This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier’s archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright
Dynamics and mechanism of HSP70 translocation induced by photodynamic therapy treatment

Feifan Zhou a, Da Xing a,*, Wei R. Chen a,b

a MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, South China Normal University, Guangzhou 510631, China
b Biomedical Engineering Program, Department of Engineering and Physics, College of Mathematics and Science, University of Central Oklahoma, Edmond, OK 73034, USA

Received 28 November 2007; received in revised form 18 January 2008; accepted 18 January 2008

Abstract

Heat shock protein 70 (HSP70) is involved in nearly all intracellular compartments. It has been recently shown to be expressed on the outer cellular membrane under photodynamic therapy (PDT) treatment. However, the mechanism and function of HSP70 translocation to the cell surface during PDT treatment are not well understood. In this study, the dynamics and mechanism of HSP70 translocation onto the cell surface and its relationship with several key intracellular events after PDT treatment were investigated using confocal microscopy. HeLa and ASTC-a-1 tumor cells were treated by PDT using different doses. In the case of PDT-induced apoptosis, cytoplasmic HSP70 rapidly translocated to the cell surface after treatment, but it was not released into the medium. Such translocation was found to be dependent on the PDT dose. Moreover, during apoptosis, the translocation of HSP70 was closely related to the changes of mitochondrial transmembrane potential (ΔΨm). Under non-lethal PDT induced surface stress, HSP70 also translocated to the cell surface, but with a slower rate and a lower final surface concentration. These findings reaffirm the HSP70 translocation onto the cell surface under PDT treatment in living cells. Our results also indicate that the function of the surface expression of HSP70, either initiated by mitochondrial disruption or direct surface stress, is to stabilize the plasma membrane integrity, although such function failed to prevent apoptosis induced by lethal PDT treatment, as evidenced in our study.

Keywords: HSP70; PDT; Apoptosis; Mitochondria

1. Introduction

Heat shock proteins (HSPs) are characterized both by the heterogeneity of physicochemical properties and the variety of functions [1]. Their chaperone functions, connected with protein folding, transport and reparation, and their protective functions to increase cell resistance to stressful conditions have been extensively studied [2–5]. HSPs have also been shown to be involved in intracellular signal transduction [6–10] and immune regulation [11–14]. Cell surface localization of HSPs under different conditions has been detected [11,15–19]. In comparison with intracellular HSPs, the functions...
of HSPs expressed on the cell surface are not well understood. One hypothesized function is that surface HSPs serve as markers of cells subject to elimination by the innate immune system [20,21]. However, such a role for surface HSPs might be a consequence of their surface localization but not an underlying cause of HSP translocation onto the plasma membrane [20,21]. Another hypothesized mechanism is the known capability of HSPs in stabilizing plasma membranes [22], which could be used by cells to reinforce the membranes in the case of potential damage [18].

Photodynamic therapy (PDT) involves administration of a tumor localizing photosensitizing agent, which could generate highly reactive oxygen radicals during light irradiation [23,24]. The mechanism of cell death induced by PDT depends on the intracellular localization of the photosensitizer [25,26]. Because the primary insult inflicted by PDT is in the form of oxidative stress, it is not surprising that this treatment was found to induce the expression of oxidative stress, it is not surprising that this treatment was found to induce the expression of various HSPs [27,28]. The PDT induced cell surface expression and release of HSPs has been studied [16]. However, the mechanism of the HSP70 surface expression during PDT has not been fully investigated.

We studied the HSP70 translocation to the cell surface during apoptosis induced by PDT in living cells using confocal microscopy. We also investigated the relationship of HSP70 translocation with certain key intracellular events, in order to understand the mechanism of the HSP70 surface expression induced by the PDT treatment.

2. Materials and methods

2.1. Chemicals and plasmids

The following fluorophore probes were used: Hoechst 33258 (10 μM, Sigma, St Louis, MO) to detect the nuclear morphology of apoptosis; Rhodamine 123 (5 μM, Molecular Probes, Inc., Eugene, OR) to monitor mitochondrial transmembrane potential (ΔΨm); MitoSOX (5 μM, Molecular Probes) to monitor the intracellular superoxide; and MitoTraker Red (100 nM, Invitrogen Life Technologies, Inc.) to label mitochondria. The optimal concentrations and incubation times for each of the probes were determined experimentally.

