Effects of HSP27 downregulation on PDT resistance through PDT-induced autophagy in head and neck cancer cells

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Abstract. We previously reported that photodynamic therapy (PDT) induces cell death in head and neck cancer through both autophagy and apoptosis. Regulation of cell death by autophagy and apoptosis is important to enhance the effects of PDT. Autophagy maintains a balance between cell death and PDT resistance. Downregulation of heat shock protein 27 (HSP27) induces PDT resistance in head and neck cancer cells. Furthermore, HSP70 regulates apoptosis during oxidative stress. However, the role of HSPs in PDT-induced cell death through autophagy and apoptosis is unclear. Therefore, in the present study, we investigated the effects of HSP27 and HSP70 on PDT-resistant cells through autophagy and apoptosis. Cancer cells were treated with hematoporphyrin at varying doses, followed by irradiation at 635 nm with an energy density of 5 mW/cm². We determined the changes in HSP expression by determining the levels of PARP-1 and LC3II. Furthermore, we assessed cell death signaling after downregulating HSPs by transfecting specific siRNAs. We observed that PDT decreased HSP27 expression but increased HSP70 expression in the head and neck cancer cells. Treatment of cells with LC3II and PARP-1 inhibitors resulted in upregulation of HSP70 and HSP27 expression, respectively. Downregulation of HSP27 and HSP70 induced cell death and PDT resistance through autophagy and apoptosis. Moreover, downregulation of HSP27 in PDT-resistant cells resulted in enhanced survival. These results indicate that the regulation of HSP27 and HSP70 plays a principal role in increasing the effects of PDT by inducing autophagic and apoptotic cell death.

Introduction

Photodynamic therapy (PDT) is an anticancer treatment involving a photosensitizer and a light source resulting in the production of reactive oxygen species (ROS) within affected tissues. PDT is an efficient inducer of apoptosis both in vitro and in vivo. It induces autophagy and apoptosis in skin cancer cells by activating MAPK (1). In a previous study, treatment with hematoporphyrin (HP) and PDT (HP-PDT) induced autophagy by increasing the levels of autophagy-related proteins such as LC3II and ATG5 and by inactivating mTOR (2). Recently, we reported that PDT stimulated the apoptosis of FaDu cells by cleaving PARP-1 and increasing caspase activity (2). To enhance the effects of PDT, it is important to maintain a balance between apoptosis and autophagy. Moreover, use of PDT may result in PDT resistance depending on the photosensitizer, light dose, and cell types used (3). Several studies have suggested that drug dose and its exposure density induce PDT resistance (4). Mechanisms underlying resistance induced by a photosensitizer are thought to be the same as those underlying drug resistance and may be related to different uptake or efflux rates, altered intracellular trafficking of the photosensitizer, and inactivation of the photosensitizer (3). In addition, alterations of proteins related to cell death and survival determine the anticancer effects of PDT (3). PDT induces oxidative stress. This transiently increases the expression of downstream early response genes and stress genes such as those encoding heat shock proteins (HSP, glucose-regulating proteins, and heme oxygenase in mammalian cells) (5,6). Mechanisms underlying PDT-induced cell death and PDT resistance are related to cell survival induced by the combined action or interaction (or both) of cell death pathways (7,8).

Defective apoptosis and autophagy are believed to play a crucial role in the sensitivity of cells to PDT (9). PDT can induce both apoptosis and autophagy. Autophagy enhances the survival of cells showing low levels of photodamage (10), thus serving as a pro-survival response by recycling damaged organelles. In contrast, autophagy induces the death of cells...
treated with high-dose PDT (11,12). Some studies have shown that PDT limits autophagy and apoptosis (13). Autophagy maintains a balance between cell death and PDT resistance during PDT (14). However, the roles of HSPs in PDT-induced autophagy are unclear.

HSPs act as intracellular chaperones of other proteins. They prevent unwanted protein aggregation and stabilize partially unfolded proteins, thus helping cells to recover from PDT-induced damage (15). Some of these signals act as mediators or promoters of apoptosis, while some signals act as stress responses that promote the repair of or increase the resistance to PDT-induced damage (16). Effects of PDT may be determined by balancing death and survival for cancer therapy resistance mechanisms (17,18). In previous study, HSP70 was found to regulate the apoptosis of chemoresistant cells by inhibiting pro-apoptotic proteins (19,20). We suggest that HSP70 expression is related to other proteins which regulate cell death and resistance in PDT. Regulation of HSP27 expression resulted in degradation of apoptotic protein and a delay in PDT-induced apoptosis by autophagy (21,22). Regulation of HSP27 expression may be one of the markers in PDT resistance and cancer therapy in oral cancer cells.

