Hsp27 Protects Adenocarcinoma Cells from UV-Induced Apoptosis by Akt and p21-Dependent Pathways of Survival

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Abstract

Transcriptional activation of p53 target genes, due to DNA damage, causes either apoptosis or survival by cell cycle arrest and DNA repair. However, the regulators of the choice between cell death and survival signaling have not been completely elucidated. Here, we report that human adenocarcinoma cells (MCF-7) survive UV-induced DNA damage by heat shock protein 27 (Hsp27)–assisted Akt/p21 phosphorylation/translocation. Protein levels of the p53 target genes, such as p21, Bcl-2, p38MAPK, and Akt, showed a positive correlation to Hsp27 level during 48 hours postirradiation, whereas p53 expression increased initially but started decreasing after 12 hours. Hsp27 prevented the G1-S phase cell cycle arrest, observed after 8 hours of post–UV irradiation, and PARP-1 cleavage was inhibited. Conversely, silencing Hsp27 enhanced G1-S arrest and cell death. Moreover, use of either Hsp27 or Akt small interference RNA reduced p21 phosphorylation and enhanced its retention in nuclei even after 48 hours postirradiation, resulting in enhanced cell death. Our results showed that Hsp27 expression and its direct chaperoning interaction increases Akt stability, and p21 phosphorylation and nuclear-to-cytoplasm translocation, both essential effects for the survival of UV-induced DNA-damaged cells. We conclude that the role of Hsp27 in cancer is not only for enhanced p53 proteolysis per se, rather it is also a critical determinant in p21 phosphorylation and translocation.

Introduction

Stress-induced expression of heat shock proteins (Hsp), the molecular chaperones, represents an emerging paradigm for the coordinated multistep regulation of apoptotic signaling events to provide protection and to facilitate cellular recovery against various stress-induced injuries. Higher levels of Hsps are observed in many malignant tissues, including breast cancer, ovarian cancer, osteosarcoma, endometrial cancer, and leukemia, compared with nontransformed cells of the same tissue (1). Hsp27, a member of the small heat shock protein family (sHSP), is constitutively expressed at low levels in many cells and tissues. Its expression increases following heat shock or other types of stress and it enhances cellular resistance (2-5). The molecular basis for overexpression of Hsp27 in malignant tissues is not completely understood. In some poorly vascularized solid tumors, Hsp27 accumulation is attributed to hypoxia, whereas oncogenic mutations could create an increased requirement for chaperone activity towards abnormally folded protein variants, leading to Hsp accumulation. Another possibility for increased Hsp27 protein level in malignant tissues could be the occurrence of functional mutations in transcription factors as observed in adenocarcinoma cell lines, where higher levels of the heat shock transcription factor 1 (HSF-1) was attributed to an increased Hsp27 protein level (6).

Several functional consequences of the higher Hsp27 levels have been identified in malignant tissues, including drug resistance (1, 4, 7-9). It may contribute to tumorigenicity through various mechanisms, which include cytotoxicity and molecular chaperone functions that could regulate cellular signaling to influence cell growth (10). Hsp27 also inhibits apoptosis in stressed cells, regulating upstream signaling pathways such as Akt activation by forming a signaling complex with p38 mitogen-activated protein kinase (MAPK) and MAPKAP-2, a kinase that phosphorylates Akt (11-14). The survival-promoting effects of Hsp27 have also been found to function through its ability to negatively regulate the Fas-mediated apoptotic pathway. Phosphorylated Hsp27 interacts directly with DAXX, preventing the association of DAXX with Fas and the protein kinase ASK1, to prevent cell death (15). Additionally, Hsp27 facilitates cell survival via enhanced degradation of proteins by the ubiquitin/proteosome pathway, which modulates the expression of death regulatory...
proteins to regulate apoptosis (16). For example, increasing the proteosome-mediated degradation of I-kBα increases the intracellular content of the transcription factor, nuclear factor-kB (NF-kB), which enhances the expression of anti-apoptotic proteins (17).

Although, the origin and functional links of Hsp27 in malignant tissues have been extensively studied, functional consequences of transiently induced Hsp27 during therapeutic regimens are not completely understood. For example, UV-C (principally 254 nm in wavelength) induces significant cellular damage, primarily producing DNA lesions such as thymidine dimers (18) and photoproducts (19, 20). Eukaryotic cells have developed a number of defense mechanisms to counteract this DNA damage, such as activation of p53 (19). On the other hand, stress-inducible proteins, such as Hsp27, are also expected to be coinduced (21-23). However, the interplay between p53 and the transiently coinduced Hsp27 due to DNA damage in cancer cells during therapeutic regimens, has not been studied, although many studies have found a correlation between the lethality due to DNA damage and Hsp27 expression levels in Hsp27 overexpressed cells (23, 24). Heat shock proteins have been found to play a role in defense mechanisms as shown in heat shock–preconditioned cells that became resistant to UV-B irradiation (25-29), possibly by functioning in nucleotide excision repair (30). This is evident by the difference in the expression of Hsp27 seen in UV-C–resistant and –sensitive human cells (31). However, the molecular mechanisms by which Hsp27 is involved in UV-C resistance have not been fully elucidated.

