Immediate and transient phosphorylation of the heat shock protein 27 initiates chemoresistance in prostate cancer cells

MATTHIAS B. STOPE1*, MARTIN WEISS1*, MELANIE PREUSS1, ANDREAS STREITBÖRGER1, CHRISTOPH A. RITTER2, UWE ZIMMERMANN1, REINHARD WALTHER3 and MARTIN BURCHARDT1

1Department of Urology, University Medicine Greifswald, Greifswald; 2Institute of Pharmacy, Ernst-Moritz-Arndt-University of Greifswald, Greifswald; 3Department of Medical Biochemistry and Molecular Biology, University Medicine Greifswald, Greifswald, Germany

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Correspondence to: Dr Matthias B. Stope, Department of Urology, University Medicine Greifswald, Ferdinand-Sauerbruch-Strasse, D-17475 Greifswald, Germany
E-mail: matthias.stope@uni-greifswald.de

*Contributed equally

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Abstract. Drug resistance minimizes the effects of prostate cancer (PC) chemotherapy with docetaxel and is generally considered to be associated with the expression of heat shock protein (HSP) 27 including various cytoprotective pathways. In the present study, we investigated the effects of HSP27 phosphorylation on PC cell growth underlying docetaxel treatment. Cell counting revealed significantly reduced cell growth during docetaxel treatment as a result of both activation of mitogen-activated protein kinase p38 (MAPK p38) and protein kinase D1 (PKD1), and, most importantly, the overexpression of the phosphorylation-mimicking mutant HSP27-3D. Further analysis revealed a docetaxel-dependent induction of HSP27 accompanied by an initial phosphorylation and rapid dephosphorylation of the protein. Based on the data, we can conclude that phosphorylation of HSP27 protein is a crucial mechanism in the initiation of chemoresistance in PC. Moreover, the results indicate a key impact of HSP27 on viability and proliferation of PC cells underlying anticancer therapy. The protective function depends on the initial phosphorylation status of HSP27 and represents a putative co-therapeutic target to prevent chemoresistance during docetaxel therapy.

Introduction

Although prostate cancer (PC) remains one of the most diagnosed malignant diseases and is the second-leading cause of tumor-associated mortality in males in the Western hemisphere (1), progression mechanisms of PC are only marginally understood. Phenotypically, it differs from a localized hormone-naive state to an advanced, castration-resistant and metastatic state that is predominantly attributed to cellular proliferation in a low-level steroid hormonal environment. The first line drug docetaxel is an option for castration-resistant PC treatment; however, efficacy varies between patients and therapeutic outcome is often unsatisfying (2). Heat shock protein (HSP) 27 has been identified as controlling anti-therapeutic mechanisms by specific alterations in proliferation, cell cycle regulation, apoptosis and general stress response in cancer (3-5). In PC, it has been demonstrated that critical events in tumor progression, such as epithelial-to-mesenchymal transition, metastasis, androgen receptor (AR) signaling and treatment resistance, are promoted by HSP27 activities. Consequently, attenuation of HSP27 by newly developed inhibitors is a promising approach in the treatment of advanced PC (6). Regulation of cell physiology as well as dysregulation in malignant cells frequently depends on protein phosphorylation controlled by a coordinated network of specific kinase and phosphatase activities. HSP27 protein contains three regulatory phosphorylation sites located at the positions serine-15, -78 and -82 which are phosphorylated in the presence of diverse cellular signals, e.g. in response to oxidative and pro-inflammatory stress (7,8). In PC cells, HSP27 is predominantly phosphorylated by the mitogen-activated protein kinase p38 (MAPK p38), mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK-2) pathway and by protein kinase D1 (PKD1) (9-11). Although HSP27 is estimated to orchestrate pivotal functions in PC progression and therapy, only little is known about HSP27 activity modulated by protein phosphorylation. Hassan et al described HSP27 phospho-status at serine-82 leads to suppress AR signaling in PC cells (9); however, further HSP27 functions regulated by protein phosphorylation remain unclear. Our previous studies of HSP functionality in PC progression showed HSP27 to have potent effects on AR activity (12,13). Since HSP27 has been suggested to play crucial roles in the initiation and development of chemoresistance, we hypothesized that HSP27 functionality may be associated with specific protein phosphorylation/dephosphorylation. In the present study, we established a PC cell model system containing PC-3 and PC-3 cells stably overexpressing HSP27 to examine the input of HSP27 phosphorylation to HSP27-driven cytoprotective properties of docetaxel-induced chemoresistance.
Materials and methods

