Increased Hsp27 after Androgen Ablation Facilitates Androgen-Independent Progression in Prostate Cancer via Signal Transducers and Activators of Transcription 3–Mediated Suppression of Apoptosis

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Abstract

One strategy to improve therapies in prostate cancer involves targeting cytoprotective genes activated by androgen withdrawal to delay the emergence of the androgen-independent (AI) phenotype. The objectives of this study were to define changes in Hsp27 levels after androgen ablation and to evaluate the functional relevance of these changes in AI progression. Using a tissue microarray of 232 specimens of hormone-naive and post-hormone ablation–treated prostate cancers including breast (10), gastric (11), ovarian and endometrial (3-5), osteosarcoma (14), glial tumors (15), and prostate (16). The elucidation of the mechanisms that mediate AI progression, including key cytoprotective molecules, is an important step towards identifying newly targeted therapeutic strategies.

Introduction

Androgen withdrawal is the most effective form of systemic therapy for men with advanced prostate cancer, producing symptomatic and/or objective responses in >80% of patients. Unfortunately, androgen-independent (AI) progression is inevitable, and the development of hormone-refractory disease and death occurs within 2 to 3 years in most men. AI progression is a multifactorial process by which cells acquire the ability to both survive in the absence of androgens and proliferate using non-androgenic stimuli for mitogenesis, and involves variable combinations of adaptive up-regulation of antiapoptotic genes, ligand-independent activation of the androgen receptor, and alternative signaling pathways including Her2/neu, epidermal growth factor receptor, transforming growth factor-β, and insulin-like growth factor-I (1–3). Several antiapoptotic genes have been functionally linked to the development of hormone resistance, including Bcl-2, Bcl-xl, and clusterin (4–6). The elucidation of the mechanisms that mediate AI progression, including key cytoprotective molecules, is an important step towards identifying newly targeted therapeutic strategies.

Many components of survival and apoptotic pathways are regulated by molecular chaperones, such as heat shock proteins (Hsp). Using array analysis to compare gene expression profiles before and after castration, we identified Hsp27 as one of the most highly expressed genes in AI prostate tumors (7). Hsp27 is a 27-kDa protein highly induced during the stress response to a wide variety of physiologic and environmental insults (8). Various roles have been proposed for Hsp27 to explain its cytoprotective effects during cellular stress, including its role as a molecular chaperone, inhibitor of protein unfolding, direct interference with caspase activation, modulation of oxidative stress, and regulation of the cytoskeleton (9). Higher levels of Hsp27 are commonly detected in various cancers including breast (10), gastric (11), ovarian and endometrial (12, 13), osteosarcoma (14), glial tumors (15), and prostate (16). Increased Hsp27 levels in breast, endometrial, and gastric cancer is associated with metastasis, poor prognosis, and resistance to chemotherapy or radiation (10, 17–19).

In prostate cancer, elevated Hsp27 expression has been linked to poor outcome, and is highly expressed in AI prostate cancer cells (7, 20–22). Bubendorf et al. (22) identified Hsp27 as an overexpressed gene in androgen-resistant CWR22 prostate xenografts. The objectives of this study were to define the changes in Hsp27 levels after androgen ablation and during AI progression in human prostate cancer, as well as to evaluate the functional relevance of these changes in AI prostate cancer. We chose the LNCaP tumor model, which closely mimics AI progression in humans by producing prostate-specific antigen (PSA)–secreting and androgen-dependent tumors when injected into male immunodeficient mice, and develop non–androgen-regulated PSA gene expression after castration as a surrogate end point of progression to AI (23).
As part of our ongoing investigations to identify key pathways mediating AI progression, we used forced overexpression for gain-of-function analyses and antisense oligonucleotides (ASOs) or short-interfering RNA (siRNA) for loss-of-function analyses to evaluate the functional role of castration-induced changes in gene expression. In this study, we characterized changes in Hsp27 after androgen ablation in LNCaP tumors and employed Hsp27 gain- and loss-of-function approaches to determine the effects of Hsp27 on LNCaP cell survival and tumor progression after androgen withdrawal in LNCaP cells in vitro and in vivo.

Materials and Methods

Tumor cell lines. The human prostatic cancer cell line LNCaP was kindly provided by Dr. Ieland W.K. Chang (Emery University, Atlanta, GA) and was maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 5% FCS.

Microarray experiments. Total RNA from each tumor sample, obtained before and 35 days post-castration, were compared on the same chip to tumor samples from intact controls. A dye-swap for each pair was done to account for dye bias. Three tumors from each post-castration time point were analyzed and results were confirmed with Northern analysis.