In an effort to understand the role of Hsp27-mediated cell survival after DNA damage, in the present study, we used UV-induced DNA damage in human adenocarcinoma cells (MCF-7) as a model system. We have found that Hsp27, which is coinduced by activation of HSF-1 along with p53 due to UV-induced DNA damage, plays an important role in protecting the MCF-7 cells in post-UV irradiation. Indeed, we found that UV-induced DNA damage activates HSF-1 and increases Hsp27 expression and facilitates the phosphorylation of p21 by Akt, followed by cell cycle arrest, DNA repair, and mitosis. Although the role of Hsp27 as a negative regulator of p53 activity via modulation of p53 stability has already been reported in the literature, the present results show a new pathway of cancer cell survival, in which late phase induction of Hsp27 enhances Akt stability and p21 phosphorylation in UV-damaged cells. Subsequently, the phosphorylated p21 relocates from the nucleus to the cytoplasm, so that the cells could pass through the checkpoints and proliferate; otherwise, they exit to the apoptotic pathway.

Materials and Methods

Cell culture and UV treatment
MCF-7 cells (American Type Culture Collection) were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (American Type Culture Collection) and antibiotics. Cells were irradiated with a germicidal lamp at a dose rate of 1 J/m²/s for 15 seconds (15 J/m² total) as measured by a Kettering model 65 radiometer (Cole Palmer Instrument, Co.). During irradiation, the cells were covered by a thin layer of PBS. Afterwards, the PBS was removed and fresh growth medium was added and the culture dish was returned to the incubator for the indicated times (i.e., postirradiation time). Control cells were treated with PBS but were not irradiated.

Hsp27 and Akt small interference RNA transfection
MCF-7 cells (2 × 10⁶/plate) were seeded in 100 mm plates and cultured for 24 hours before transfection. After growing in regular growth medium for 24 hours, cells were irradiated with UV and the medium was replaced with small interference RNA (siRNA) containing medium and maintained until they are used at different postirradiation times. Cells were transfected with either human Hsp27 siRNA (5′ GAUCA CCAUCCCGUC ACC 3′) with two bases overhanging at the 3′ end of the antisense strand (Ambion, Inc.; ref. 32) or Akt siRNA (SignalSilence Akt siRNA II). siRNAs were mixed with DharmaFect 4 transfection agent (Ambion) in antibiotic-containing serum-free medium for 20 minutes and then added to the cells at a final concentration of 2.5 nmol/L for Hsp27 and 50 nmol/L for Akt siRNA. A scrambled Hsp27 siRNA (5′ AAUA CAAACUG UUGU CAGCGCUG 3′) and SignalSilence control siRNA were used as controls for Hsp27 and Akt, respectively.

Clonogenic assay
After UV-C irradiation and siRNA treatment, the colony-forming ability of the MCF-7 cells was determined by clonogenic assay as described previously (33).

Western blot analysis
At the indicated times of post-UV irradiation, the cells were trypsinized, washed twice with ice-cold PBS, and lysed in radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitor cocktail. Sixty micrograms of total protein was resolved by 4% to 12% SDS-PAGE, unless stated otherwise, and transferred to polyvinylidene difluoride membranes. Western blot analyses were done using primary antibodies for Hsp27 (Stressgen), p-Hsp 27, Bax, Bcl-2, p21, p53, p15, PARP-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Akt, p-p38MAPK, and p-MAPKAP-2 (Cell Signaling) and enhanced chemiluminescence reagent (Pierce Protein Research Products).

Antibody conjugation and immunoprecipitations
Primary antibody for Hsp27 was conjugated overnight with protein G-agarose beads in PBS at 4°C. The antibody-conjugated beads were washed four times in radioimmunoprecipitation assay lysis buffer and these antibody-conjugated beads were used for immunoprecipitation. For each treatment, 1 mg of total protein was incubated with 8 μL of antibody-conjugated beads in a total volume of 300 μL of lysis buffer. Incubation was carried out for 3 hours.
at 4°C; beads were then washed four times with lysis buffer before being resolved by 4% to 12% SDS-PAGE. Immunoblots were carried out as described above. The reciprocal pull-down experiment was done using the antibody against Akt.