The following chemicals and commercially available kits were used: photosensitizer Photofrin (Sinclair Pharmaceuticals, GU7 2AB, UK); CCK8 (Dojindo Laboratories, Kumamoto, Japan); and HSP70 ELISA Kit (R&D Systems, Minneapolis, MN).

In addition, we used Lipofectin reagent (Invitrogen) to transfect YFP-HSP70 into cells. The YFP-HSP70 plasmid was given by Dr. Richard I. Morimoto of Northwestern University [29,30].

2.2. Cell culture and transfection

Human epithelial carcinoma cells (HeLa) and human lung adenocarcinoma cells (ASTC-a-1) were used for the experiments. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 15% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO2, 95% air at 37°C in a humidified incubator. For the experiments, cells were transfected with 1 μg plasmid DNA of YFP-HSP70 and 2 μl of Lipofectin reagent per 100 ml of serum-free medium at 37°C for 4 h, then cultured in DMEM at 37°C for 24 h before experiments.

2.3. Photodynamic therapy

The light source was a He–Ne laser (HN-1000, Guangzhou, China; 632.8 nm). Cells (1 × 10^5 per well) growing in 35 mm Petri dishes were incubated in the dark with Photofrin in complete growth medium. The incubation times were 20 h or 20 min, and the cells were rinsed with PBS. The long incubation period was used to ensure the mitochondrial localization of Photofrin, and the short incubation period was used for the cell surface localization of Photofrin. Different irradiation fluences (1–15 J/cm²) and various Photofrin doses (5–20 μg/ml) were used in this study. For the following experiments, PDT light was delivered at a fluorescence rate of 10 mW/cm² for 8 min. For the control group, cells were incubated in the same medium without Photofrin and without light exposure.

To study PDT induced cell apoptosis, damage dose (5 J/cm²; 10 μg/ml, 20-h incubation) was used. To study PDT induced non-lethal cell stress, stress dose (5 J/cm²; 10 μg/ml, 20-min incubation) was used.

2.4. Confirmation of cell death

Cell cytotoxicity assay was assessed with a colorimetric tetrazolium salt-based assay, Cell Counting Kit-8 (CCK8). WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt] is reduced by dehydrogenases in cells to give a yellow colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by the activity of dehydrogenases in cells is directly proportional to the number of living cells. The absorbance at 450 nm of the formazan dye is proportional to the number of viable cells in the medium, and the number of viable cells can be determined using the absorbance value of a previously prepared calibration curve.
Cells were cultured in a 96-well microplate at a density of $1 \times 10^3$ cells per well for 24 h and then coincubated with 10 $\mu$g/ml Photofrin for 20 h at 37 °C in the dark. The samples were then divided into five groups and exposed to semi-conductor laser irradiation at fluence of 0, 1, 3, 5, and 10 J/cm$^2$, respectively. After the irradiation, 96-well microplates were returned to the incubator for a further culture. Cell cytotoxicity assay was assessed with Cell Counting Kit-8. OD$_{450}$, the absorbance value at 450 nm, was read with a 96-well plate reader (INFINITE M200, Tecan, Switzerland), to determine the viability of the cells. The viability of cells was calculated as: cell viability (% of control) = OD$_{Tm}$/OD$_{Con} \times 100\%$ (where OD$_{Tm}$ was the absorbance value at 450 nm of treated cells and OD$_{Con}$ was the absorbance value at 450 nm of control cells).

To assess the changes in nuclear morphology of apoptosis, cells ($1 \times 10^4$ per well) were cultured in 35 mm glass bottomed dishes. Three hours after PDT treatment under different doses, the cells were stained with Hoechst 33258 for 10 min at room temperature and washed twice with PBS. The cell samples were visualized under a Nikon fluorescent microscope with a hydargyrum lamp as the light source. Hoechst 33258 was excited with a light source through a 330–380 nm band-pass filter and emitted light was detected through a 450–490 nm band-pass filter.

