced apoptosis of tumor cells have been reported and suggested to be significant to assistant

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cancer therapy. The first report revealed high intensity pulsed ultrasound could cause cell membrane pore, DNA damage and then induce apoptosis in human leukemia cell lines [4]. Then Lagneaux and Meulenaer [5] further indicated that the low intensity ultrasound has more potential in the cancer therapy by triggering apoptosis. Among the studies on its mechanism, it is revealed that inertial cavitation plays major role in the ultrasound induced early apoptosis and necrosis [6], which could also be proved by the enhanced cell killing effects by the introduction of ultrasound contrast agent [7]. Honda et al. also suggested that cavitation can induces the DNA and cell membrane damage by the action of residual hydrogen peroxide [8, 9].

The mechanisms related to US induced apoptosis have been suggested, including three apoptotic signal processes. However, to make sure what happened exactly after the ultrasound exposure in tumor cells or tissues, further experimental system need to be introduced to give us more information about how the metabolic process was activated and developed.

Genes firstly respond to ultrasound exposure via regulating its expressing characteristics. Tabuchi, et al [10, 11] reported two gene networks response to ultrasound exposure and indicated the potential signaling pathway. However, compared to the genes, proteins are the real main participators involved in the different metabolic processes, both in the tumor pathogenesis, growth, metastasis, and after stimulation by physical and chemical factors [12]. So the changes of proteins expression are always related to how the simulated metabolic process happen and develop. To detect the interesting proteins and analyze their functions can also give more information about how the tumor cells or tissues respond to the outer physical and chemical stimulation. In this paper, proteome analysis techniques are useful to filtrate the changed proteins and identify them. With the following functional analysis from protein database, this experimental system is very useful to analyze the metabolic process happened in the tumor cells or tissues simulated by the biomedical ultrasound. And then the results could supply the information to develop new approaches used in the cancer therapy.

In this paper, combined with double staining flow cytometry and morphologic observation, the comparative proteomic analysis techniques were used in the study on the apoptosis induced by focused ultrasound. The results show us that ultrasound indeed induces the cell apoptosis. Moreover, among the proteins which change their expressing characteristics, there are someone involved in the metabolic process and signaling pathway of apoptosis. The comparative proteomic analysis techniques, introduced in the study on ultrasound induced apoptosis by my lab firstly, are proven to be feasible. In the meantime, it also could be extended to the researches of effects from other physical and chemical factors on biological cells or tissues.

2. Material and method

2.1. Cell culture and ultrasound exposure

The SMMC-7721 human hepatocarcinoma cell line was obtained from the Center of Laboratory Animals of the Forth Military Medical University. Cells were grown in RPMI 1640 culture medium supplemented with 10% fetal serum. After 5 generations, the cultured cells were divided into two groups, one is control and another is irradiated by the ultrasound at the intensity of 1.2 W/cm^2. The ultrasound exposure set is shown in Figure 1.

After irradiation, two groups of cells were washed and harvested to get the cell pellet via centrifugation 1500 g for 10 minutes. The cell pellet was prepared for the following 2D electrophoresis analysis.

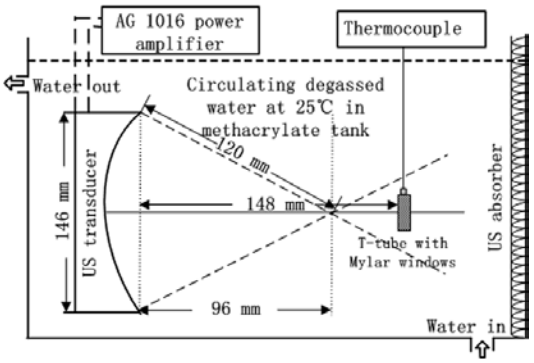

Fig. 1. Experimental set of US exposure.

2.2. 2D electrophoresis

The proteins were extracted from the cells by the freeze-thaw lysis method in the cell lysis solution (8 M urea, 4% CHAPS and 2% Pharmalyte 3-10) with added 0.1 mM PMSF to inhibit the activity of protease. Finally, Centrifugation at 40,000 g for 1 hour at 4℃ was used to remove the precipitation. The protein concentration was measured by the Bradford assay.

The reswelling buffer (8 M urea, 2% CHAPS (w/v), 0.7 mg dithiothrietol (DTT), 1 uL IPG buffer and 0.002% bromophenol blue) was completely mixed with proteins by Vortex Mixer for 2 minutes, and any remnant debris were removed by centrifugation at 2000 g for 2 minutes at 4℃. In every strip holder, about 200 ug proteins in 250 uL supernatant was added in the IPG strip which is covered by Mineral Oil. The immobilized IPG strip with nonlinear pH gradient from 3 to 10 was used in isoelectric focusing process. The rehydration lasted for 12 hourswith the sample in reswelling buffer, including 1 hour without current followed by 5 hour at 30 V and then 6 hour at 60 V. The isoelectric focusing included the process of removing any ions for 3 hours each at 200 V and 500 V, and then a total of 35000 Vhs for protein isolation.

