Heat Shock Protein 27 Increases after Androgen Ablation and Plays a Cytoprotective Role in Hormone-Refractory Prostate Cancer

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ABSTRACT

Heat shock protein 27 (Hsp27) is a chaperone implicated as an independent predictor of clinical outcome in prostate cancer. Our aim was to characterize changes in Hsp27 after androgen withdrawal and during androgen-independent progression in prostate xenografts and human prostate cancer to assess the functional significance of these changes using antisense inhibition of Hsp27. A tissue microarray was used to measure changes in Hsp27 protein expression in 232 specimens from hormone naive and posthormone-treated cancers. Hsp27 expression was low or absent in untreated human prostate cancers but increased beginning 4 weeks after androgen-ablation to become uniformly highly expressed in androgen-independent tumors. Androgen-independent human prostate cancer PC-3 cells express higher levels of Hsp27 mRNA in vitro and in vivo, compared with androgen-sensitive LNCaP cells. Phosphorothioate Hsp27 antisense oligonucleotides (ASOs) and small interference RNA potently inhibit Hsp27 expression, with increased caspase-3 cleavage and PC3 cell apoptosis and 87% decreased PC3 cell growth. Hsp27 ASO and small interference RNA also enhanced paclitaxel chemosensitivity in vitro, whereas in vivo, systemic administration of Hsp27 ASO in athymic mice decreased PC-3 tumor progression and also significantly enhanced paclitaxel chemosensitivity. These findings suggest that increased levels of Hsp27 after androgen withdrawal provide a cytoprotective role during development of androgen independence and that ASO-induced silencing can enhance apoptosis and delay tumor progression.

INTRODUCTION

Prostate cancer is the most common male cancer in North America with >200,000 new cases and 30,000 deaths/year in 2002 from metastatic hormone-refractory prostate cancer (1). Androgen withdrawal therapy remains the only effective treatment for advanced prostate cancer, resulting in durable responses lasting 18 to 24 months. A rise in serum prostate-specific antigen and a return of clinical symptoms, most prominently painful bone metastases, herald the onset of hormone-refractory prostate cancer, a chemoresistant disease with few treatment options. Recently, docetaxel is emerging as an active chemotherapeutic with 50% response rates (2).

Progression to androgen independence is a multifactorial process by which cells acquire the ability to both survive in the absence of androgens and proliferate using nonandrogenic stimuli for mitogenesis and involves variable combinations of clonal selection, adaptive up-regulation of antiapoptotic survival genes, androgen receptor transactivation in the absence of androgen from mutations or increased levels of coactivators, and alternative growth factor pathways, including Her2/neu, epidermal growth factor receptor, transforming growth factor β, and insulin-like growth factor I (3–8). Several genes have been functionally linked to the development of hormone resistance, including Bcl-2, clusterin, insulin-like growth factor binding protein 2, and insulin-like growth factor binding protein 5 (7–11). Recent data also links increased heat shock protein 27 (Hsp27) with hormone resistance and poor outcome in prostate cancer (12). Hsp27 belongs to a family of chaperone proteins that modulate a diverse range of cytoprotective, homeostatic, and pathogenic intracellular activities (13–17). In neoplasia, Hsps are associated with multidrug resistance (18) and apoptosis (19, 20) and are functionally linked to increased tumorigenicity and treatment resistance in breast (21–24) and colon (25, 26) cancers.

Increased Hsp27 expression in hormone-refractory prostate cancer suggests that Hsp27 may confer resistance to androgen withdrawal by blocking apoptotic signals from androgen ablation (27, 28). Moreover, Hsp27 expression in hormone-naive prostate cancer may increase resistance to androgen ablation because a higher proportion of nonresponders or early relapses to hormonal therapy occurred in patients strongly expressing Hsp27 (12). Blockage of apoptosis is important in hormone-refractory prostate cancer and is associated with the differential expression of cell survival genes like Bcl-2 and clusterin (29). Hsp27 and Bcl-2 act at different levels to regulate apoptosis depending on the type of apoptotic signal (30). Hsp27 is a predictor of poor outcome and response to therapy in breast cancer (31–33) but has not been extensively studied in prostate cancer. Hsp27 levels are higher in AI PC-3 and DU145 cell compared with androgen-sensitive LNCaP cells (12) and among the most consistently overexpressed genes in hormone-refractory prostate cancer xenografts (27).