Microarrays of 13,791 (70-mer) human oligos (Orogen, Huntsville, AL) printed in duplicate in 3× SSC onto amine-coated slides (Ezray, Agpent, Fisher Scientific, Ottawa, Ontario, Canada) were supplied by the Array Facility of the Prostate Centre at Vancouver General Hospital. Slides were printed with a BioRobotics Microgrid II (Harvard Biosciences and Genomic Solutions, Ann Arbor, MI) under 60% humidity at 22°C; then UV cross-linked using 3,000 μJ (Stratalinker, Stratagene, La Jolla, CA). Slides were prehybridized in 5× SSC, 0.1% SDS, and 0.2% bovine serum albumin (BSA; Sigma, St. Louis, MO) at 48°C for 45 minutes, washed in deionized water, dipped in isopropanol, and dried in a centrifuge at 2,000 rpm for 2 minutes. Arrays were hybridized in a humid Hybrid oven (ThermoHybaid, England) with reverse-transcribed fluorescently labeled (Cy3- or Cy5-dUTP; Amersham Pharmacia Biosciences Inc., Quebec, Canada) CDNA (from 20 μg of total RNA) at 42°C for 16 hours in a hybridization buffer consisting of 50% formamide, 5× SSC, 0.01% SDS, and 0.2% sodium bovine serum albumin (BSA; Sigma, St. Louis, MO) at 48°C for 45 minutes, washed in deionized water, dipped in isopropanol, and dried in a centrifuge at 2,000 rpm for 2 minutes. Arrays were hybridized in a humid Hybrid oven (ThermoHybaid, England) with reverse-transcribed fluorescently labeled (Cy3- or Cy5-dUTP; Amersham Pharmacia Biosciences Inc., Quebec, Canada) CDNA (from 20 μg of total RNA) at 42°C for 16 hours in a hybridization buffer consisting of 50% formamide, 5× SSC, 0.01% SDS, 8 μg BSA, 25 μg yeast tRNA, and 20 μg salmon testes DNA. Following stringent washes (1× SSC and 0.1% SDS, then 0.1× SSC), fluorescent images of the slides were acquired using an ArrayWoRx, Microarray Scanner (Applied Precision, Seattle, WA). Signal quality and quantity were assessed using ImageJ 1.46 (BioDiscovery, San Diego, CA). Data from ImageJ were analyzed in GeneSpring 6.1 using a quality and quantity were assessed using Imagene 5.6 (BioDiscovery, San Diego, CA). Data from Imagene were analyzed in GeneSpring 6.1 using a microarray analysis. Sections were deparaffinized and rehydrated through xylene and ethanol, then transferred to the 0.02% triton for permeabilization. Slides in citrate buffer (pH = 6) were heated in the steamer for 30 minutes. After cooling for 30 minutes and washing thrice for 5 minutes in PBS, the slides were incubated in 3% BSA for a further 30 minutes. The slides were successively transferred to 3% H2O2 for 10 minutes and then were incubated overnight with anti-Hsp27 antibodies from Nova Castra (Newcastle upon Tyne, United Kingdom) at the concentration of 1:400 in 1% BSA. The next day, the primary antibody was washed extensively with PBS and the LSAB+ kit (Dako, Carpinteria, CA) was used as the detection system. Chromogen Nova-red (Vector Laboratories, Burlingame, CA) was applied for 2 minutes and counterstaining was done with H&E (Vector Laboratories). After ethanol rehydrating, the slides were covered with a cover glass with Cytoseal, a xylene-based mounting medium (Stephen Scientific, Riverdale, NJ). Negative control slides were processed in an identical fashion to that above, with the substitution of 1% BSA for the primary antiserum. Photomicrographs were taken through a Leica DMLS microscope coupled to a digital camera (Photometrics CoolSNAP, Roper Scientific, Inc., Glenwood, IL).

Scoring of Hsp27 staining. The staining intensity of malignant tissue was evaluated and scored by one pathologist (L. Fazli) and automated quantitative image analysis was done with pro-plus image software (MediaCybernetics, San Diego, CA). Sections were graded from 0 to +3 intensity representing the range from no staining to heavy staining. The overall percentage of cancer cells showing staining (0-100%) was also indicated. All comparisons of staining intensities and percentages were made at ×200 magnification.

Lentiviral infection of Hsp27 into LNCaP cells. The full-length cDNA for human Hsp27 was subcloned into the lentiviral vector pHR-cytomegalovirus (CMV)-enhanced green fluorescent protein (EGFP) at the BamHI and XhoI sites. Two vectors were created for study: pHR-CMV-Hsp27 and pHR-CMV (empty vector). Clone identity was verified using restriction digest analysis and plasmid DNA sequencing. Infectious lentivirus was generated by cotransfection of 1.5 × 10^6 293T cells with target plasmids with pCMV8.R8.2 (carries sequence necessary for viral assembly of lentivirus) and pMD.G, which expresses the vesicular stomatitis virus envelope glycoprotein G pseudotype. The 293T cells were transfected for 12 to 15 hours, after which fresh medium were added for 24 hours. After this, the virus containing media was collected and filtered through a 0.45-μm filter. Early passage LNCaP cells (passage 30) were plated in 10 cm plates, and competent retrovirus was added to 30 to 40 multiplicity of infection. Cells were harvested for UV microscopy to verify green fluorescent protein expression, and Western blotting was used to verify Hsp27 expression.

For in vitro growth assays, LNCaP-Hsp27 and LNCaP-empty were plated at different densities, 2.5, 10, and 50, and 100 × 10^3 cells into a 75 cm² flask in RPMI 1640 supplemented with 5% FCS. The following day, cells were treated in RPMI 1640 serum-free media. After 5 weeks, cells were harvested for flow cytometry analysis as described below.
Antisense oligonucleotides and short-interfering RNA sequences. Hsp27 phosphorothioate ASO targeting the human Hsp27 translation initiation site (5'-GGGACGCGCGCGTGGTGT-3') was purchased from Qiagen Operon (Alameda, CA). Human signal transducers and activators of transcription 3 (Stat3) ASO (5'-GCTTCAGCATCTGGTCC-3') was generously provided by Dr. B. Monia at Isis Pharmaceuticals (Carlsbad, CA). Scrambled control oligonucleotide (5'-CACGCGTCACAGGTTTTC-3') was purchased from Qiagen Operon. Small RNA interference siACE-RNA was purchased from Dharmacon, Inc. (Lafayette, CO). The sequence of Hsp27 siRNA duplex used corresponded to the human Hsp27 site (5'-GUCCUAUGAAUUGUCGACG-3'). A scrambled siRNA duplex (5'-CAGGCCGUGACAACAGGUCU-3') was used as a control.