After two-steps equilibration for 15 minutes each, the second dimension separation was performed in 2D gels (14×15 cm, 1 mm thickness) which contained 12% acrylamide using Hoefer SE 600 standard vertical system. The current of 10 mA per strip for 20 minutes, followed by 20 mA per strip for 5 hours could separate proteins completely. The 2D gels were stained using silver staining.

2.3. Image analysis and protein identification

The 2D gel images were scanned and analyzed by the Image Master 2D Elite software (Pharmacia, Amersham Biotech). The images were analyzed by intensity calibration, spot detection, background subtraction, normalization and one dimension calibration. Then the volume value, pI and molecular weight of each protein spot were calculated. Compared to the control gel, the corresponding protein spots have at least two-fold increase or decrease in volume value after ultrasound exposure in 3 repeated experiments (P<0.05) were regarded as up or down regulated.

After tryptic digestion, peptide mass fingerprinting was used to identify the proteins. The peptide information are matched in ProFound-Peptide Mapping and analyzed by Data explore (Applied Biosystems). Score higher than 1.65 which means the search is in the 95th percentile is regarded as a correct identification.

2.4. Statistical analysis

All the experiments were three times repeated. All the data are presented as the mean ± standard deviation (SD). Difference between the two groups were assessed with student's t-test at 95% confidence interval; $P<0.05$ was considered to be significant.

3. Results and discussion

3.1. Morphologic changes of irradiated cells

The irradiated cells show the distinct morphologic characteristics of apoptosis. As shown in Figure 2(b), the condensation of nucleus, as well as chromatin margination, were found after the cells were irradiated by ultrasound at intensity of 1.2 W/cm^2, while no these characteristics were observed in the control one.

3.2. Functional detection of apoptotic cells

The double staining flow cytometry (FITC-labelled Annexin V/PI) are based on the functional assays of cell in different states. The viable cells can't be stained by either Annexin V or PI, so it shows characteristics of Annexin V^-/PI^-. Early apoptotic cells can only be stained by Annexin V, which is Annexin V^+/PI^-. Secondary necrosis cells, stained by both Annexin V and PI, are Annexin V^+/PI^+. The percentage of cells in different styles is shown in Figure 3. It is obviously that the viable cells decreased after the ultrasound exposure, in the meantime, the early apoptotic cells and secondary

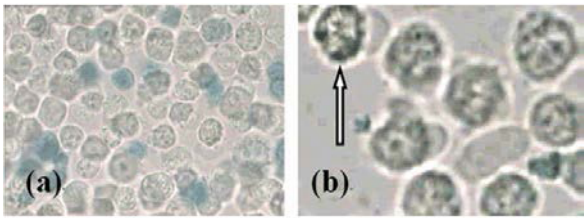

Fig. 2. Morphologic characteristics of SMMC-7721 cells.

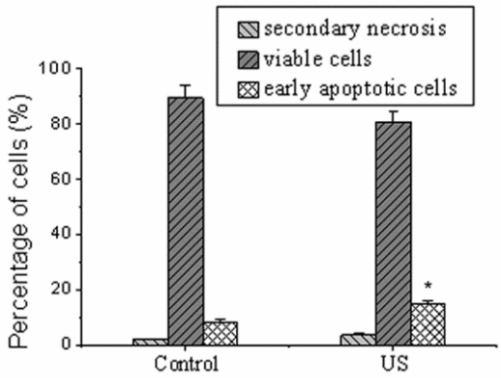

Fig. 3. Early apoptosis induced by the ultrasound exposure (*: $P<0.05$).

necrosis cells increased. The percentage of early apoptosis increased 8±0.4% (P<0.05) compared with the cells before ultrasound exposure, which proved the apoptosis of cells was induced by ultrasound indeed. However, further studies, such as the analysis of the changed protein expression and their functions, are needed to make sure the related metabolic process and analyze the potential mechanism of ultrasound induced cell apoptosis.

3.3. Two-dimensional electrophoresis analysis

There are 987±19 protein spots separated successfully in the human hepatocarcinoma cells SMMC-7721, in which 672±6 protein spots have been matched well with those in the 2D map of treated cells. In the mean time, among the matched proteins, 59±3 proteins were up-regualted and 16±1 proteins were down-regulated significantly (P<0.05) after ultrasound exposure according to the volume value of protein spots. Some of them were shown in Figure 4 with control cells on left and ultrasound treated cells on right.