The aim of this study was to investigate the mechanisms which control PDT resistance during PDT-induced cell death by regulating the expression of HSPs. Thus, this study investigated the effects of PDT on HSP expression to control PDT resistance.

Materials and methods

Chemicals and reagents. HP, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange, 3-methyadenine (3-MA), and 3,4-dihydro-5-[1-piperidinyl]butoxy]-1(2H)-isoquinoline (DPQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. Human oral cancer FaDu cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY, USA) supplemented with antibiotics and antimycotics (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B) (antibiotics and antimycotics; Gibco-BRL) and 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

Photosensitizer and irradiation treatments. Cells were treated with HP at varying concentrations in the range of 0-6 µM. HP-treated cells were then irradiated using a light-emitting diode (LED; 4.5 mW/cm²) for 15 min. Effects of PDT on FaDu cells were investigated after 24 h. To determine the effect of an autophagy inhibitor, FaDu cells were treated with 5 mM 3-MA and HP at 2 and 4 µM for 24 h. The cells were treated again with 3-MA along with irradiation for 24 h. After irradiation, the PDT-treated cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air. To determine the effect of an apoptosis inhibitor, FaDu cells were treated with 20 µM DPQ in the same way as the autophagy inhibitor.

Induction of HP-PDT resistance. PDT-induced variants of FaDu cells were modified as previously reported (23). Briefly, FaDu cells cultured in 100-mm dishes were incubated with 2 µM HP in medium for 24 h, followed by a 15-min irradiation using an LED (4.5 mW/cm²). Surviving cells were recovered by a 24-h incubation in complete medium. After recovery, the PDT-treated cells were exposed to HP-PDT. The cells that survived the second cycle of PDT were repeatedly (15 times) exposed to PDT before the isolation of PDT-resistant cells. The surviving PDT-resistant cells were then treated 15 times with HP-PDT.

Cytotoxicity assay. Cytotoxicity was determined by measuring the reduction of MTT to insoluble formazan. FaDu cells were cultured (1x10⁵ cells/ml) in 96-well plates for 24 h. HP was added to the culture medium, and the cells were incubated for 24 h. Light irradiation was performed for 15 min. After irradiation, fresh medium was added, and the cells were incubated for 24 h. The cells were then incubated in PBS containing 30 ml MTT at 37°C for 3 h. The formazan produced was solubilized by adding 50 ml dimethyl sulfoxide. Optical density was measured at 570 nm by using an ELx800uv ELISA reader (Bio-Tek Instruments, Winooski, VT, USA).

Western blotting. Proteins from the cell cultures were extracted using lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 1% protease inhibitor cocktail. Next, 100 µg of the protein extract was mixed with a sample buffer, and proteins present in the extract were separated by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel. The proteins were transferred onto a membrane, and non-specific binding sites on the membrane were blocked using 2% non-fat milk. Next, the membranes were incubated overnight with primary antibodies against HSP27, HSP70, HSP90, and GAPDH (1:500 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and with primary antibodies against LC3II and PARP-1 (1:1,000; Cell Signaling Technology, Beverly, MA, USA) in a blocking solution at 4°C. After washing with 1X TBS-T, the membranes were incubated with HRP-conjugated anti-mouse or anti-rabbit antibodies for 2 h at room temperature. The membranes were then washed 4 times with TBS-T (5 min/wash), and the immunoblotted proteins were visualized using enhanced chemiluminescence (Santa Cruz Biotechnology), according to the manufacturer’s instructions.

siRNA transfection. FaDu cells were grown to 70% confluence in antibiotic-free DMEM supplemented with 0.2% FBS. The cells were then transfected with siRNAs against genes encoding HSP27 or HSP70 (L-005269-00 or L-005168-00, respectively) or with siControl non-targeting siRNA #1 (all from Dharmacon, Lafayette, CO, USA) for 24 h. Transfection was performed using HiPerFect transfection reagent (Qiagen, Chicago, IL, USA) to facilitate the uptake of siRNAs into the cells, according to the manufacturer’s protocol. At 48 h after PDT, the cells were collected and used for further analysis.