Treatment of cells with antisense oligonucleotides or short-interfering RNA. Cells were plated at a density of 30,000 cells per 1.9 cm² and treated the day after with the indicated siRNA or ASO for 1 or 2 days, respectively. OligofectAMINE, a cationic lipid (Invitrogen, Life Technologies), was used to increase ASO or siRNA uptake into the cells. LNCaP cells were treated with increasing concentrations of ASO or siRNA after a preincubation for 20 minutes with 3 mg/mL OligofectAMINE in serum-free OPTI-MEM (Life Technologies). Four hours after the beginning of the incubation, the medium was replaced with standard culture medium described above.

Western blot analysis. Hsp27 and caspase-3 cleavage Western blot analysis was done as described previously (7). Stat3 and c-fos were detected by Western blotting using 40 µg of proteins. Rabbit polyclonal antibody (Santa Cruz Biotechnology, Inc., Delaware, CA) was used to detect total Stat3 (92 kDa) and chicken polyclonal antibody (Abcam, Cambridge, MA) was used to detect c-fos (46 kDa).

Immunofluorescence. LNCaP-empty and -Hsp27 cells were grown on glass coverslips in RPMI plus 5% fetal bovine serum for 48 hours. Subsequently, cells were fixed with cold 3% acetone in methanol at −20 °C and permeabilized in 0.2% Triton in PBS. Slides were incubated in blocking solution, 3% milk in PBS for 1 hour, and simultaneously treated overnight with primary antibodies, mouse monoclonal Hsp27 (StressGen) and rabbit polyclonal Stat3 (Santa Cruz Biotechnology). Secondary fluorescent antibodies antimouse FITC and antirabbit Texas red conjugated were added for 1 hour at room temperature with three 5-minute washes (0.1% Triton in PBS). Cells examined for localization of red and green protein were mounted with fluorescent 4,6-diamidino-2-phenylindole vectashield mounting medium (Vector Laboratories). Images were captured using a Zeiss Axioplan II fluorescence microscope at ×63 magnification followed by analysis with imaging software (Northern Eclipse, Empix Imaging, Inc., Mississauga, Ontario, Canada). Analysis of focal colocalization was also done with Northern Eclipse and Adobe Photoshop 5.5 software with an assignment of yellow (Y) for colocalized foci and green (G) or red (R) as non-colocalization.

Immunoprecipitation. LNCaP cells were lysed in radioimmunoprecipitation assay buffer without SDS containing complete protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). Lysates from LNCaP-Hsp27, LNCaP-empty, or LNCaP-Hsp27 was incubated with 5 µg anti-Stat3 (Santa Cruz Biotechnology, anti-Hsp27 (StressGen), and anti-IgG antibodies (Santa Cruz Biotechnology). After 12 hours of incubation, 50 µL of protein A beads (Amersham Pharmacia Biosciences) were added into the reaction tubes and incubated for 2 hours. The beads were washed three times using radioimmunoprecipitation assay lysis buffer and resolved in 5× loading buffer (MBI, Fermentas Inc., Burlington, Canada).

In vitro mitogenic assay. The in vitro growth-inhibitory effects of Hsp27 ASO on LNCaP cells were assessed using the crystal violet assay as previously described (4). Cells were treated once daily with 20 nmol/L of oligonucleotide for 2 days or 1 nmol/L siRNA for 1 day. Every 24 hours, over a period of 4 days, crystal violet assays were done. Each assay was done in triplicate.

Immunocytochemistry for in situ apoptosis. LNCaP cells were plated in 9.87 cm² labtek (Nunc, Roskilde, Denmark) and treated with oligonucleotides or siRNA as described above. Two days after transfection, cells were harvested and fixed with methanol for 10 minutes. Slides were then transferred into a coplin jar containing 50 mL of 50% formalamide (v/v distilled H2O) in a water bath at 56°C to 60°C for 20 minutes. Slides were then incubated for 10 minutes in 0.2% triton, and endogenous peroxidase quenched in 3% hydrogen peroxide for 5 minutes. Detection of cleaved apoptotic DNA fragments were done using mouse monoclonal antibody MAB3299 (Chemicon International, Temecula, CA).

Flow cytometric analysis. Flow cytometric analysis of propidium iodide–stained nuclei was done as described previously (7). Briefly, the LNCaP cells were plated in 75 cm² dishes and were treated as described above the following day. The cells were trypsinized 2 days after ASO treatment and analyzed for relative DNA content on a dual laser flow cytometer (Beckman Coulter Epics Elite, Beckman, Inc., Miami, FL). Each assay was done in triplicate.

Measurement of caspase-3 cleavage. Caspase-3 cleavage was detected by Western blotting as described above using 40 µg of proteins. Polyclonal antibody (New England BioLabs, Inc., Mississauga, Ontario, Canada) was used to detect full length (32-35 kDa) and large fragments of activated caspase-3 (17-20 kDa) which results from cleavage after Asp175.