Tumor cell lines and chemicals. Human epithelial PC cancer cell line PC-3 (Cell Lines Service; CLS, Eppelheim, Germany) was grown in RPMI-1640 media with 10% fetal bovine serum and 1% penicillin/streptomycin (PAN-Biotech, Aidenbach, Germany) in a 5% CO₂ atmosphere at 37°C. PC-3-HSP27 cells stably overexpressing HSP27 were selected with 400 µg/ml G418 (Carl Roth, Karlsruhe, Germany) as previously described (12). For co-transfection experiments, docetaxel was purchased from Sigma-Aldrich (Munich, Germany), bryostatin-1 (3x10⁻⁶ M) from Tocris Bioscience (Minneapolis, MN, USA), CID755673 (5x10⁻⁵ M) from Axon Medchem (Groningen, Netherlands), SB203580 (10⁻⁵ M) from Selleckchem (Munich, Germany) and sorbitol (3x10⁻¹ M) was acquired from Carl Roth. Incubation with sorbitol was limited to 30 min/day since continuous incubation revealed cell damaging effects (14). Before the hyperosmolar shock with sorbitol, culture supernatant was removed, collected and re-added after sorbitol treatment. Drug treatment was generally initiated in adherent cells seeded 24 h before.

Transfection experiments. One day before transfection, cells were plated into 6-well (150,000 cells/well) or 24-well (30,000 cells/well) culture plates. For overexpression experiments, cells were transfected with 1 µg DNA (24-well) and pHSP27-3D were kindly provided by C. Kubisch (Munich, Darmstadt, Germany). The vectors pHSP27 wt, pHSP27-3A and pHSP27-3D were kindly provided by C. Kubisch (Munich, Germany) (15). pcDNA3.1 (Invitrogen) was used as empty control vector.

Western blotting. Cells were lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 10 mM K₂HPO₄, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.05% sodium dodecyl sulfate, 1 mM Na₂VO₄, 20 mM Naf, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM 2-phosphoglycerate and complete protease inhibitor cocktail from Roche Applied Science; Mannheim, Germany]. Determination of protein concentration was carried out utilizing Bradford reagent (Bio-Rad) and equal amounts of protein were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Whatman, Dassel, Germany). Proteins of interest were detected by specific primary antibodies directed against HSP27, P-Ser₁₇₀-HSP27, P-Ser₁₇₇-HSP27, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Danvers, MA, USA) and P-Ser₁₅-HSP27 (Thermo Scientific, Waltham, MA, USA) incubated overnight and peroxidase-coupled secondary antibodies directed against mouse and rabbit (Cell Signaling Technology) incubated for 1 h. Proteins were visualized by using SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific) and a ChemiDoc system (Bio-Rad). Quantification of protein signals was carried out by Image Lab 3.0 software (Bio-Rad).

Cell viability assay. To verify effects on cell viability, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For MTT assays, cells were incubated with 0.1 µg/ml aqueous MTT solution (Carl Roth) for 2 h at 37°C. Formazan complexes were solubilized by the addition of 120 µl dimethyl sulfoxide lysis buffer (DMSO; containing 10% SDS, 0.2% HCl) and dye formation was measured in a plate reader Infinite 200 PRO (Tecan, Männedorf, Switzerland) at 550 nm wavelength.

Cell proliferation assay. Cellular proliferation was examined by cell counting by using a CASY Cell Counter and Analyzer Model TT (Roche Applied Science). Therefore, adherent cells were detached by trypsin treatment, suspended in CASYton (Roche Applied Science) as 1:100 dilution. Measurement of 400 µl cell suspension was performed in 3 cycles using a capillary of 150 µm in diameter and 7.20/15.45 nm as gate settings to discriminate between living PC-3 and dead cells, as well as cellular debris.