Autophagosome staining. FaDu cells were seeded in 4-well plates and were grown to 70% confluence. The cells were then irradiated and treated with 2 or 4 µM HP for 24 h. At designated times, the cells were incubated with 1 µg/ml acridine orange and BF-554 (a specific fluorescence probe for autophagosomes) for 30 min. After washing twice with PBS, the cells were analyzed using a confocal laser scanning microscope (Zeiss LSM 510, Jena, Germany) equipped with a ×100 objective (1.35 NA).
orange (Molecular Probes, Eugene, OR, USA) in serum-free medium for 15 min. After incubation, acridine orange was removed and the cells were observed under a confocal fluorescence microscope (Carl Zeiss, Oberkochen, Germany).
The cytoplasm and nucleus of the stained cells appeared bright green, and the acidic autophagic vacuoles appeared bright red.

**TUNEL assay.** TUNEL assay (DeadEnd™ Fluorometric TUNEL system; Promega, Madison, WI, USA) was performed to detect the breaks in DNA strands. Cells were fixed with 4% methanol-free formaldehyde and were permeabilized with 0.2% Triton X-100. The cells were then incubated with 100 µl reaction mixture containing biotinylated nucleotide mix and recombinant terminal deoxynucleotidyl transferase (rTdT) in equilibration buffer at 37˚C for 1 h. Next, the cells were incubated in 2X SSC at room temperature. After washing, the cells were mounted with Vectashield® including DAPI to stain the nuclei. The reaction without the rTdT enzyme was used as a negative control. The stained cells were analyzed using a confocal microscope (Carl Zeiss).

**Statistical analysis.** Data are expressed as mean ± standard deviation. All of the experiments were performed 3 times. Differences between the groups were evaluated using Student’s t-test and Tukey’s multiple comparison test by using GraphPad software version 5 (GraphPad Software, San Diego, CA, USA). Null hypotheses of no difference were rejected if p-values were <0.05.

**Results**

**Alteration of HSP expression in PDT-treated head and neck cancer cells.** To investigate the alteration in HSP expression during PDT, cancer cells were irradiated at 625 nm after HP treatment. HP treatment decreased the expression of HSP27 and increased the expression of HSP70 in a dose-dependent manner. These results suggested a negative correlation between HSP27 and HSP70 expression during HP-PDT (Fig. 1).

**Effects of HSP expression on PDT-induced cell death and autophagy.** In a previous study, PDT induced apoptosis as well as autophagy (2). Therefore, we investigated the alteration in HSP expression by treating FaDu cells with an autophagy inhibitor (Fig. 2). Treatment with the combination of 3-MA and PDT decreased HSP27 expression compared with PDT alone. In contrast, treatment with 3-MA and PDT increased HSP70 and HSP90 expression. In a previous study, we found that PDT induced apoptosis by PARP-1 (2). We suggested that PARP-1 dysfunction was related to PDT resistance by changes in HSP expression. To investigate the effect of a PARP-1 inhibitor on HSP expression, FaDu cells were treated with or without the PARP-1 inhibitor DPQ during PDT (Fig. 3). Inhibition of apoptosis by the PARP-1 inhibitor increased the expression of HSP27 during PDT. At 4 µM, HSP70 and HSP90 were
decreased as 20 µM DPQ was administered. Therefore, we concluded that apoptotic and autophagic signaling regulated HSP expression. The autophagy inhibitor showed maximized effects with HSP expression levels in PDT. DPQ as a PARP-1 inhibitor reversed the HSP expression levels in PDT.

Effects of HSP downregulation on PDT resistance. To evaluate the effects of HSP downregulation on PDT resistance, FaDu cells were transfected with siRNAs against genes encoding HSP27 and HSP70. Treatment with 4 µM PDT increased the survival of cells transfected with the siRNA against the gene