3.4. Proteins identification and analysis

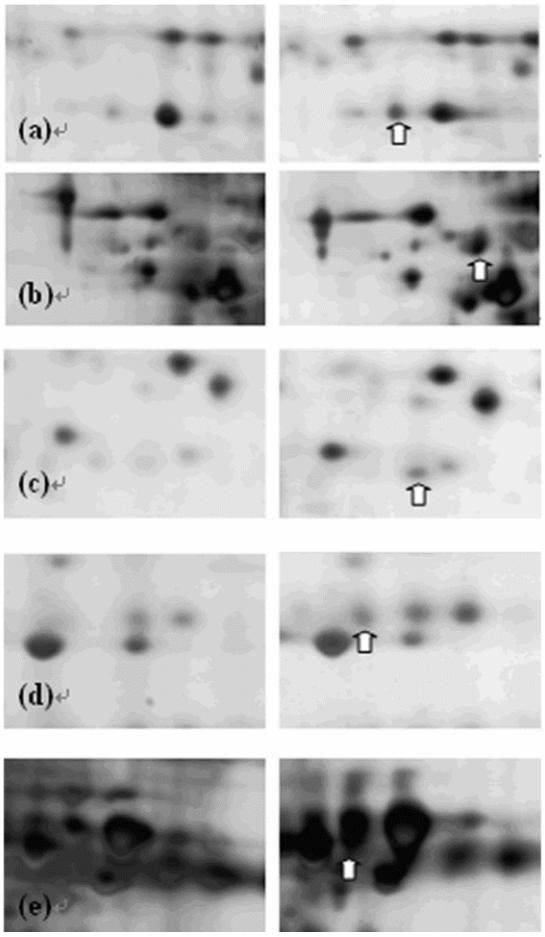

Fig. 4. Different expressing characteristics of proteins before and after ultrasound exposure (at least two-fold increase or decrease in volume value, P<0.05). (a) Protein p53; (b) Bip; (c) calreticulin; (d) CH60; (e) HSP70.

Two methods are introduced into identifying the protein, one is from the calculated pI and Mw of protein and another is from the identification by MALDI-TOF mass spectrometry. In this study, p53 protein, a tumor suppressor and pro-apoptotic protein, is found to be 3 times expressed in the 2D map of the ultrasound irradiated sample ($P<0.01$). It means the increased expression of protein p53 may be the result of ultrasound exposure and related to the metabolic process happened after the ultrasound exposure in SSMC-7721 cells. Protein p53 is always involved in the apoptotic activation and development. In fact, it is one key point in the signal process of apoptosis. The increased expression of protein p53 can stimulate Bax protein, one BCL-2 family member, which has a p53-binding site on its promoter site. Bax can promote the loss of integrity of mitochondrial membrane, and then making the cytochrome C release from mitochondrial to cytosol. The released cytochrome C always is associated with the activation of caspase cleavage, which is the key step to begin the apoptosis in cells. Furthermore, the increased expression of protein p53 also can improve the production of reactive oxygen species (ROS), which can activate mitochondrial damage and then induce apoptosis directly via caspase-3. Both the processes are related to mitochondria activation, so it seems that the apoptotic signal process induced by focused ultrasound exposure in this study should be mitochondrial-dependent, and also caspase-dependent.

In this study, some up or down regulated proteins are also associated with the cell apoptosis, like heat shock proteins (HSPs) and calcium ion channel related proteins. It has been well known that HSPs work at several functional points in the apoptotic signaling pathway. For example, HSP could inhibit the key steps in apoptotic process because they have the cytoprotective role. In the other hand, HSPs could also serve as chaperones of a key apoptotic signaling protein or promote apoptosis directly. Vykhodtseva indicated that the production of heat shock proteins, including CH60, HSP70 and Bip, is the potential mechanism of focused US exposure induced apoptosis [13]. Such as, CH60 promotes apoptosis by helping the maturation of precaspase-3, which belongs to a group of enzymes involved in the apoptotic process; HSP70, normally suppresses the apoptosis happened in cells and should has a lower expression in apoptotic process, increased its expression in ultrasound irradiated cells. The reason may be that the quantity of HSP70 was accumulated in the process of preventing apoptosis when apoptotic signal process has begun and became irreversible already. In the study, ER stress could be also involved because two proteins, Bip and Calreticulin, were identified up regulated by ultrasound exposure and they are the key proteins mediate the ER-initiated apoptosis. Calreticulin is also related to the calcium transportation through membrane and the calcium homeostasis is very important for the activation of mitochondria and apoptosis.

4. Conclusion

Combined with the morphologic observation and double staining flow cytometry, comparative proteomic analysis were performed to filter and analyze the key proteins closely related with ultrasound exposure in human hepatocarcinoma SMMC-7721 cells. Some key proteins were identified and analyzed according to their relationship with apoptotic signaling pathway, including protein p53, heat shock proteins and calreticulin. It proved that US induced apoptosis was initiated in a mitochondrial-dependent apoptotic signaling pathway, related to the mitochondria activation during which process several key proteins participated. The comparative proteomic method could directly get and analyze the proteins expressed and changed in mitochondria. It would be very helpful to analyze the protein-protein interaction in the mitochondrial-dependent apoptotic signal process induced by ultrasound exposure.

Acknowledgment

Work supported by the National Natural Science Foundation of China (Grant No. 81201105); New Teachers' Fund for Doctor Stations, Ministry of Education (No. 20110201120053) and the Fundamental Research Funds for the Central Universities (No. xjj2015070).

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