2.5. Confocal laser scanning microscopy

Fluorescent emissions from YFP, Rhodamine 123, MitoSOX, Photofrin, and MitoTraker Red were monitored confocally using a commercial laser scanning microscope (LSM 510/ConfoCor 2) combination system (Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40×/1.3 NA Oil DIC objective. Excitation wavelength and detection filter settings for each of the fluorescent indicators were as follows: YFP fluorescence was excited at 514 nm with an Ar-Ion laser (reflected by a beam splitter HFT 458/514 nm), and emission was recorded through a 530–550 nm IR band-pass filter. Rhodamine 123 was excited at 488 nm with the Ar-Ion laser (reflected by a beam splitter HFT 488 nm), and the fluorescence emission was recorded through a 500–530 nm IR band-pass filter. MitoSOX fluorescence was excited at 514 nm with the Ar-Ion laser, and emitted light was recorded through a 560 nm long-pass filter. Photofrin fluorescence was excited at 458 nm with the Ar-Ion laser (reflected by a beam splitter HFT 458/514 nm), and emitted light was recorded through a 600–650 nm IR band-pass filter. MitoTraker Red fluorescence was excited at 633 nm with a He-Ne laser, and emitted light was recorded through a 650 nm long-pass filter. For intracellular measurements, the desired measurement position was chosen in the LSM image. To quantify the results, the average emission intensities of the desired measurement position were processed with Zeiss Rel3.2 image processing software (Zeiss, Jena, Germany).

2.6. Detection of mitochondrial potential depolarization and intracellular superoxide

Rhodamine 123 was used as an indicator of the mitochondrial potential depolarization ($\Delta \Psi_{m}$). Cells ($1 \times 10^4$ per well) growing in 35 mm Petri dishes were loaded with Rhodamine 123 for 30 min at 37 °C, rinsed three times with PBS prior to fluorescence measurement. Intracellular accumulation of superoxide was estimated using MitoSOX, which selectively targets mitochondria and is oxidized by superoxide, emitting red fluorescence upon binding to nucleic acids. Cells ($1 \times 10^4$ per well) growing in 35 mm Petri dishes were loaded with MitoSOX for 10 min at 37 °C, rinsed with PBS once prior to fluorescence measurement by a confocal microscope.

2.7. Detection of HSP70 release

To detect the release of HSP70 from PDT-treated tumor cells, a commercially available kit HSP70 ELISA was used. The supernatants of PDT-treated cell cultures were collected at different post-PDT times for ELISA analysis by adding to the microtiter wells coated with human anti-HSP70 monoclonal antibody. The captured HSP70 was detected with a HSP70-specific biotinylated rabbit polyclonal antibody that was subsequently bound by an avidinhorseradish peroxidase conjugate.

3. Results

3.1. Photodynamic therapy-induced apoptosis in HeLa cells and ASTC-a-1 cells

To establish a proper irradiation fluence of PDT to induce apoptosis, CCK-8 was used to measure the OD$_{450}$ value, an indicator of cell apoptosis. Using the same photosensitizer dose (10 $\mu$g/ml, 20-h incubation), the OD$_{450}$ value was statistically significantly lower for cells irradiated with 5 J/cm$^2$ (Student’s paired t-test, $P < 0.001$) than with 1 and 3 J/cm$^2$, as shown in Fig. 1A. The OD$_{450}$ value further decreased as the irradiation fluence increased. Under 10 $\mu$g/ml Photofrin dose and 5 J/cm$^2$ irradiation fluence, the cells showed clear shrinkage 140 min after PDT treatment; cell apoptosis was completed 160 min after PDT treatment (Fig. 1B). As a control group, the HeLa cells expressing YFP-HSP70 were exposed to room air at 37 °C; no morphological changes were observed (Fig. 1B). Therefore, this PDT treatment protocol (10 $\mu$g/ml Photofrin, 5 J/cm$^2$ of 632.8 nm light) was chosen as apoptotic dose in this study. To further confirm the selection of the apoptotic PDT
dose, Hoechst 33258 staining assays were used for HeLa cells and ASTC-a-1 cells 3 h after PDT treatment. The fluorescent images of the treated cells show clear cell apoptosis (Fig. 1C).

3.2. Real-time detection of HSP70 distribution in living cells after PDT treatment

To monitor the dynamic distribution of HSP70 after Photofrin-PDT treatment in real time, a confocal microscope was used. Fluorescence emission from cells expressing YFP-HSP70 showed a significant position shift less than 30 min after PDT treatment from inside of the cell to the surface of the cell in both cell lines (Fig. 2A). The control cells expressing YFP-HSP70 showed a stable YFP fluorescence emission inside the cell for more than 4 h (Fig. 2A). Fluorescence intensities of YFP from HeLa cells after PDT treatment showed a continuous decrease with time inside the cytoplasm and a continuous increase on the cell surface, reflective of HSP70 translocation, reaching a stable stage after 30 min (Fig. 2B).