Electrolytic mobility shift assay

The cells were collected at different postirradiation times and nuclear proteins were extracted using a nuclear protein extraction kit (NE-PER) from Pierce. Electrolytic mobility shift assay (EMSA) for HSF-1 was done using an oligonucleotide probe corresponding to a double-stranded heat shock element (HSE) consensus sequence, labeled with biotin in the 5′ end (5′-biotin-CTAGAAGCTTCTAGAAGCTTCTAG-3′). For each sample, 15 μg of extracted nuclear protein was added with 20 fmol of HSE, 2.5% glycerol, 50 ng/μL of poly(dI-dC) and 5 mmol/L of MgCl₂ provided in the EMSA kit (Pierce). This sample complex was incubated at 25°C for 20 minutes and loaded onto 6% precast DNA retardation gel (Invitrogen) and transferred to a nylon membrane. The transferred DNA was cross-linked to the membrane with a hand-held UV lamp for 5 minutes. Finally, the biotin-labeled DNA was detected by chemiluminescence as per the protocol in the EMSA kit (Pierce).

Analysis of p21 using immunofluorescence microimaging

MCF-7 cells were grown on coverslips, UV-C-irradiated with 15 J/m², at indicated times, fixed for 30 minutes with 4% paraformaldehyde in PBS, and permeabilized for 10 minutes with 1% Triton X-100. Cells were then
blocked with 5% bovine serum albumin in PBS containing 0.1% Triton X-100 for 1 hour at room temperature. Next, primary monoclonal p21 (1:100) antibody prepared in PBS containing 0.1% Tween 20 buffer was added, and incubated on the coverslips for 1 hour at room temperature. After washing with PBS, fluorescent Texas red tag–conjugated goat anti-mouse IgG (1:200) secondary antibody was added, and the slides were incubated at room temperature for 1 hour. Slides were further stained for nuclei with 4′,6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were acquired at room temperature with a Nikon fluorescence microscope E80i (Nikon) fitted with filters for Texas red and DAPI. The digital images were captured with a CCD camera and processed using SPOT software (Diagnostic Instruments).

**Cell cycle analysis**

Control and UV-C–irradiated MCF-7 cells were cultured, treated with or without Hsp27 and Akt siRNA, harvested at the indicated times, fixed in ice-cold ethanol (75%) and left at −20°C overnight. Then cells were stained with propidium iodide 10 μL (10 mg/mL) in the presence of 10 μg/mL of RNase A, and analyzed using BD Calibur.

**MTT assay**

Cell viability against UV irradiation after Akt siRNA silencing and control siRNA was determined using (3,4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 96-well plate as described previously (34).

**Results**

**Downregulation of Hsp27 enhances MCF-7 cells susceptibility to UV-C–induced apoptosis**

MCF-7 cells were irradiated with UV (15 J/m²) to create DNA damage. The UV-irradiated cells were analyzed at various postirradiation time points, between 2 and 48 hours. Western blots showed that p53 level peaked at 8 hours postirradiation and reduced gradually from 12 to 48 hours (Fig. 1A and D). The Hsp27 level increased (Fig. 1A and E) during this time showing a negative correlation between Hsp27 and p53 expression in UV-irradiated cells. The Ser82 phosphorylated Hsp27 (Fig. 1A) was also observed to increase with postirradiation time indicating that MAPKAP-2 activity is higher in UV irradiated cells, as it is known to phosphorylate the Ser82 of Hsp27 (35). Hsp27 expression is rapidly upregulated to provide an endogenous defense mechanism to cells. The elevated synthesis of Hsp27 is facilitated by the stress-induced activation of HSF-1, a transcription factor of inducible Hsp27. EMSA confirmed that HSF-1 is activated upon treating with UV and its DNA binding was increased (Fig. 1B).
complex (36), confirming UV irradiation to be a potent inducer of HSF-1 binding to DNA, leading to transcriptional activation of the inducible Hsp27.

The negative correlation of Hsp27 and p53 implies that Hsp27 potentiates the degradation of p53 after 8 hours of postirradiation. It is known that Hsp27 affects MDM-2 to accelerate proteolysis of p53 in drug-treated cancer cells (37). To confirm the role of Hsp27 in p53 degradation in UV-treated cells, Hsp27 was silenced with siRNA after the UV irradiation. In siRNA-treated cells, p53 continued to accumulate up to 48 hours of postirradiation (Fig. 1C and D), although there was obviously no significant accumulation of Hsp27 observed during this time (Fig. 1C and E). However, in scrambled Hsp27 siRNA–treated cells, the Hsp27 and p53 expression patterns were similar to Fig. 1A, D, and E (Fig. 1C). These results confirm that transiently expressed Hsp27, in response to UV-induced DNA damage, does accelerate the degradation of p53. Figure 2A shows colony density in 10 cm² plates, stained with crystalline violet after 24 and 48 hours of postirradiation in UV-irradiated and in UV-irradiated/Hsp27 siRNA–treated cells. As seen in Fig. 2A, in the UV irradiated case, the cells repopulated (almost 100%). However, Hsp27-silenced MCF-7 showed enhanced cell death in response to irradiation. Quantitative estimation (Fig. 2B) shows that the UV-treated cells repopulated almost an equivalent number of colonies, compared with preirradiation, due to the survival of these cells from UV-induced DNA damage.