Assessment of in vivo tumor growth. For in vivo studies, using a 27-gauge needle, 10⁶ LNCaP cells were inoculated s.c. with 0.1 mL of Matrigel (Becton Dickinson Labware, Franklin Lakes, NJ) in the flank region of 6- to 8-week-old male athymic nude mice (Harlan Sprague-Dawley, Inc., Indianapolis, IN) under halothane anesthesia. All animal procedures were done according to local guidelines on animal care and with appropriate institutional certification. Tumors were measured twice weekly and their volumes were calculated by the formula: length × width × 0.5236. Mice bearing tumors between 200 and 300 mm³ in volume were castrated via scrotal approach and randomly assigned to a treatment arm. Mice were treated beginning 1 day after castration with 10 mg/kg of ASO or control oligonucleotide i.p. once daily. Tumor volume...
and serum PSA measurements were done weekly. Data points for both sets of experiments were expressed as average tumor volume ± SE of the mean based on 10 determinations.

Statistical analysis. All of the results were expressed as the mean ± SE. Statistical analysis was done with a one-way ANOVA followed by Fisher’s protected least significant difference test (StatView 512, Brain Power, Inc., Calabasas, CA). *, P ≤ 0.05 was considered significant; **, P ≤ 0.01; ###, P ≤ 0.001.

Results

Hsp27 mRNA expression increases after androgen ablation in LNCaP xenografts. Expression profiling using 13,791 (70-mer) human oligos (Operon) printed in duplicate onto amine-coated slides were used to quantify changes in Hsp27 expression during castration-induced apoptotic stress and AI progression, comparing Hsp27 mRNA levels in hormone-dependent LNCaP xenografts collected at baseline with AI tumors 35 days after castration. Log² plots from comparative microarray analyses of tumors at baseline and 35 days post-castration identified Hsp27 within the top 1% of overexpressed genes in Al tumors. Changes in Hsp27 correlated highly (95%) with changes in tumor PSA mRNA levels (data not shown). Northern blot analysis of tumors confirmed that Hsp27 mRNA increased by 77.6% 35 days after castration (**, P ≤ 0.01) compared with the precastrate levels (Fig. 1A and B). Changes in Hsp27 mRNA expression also paralleled changes in serum PSA in tumor-bearing mice, the main end point of AI progression in the LNCaP model (Fig. 1B).

Hsp27 immunostaining increases after androgen withdrawal in human prostate cancer. To determine whether Hsp27 levels also increase after androgen- ablation or during AI progression in human prostate cancer, Hsp27 immunostaining was evaluated in human prostate tissue microarrays. A-i, neoplastic glands in nontreated tumors: a few foci of weakly positive cancer cells are visible but most of tumor is not immunoreactive. A-ii and iii, strong immunoreactivity of cancer cells after 3 and 6 months of androgen ablation, respectively. A-iv, sheets of uniformly and intensely reactive tumor cells characteristic of Al tumors. B, mean Hsp27 staining after androgen ablation. Specimens spotted on the tissue microarray were graded from 0 to +3 intensity, representing the range from no staining to heavy staining by visual scoring and automated quantitative image analysis by pro-plus image software. Data from 232 samples were used to calculate average ± SE. All comparison of stain intensity was made at 200× magnification. **, P ≤ 0.01; ###, P ≤ 0.001, indicate significance between groups. C and D, highly intensive and uniformly reactive tumors characteristic of hormone-refractory bone and liver metastases, respectively.

Figure 2. Changes in Hsp27 immunostaining in human prostate cancer tissue microarray. A-i, neoplastic glands in nontreated tumors: a few foci of weakly positive cancer cells are visible but most of tumor is not immunoreactive. A-ii and iii, strong immunoreactivity of cancer cells after 3 and 6 months of androgen ablation, respectively. A-iv, sheets of uniformly and intensely reactive tumor cells characteristic of Al tumors. B, mean Hsp27 staining after androgen ablation. Specimens spotted on the tissue microarray were graded from 0 to +3 intensity, representing the range from no staining to heavy staining by visual scoring and automated quantitative image analysis by pro-plus image software. Data from 232 samples were used to calculate average ± SE. All comparison of stain intensity was made at 200× magnification. **, P ≤ 0.01; ###, P ≤ 0.001, indicate significance between groups. C and D, highly intensive and uniformly reactive tumors characteristic of hormone-refractory bone and liver metastases, respectively.
human disease, 232 prostate cancer specimens spotted on a tissue microarray were stained for Hsp27 by immunohistochemistry (Fig. 2; Table 1). Hsp27 protein was present in the cytoplasm of the epithelial cells (Fig. 2A). More specifically, Hsp27 staining was limited to the basal layer of the benign prostate glands, and there was very weak or no staining in untreated prostate cancer. Hsp27 staining increases 4-fold after neoadjuvant hormone therapy and was significantly higher at all time points after hormone therapy compared with untreated hormone-naive prostate cancer (***, \(P < 0.001\)). The mean intensity of positive cells in the untreated, <3 months, 3 to 6 months, >6 months, and AI were 0.66, 1.29, 1.76, 1.99, and 2.08, respectively, when scored as described in Materials and Methods (Fig. 2B). AI tumor tissue from prostate and metastatic sites exhibited uniform and highly positive Hsp27 staining in all specimens (Fig. 2C and D).