To determine whether HSP70 was released into culture supernatants of PDT treated cells during apoptosis, HSP70 ELISA was used. The result did not show a signif-
Fig. 2. Dynamics of HSP70 translocation after PDT treatment. (A) Fluorescent image series and confocal images of HeLa cells and ASTC-a-1 cells expressing YFP-HSP70 after PDT treatment. Bar = 10 μm. (B) Temporal profiles of YFP emission intensities of different organelles in HeLa cells after PDT treatment. The desired measurement position was labeled as in (A). (n = 4). (C) HSP70 released into culture supernatants from HeLa cells treated by PDT using ELISA. At the indicated times post-treatment, the cell culture supernatants were collected for the determination of HSP70 release. (D) Temporal profiles of YFP emission intensities from the surface of HeLa cells after PDT treatment with different photosensitizer doses (5, 10, 20 μg/ml Photofrin, respectively, 5 J/cm²). Bars, SD (n = 4).
significant increase of HSP70 in PDT-treated culture supernatants (Fig. 2C). Furthermore, we found that the rate of HSP70 increase on the cell surface depended on the photosensitizer dose (Fig. 2D). The higher the dose, the faster the translocation of HSP70 onto the cell surface. Similar results were also obtained in ASTC-a-1 cells (data not shown).

3.3. Relationship of surface HSP70 expression with mitochondrial potential depolarization and intracellular superoxide concentration during cell apoptosis induced by PDT

To determine whether surface HSP70 expression is related to certain key mitochondrial events, we monitored the dynamic changes in intracellular superoxide concentration and mitochondrial potential depolarization (ΔΨm) during HeLa cell apoptosis induced by Photofrin-PDT. Measurement of simultaneous emissions of Rhodamine 123/MitoSOX and YFP-HSP70/MitoS-OX showed that the fluorescent intensity of MitoSOX increased immediately after PDT treatment, then decreased gradually from 8 to 25 min post-treatment (Fig. 3A middle panels and Fig. 3C). The decrease of fluorescent intensity of MitoSOX indicates the permeabilization of mitochondria (Fig. 3A lower panels). Corresponding to the decrease of MitoSOX emission, fluorescent intensity of Rhodamine 123 also decreased quickly (Fig. 3A top panels and Fig. 3C) while that of YFP-HSP70 increased sharply (Fig. 3B and C). Similar results were also obtained in ASTC-a-1 cells (Fig. 3D). These experimental results indicate that the mitochondrial membrane is permeabilized about 8 min after PDT treatment, causing the decrease of mitochondrial membrane potential, and the increase of HSP70 on the cell surface.

3.4. Relationship of surface HSP70 expression with cell surface stress

To determine the effect of non-lethal surface stress on HSP70 translocation, we irradiated HeLa cells expressing YFP-HSP70 with 5 J/cm² after the cells were incubated with 10 μg/ml of Photofrin for 20 min. Compared to cells incubated with Photofrin for 20 h, the short incubation resulted in a surface localization of Photofrin, as shown in Fig. 4A. The surface stress did not induce apoptosis, as shown in Fig. 4B. In addition, the fluorescent intensity of Rhodamine 123 remained unchanged after PDT stress treatment (Fig. 4C). The PDT-induced cell surface stress caused a mild increase of HSP70 fluorescence from the cell surface, far less than the PDT-induced lethal damage, as shown in Fig. 4D. Similar results were also obtained in ASTC-a-1 cells (Fig. 4E). These experimental results indicate that the mitochondrial membrane disruption induced significant HSP70 translocation after PDT damage treatment; without such a disruption, non-lethal direct surface stress only induced mild HSP70 translocation.

4. Discussion

The phenomenon of translocation of cytoplasmic HSP70 onto the cell surface induced by PDT was previously described [16]. However, the mechanism and function of the HSP70 translocation are not well understood. We used recombinant YFP-HSP70 and a confocal laser scanning microscope to study the HSP70 translocation after PDT treatment. We also analyzed the relationship between HSP70 translocation and several key cellular events during PDT-induced apoptosis.