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**Figure 4. HP-PDT-induced cell death through autophagy and apoptosis in cells transfected with siRNAs against genes encoding HSP27 and HSP70.** (A) Cells transfected with siRNAs against the genes encoding HSP27 or HSP70 were treated with HP-PDT. Viability of these cells was assessed after HP-PDT. (B) Acridine orange staining of cells transfected with siRNA against the genes encoding HSP27 or HSP70 showed that HP-PDT affected autophagy compared with that in the non-transfected cells. Red or orange colors after acridine orange staining indicate autophagy. (C) TUNEL assay of cells transfected with siRNA against the genes encoding HSP27 or HSP70 showed that HP-PDT affected apoptosis. Non-transfected cells, cells treated with 4 µM HP-PDT, cells transfected with siRNA against the gene encoding HSP27, cells transfected with siRNA against the gene encoding HSP70 and treated with 4 µM HP-PDT were used. Green fluorescence indicates cells showing apoptosis, and blue fluorescence indicates nuclei. Scale bar, 20 µm. Statistical difference compared to HP-PDT only (*P<0.05).
encoding HSP27 in a dose-dependent manner, indicating that downregulation of HSP27 increased PDT resistance (Fig. 4A left panel). In contrast, PDT decreased the survival of cells transfected with siRNA against the gene encoding HSP70, indicating that downregulation of HSP70 induced cell death during PDT (Fig. 4A right panel). To confirm the regulation of cell death by HSPs, cells were stained with autophagic and apoptotic markers. PDT induced both autophagy and apoptosis in a dose-dependent manner. Positive autophagic staining by acridine orange staining was observed at 2 and 4 µM (Fig. 4B). The number of autophagic cells peaked after treatment with 2 µM PDT, and the number of cells transfected with siRNA against the gene encoding HSP27 peaked after treatment with 4 µM PDT (Fig. 4B). Cells transfected with siRNA against the gene encoding HSP70 and not treated with PDT showed autophagy (Fig. 4B). Downregulation of HSP70 did not affect PDT-induced autophagy. We also examined the effects of HSP27 and HSP70 downregulation on apoptosis during PDT. Downregulation of HSP27 decreased the apoptosis of cells while downregulation of HSP70 increased the apoptosis of cells during PDT compared with that of the non-transfected cells (Fig. 4C). These results indicated that PDT induced apoptosis along with autophagy. In addition, these results indicated that downregulation of HSP27 stimulated autophagy while that of HSP70 induced apoptosis. Thus, these results revealed that HSP27 and HSP70 regulated PDT resistance.

**Effects of HSP27 and HSP70 downregulation on cell death proteins.** To observe the alteration in cell death proteins after HSP downregulation, we determined the differential expres-
sion of LC3 and PARP-1. In the cells transfected with siRNA against the gene encoding HSP27, treatment with 4 µM PDT induced LC3II conversion; however, this was not observed in the non-transfected cells (Fig. 5A). Moreover, HSP27 downregulation decreased HSP70 expression. However, PDT cleaved PARP-1 regardless of the transfection of siRNA against the gene encoding HSP27. In cells showing HSP70 downregulation, PDT increased the expression of LC3II. PARP-1 cleavage increased after HSP70 downregulation. HSP27 expression had no effect on the cells transfected with siRNA against HSP70 (Fig. 5B). These results indicated that HSP27 mediated the autophagic signal in association with LC3II and that HSP70 regulated PARP-1, which was associated with apoptosis.

Alteration in apoptotic protein expression during PDT resistance. To investigate the alteration in the expression of cell death proteins during PDT resistance, we obtained PDT-resistant cells that remained alive after repeated PDT. Cell lysates were immunoblotted using antibodies against LC3, HSPs, and PARP-1. Since PDT-resistant cells showed decreased HSP27 expression, we concluded that PDT inhibited HSP27 expression (Fig. 6). HSP70 levels were unchanged in the PDT-resistant and non-resistant cells. HSP90 expression was slightly higher in the PDT-resistant cells than in the PDT-treated FaDu cells. Remarkably, LC3II levels increased after PDT in the PDT-resistant cells. Downregulation of HSP27 increased the level of active LC3II, which was associated with autophagy. This result indicated that HSP27 plays a key role in PDT resistance and cell death by controlling autophagy. PDT induced the cleavage of PARP-1 in FaDu cells. Furthermore, PARP-1 expression and cleavage were lower after PDT in PDT-resistant cells than in the non-resistant cells. Repeated PDT downregulated HSP27 in the PDT-resistant cells which in turn increased PDT resistance through autophagy. Moreover, PDT sensing was decreased in PDT-resistant cells. These results indicated that HSP27 downregulation regulated PDT resistance through autophagy and apoptosis.

Discussion

Regulation of HSP expression levels may play critical roles in cancer therapy. PDT has destructive power with reactive oxygen species generated by light irradiation on a photosensitizer, followed by accumulated cancerous cells. Effect of PDT is regulated by HSPs, i.e., by the upregulation of HSP70 and downregulation of HSP27. The potent cytoprotective and folding properties of HSPs are associated with oncogenesis since high expression of HSPs facilitates tumor growth and survival (23). In these patients, low HSP27 expression was correlated with non-responsiveness to the chemotherapy regimen, which was in contrast to that observed in patients.
with breast cancer (23). This is one of the few studies, which showed that low HSP27 expression was correlated with a negative outcome in patients with cancer.