**Table 1.** Hsp27 staining in prostate cancer before and after androgen ablation

<table>
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<tr>
<th>Prostate cancer</th>
<th>No. of cases</th>
<th>Immunohistochemistry score</th>
<th>SD</th>
<th>SE</th>
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<tr>
<td>Not treated</td>
<td>35</td>
<td>0.66</td>
<td>0.14</td>
<td>0.07</td>
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<tr>
<td>Neoadjuvant hormone therapy–treated (mo)</td>
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<td></td>
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<tr>
<td>&lt;3</td>
<td>58</td>
<td>1.29</td>
<td>0.20</td>
<td>0.09</td>
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<tr>
<td>3-6</td>
<td>52</td>
<td>1.76</td>
<td>0.22</td>
<td>0.11</td>
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<tr>
<td>&gt;6</td>
<td>57</td>
<td>1.99</td>
<td>0.507</td>
<td>0.10</td>
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<tr>
<td>Hormone-refractory prostate specimens</td>
<td>16</td>
<td>2.08</td>
<td>0.25</td>
<td>0</td>
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<tr>
<td>Hormone-refractory metastasis</td>
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<td></td>
<td></td>
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<tr>
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<td>0.48</td>
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<td>0.308</td>
<td>1.2</td>
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<tr>
<td>Non-osseous distant metastasis</td>
<td>5</td>
<td>1.7</td>
<td>0.2885</td>
<td>0.55</td>
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**Figure 3.** Effect of Hsp27 overexpression on AI LNCaP cell survival in vitro and tumor growth in vivo. A, the full-length cDNA for human Hsp27 was subcloned into the lentiviral vector pHR-CMV-EGFP. After 1 week, protein was extracted from cultured cells and Hsp27 and glyceraldehyde-3-phosphate dehydrogenase protein levels were analyzed by Western blotting. B, LNCaP-Hsp27 and -empty vector cells were cultured at different densities in RPMI 1640 serum-free medium for 5 weeks and apoptotic rates quantified by flow cytometry. The results are expressed in percentage and the value obtained for LNCaP-mock at 2, 5.10^4 density of cells was chosen as control (**, \(P < 0.05\); ***, \(P < 0.001\)). C, effects of Hsp27 overexpression on LNCaP tumor growth post-castration. Twenty nude mice were injected with LNCaP-Hsp27 or LNCaP-empty (10^6 cells). The mice were castrated when PSA increased >50 ng/mL. **, mean tumor volume increased significantly in LNCaP-Hsp27 compared with LNCaP-empty tumors beginning 1 week after castration. D, effects of Hsp27 overexpression on serum PSA levels post-castration. Mean serum PSA levels increased significantly faster in the LNCaP-Hsp27 group compared with LNCaP-empty group beginning 1 week after castration.
in LNCaP-Hsp27 cells than the LNCaP-empty control cells (Fig. 3B). LNCaP-Hsp27 cells were also more resistant to paclitaxel-induced apoptosis compared with LNCaP-empty cells (P ≤ 0.001, data not shown).

We next compared the rate of AI progression in LNCaP tumors in vivo after castration in LNCaP-Hsp27- (n = 10) and control-transfected (n = 10) tumors. Beginning 1 week after castration, tumor volume and serum PSA levels increased more rapidly in LNCaP-Hsp27 tumors compared with mock-transfected controls. At the time of sacrifice, tumor volume (Fig. 3C) was 4.3-fold higher in the LNCaP-Hsp27 group (1,912 ± 330 mm³) compared with the LNCaP-empty control group (437 ± 57 mm³; **; P ≤ 0.01), and serum PSA (Fig. 3D) was 10-fold higher in the LNCaP-Hsp27 group (466 ± 114 ng/mL) compared with the LNCaP-empty control group (46 ± 21.8 ng/mL; *, P ≤ 0.05). These data imply that increased Hsp27 levels protect androgen-dependent prostate cancer cells from treatment stress induced by androgen withdrawal and facilitate AI tumor progression after castration.

Sequence-specific and dose-dependent inhibition of Hsp27 expression by antisense oligonucleotides and short-interfering RNA. To further study the functional relevance of castration-induced increases in Hsp27, inhibition of Hsp27 gene expression using Hsp27 ASO or siRNA was employed with the appropriate scrambled controls. As shown in Fig. 4A and B, ASO treatment of LNCaP cells reduced Hsp27 mRNA levels in a dose-dependent manner up to 73% at 50 nmol/L (**, P ≤ 0.01), whereas Hsp27 mRNA expression was not significantly suppressed by scrambled controls.