Statistical analysis. Statistical comparisons of at least three independent measurements were performed using the unpaired Student's t-test with 95% confidence interval. For all statistical analyses, results of ps0.05 were considered statistically significant. Data are provided as means ± SD.

Results

Establishment of an in vitro cell model system for HSP27-driven chemoresistance in PC. High expression of HSP27 is known to induce pro-oncogenic effects in PC (16). We used an experimental system composed of the PC cell line PC-3 and PC-3 cells stably overexpressing HSP27 (PC-3-HSP27; inset Fig. 1B) as a suitable model in which to study drug-induced and HSP27-driven effects of acquired chemoresistance. As shown by MTT assays, various concentrations of docetaxel revealed a concentration-dependent cytostatic effect in both cell lines after 72 h of incubation, although docetaxel sensitivity was obviously lower in PC-3-HSP27 cells for all chosen docetaxel concentrations (Fig. 1A). Overexpression of HSP27 in PC-3-HSP27 cells led to significantly increased survival rates in the presence of docetaxel at concentrations of 10⁻⁶ M (1.9-fold, p=0.0258), 10⁻⁵ M (1.8-fold, p=0.0080) and 10⁻⁴ M (1.3-fold, p=0.0170) compared to maternal PC-3 cells. These findings were validated by growth kinetics utilizing a CASY Cell Counter and Analyzer model TT for daily measurement over a period of 144 h (Fig. 1B). Similarly, elevated HSP27 levels resulted in enhanced cytoprotective effects during drug treatment confirmed by significantly higher survival of PC-3-HSP27 cells (48 h, 1.3-fold, p=0.0078; 72 h, 1.4-fold, p=0.0022; 120 h, 1.5-fold, p=0.0392; 144 h, 1.4-fold, p=0.0327).

Activation of MAPK p38 and PKD1 reduce PC cell growth. MAPK p38 and PKD1 are known to be involved in important cellular regulation pathways by phosphorylation of diverse proteins, including HSP27 (9-11,17). Therefore, we assessed changes in chemosensitivity of docetaxel-treated PC-3-HSP27 cells co-incubated with modulators of MAPK p38 and PKD1 activity. Specific protein phosphorylation was activated by the use of 3x10⁻¹ M sorbitol (sorb; MAPK p38) and 3x10⁻⁸ M bryostatin-1 (bryo; PKD1) and inhibited by the use of 10⁻³ M SB203580 (SB; MAPK p38) and 5x10⁻⁵ M CID755673 (CID; PKD1), respectively. Studies with PC-3-HSP27 cells treated with docetaxel and exclusively incubated with a single kinase activator or inhibitor exhibited no statistically significant differences for cellular growth compared to controls (Fig. 2A). Thus,
we compared combinations of both activators (sorb + bryo) and both inhibitors (SB + CID). As a result, co-activated MAPK p38 and PKD1 led to increased docetaxel sensitivity of cells (Fig. 2B), measured by significantly decreased cell numbers (24 h, 1.9-fold, p=0.0438; 48 h, 1.6-fold, p=0.0313; 72 h, 1.6-fold, p=0.1550; 96 h, 1.6-fold, p=0.0271; 120 h, 2.0-fold, p=0.0171; 144 h, 2.0-fold, p=0.1383). In addition to other putative regulatory pathways, our data indicated kinase-dependent control of HSP27 functions in chemoresistance, assuming higher phosphorylated HSP27 responsible for increasing sensitivity to docetaxel treatment.

