Physiological and Genetically Engineered Expression Modulation Methods Do Not Affect Cellular Levels of the Heat Shock Protein HSP60 in Prostate Cancer Cells

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Abstract. *Background/Aim:* Heat shock proteins (HSP) play a crucial role in the cellular responses during stressful conditions. In addition, HSP are involved in the regulation of a variety of important signaling pathways and processes as well as many pathological conditions, including cancer. In prostate cancer (PC), HSP60 is associated with poor differentiation and prognostic clinical parameters, such as high Gleason score, initial serum prostate-specific antigen levels, and lower cancer-specific survival. In this study, we investigated the regulation of HSP60 protein in PC. *Materials and Methods:* LNCaP or PC3 cells were treated with androgens or transfected with vectors containing microRNA-1 (miR-1), HSP60, HSP60-specific short-hairpin RNA (shHSP60), or a miR-1 inhibitor. The change in HSP60 protein levels was examined using Western blot. *Results:* Treatment of PC cells with androgens did not alter the HSP60 protein levels. Modulation of miR-1 levels in LNCaP cells also did not affect the HSP60 protein. Furthermore, HSP60 levels could not be modified by overexpression or short hairpin RNA. *Conclusion:* It was found that neither physiological factors, such as androgens and the HSP60-specific miR-1, nor overexpression and knockdown systems could influence the HSP60 protein levels. These results suggest an essential role of HSP60 in PC cells, as its protein expression status is regulated very precisely.

The members of the heat shock protein (HSP) family are induced under stress conditions, such as heat and cold shock, toxic agents, and UV radiation (1). However, in addition to their primarily cytoprotective function, HSPs also exert important roles in physiological signal transduction. For example, HSP27 can interact with transcription factors, such as the signal transducer and activator of transcription 5 (STAT5) and the androgen receptor (AR) and modulates their transcriptional activities (2, 3).

HSP60 is involved in numerous pathological processes, including inflammation, autoimmune diseases, neurodegenerative diseases, and cancer (4). The protein is primarily localized in mitochondria but is also found in the cytosol and other cell organelles (4, 5). Mitochondrial HSP60 is critical for maintaining the respiratory chain functionality and, thus, has essential roles in cellular energy metabolism (5, 6). In tumor tissues, HSP60 can be both upregulated (breast cancer, ovarian cancer) and downregulated (renal cell carcinoma, bladder cancer) (7-11) and elicit cytoprotective as well as pro-apoptotic cell responses (12, 13). In prostate cancer (PC), HSP60 expression correlates with poor differentiation and clinical parameters, such as Gleason score, serum prostate-specific antigen, and tumor-specific survival (7). Furthermore, HSP60 suppresses the activity of apoptosis-specific caspases and, thus, the induction of apoptosis (14).

The present study aimed to characterize the stability of HSP60 protein expression in an *in vitro* PC cell model. For this purpose, we attempted to modulate HSP60 expression levels by physiological noxious agents and genetic engineering techniques.

Materials and Methods

Cell culture. Human PC cells, LNCaP and PC3, were obtained from the American Type Culture Collection (ATCC, Rockville, USA) (15). The cells were cultured in RPMI 1640 medium (Life

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Figure 1. Cloning strategy of (A) pGEX-6P-1-HSP60, (B) pcDNA3.1(+)-HSP60, and (C) pExpress-HSP60.

Technologies, Darmstadt, Germany) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES-buffer, 1% GlutaMax, and 1% penicillin/streptomycin (Fisher Scientific, Schwerte, Germany) at 37°C and 5% CO₂. For stimulation with dihydrotestosterone (DHT), cells were incubated for 72 h (37°C, 5% CO₂) in the presence of 10 µM DHT (Sigma-Aldrich, Munich, Germany).