**Figure 4.** Sequence-specific and dose-dependent suppression of Hsp27 mRNA and protein levels by Hsp27 ASO and siRNA. A, LNCaP cells were treated daily with various concentrations of Hsp27 siRNA or ASO or a scrambled control for 1 or 2 days, respectively. One day after treatment, total RNA was extracted from cultured cells, and Hsp27 and 28S levels were analyzed by Northern blotting. Oligofectamine, cells treated with Oligofectamine only. B, quantitative analysis of Hsp27 mRNA levels after normalization to 28S rRNA levels by densitometry analysis in LNCaP cells after treatment with various concentrations of Hsp27 ASO or scrambled control. Points, means of triplicate analysis; bars, SE. *, P ≤ 0.05; **, P ≤ 0.01; difference from scrambled controls using Student’s t test. C, LNCaP cells were treated once with various concentrations of Hsp27 siRNA or scrambled control. Two days after treatment, total RNA was extracted from cultured cells, and Hsp27 and 28S levels were analyzed by Northern blotting as described above. D and E, LNCaP cells were treated daily with various concentrations of Hsp27 ASO or siRNA or scrambled control. Three days after the first treatment, proteins were extracted from cultured cells, and Hsp27 and vinculin protein levels were analyzed by Western blotting.
oligonucleotide. Similar dose-dependent inhibition was observed using siRNA between $10^{-3}$ and 1 nmol/L (Fig. 4C). Significant inhibition of Hsp27 protein levels in LNCaP cells were also detected after treatment with Hsp27 ASO (Fig. 4D) and siRNA (Fig. 4E).

**Hsp27 antisense oligonucleotides and short-interfering RNA induce apoptosis and increase caspase-3 cleavage.** Hsp27 ASO treatment significantly reduced LNCaP cell growth by 29% (**, $P \leq 0.01$), 31% (**, $P \leq 0.01$), 40% (**, $P \leq 0.01$), and 63% (***, $P \leq 0.001$) 1, 2, 3, and 4 days posttreatment, respectively, compared with scrambled controls (Fig. 5A). Similar growth suppression occurred using 1 nmol/L Hsp27 siRNA (data not shown). Apoptosis, detected by ssDNA nuclear staining (MAB 3299, Chemicon), increased 8.9-fold after treatment with Hsp27 ASO (**, $P \leq 0.001$) in LNCaP cells compared with those treated with control oligonucleotide (Fig. 5B and C).

The induction of apoptosis by Hsp27 ASO was also clearly shown using flow cytometry. After the same treatment schedule described above, the fraction of cells undergoing apoptosis (sub-G$_0$-G$_1$) was significantly higher after treatment with Hsp27 ASO 20 nmol/L (35.2% versus 18.9% **, $P \leq 0.01$), compared with scrambled controls (Fig. 5D).

Hsp27 has been reported to interact with and inhibit caspase-3 activation (25). Using coimmunoprecipitation (data not shown), we confirmed that Hsp27 interacts with several regulators of the mitochondrial apoptotic pathway (bax and cytochrome c), and
that caspase-3 cleavage increases significantly within 2 days after Hsp27 ASO treatment (Fig. 5E). Increased caspase-3 cleavage after Hsp27 ASO was also detected by immunostaining using an antibody specific for the cleaved caspase-3 fragment (data not shown). Hsp27 siRNA (1 nmol/L) resulted in similar increases in DNA fragmentation, sub–G₀-G₁ fraction, and caspase-3 cleavage (data not shown). Collectively, these data suggest that Hsp27 knockdown increases the rate of apoptosis by interfering with Hsp27-regulated inhibition of caspase-3 activation.

Hsp27 antisense oligonucleotides inhibits proliferation and induces apoptosis via inhibition of signal transducers and activators of transcription 3 activity. In many human cancers, aberrant constitutive activation of Stat3 is sufficient to induce tumorigenesis through activation of proto-oncogenes like c-fos and sPLA₂-IIA (26–28). Stat3 enhances cell survival and oncogenesis in breast carcinoma cells (18), whereas Stat3 signaling is implicated in the development of AI progression (29, 30). To determine whether androgen ablation–induced changes in Hsp27 could act as an upstream regulator, Stat3, LNCaP, and PC-3 cells were treated with increasing concentrations of Hsp27 ASO or control oligonucleotides. RNA and protein were extracted 2 and 3 days after treatment, respectively, and analyzed for Stat3 expression levels and the downstream Stat3-regulated genes c-fos and sPLA₂-IIA. Stat3 protein levels decreased within 2 days in LNCaP (Fig. 6A) and PC-3 cells (data not shown) after treatment with Hsp27 ASO. In addition, the Stat3-regulated genes c-fos protein and sPLA₂-IIA mRNA also decreased after the same treatment (Fig. 6B and C). Moreover, when a Stat3 ASO was used to specifically knock down Stat3 levels in LNCaP-empty and LNCaP-Hsp27 cells, the cytoprotection to in vitro androgen withdrawal or paclitaxel treatment normally conferred by Hsp27 overexpression in LNCaP-Hsp27 cells was no longer detected (Fig. 6D). Collectively, these data suggest that the increased rate of apoptosis after Hsp27 knock down is in part due to attenuation of Hsp27-mediated regulation of Stat3 activity.

Hsp27 colocalizes and interacts with signal transducers and activators of transcription 3. To further define the role of Hsp27 in the regulation of, or interaction with, Stat3, we examined whether Hsp27 colocalizes and interacts with Stat3 using immunofluorescence and coimmunoprecipitation. Figure 7A illustrates that Hsp27 colocalizes in the cytoplasm with Stat3 in LNCaP-empty and LNCaP-Hsp27 cell lines, and that the intensity of Stat3 staining and colocalization is higher in Hsp27 transfectants relative to mock-transfected cells. Using coimmunoprecipitation, Hsp27 coimmunoprecipitated with Stat3 in both LNCaP-mock and LNCaP-Hsp27 and higher levels of Stat3 were detected in Hsp27-transfectants (Fig. 7B). Collectively, the results illustrated in Figs. 6 and 7 indicate that Hsp27 interacts, either directly or indirectly as part of a protein complex, with Stat3, and that Stat3 levels and transcriptional regulation of c-fos and sPLA₂-IIA directly correlate with Hsp27 levels.