**FIGURE 3.** Hsp27 knockdown enhances apoptosis after UV irradiation of MCF-7 cells. Western blots of Bax, BCl-2, and p21 in UV-treated cells (A) and in UV and Hsp27 siRNA or scrambled siRNA–treated cells (B), determined at different postirradiation times as indicated. In Hsp27 knockdown cells, the Bax increased but BCl-2 is downregulated with postirradiation time. C and D, quantitative plots of Bax and Bcl-2, obtained in three independent experiments. GAPDH intensity was used to normalize the protein loading. E and F, Western blots of p21 and T145 phosphorylated p21 in UV-irradiated and UV and siRNA-treated cells, respectively. The p21 increased up to 48 h of postirradiation. But T145-phosphorylated p21 was downregulated in Hsp27 siRNA–treated cells.
DNA damage and recovering the active proliferation. However, in Hsp27-silenced cells, >40% colony-forming activity was re-established, whereas in scrambled Hsp27 siRNA–treated cells almost 100% survival was obtained (Fig. 2B). To confirm whether similar results could be obtained with the use of chemotherapeutic agents, normal and Hsp27 silenced MCF-7 cells were treated with doxorubicin and the colony density was determined. As shown in Fig. 2C, the results are similar to UV irradiation, illustrating that the fundamental mechanism is same in both UV induced DNA damage or antineoplastic agents induced DNA damage. These results show that silencing transiently expressed Hsp27 potentiates cell death by UV irradiation in MCF-7 cells through a mechanism involving Hsp27 and p53.

**Hsp27-dependent p53 target genes and G1–S cell cycle arrest**

Because the stability of p53 after UV irradiation is dependent on Hsp27, the level and dependence on Hsp27 of p53 target proteins, such as Bax, Bcl-2, and p21 were determined. As shown in Fig. 3A, the levels of Bax and Bcl-2 increased with time, whereas in Hsp27-depleted cells, Bcl-2 completely disappeared 36 hours post–UV irradiation (Fig. 3B). However, in scrambled Hsp27 siRNA–maintained cells, there was no such loss of Bcl-2 even after 48 hours postirradiation. Quantitative determination showed that Bax and Bcl-2 ratios remained almost the same in UV-irradiated cells (Fig. 3C). However, compared with Bcl-2, a very high level of Bax (∼5-fold increase) was observed in UV-irradiated/Hsp27 siRNA–treated cells (Fig. 3D). The p21 level increased from 8 to 48 hours postirradiation in UV-irradiated/Hsp27 siRNA–treated cells (Fig. 3E and F). Although an increase in p21 was observed in both cells, no phosphorylated p21 could be detected in Hsp27-silenced cells (Fig. 3F). These results indicate that Bax and Bcl-2 induction occurs as an immediate response to UV-C irradiation and the proapoptotic protein Bax increases continuously with time after UV irradiation and that a high level of Bax remains in the presence of Hsp27 siRNA, leading to the conclusion that the Hsp27-dependent p53 target genes and G1–S cell cycle arrest are related to Bax induction. This suggests that silencing Hsp27 potentiates cell death by UV irradiation in MCF-7 cells through a mechanism involving Hsp27 and p53.
irradiation, both in UV-irradiated and UV-irradiated/Hsp27 siRNA–treated cells. However, in UV-irradiated only cells, such an increase in Bax is compensated by an increase in Bcl-2 (Fig. 3A), and the observed downregulation in Bcl-2 in Hsp27 silenced cells (Fig. 3B) increases apoptosis.

Because large differences in the phosphorylation of cell cycle–related proteins such as p21 were noticed (among UV alone–treated and UV-irradiated/Hsp27 siRNA–treated cells), cell cycle analysis after UV-C irradiation was carried out using fluorescence-activated cell sorting. In UV-irradiated cells, G1-S phase cell cycle arrest was observed (Fig. 4A) at 8 hours after exposure to UV irradiation. However, the G1-S phase accumulation was depleted 24 hours postirradiation. Quantitative analysis (Fig. 4B) showed that cells in G1 increased from 50% to 70% by 8 hours postirradiation and decreased to 50% by 24 hours. The G2 and S phase populations decreased from 30% to 20% and 20% to 10%, respectively, and increased back. No apoptotic cell population (sub-G1 phase) was accumulated after 24 and 48 hours post–UV irradiation. In Hsp27 silenced cells, however, there was no obvious cell cycle arrest (Fig. 4C and D). But the apoptotic cell population (sub-G1, apoptotic cells) gradually accumulated with increasing postirradiation time (increased from 2% in unirradiated cells to 6% and 29% after 24 and 48 h of post–UV-C irradiation, respectively; Fig. 4D), whereas the G2-M peak dropped after the onset of apoptosis (Fig. 4C and D). Increased cell death (Fig. 2) and cell cycle arrest upon treating with Hsp27 siRNA indicate that these cells exit to an apoptotic pathway rather than DNA repair or survival. To further substantiate that the cells undergo increased apoptosis upon silencing Hsp27, PARP-1 integrity was determined at various postirradiation times. It has been very well established that caspase-3 activation leads to the cleavage of PARP-1, which can be detected in Western blots by the appearance of a distinct band at 89 kDa indicating cleaved PARP-1 (38). The UV-irradiated MCF-7 showed this cleaved band, but its intensity decreased with time. Indeed at 24 and 48 hours of postirradiation, the uncleaved PARP-1 was higher than the cleaved band intensity (Fig. 5A). Conversely, in Hsp27–silenced cells, the intensity of the cleaved PARP-1 band increased through 48 hours of post–UV irradiation (Fig. 5B), and scrambled siRNA–treated cells showed no change at 24 or 48 hours of postirradiation. Taken together, these results suggest that the loss of Hsp27 enhances cellular susceptibility to UV-C–induced apoptosis. Therefore, Hsp27 is a critical determinant in cell susceptibility to apoptosis induced by UV-C–induced DNA damage.