HSP27-driven chemoresistance is diminished by overexpression of non-phosphorylatable HSP27-3D mutant. Considering several cellular pathways potentially being targeted by MAPK p38 and PKD1 activities and therefore being responsible for the changes in chemosensitivity, we further analyzed specific effects of HSP27 phosphorylation on chemoresistance. Cellular functionality of HSP27 depends on phosphorylation of the three regulatory phosphorylation sites, namely serine-15, -78 and -82 (18). PC-3 cells were transiently transfected with DNA plasmids encoding for HSP27 wild-type (HSP27 wt) protein, PC-3-HSP27 cells were incubated over a period of 144 h with 3x10^{-1} M MAPK p38 activator sorbitol (sorb), 3x10^{-8} M PKD1 activator bryostatin-1 (bryo), 10^{-5} M MAPK p38 inhibitor SB203580 (SB), and 5x10^{-5} M PKD1 inhibitor CID755673 (CID), respectively. Cell numbers were measured at indicated time points using a CASY Cell Counter and Analyzer Model TT. Results were standardized to untreated control cells and illustrated as the means ± SD with p-values determined by Student's t-test. Due to negligible effects on cellular growth of cells treated with individual activators or inhibitors, the experiments were replicated applying combinations of both activators (sorb + bryo) and both inhibitors (SB + CID). Given results are indicated as the means ± SD of cell count and cell viability, respectively, and were compared to control cells. *p≤0.05, as determined by Student's t-test. MAPK p38, mitogen-activated protein kinase p38; PKD1, protein kinase D1; HSP, heat shock protein.
incubation with docetaxel, however, caused notable reductions in proliferation of PC-3 cells overexpressing phosphorylation mimicking HSP27-3D compared to cells overexpressing HSP27 wt and non-phosphorylatable HSP27-3A (Fig. 3C). Compared to HSP27 wt and HSP27-3A, respectively, HSP27-3D-expressing cells exhibited significantly reduced cell proliferation (72 h, 1.2-fold reduction, p=0.0329; 96 h, 1.2-fold reduction, p=0.0226; 120 h, 1.3-fold reduction, p=0.0005). As expected, similar findings were noted by further analyses of cellular viability using MTT assay (Fig. 3D). Again, HSP27-3D revealed poorer cellular viability of transfected, docetaxel-treated cells, when comparing overexpression of HSP27-3D with HSP27 wt and HSP27-3A (96 h, 1.2-fold reduction, p=0.0415; 120 h, 1.3-fold reduction, p=0.0087). Analysis of cell counting (Fig. 3C) as well as cellular viability (Fig. 3D) of HSP27 wt- and HSP27-3A-transfected cells showed no differences.

**Discussion**

In the present study, we demonstrated that treatment of PC cells with docetaxel, a first-line therapy drug for castration-resistant PC, induces phosphorylation of HSP27, which is a cytoprotective factor counter-acting antiproliferative cancer therapies (18). Markedly, we detected a rapid phosphorylation within the first day of docetaxel incubation at all of the three regulatory phosphorylation sites immediately followed by dephosphorylation. Furthermore, permanently phosphorylated HSP27 was associated with attenuated cytoprotective proper-
ties, strongly supporting the theory that rapid but short-termed phosphorylation of HSP27 is an important step in HSP27-driven initiation of chemoresistance.

HSP27 induction has been detected in various solid tumors. Hence, HSP27 may be regarded as a pivotal factor for progression and treatment resistance. In PC cells, HSP27 has a crucial role in tumor-specific and therapy-induced cytoprotection enabling tumor growth in the setting of hormonal ablation and cytostatic therapy. It is well recognized that HSP27 properties control activities of various signaling molecules, such as β-catenin, E-cadherin, interleukins, transforming growth factor β and AR, with all these effector proteins frequently dysregulated in PC and shown to exert oncogenic transformation (12,19-22). As shown in Fig. 1, stable overexpression of HSP27 in PC-3 cells revealed significantly enhanced resistance to docetaxel treatment in a concentration- and time-dependent manner. These findings, in accordance with previous studies (14), reflect HSP27-driven cytoprotection in PC.

Biological functions of HSP27 are mainly regulated by protein phosphorylation, suggesting that HSP27 phosphorylation may concur with chemoresistance mechanisms in PC cells. Therefore, it is reasonable to assume that temporal and spatial phosphorylation/dephosphorylation of HSP27 by kinase and phosphatase activities are an essential part of HSP27 in the regulation of treatment-resistant processes.