Figure 6. Effect of Hsp27 knock-down on Stat3 signaling pathway. LNCaP cells were treated daily with various concentrations of Hsp27 ASO or scrambled control for 2 days. A and B, 3 days after the first treatment, proteins were extracted from cultured cells, and Stat3, c-fos, and vinculin protein levels were analyzed by Western blotting. C, 2 days after the first treatment, total RNA was extracted from cultured cells, and sPLA₂-IIA and 28S levels were analyzed by Northern blotting. Oligofectamine, cells treated with Oligofectamine only. D, LNCaP-Mock and -Hsp27 were treated with 500 nmol/L of Stat3 ASO or scrambled control for 2 days. Serum-free media and 10 nmol/L of paclitaxel were added after transfection for 24 hours and the cells were then analyzed for their growth properties by crystal violet assay (OD, 560 nmol/L). The experiment was repeated in triplicate. **, P < 0.01; *, P < 0.05, significance of differences between Stat3 ASO- and scrambled control-treated groups.
Hsp27 antisense oligonucleotides delay LNCaP tumor progression after castration in vivo. Twenty male mice bearing LNCaP tumors were castrated 6 to 8 weeks after tumor implantation and were randomly selected for treatment with Hsp27 ASO versus scrambled control. Mean tumor volume and PSA were similar in both groups at the beginning of the treatment. Beginning 1 day after castration, 10 mg/kg of oligonucleotide was diluted in PBS and administered once daily by i.p. injection for 10 weeks. As shown in Fig. 8, LNCaP tumor volume (A) and serum PSA levels (B) decreased more rapidly in mice treated with Hsp27 ASO, compared with those treated with scrambled controls. All mice (n = 10) treated with castration plus Hsp27 ASO had a significant inhibition of AI tumor growth during the 10 weeks of analysis. At sacrifice, mean PSA levels were 3.2-fold higher in the scrambled control (42.67 ± 8.2 ng/mL) compared with the Hsp27 ASO–treated group (13.12 ± 3 ng/mL; **, P ≤ 0.01), whereas tumor volume was 2.3-fold higher in scrambled control (506.51 ± 106.8 mm³) compared with the Hsp27 ASO–treated group (214.27 ± 57.45 mm³; *, P ≤ 0.05). No side effects were observed with Hsp27 ASO or scrambled control treatment.

Discussion
Androgen ablation induces apoptotic prostate cancer cell death, but despite the initially high response rates, remissions are temporary because surviving tumor cells eventually recur within several years. Cellular and molecular events that mediate AI progression are complex and multifactorial, and include adaptive changes in the expression of various apoptosis-regulating genes, including androgen receptor, bcl-2, clusterin, IGFBP-2, and IGFBP-5 (1–6).

Hsp27 expression is induced by various stressors such as chemotherapy and can inhibit cell death triggered by various stimuli when overexpressed (31). Hsp27 prevents formation of the apoptosome and the subsequent activation of caspases through direct sequestration of cytochrome c released from the mitochondria into the cytosol (32). When expressed at high levels, Hsp27 also interferes with caspase activation upstream of the mitochondria, for instance, by preventing the caspase-8-triggered activation of the proapoptotic Bcl-2 family member, Bid (33). Hsp27 may also inhibit cytochrome c–mediated caspase activation by sequestering both pro-caspase-3 and cytochrome c (34).

Figure 7. Hsp27 colocalizes and interacts with Stat3. A, LNCaP-empty and -Hsp27 cells were grown on glass coverslips on RPMI with 5% fetal bovine serum for 48 hours. Subsequently, cells were fixed and incubated with mouse monoclonal Hsp27 (green) and rabbit polyclonal Stat3 (red) antibody simultaneously. Analysis of focal colocalization was done with Northern Eclipse and Adobe Photoshop 5.5 software with an assignment of yellow (Y) for a colocalized foci and green (G) or red (R) as non-colocalization. B, LNCaP-empty and LNCaP-Hsp27 cells were harvested and cell lysate was used to immunoprecipitate Hsp27 and Stat3 using anti-Stat3 and anti-Hsp27 antibodies, respectively. After resolving immuno-complexes on 10% SDS-PAGE and transfer to polyvinylidene difluoride membrane, the membrane was probed with anti-Stat3 and anti-Hsp27 antibodies, respectively.
Castration was done when tumors reached a mean of 260 mm³. Hsp27 ASO or castration, for 10 weeks. Tumor volume (Cancer Res 2005; 65: (23). December 1, 2005 11092 www.aacrjournals.org) of prostate cancer cells is accompanied by interleukin-6 (IL-6) in several prostate cancer cells is accompanied by the activation of Stat3. For example, androgen-independent DU145 prostate cancer cell lines constitutively express activated Stat3 as a result of autocrine production of IL-6, whereas androgen-sensitive LNCaP cells increase Stat3 activity following paracrine-mediated IL-6 stimulation via Janus-activated kinase (37, 38). Increased Stat3 signaling has been linked to AI progression, whereas constitutively active Stat3 signaling can antagonize androgen deprivation–induced prostate cell death (39, 40). IL-6-induced proliferation of prostate cancer cell lines is associated with the activation of Stat3, whereas inhibition of IL-6 activation of Stat3 inhibited cell growth and induction of apoptosis (35, 37). In this study, we found that Hsp27 colocalizes with Stat3 and that Stat3 protein levels correlate directly with changes in Hsp27. Our findings suggest that increased apoptotic rates after Hsp27 silencing is mediated, in part, via reduced Stat3 protein levels and Stat3-regulated genes c-fos and sPLA₂-IIA.