**Hsp27-dependent Akt stabilization and p21 phosphorylation**

As shown in Fig. 6A, the Akt level increased between 8 and 48 hours post–UV irradiation. Interestingly, during this time, Hsp27 was observed to be high (Fig. 1A). However, there was no such increase in Akt level (Fig. 6B) in Hsp27–silenced cells, suggesting that Hsp27 is required for Akt stabilization in DNA-damaged cells, as an integral part of the DNA repair process in UV-irradiated cells. Moreover, a correlation plot between Hsp27 and Akt blot intensities (pooled together from normal and Hsp27 silenced cells after UV irradiation) shows that there is more or less linear correlation ($r^2 = 0.98$), confirming that Akt stabilization is proportionate to the Hsp27 expression (Fig. 6C). These observations suggest that Hsp27 is pivotal in governing cellular sensitivity to genotoxic stress through orchestrating Akt activity.

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**FIGURE 5.** PARP-1 cleavage upon treating with UV-C. MCF-7 cells were cultured and treated as described in Fig. 1. Western blots of intact PARP-1 and cleaved PARP-1 in UV-treated and UV + siRNA–treated cells. PARP-1 fragment, observed at 89 kDa due to caspase 3 activation, was high at 8 h of postirradiation time, but continued to decrease with postirradiation time. However, in siRNA-treated cells, the intensity of this fragment was increased with time up to 48 h.
To investigate whether there is any direct interaction between Hsp27 and Akt in DNA-damaged cells, immunoprecipitation and immunoblotting experiments were carried out. In UV-treated cell lysates, Hsp27 was immunoprecipitated and immunoblotted with Hsp27 and Akt antisera. In the immunoprecipitate of Hsp27, the Akt was detected and vice versa, showing that they coimmunoprecipitate each other (Fig. 7A and B) due to a protein/protein interaction between them. The Hsp27/Akt interaction increased with increasing Hsp27 levels after postirradiation (Fig. 7A). A positive interaction between Hsp27 and p53 was also observed (Fig. 7A). Such an interaction may lead to proteolytic degradation of p53 as previously established (3). Obviously, there was no such Hsp27/Akt association detected in Hsp27-silenced cells (data not given). A similar interaction was observed with other cell cycle regulators including CDC-25C and p15, and there was a systematic increase in p15 levels but not in CDC-25C with time, as observed for Akt (Fig. 7A).

Cell cycle progression is tightly regulated by the family of cyclin-dependent kinase inhibitors and its expression is induced by the activation of wild-type p53 (39, 40). The cell growth–inhibiting activity of p21 is strongly correlated with its nuclear localization (39-41). Phosphorylation of p21 at T145 by Akt is known to initiate translocation from the nucleus to the cytoplasm, thereby regulating the growth-regulating activity of p21. Because G1-S phase cell cycle arrest was observed in UV-C–irradiated cells (Fig. 4), we determined the localization of p21 at different postirradiation times using fluorescent microscopic imaging. Figure 8A shows microscopic images of dual staining (DAPI and Texas red–tagged IgG against the p21) for both UV-irradiated and UV-irradiated/Hsp27 siRNA cells.
In UV-irradiated cells, p21 was observed in the nuclei for the first 8 hours postirradiation, but disappeared from the nuclei afterwards. But in the case of UV-irradiated/Hsp27 siRNA-treated cells, p21 continued to be present in the nuclei even after 48 hours postirradiation (Fig. 8B). These results indicate that in siRNA-treated cells, the possible T145 phosphorylation of p21 did not occur and thus the translocation of p21 to the cytoplasmic region did not proceed to remove cell cycle arrest, as observed in fluorescence-activated cell sorting analysis (Fig. 4).

At about 24 h onwards, no significant p21 was detected in the nuclei suggesting that Hsp27-assisted phosphorylation of p21 by Akt removes it from the nucleus. In UV + siRNA-treated cells (bottom), p21 was localized in the nucleus even after 48 h, suggesting that the absence of Hsp27 does not favor p21 phosphorylation by Akt and the p21 resides in the nucleus for longer times, forcing the cells to exit to the apoptotic pathway.