It has been reported that kinase pathways of MAPK p38 and PKD1 are major regulators of HSP27 properties in PC (9,23). Since we showed that the pharmacological activation of MAPK p38 and PKD1 kinases led to elevated docetaxel sensitivity (Fig. 2B), it is conceivable that this sensitizing effect, at least to some extent, is mediated through HSP27 phosphorylation. Accordingly, HSP27 phosphorylation by MAPK p38 and PKD1 was already found in PC and other cancer entities (9-11,16). However, due to both kinases operating in numerous signaling pathways, we cannot exclude the possibility that both kinases affect additional cellular targets whose modulation may...
contribute to enhanced antiproliferative effects in the presence of docetaxel. To examine this possibility, we focused on transfection experiments utilizing DNA plasmids encoding for the well-approved HSP27 mutants HSP27-3A and HSP27-3D representing an unphosphorylatable and a phospho-mimicking type of HSP27, respectively. Expression of HSP27-3A obtained no alteration on docetaxel sensitivity whereas expression of HSP27-3D resulted in significantly increased sensitivity to docetaxel (Fig. 3C and D). These observations were supported by western blot analysis using an experimental chemotherapy model which exhibited rapid and transient phosphorylations of HSP27, a mechanism similar to HSP27-driven drug resistance described in breast and pancreatic cancer cells (24, 25).

In particular, we detected docetaxel-induced phosphorylation of HSP27 amino acids serine-15, -78 and -82 after 24 h of incubation (Fig. 4). Experiments in high time resolution (1-24 h) exhibited a rapid but transient HSP27 phosphorylation within the first 8 h (data not shown).

Although our experiments clearly indicate that permanently phosphorylated HSP27 increases chemosensitivity, our current understanding of HSP27-phospho-regulation is still limited. Nakashima et al. showed enhanced chemosensitivity in gemcitabine-treated pancreatic cancer cells with phosphorylated HSP27 protein (25), confirming our own observations. In contrast, Tabá et al. reported converse effects of phosphorylated HSP27 in pancreatic cancer cells in the presence of gemcitabine, which was confirmed in a cell model system of 5-fluorouracil-resistant colon cancer (26, 27).

Furthermore, some studies provide examples for growth regulatory properties of phospho-HSP27 in the absence of chemotherapeutics (28, 29). Collectively, regulation of HSP27 appears to be part of a complex regulatory network controlled by kinases and phosphatases and may be induced in a drug- and cell type-specific manner.

Protein phosphorylation at multiple sites is often catalyzed in a fixed order. However, human HSP27 phosphorylation does not occur in an obligatory sequence (11). Although HSP27 phosphorylation has been shown in response to various stimuli, such as cytokines, receptor ligands, glucose and metals (18), to our knowledge this study is the first to report HSP27 phosphorylation initiated by drug treatment of PC cells. Due to the transient character of the HSP27 phosphorylation which lasts several hours, strongly orchestrated activities of kinases and phosphatases are required. According to our observations and the data from previous studies, most probably MAPK p38 and PKD1 pathways exert HSP27 phosphorylation (18). Since several kinases were described using HSP27 as substrate for protein phosphorylation, e.g. various MAPK-activated kinases and protein kinase C (30-32), it cannot be excluded that further kinase pathways contribute to HSP27 phosphorylation. Despite the increase in molecular insights into HSP27 phosphorylation in cancer mechanisms, functions of HSP27 dephosphorylation are for the most part unknown. Several studies have shown that inhibition of protein phosphatase 2A (PP2A) affects the phospho-status of human HSP27 (33, 34). Notably, Liu et al. recently described that dual specificity protein phosphatase 1 (DUSP1) controls treatment resistance in pancreatic cancer by affecting MAPK pathways (35), thus, it is consistent with our observations indicating potential targets for further examinations.

In conclusion, our findings enhance our understanding of survival mechanisms in drug-resistant PC cells and qualify HSP27 phosphorylation pathways as appropriate targets for new anticancer strategies. Future research will clarify if kinase activators as well as phosphatase inhibitors are a potent opportunity to support existing HSP27-targeting anticancer therapies.

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References