Collectively, these findings link increased Hsp27 levels with the hormone-refractory phenotype and indicate that inhibition of Hsp27 up-regulation after androgen withdrawal can inhibit AI progression. Hsps have attracted attention as new therapeutic targets for cancer, especially since the discovery and characterization of geldanamycin as an inhibitor of Hsp90 (41), and the targeting of the clusterin gene (6, 42, 43), whose product has small Hsp-like function. We are targeting cell survival or antiapoptotic genes up-regulated after androgen withdrawal as one strategy to delay tumor progression (44), and recently reported results of a proof-of-concept phase I trial demonstrating that OGX-011, a second-generation ASO targeting clusterin, is well-tolerated and potently inhibits clusterin expression in prostate cancers (43). Phosphorothioate ASOs are chemically modified stretches of ssDNA complementary to mRNA regions of a target gene, thereby inhibiting gene expression and providing a useful tool for in vitro and in vivo functional genomics, and can also serve as preclinical proof-of-principle to support the clinical development of a drug candidate (44, 45). Although siRNA offers an alternative and more potent method of sequence-specific posttranscriptional gene silencing, few chemical modifications have been identified that support systemic activity (46, 47). In this report, suppression of Hsp27 using ASO after castration provides in vivo proof-of-principle that inhibition of castration-induced increases in Hsp27 can delay AI progression. LNCaP tumor volume and serum PSA decreased more rapidly after Hsp27 silencing is mediated, in part, via reduced Stat3 protein levels and Stat3-regulated genes c-fos and sPLA₂-IIA.

In summary, the results of this study support the hypothesis that increased Hsp27 after androgen ablation is an adaptive response induced by castration to enhance cell survival, and thereby promote AI tumor growth. Hsp27 silencing using ASO and siRNA technology alters Stat3 signaling, enhances caspase-3 activation and apoptosis, and offers a treatment strategy to delay progression of prostate cancer after androgen withdrawal.

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We thank Virginia Yago, Mary Bowden, and Eleyna Gomez for their excellent technical assistance in animal experimentation.

**Figure 8.** Effect of Hsp27 ASO treatment on LNCaP tumor growth in vivo after castration. A and B, 20 male mice bearing LNCaP tumors were randomly selected for treatment with Hsp27 ASO or scrambled control oligonucleotides. Castration was done when tumors reached a mean of 260 mm³. Hsp27 ASO or scrambled controls were injected (10 mg/kg/mice) daily, starting 1 day after castration, for 10 weeks. Tumor volume (A) and serum PSA levels (B) were measured once weekly. Points, mean tumor volume in each experimental group containing 10 mice; bars, SE. *, P < 0.05; **, P < 0.01, differ from scrambled control and by Student’s t test.

The role of Hsp27 in prostate cancer progression has not been well-defined, and only recent studies report its association with hormone-refractory disease (7, 22). In this study, we identified Hsp27 as highly overexpressed in AI LNCaP tumors using a gene microarray of 13,791 genes, and confirmed in tissue microarrays that Hsp27 levels increased significantly in human disease after neoadjuvant hormone treatment to become uniformly and highly expressed in AI tumors. The highly uniform expression of Hsp27 in metastatic hormone-refractory lesions obtained from rapid autopsy specimens further underscores the association of Hsp27 with the lethal component of this disease. We extend these observations into functional data by showing that increased Hsp27 levels following androgen withdrawal confers an ant apoptotic survival advantage to enhance the AI growth of LNCaP tumors. Importantly, Hsp27 knockdown using ASO or siRNA increased active cleaved caspase-3 and apoptotic rates, supporting the negative regulation of caspase-3 activation by Hsp27 (25).

In this report, we identify a mechanism by which castration-induced changes in Hsp27 expression serves as an upstream regulator of Stat3 activity. Considerable evidence suggests that activated Stat3 functions as an oncogenic protein in many cancers, including prostate cancer (35, 36). The AI phenotype induced by interleukin-6 (IL-6) in several prostate cancer cells is accompanied by the activation of Stat3. For example, androgen-independent DU145 prostate cancer cell lines constitutively express activated Stat3 as a result of autocrine production of IL-6, whereas androgen-sensitive LNCaP cells increase Stat3 activity following paracrine-mediated IL-6 stimulation via Janus-activated kinase (37, 38). Increased Stat3 signaling has been linked to AI progression, whereas constitutively active Stat3 signaling can antagonize androgen deprivation–induced prostate cell death (39, 40). IL-6-induced proliferation of prostate cancer cell lines is associated with the activation of Stat3, whereas inhibition of IL-6 activation of Stat3 inhibited cell growth and induction of apoptosis (35, 37). In this study, we found that Hsp27 colocalizes with Stat3 and that Stat3 protein levels correlate directly with changes in Hsp27. Our findings suggest that increased apoptotic rates after Hsp27 silencing is mediated, in part, via reduced Stat3 protein levels and Stat3-regulated genes c-fos and sPLA₂-IIA.

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