Bcl-2 expression was found to be affected by silencing Akt. As seen in Fig. 9B, Bcl-2 decreased with increasing postirradiation time, whereas control Akt siRNA (Fig. 9B) or no siRNA treatment showed (Fig. 3A) increased Akt with increasing postirradiation time. However, total Bax and p21 levels were unaffected by Akt siRNA (Fig. 9B). Interestingly, p21 phosphorylation is completely inhibited by Akt silencing (Fig. 9B), whereas there was no change in the pattern of its expression in control siRNA–treated cells (Fig. 9B) and no siRNA–treated cells (Fig. 3E). These results, in combination with the data from Hsp27-silenced cells (Fig. 3E), show that Hsp27 is required for protection from degradation/denaturing of Akt and maintaining the phosphorylation of p21. Furthermore, p21/DAPI immunofluorescence confirmed that the Akt siRNA–treated cells retained p21 in nuclei (Fig. 9C). The MTT assay summarized in Fig. 9D showed increased loss of cells in Akt siRNA–treated cells. Finally, >10% of the cell population was found to be apoptotic as detected

Hsp27 expression is independent of Akt in UV-treated MCF-7 cells. However, Bcl-2 expression was found to be affected by silencing Akt. As seen in Fig. 9B, Bcl-2 decreased with increasing postirradiation time, whereas control Akt siRNA (Fig. 9B) or no siRNA treatment showed (Fig. 3A) increased Akt with increasing postirradiation time. However, total Bax and p21 levels were unaffected by Akt siRNA (Fig. 9B). Interestingly, p21 phosphorylation is completely inhibited by Akt silencing (Fig. 9B), whereas there was no change in the pattern of its expression in control siRNA–treated cells (Fig. 9B) and no siRNA–treated cells (Fig. 3E). These results, in combination with the data from Hsp27-silenced cells (Fig. 3E), show that Hsp27 is required for protection from degradation/denaturing of Akt and maintaining the phosphorylation of p21. Furthermore, p21/DAPI immunofluorescence confirmed that the Akt siRNA–treated cells retained p21 in nuclei (Fig. 9C). The MTT assay summarized in Fig. 9D showed increased loss of cells in Akt siRNA–treated cells. Finally, >10% of the cell population was found to be apoptotic as detected

(presented images are superimposed red and blue fluorescence). In UV-irradiated cells, p21 was observed in the nuclei for the first 8 hours postirradiation, but disappeared from the nuclei afterwards. But in the case of UV-irradiated/Hsp27 siRNA–treated cells, p21 continued to be present in the nuclei even after 48 hours postirradiation (Fig. 8B). These results indicate that in siRNA-treated cells, the possible T145 phosphorylation of p21 did not occur and thus the translocation of p21 to the cytoplasmic region did not proceed to remove cell cycle arrest, which occurred in Hsp27-unsilenced cells. Thus, the cells are forced to undergo apoptosis when treated with Hsp27 siRNA. These results also indicate that Hsp27-dependent p21 phosphorylation at T145 by Akt is critical in determining its cellular localization.

The addition of Akt siRNA further confirmed the results of p21 phosphorylation, described above. Akt was observed to decrease with postirradiation time; whereas it increased in control siRNA maintained cells (Fig. 9A). Also, neither Hsp27 expression nor Hsp27 phosphorylation pattern was affected by Akt silencing, suggesting that Hsp27 Regulates Akt/p21-Assisted Cell Survival

Mol Cancer Res; 8(10) October 2010

www.aacrjournals.org

Published OnlineFirst September 21, 2010; DOI: 10.1158/1541-7786.MCR-10-0181
by flow cytometry analysis in Akt siRNA–treated cells (Fig. 9E). Overall, results in Fig. 9 establish that Hsp27-assisted Akt phosphorylation of p21 is required for survival after UV irradiation.

The levels of protein kinases that can phosphorylate Hsp27, i.e., p-p38MAPK and its downstream p-MAPKAP-2, were also determined in UV-irradiated cells, as summarized in Fig. 10. The p-p38MAPK started to appear at 8 hours postirradiation and increased with time (Fig. 10A). This increase was in parallel with the Hsp27 expression (Fig. 1A and B). However, in Hsp27 siRNA–treated cells, p-p38MAPK was not increased at any of the time points studied (Fig. 10B). Similarly, p-MAPKAP-2 was also observed to be high at 8 hours postirradiation, whereas in siRNA-treated cells there was no UV irradiation–induced change in p-MAPKAP-2 (Fig. 10A and B).

**Discussion**

The primary finding of the present work is that the survival of MCF-7 cancer cells, through p53 activation...
expression of Hsp27 did not confer resistance to UV-A
controversial. T rautinger et al. (27, 28) reported that over-
the deleterious effects of UV irradiation is somewhat
senescence (37).