Protein analysis. After the indicated incubation times, cells were harvested by treating with 100 µl lysis buffer [50 mM Tris-HCl (pH 6.8, v/v), 2% sodium dodecyl sulfate (w/v), 10% glycerol (v/v), 0.01% bromophenol blue (w/v), 5% 2-mercaptoethanol (v/v)]. The protein lysate (20 µl) was separated using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (Mini-Protein System, BioRad, München, Germany). The proteins were subsequently transferred to a Protean nitrocellulose membrane (Whatman, Dassel, Germany) using the semi-dry Trans-Blot SD transfer cell (BioRad). After blocking the membranes with Roti-block blocking solution (Carl Roth, Karlsruhe, Germany), proteins were detected using specific antibodies. Signals were visualized using SuperSignal West Dura chemiluminescent substrate (Thermo Scientific; Waltham, MA, USA) in a ChemiDoc system (BioRad) with Image Lab 5.1 beta software (BioRad). The antibodies that were used in this study are: HSP60 (D307) Antibody #4870 (1:1,000, Cell Signaling

Technology, Danvers, MA, USA); GAPDH (14C10) Rabbit mAb #2118 (1:10,000, Cell Signaling Technology); Anti-rabbit IgG, HRP-linked Antibody #7074 (1:5,000, Cell Signaling Technology).

Cloning of HSP60-specific DNA vectors. To clone the full-length sequence of HSP60, total RNA was isolated from LNCaP cells (peqGOLD TriFast, Peqlab, Erlangen, Germany), transcribed into cDNA (RevertAid First Strand cDNA Synthesis Kit, Fermentas, St. Leon-Rot, Germany) and cloned into the DNA vector pGEX-6P-1 (NovoPro Bioscience, Shanghai, PR China; Figure 1A). HSP60 cDNA was amplified using specific oligonucleotides binding at the 5' end (5'-AAAAGGATCCATGCTTCGGTTACCCACAGTC-3') and the 3' end (5'-AAACTCGAGTTAGAACATGCCACCTCCCATAC-3') of the open reading frame of HSP60. The oligonucleotides contained specific recognition sites for restriction endonucleases (5' end: BamHI; 3' end: XhoI). The BamHI-HSP60-XhoI PCR product was double digested (BamHI/XhoI; Fermentas), purified (NucleoSpin Gel and PCR Cleanup Kit; Macherey-Nagel, Düren, Germany) and ligated (T4 DNA Ligase; Fermentas) into a BamHI/XhoI linearized pGEX-6P-1 vector. Vector amplification was carried out in transformed XL-1 Blue competent E. coli (Agilent Technologies, Santa Clara, CA, USA) propagated in Luria-Bertani (LB) medium containing 100 µg/ml ampicillin (Carl Roth).

The HSP60 open reading frame sequence was re-cloned into eukaryotic expression vectors using the standard methods of recombinant DNA technology described above. For this purpose, the pGEX-6P-1-HSP60 vector DNA was digested with BamHI/XhoI and HindIII/NheI (all Fermentas) and cloned into the corresponding linearized vectors pcDNA3.1(+) (Thermo Scientific; Figure 1B) and pExpress (NovoPro Bioscience; Figure 1C), respectively.

For knockdown experiments, a customized HSP60-specific shRNA vector was constructed using the standard cloning methods described below. Two oligonucleotides containing the cDNA sequence of shHSP60 (5'-GATCCCCTGTACAAAGTAGAGAA GTATTCAAGAGATACTTCTCTACTTTGTACATTTTTA; 5'-TCG ATAAAAATGTACAAAGTAGAGAAAGTATCTCTTGAATACTTC TCTACTTTGTACAGGG) were hybridized, resulting in overhangs that mimicked BglII- and XhoI-cleaved ends (Figure 2). After purification (NucleoSpin Gel and PCR Clean-up Kit; Macherey-Nagel), the hybridization product was ligated (T4 DNA Ligase; Fermentas) into a BglII/XhoI-digested pSuperior vector (OligoEngine, Seattle, WA, USA). DNA vector amplification was done in XL-1 Blue competent *E. coli* (Agilent Technologies) in LB Medium with 100 µg/ml ampicillin (Carl Roth) and the correct insert was verified using commercial sequencing (Qiagen, Hilden, Germany). For the overexpression of miR-1, the expression vector pmiR-1 was used (16).

Transfection experiments. For transfection, 150,000 cells/well were seeded into 6-well cell culture plates. Cells were transfected using Lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) with a total amount of 5 µg plasmid DNA or using siLentFect (BioRad) with 40 nM miR-1 inhibitor (Qiagen), respectively. Empty vectors, pcDNA3.1(+) (Thermo Scientific), pExpress (NovoPro Bioscience), and pSuperior (OligoEngine), served as control.