In this study, for the first time, we observed the dynamic translocation of HSP70 from cytoplasm onto the cell surface at the single-cell level during PDT treatment. When the cells were treated with a typical PDT dosage to induce apoptosis of cells expressing YFP-HSP70, both fluorescent images (Fig. 2A) and spectrofluorometric analysis (Fig. 2B) showed an instantaneous increase in the YFP emission from the cell surface and a corresponding emission decrease in cytoplasm, reflective of the translocation of HSP70. The translocation reached a stable stage 30 min after PDT (Fig. 2B), and there was no significant increase of HSP70 release from PDT induced apoptotic cells (Fig. 2C). These results imply that cytoplasmic HSP70 immediately translocated onto and remained on the cell surface after PDT treatment.

What is the possible mechanism of translocation of cytoplasmic HSP70 onto the cell surface during apoptosis induced by PDT? Previous studies suggested that surface localization of HSP70 during apoptosis could be considered as an intermediate stage for the transition of HSP70 from cells into extracellular space [21,31]. However, our data show the lack of HSP70 release into the culture supernatants, suggesting that the HPS70 translocation to the cell surface may be an end point of HSP70 during PDT-induced apoptosis (Fig. 2C). Others have suggested that the surface translocation is connected to the known capability of HSP70 in stabilizing plasma membranes [18,21]. Our data show that the extent of the initial surface HSP70 expression is related to the PDT dose (Fig. 2D); the higher the Photofrin dose, the faster the increase of intracellular ROS and the more severe the mitochondrial lesion. Since Photofrin-PDT directly impacts mito-
Fig. 3. Relationship between surface HSP70 expression and mitochondrial potential depolarization (ΔΨm) during cell apoptosis induced by PDT. (A) Fluorescent image series of Rhodamine 123 (representing mitochondrial potential depolarization, upper panel) and MitoSOX (representing intracellular superoxide concentration, hence mitochondrial disruption, middle and lower panels) in HeLa cells after PDT. HeLa cells were stained with Rhodamine 123 and MitoSOX before laser irradiation. (B) Fluorescent image series of YFP-HSP70 (representing distribution of HSP70, upper panel) and MitoSOX in HeLa cells after PDT. Temporal profiles of fluorescent emission intensities of YFP-HSP70, Rhodamine 123, and MitoSOX in apoptotic HeLa cells (C) and apoptotic ASTC-a-1 cells (D) after PDT. The desired measurement position was labeled as in (A) and (B). Bars, SD (n = 4).
chondria, the initial surface HSP70 expression is associated with the degree of cell lesion, as shown in Fig. 2 D.

To study the mechanism of HSP70 translocation, we analyzed the relationship between HSP70 expression, mitochondrial transmembrane potential, and intracellular superoxide concentration. Our study showed that the mitochondrial membrane was permeabilized about 8 min after PDT treatment, causing the decrease of mitochondrial membrane potential, and the increase of HSP70 on the cell surface (Fig. 3). There was a drastic increase of HSP70 on the cell surface when the mitochondrial membrane was permeabilized. This suggests that HSP70 translocates onto the cell surface to stabilize the plasma membrane integrity under the induced “danger signal” [32], although the protection function of HSP70 failed to prevent apoptosis due to the mitochondrial disruption.

To further investigate its protective role of the HSP70, a “non-lethal dose” was used in our study as a comparison with the results of the “lethal dose”. For the non-lethal dose, a short drug-cell incubation duration (20 min) was used to ensure
that the drug was localized only on the cell membrane. With such a low concentration of the PDT drug, the light treatment could only induce a stress on the cell membrane, without causing cell death, since it is well known that PDT-induced cell death is initiated from the mitochondrial damage. The results are shown in Fig. 4. It is clearly demonstrated that HSP70 also translocated to the cell surface immediately (Figs. 4D and E) after the treatment with the “non-lethal dose”. However, the cell surface integrity and cell viability were maintained. These results support the reported role of HSPs to reinforce the membranes in the case of their destabilization [22].

In summary, we directly observed the dynamic redistribution of HSP70 under PDT treatment. Our results indicate that the overall function of HSP70 translocation is to stabilize the plasma membrane, although this protective role failed to prevent apoptosis due to mitochondrial membrane disruption. Therefore, this study sheds some light on the function and mechanism of this important protein.

Acknowledgements

This research is supported by the National Natural Science Foundation of China (30470494; 30627003), the Natural Science Foundation of Guangdong Province (7117865), and by a grant from the US National Center of Health (P20 RR016478 from the INBRE Program of the National Center for Research Resources).

References