Recently, it was shown that overexpression of
drugs. Recently, it was shown that overexpression of
Hsp27 has a protective role against the genotoxicity
induced upon treatment with UV-C (Fig. 1A). Although the Hsp27 level is increased after 8 hours of post–
UV irradiation, induced p53 (Fig. 1A) degraded during this
period of time. Hsp27-assisted proteosomal degradation of
p53 has been reported in many previous studies; most of
these studies used Hsp27-overexpressing cells. Similarly,
silencing Hsp27 using siRNA has been found to increase
the stability of p53 and to accelerate cancer cell death
(37). However, the exact mechanism of the transient induc-
tion of Hsp27 in response to DNA damage such as UV
irradiation, and its interaction with p53, has not been stud-
before. Recently, it was shown that Hsp27 inhibited
radiation-induced apoptosis by inhibiting protein kinase C
(PKC)-mediated reactive oxygen species (46). Other studies
have reported that Hsp27 is induced by p53, and as such,
Hsp27 is one of the p53 target genes (47). Contrarily,
Hsp27 has been found to be independently induced and
play an important role in transactivating p53 (48). In
the present work, we provide evidence that Hsp27 is inde-
pendently induced and indeed that Hsp27 plays an impor-
tant role in p53 degradation after UV irradiation. First,
we observed that HSF-1, the transcription factor that is
responsible for Hsp27 induction, is activated (Fig. 1B).
Second, we found that silencing Hsp27 preserved the p53
protein level (Fig. 1C). We observed a negative correlation
between the Hsp27 and p53 protein levels in the present
work (Fig. 1A). It was also found that p21 phosphoryla-
tion by Akt is dependent on Hsp27 level (Fig. 3E and F). Thus, it
seems that the transient increase in Hsp27 in cancer cells not
only serves to transactivate p53 and subsequently suppress
apoaptosis but also regulates the activities of p53 target genes
such as p21. In spite of the fact that p53 is stabilized in
Hsp27-silenced cells (Fig. 1C), the Akt level is also depleted
(Fig. 6C).

Another important observation made in the present
study is the differential response of the antiapoptotic pro-
tein Bcl-2 expression and cell cycle arrest, depending on
the level of Hsp27 in UV irradiated MCF-7 adenocarci-
oma cells (Fig. 4A and C). The p53 target proteins such as
p21, Bax, and Bcl-2 are indirectly dependent on the level
of Hsp27 in UV irradiated MCF-7 adenocarcino-
ma cells (Fig. 4A and C). The p53 target proteins such as
p21, Bax, and Bcl-2 are indirectly dependent on the level
of Hsp27 (Fig. 3). Because the life span of p53 is influ-
enced by Hsp27, the Bcl-2 level is decreased in siRNA-
treated cells, an indication that p53 transcriptional activity
is modulated by Hsp27. Although p21 expression is unaf-
fected upon silencing Hsp27, the T145-phosphorylated
p21 level is reduced in these Hsp27-silenced cells (Fig. 3F).

The involvement of HSPs in cellular resistance to the deleterious effects of UV irradiation is somewhat
controversial. T rautinger et al. (27, 28) reported that over-
expression of Hsp27 did not confer resistance to UV-A–
and UV-B–induced cell death in squamous cell carcinoma.
On the other hand, another study showed that human
cells tolerate damage caused by UV-B irradiation if they
are pretreated with heat (26, 43, 44). T rautinger et al. (27)
showed that inducible HSPs play roles in cellular
responses after UV irradiation.

p53 accumulation and expression of the mRNAs of
matrix metalloproteinases and collagenase were reported
to be induced following UV irradiation in human cells
(21, 45). In the present work, we observed that p53 and
Hsp27 are coinduced upon treatment with UV-C (Fig. 1A).

Second, we found that silencing Hsp27 preserved the p53
protein level (Fig. 1C). We observed a negative correlation
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study is the differential response of the antiapoptotic pro-
tein Bcl-2 expression and cell cycle arrest, depending on
the level of Hsp27 in UV irradiated MCF-7 adenocarci-
oma cells (Fig. 4A and C). The p53 target proteins such as
p21, Bax, and Bcl-2 are indirectly dependent on the level
of Hsp27 (Fig. 3). Because the life span of p53 is influ-
enced by Hsp27, the Bcl-2 level is decreased in siRNA-
treated cells, an indication that p53 transcriptional activity
is modulated by Hsp27. Although p21 expression is unaf-
fected upon silencing Hsp27, the T145-phosphorylated
p21 level is reduced in these Hsp27-silenced cells (Fig. 3F).

This is once again in spite of the fact that p53 is stabilized
even after 24 hours postirradiation in Hsp27-silenced cells.