Statistics. Statistical analyses were conducted using the unpaired Student's t-test. Data are expressed as mean±SD, and results of $p < 0.05$ were considered significant.

Results

As androgens play a critical role in PC, we investigated the influence of DHT on HSP60 protein expression. For this purpose, following treatment of LNCaP and PC-3 cells with 10 µM DHT for 48 h alterations in intracellular HSP60 levels were examined by Western blot (Figure 3A). Quantification of the results showed only highly modest and statistically insignificant changes in the HSP60 expression in the presence of DHT.

Besides hormones, microRNAs are also critical regulators of cell physiology by controlling the translation or stability of their target proteins (17). For example, MicroRNA miR-1 has been described as a direct regulator of the HSP60 expression in cardiomyocytes (18). To evaluate whether miR-1 also controls the HSP60 expression in PC cells, transfection experiments were performed using a specific miR-1 inhibitor and a miR-1 overexpression vector. Our analysis showed that both inhibition and overexpression of miR-1 did not affect cellular HSP60 protein levels in PC cells (Figure 3B).

Figure 2. Cloning strategy of pSuperior-shHSP60.

Since affecting physiological factors did not cause detectable alterations in HSP60 expression, we attempted to modulate HSP60 levels using genetic engineering. Two established overexpression vectors [pcDNA3.1(+) and pExpress] as well as a knockdown vector, optimized for the expression of short shRNA (pSuperior), were used for this purpose. The custom cloned HSP60-specific vectors were transfected into LNCaP cells and intracellular HSP60 protein levels were measured. In the first 24 h following pcDNA3.1(+)-HSP60 transfection, there was a tendential but insignificant increase in HSP60 expression (Figure 4A), however, this could not be confirmed 48 h and 72 h after transfection. The use of the vector pExpress-HSP60 also did not lead to an increase in HSP60 levels (Figure 4B). In a *vice versa* approach, we pursued the downregulation of HSP60 expression, by transfecting PC cells with the HSP60 knockdown vector pSuperior-shHSP60. Interestingly, no decrease in intracellular HSP60 levels could be detected during the incubation period (Figure 4C).

Figure 3. DHT and miR-1 have no effect on HSP60 protein levels in PC cells. Relative HSP60 Western blot analysis of (A) LNCaP and PC-3 cells after incubation with 10 μ M DHT and (B) LNCaP cells after inhibition (miR-1 Inh) and overexpression (miR-1 OExp) of the regulatory microRNA miR-1. HSP60 expression was normalized to the reference protein GAPDH and presented as mean $\pm$ SD. All experiments were performed at least 3 times.

Discussion

The primary function of HSPs is to protect cells under sublethal stress (19). In addition, members of the HSP family have been associated with cancer growth as they can promote tumor cell proliferation and inhibit cellular death pathways. High expression of HSP27 and HSP70 have, in addition, been associated with drug resistance as well (20). In PC, HSP27 levels increase following androgen ablation, and high HSP27 levels promote the development of castration-resistant PC (21). Increased HSP27 and HSP60 protein expression has also been identified as a predictor of biochemical recurrence after radical prostatectomy, and modulation of HSP expression has been discussed as a potential new therapeutic approach to control tumor cell proliferation, including improved survival (22, 23). Suppression of HSP27 leads to the downregulation of AR in PC cells and, thus, inhibits its oncogenic activity (2). An increase in HSP60 has also been found in PC, especially in patients with poor prognosis (7, 22, 23). Taken together, these data suggest that HSP60 could also be a potential therapeutic target in these patients. This study, however, indicates that, unlike HSP27, the protein expression of HSP60 is probably not controlled by androgens (21). Because androgens primarily regulate tissue-specific genes, this finding suggests that HSP60 plays a general role in cell homeostasis. In addition, there are no data that other therapeutic agents affect HSP60 protein levels in PC or even other tumors, such as ovarian cancer, osteosarcoma, and bladder cancer (24-27). Despite this, there is strong evidence that HSP60 is upregulated in chemoresistant tumor cells and is involved in ovarian cancer chemo resistance (14, 28).