**Downregulation of HSP27 affects PDT-induced cell death.** The present study suggested that alteration of HSP expression by PDT plays a critical role in tumor cell death and PDT resistance. As shown in Fig. 1, PDT decreased the expression of HSP27 and increased the expression of HSP70 and HSP90. Moreover, downregulation of HSP27 induced PDT resistance by activating autophagy (Fig. 4). HSP27 (encoded by **HSPB1**) is usually overexpressed in patients with breast cancer and affects the disease outcome and sensitivity of these patients to chemotherapy and radiotherapy (24, 25). A recent study reported that histone deacetylase 6, transcription factor STAT2, and pro-caspase-3 were degraded in human cancer cells in which HSP27 was downregulated by siRNA transfection, suggesting that these are target proteins of HSP27 (21). A decrease in HSP27 expression by PDT may exert protective effects against PDT-induced cell death in oral cancer cells. HSP27 knockdown induced autophagy and attenuated PDT-induced apoptosis of oral cancer cells (22). In the present study, we confirmed that downregulation of HSP27 expression induced autophagy through LC3II in PDT-resistant cells (Fig. 6). We also found that HSP27 participated in cancer cell survival. These results elucidated the mechanisms involved in PDT resistance.

**HSP70 plays a fundamental role in protecting cells against PDT.** Kayama et al reported the role of HSP70 in the molecular interplay between pro-survival and pro-apoptotic pathways after photoreceptor stress (26). In the present study, PDT induced the overexpression of HSP70 and HSP90 (Fig. 1). HSP70 upregulation prevents photoreceptors from undergoing immediate apoptosis. Downregulation of HSP70 by siRNA increased PDT-induced apoptosis through PARP-1 cleavage (Fig. 5). Furthermore, HSP70 prevented PDT resistance and inhibited the activation of apoptotic pathways. Transient knockdown of HSP70 expression was found to potentiate apoptosis, and its overexpression prevented OSU-03012-induced increase in cytotoxicity and autophagy (27). However, the role of HSP70 in ROS-induced autophagy remains known. In our study, downregulation of HSP70 inhibited autophagy by decreasing LC3II and induced apoptosis by cleaving PARP-1 (Fig. 5). Upregulation of PAPR-1 and the PARP-1 inhibition which blocks the cleaved form induced PDT resistance against PDT. HSP70 regulated PARP-1 activation. Therefore, we suggest that downregulation of HSP70 may inhibit PDT resistance by activating PARP-1.

**Autophagy is related to PDT resistance through HSP27 downregulation.** PDT concurrently induces both autophagic and apoptotic pathways in cancer cells (2, 13). Autophagy acts as both a tumor suppressor and a promoter. Autophagy may delay apoptosis and be activated in response to PDT contributing to cell resistance (28, 29). Therefore, inhibitors of autophagy may re-sensitize resistant cancer cells to anticancer therapies. In the present study, PDT decreased HSP27 expression, which was associated with autophagy induction by LC3II after PDT resistance (Fig. 6). It has been suggested that regulation of autophagy may play a critical role in maintaining a balance between PDT resistance and PDT-induced apoptosis. Therefore, it appears that regulation of autophagic signals may re-sensitize PDT-resistant cells to PDT. HSP70 exerted protective effects against PDT in oral cancer cells. Downregulation of HSP70 induced both apoptosis and autophagy. Moreover, HSP70 sensitized oral cancer cells to ROS stress but had no effect on PDT-resistant cells treated with PDT (Fig. 6). Therefore, autophagy induction by HSP27 decreased PDT sensitivity and PDT resistance. Downregulation of HSP70 increased PDT sensitivity and mediated PDT-induced apoptosis. Wei et al reported that autophagy is associated with PDT resistance in colorectal cancer stem-like cells (30). Autophagy and apoptosis may be triggered by common signals, resulting in combined autophagy and apoptosis. Autophagic and apoptotic signals may also complement each other. Several molecules regulate the crosstalk between apoptotic and autophagic pathways; however, the exact molecules are unknown. Our study suggests that a balance between autophagy and apoptosis that is regulated by HSPs may be responsible for the effects of PDT.

Taken together, PDT resistance occurs due to crosstalk between autophagic and apoptotic pathways in oral cancer cells. In the present study, PDT altered the expression of HSP27 and HSP70. Downregulation of HSP27 was associated with autophagy, which promoted the survival of cancer cells. HSP70 was inhibited during PDT-induced apoptosis as shown in our study and by others. Therefore, HSP27 and HSP70 may play a critical role in the cross-regulation of PDT-induced autophagy and apoptosis during cancer treatment.

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