Figure 10. p-p38MAPK and p-MAPKAP-2 in UV-irradiated MCF-7 cells.
A, Western blots of p-p38MAPK and p-MAPKAP-2 in UV-irradiated
and UV + Hsp27 siRNA-treated cells. In UV-treated cells, p38MAPK
is increasingly phosphorylated up to 48 h of postirradiation. Also,
p-MAPKAP-2 increased with postirradiation time. B, same as A, but
maintained in Hsp27 siRNA medium. Very low p-p38MAPK and no
p-MAPKAP-2 were noticed.
Thus, it is clear that Hsp27 is essential for Akt activity in DNA-damaged cells, leading to the phosphorylation of p21 as a survival pathway. Furthermore, Hsp27 is found to coimmunoprecipitate with Akt (Fig. 7A). Indeed, the results indicate that the protein level of Hsp27 correlates with the extent of cell survival after UV irradiation. Hsp27 had been observed to protect Akt and enhance its phosphorylation of p21 (Fig. 3A and B). Silencing Hsp27 depleted Akt and increased apoptosis (Fig. 4A and C). Recently, it was shown that phosphorylation of Akt by MAPKAP-2 was enhanced by the Hsp27 association (13, 14, 49). Prior dissociation of Hsp27 before the phosphorylation was shown to induce apoptosis. Also, it was shown that cisplatin-resistant L929 cells survived by overexpressing Hsp27 through Akt activation (50). In the present study, we show that the interactions of Hsp27 with Akt is a critical determinant of Akt stabilization, as the removal of Hsp27 prevents Akt stabilization and results in apoptosis in UV-C-irradiated MCF-7 cells (Fig. 4A and C).

The cellular localization of p21 was recently proposed to regulate p21 function (51). The mechanism of cellular translocalization of p21 was shown by Zhou et al. (39), wherein phosphorylation of p21 by Akt at T145 was found to relocate p21 to the cytoplasm and suppress growth-inhibitory activity. Cytoplasmic p21 has been shown to form a complex with ASK1 to inhibit the stress-induced MAPK cascade, which results in resistance to apoptosis induced by many stimuli (52). Thus, our results, together with the previous findings (52), support this mechanism for the Hsp27-assisted antiapoptotic effect of T145-phosphorylated p21. Based on our results, we propose a novel mechanism of survival of MCF-7 adenocarcinoma cells after UV-induced DNA damage, orchestrated by Hsp27, as shown in Fig. 11.

**FIGURE 11.** Schematic illustration of Hsp27 acting as a switch between cell death and survival after UV-C irradiation. UV irradiation triggers three major signaling events that are relevant to the present work: (1) it causes DNA damage and p53 activation, (2) HSF-1 activation and transcription of heat shock proteins such as Hsp27, and (3) activation of MAPK. The UV-induced p53 is transactivated to induce its target genes such as Bax, Bcl-2, p21, etc. p21 involves cell cycle arrest at G1–S phase and leads to apoptosis (cell death pathways). On the other hand, UV-induced Hsp27 is phosphorylated by p38MAPK/MAPKAP-2 (also induced by UV). The p-Hsp27 plays multiple roles. It can negatively regulate p53. Also, the p-Hsp27 chaperones Akt and stabilizes it. The Hsp27-stabilized Akt phosphorylates p21 and translocates it to the cytoplasm, removing the cell cycle arrest so that the cells can actively go through the checkpoints (cell survival pathway). Silencing Hsp27 allows p53 to survive longer and the continued presence of p21 in the nucleus for longer times, arresting the cell cycle and leading to cell death. Thus, Hsp27 acts as a switch between cell death and survival and indeed removal of transiently induced-Hsp27 could be an effective approach in UV-C therapy.
In summary, the results of the present study illustrate that transcriptional activity of UV-induced p53 is regulated by coinduced Hsp27, so that DNA damage could be fixed and cells can survive. Hsp27, appearing after the depletion of p53, enhances Akt stabilization and the phosphorylation of p21. Such a phosphorylation results in the translocation of p21 from the nuclei to the cytoplasm, removing the cell cycle arrest, so that the cells proliferate normally (Fig. 11). Depletion of Hsp27 enhances cell death due to inadequate phosphorylation of p21 and its inability to translocate to the cytoplasm to facilitate active proliferation, and hence, the cells exit to apoptosis. Thus, Hsp27 acts as a switch between apoptosis and survival by modulating Akt stability. A limitation of the present work, however, is that knockdown of Hsp27 affects a broad spectrum of proteins in terms of stability/folding. This study illustrates a mechanism concerning Hsp27/Akt, whereas the other possibilities cannot be ruled out.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The services of DHLRI core facilities, namely, Proteomics, Flow Cytometry and Microscopy, are gratefully acknowledged.

Grant Support

NIH grant R21 EB004658 and American Heart Association grant BGIA 0365203B (G. Ilangoan).

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Received 04/27/2010; revised 07/27/2010; accepted 08/16/2010; published OnlineFirst 09/21/2010.

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Mol Cancer Res 2010;8:1399-1412. Published OnlineFirst September 21, 2010.

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