One of the direct regulators of HSP60 is miR-1, with even low miR-1 levels being related to recurrent PC (18, 29, 30). Consistent with the low miR-1 expression, increased HSP60 protein has been correlated with the biochemical recurrence of PC (7, 22, 23). Thus, it can be suggested that miR-1 regulation plays a role in altering HSP60 protein levels in PC. However, direct regulation of HSP60 by miR-1 was not detected in the PC cell line LNCaP. The fact that the protein levels did not change after treatment with an intrinsic HSP60 regulator suggests a robust regulatory mechanism that maintains HSP60 protein levels at a constant level, possibly using an alternative pathway in the presence of miR1. This steady-state regulation for constantly expressing a protein with pivotal function has also been previously demonstrated for other factors, such as tumor suppressor p53, thiamine thiazole synthase (THI1), and genomes uncoupled 4 (GUN5) (31-33).

Since our effort to regulate HSP60 protein levels using physiological factors had no effect, we used genetic engineering. Using two different overexpression systems, however, detected no significant changes in the HSP60 protein levels. Because both expression systems are based on the cytomegalovirus (CMV) promoter, which is considered as one of the strongest promoters *in vitro*, this result suggests a strong counter-regulation of HSP60 upon its overexpression (34). However, there is a possibility that the CMV promoter is silenced in cancer cells (35). Therefore, an HSP60-specific shRNA was expressed in LNCaP cells, controlled by an H1 RNA polymerase III promoter. This knockdown approach also showed no alteration in HSP60 expression. However, it was striking that similar to 24 h after transfection of the pcDNA3.1(+)-HSP60 overexpression vector, a trend towards a decrease in HSP60 level was also

Figure 4. The genetically engineered modulation of HSP60 expression by short hairpin RNA technology did not affect intracellular HSP60 levels in LNCaP cells. Relative HSP60 Western blot analysis of LNCaP cells after transfection with (A) the HSP60 overexpression vectors pcDNA3.1(+)-HSP60 (B) and pExpress-HSP60 (C) and the HSP60 knockdown vector pSuperior-shHSP60. HSP60 expression was normalized to the reference protein GAPDH and presented as mean $\pm$ SD.

observed 24 h following the knockdown approach. This result could imply that the genetically engineered modulation of HSP60 expression is very rapidly counter-regulated.

Li *et al.* have used synthetic double-stranded siRNA molecules directed against HSP60 mRNA, transfected into rat pancreatic tissue cells (36). Although a significant reduction of HSP60 mRNA to 0.5-fold was exhibited, at the protein level the down-regulation was only about 0.7-fold of the control approach. In breast cancer cells, a modest suppression of HSP60 expression was also detectable after transfection with commercial HSP60-specific siRNA molecules, but the authors did not quantify and statistically analyze their data (37). In the case of the pSuperior-shHSP60 vector used in the present study, it cannot be excluded that the effect of the HSP60-specific shRNA was not efficient enough. miR-1 is a specific physiological repressor of HSP60 expression (18). Using the miR-1 overexpression vector pmiR-1, which is also based on pSuperior, no downregulation of HSP60 protein expression was demonstrated either. Since the sequences of the pSuperior constructs for the knockdown of HSP60 as well as for the expression of miR-1 were verified by sequencing, sequence

errors in the regulatory and coding regions of the DNA plasmids can be excluded. Furthermore, the RNA expression system pSuperior is an established commercial vector that has also been frequently used successfully by our group for expression modulation (16, 38-42). The protein overexpression vectors pcDNA3.1(+) and pExpress used for overexpression of HSP60 are also well-characterized and very effective protein expression systems. Nevertheless, neither overexpression system showed significant upregulation of HSP60 synthesis in PC cells. This result supports the hypothesis that constant HSP60 expression in PC cells is important for cellular physiology and is, therefore, very tightly regulated.

In this study, HSP60 protein regulation was investigated in different PC cell lines. It was found that neither physiological factors, such as androgens and the HSP60-specific miR-1, nor overexpression and knockdown systems could influence its protein levels. These data may indicate that a constant expression level of HSP60 protein in progression in PC is important for cell malignancy. Deviations from this constant expression level appear to be counter regulated by as yet unknown cellular mechanisms.

Conflicts of Interest

The Authors declare that they have no competing interests.

Authors' Contributions

Conceptualization: HHHE, MBS; methodology: HHHE, AS; formal analysis: HHHE, AS, AM, MBS; supervision: AM, MBS; writing original draft: HHHE, MBS; original draft review and editing: HHHE, AM, MBS.

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