Accumulating evidence links rising Hsp27 levels with hormone-refractory prostate cancer and development of treatment resistance and therefore identifies Hsp27, a potential therapeutic target. However, the functional significance of changes in Hsp27 associated with drug resistance and hormone-refractory prostate cancer remains undefined. Antisense oligonucleotides (ASOs) and small interference RNA (siRNA) duplexes are useful tools in functional genomics. Phosphorothioate ASOs are stabilized to resist nuclease digestion by substituting one of the nonbridging phosphoryl oxygen of DNA with a sulfur and useful for both in vitro and in vivo models (34, 35). siRNA are double-stranded RNA that potently block gene expression by a process of sequence-specific posttranscriptional gene silencing (36). These siRNAs are incorporated into a multiprotein RNA-induced silencing complex, where the siRNA duplex is unwound, leaving the antisense strand to guide the silencing complex to its homologous mRNA targets for endonucleolytic cleavage (37). The aim of this study was to characterize changes in Hsp27 after androgen withdrawal and during androgen-independent progression in human prostate cancer and to assess the effects of antisense inhibition of Hsp27 in vitro and in vivo on rates of prostate cancer apoptosis and tumor progression.

MATERIALS AND METHODS

Tumor Cell Lines. Human prostate cancer PC-3 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM (Invitrogen-Life Technologies, Inc., Burlington, Ontario, Canada), supplemented with 5% fetal calf serum. LNCaP cells were kindly provided by
Dr. Leland W. K. Chung (University of Virginia, Charlottesville, VA) and maintained in RPMI 1640 (Invitrogen-Life Technologies, Inc.) supplemented with 5% FCS.

Chemotherapeutic Agents. Paclitaxel was purchased from Biolyse Pharma (St. Catharines, Ontario, Canada). Stock solutions of paclitaxel were prepared with PBS to the required concentrations before each in vitro experiment. Dr. Helen M. Burt (Pharmaceutical Sciences, University of British Columbia, Vancouver, Canada) generously supplied polymeric micellar paclitaxel used for in vivo studies.

Hsp27 ASO and siRNA. Phosphorothioate ASOs used in this study were purchased from Qiagen Operon (Alameda, CA). The sequence of Hsp27 ASO used corresponded to the human Hsp27 translation initiation site (5'-GG-GACGCGCCGGCGCCTGCT-3'). A scrambled oligonucleotide antisense ASO (5'-CACGCGTCAGACAGCTTCTT-3') was used as a control. The sequence of Hsp27 siRNA used corresponded to the human Hsp27 site (5'-GUCUCAUGCGAUUUACAGC-3'; Drharmacon, Lafayette, CO). A scrambled siRNA duplex (5'-CACGCGGUGACACACGUUUCAU-3') was used as a control.

Treatment of Cells with Hsp27 ASO and siRNA. Cells were plated at the density of 4000 cells per 1.9 cm² and treated 1 day later for 1 or 2 days with siRNA or ASO, respectively. Oligofectamine, a cationic lipid (Invitrogen-Life Technologies, Inc.), was used to increase the ASO or siRNA uptake into the cells. PC-3 cells were treated with various concentrations of ASO or siRNA after a preincubation for 20 min with 3 mg/ml oligofectamine in serum free OPTI-MEM (Invitrogen-Life Technologies, Inc.). Four hours after the beginning of the incubation, the medium was replaced with standard culture medium described above.

Northern Blot Analysis. Total RNA was isolated from cultured PC-3 and LNCaP cells and tumor tissues using the Trizol method (Invitrogen). Electrophoresis, hybridization, and washing conditions were carried out as reported previously (11). Human Hsp27 cDNA probe was obtained from StressGen (Victoria, British Columbia, Canada). Density of bands for Hsp27 was normalized against that of 28S rRNA by densitometric analysis (Quantity One; Bio-Rad, Hercules, CA). Each assay was performed in triplicate.

Western Blot Analysis. Samples containing equal amounts of protein (15 μg) from lyses of cultured PC-3 cells underwent electrophoreses on a SDS-polyacrylamide gel and transferred to a nitrocellulose filter. Filters were blocked in PBS containing 5% nonfat milk powder at 4°C overnight and then incubated for 1 hour with a 1:5000-diluted antihuman Hsp27 rabbit polyclonal antibody (StressGen) or 1:2000-diluted antihuman Vinculine monoclonal antibody (StressGen) overnight. Filters were then incubated for 30 minutes with 1:5000-diluted horseradish peroxidase-conjugated anti-rabbit or mouse monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Specific proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Life Sciences, Arlington Heights, IL). MAB3299 that specifically reacts with single-stranded DNA, permitting detection of apoptotic cells and not necrotic cells (39).

Flow Cytometric Analysis. Flow cytometric analysis of propidium iodide-stained nuclei was performed as described previously (40). Briefly, the PC-3 cells were plated in 75-cm² dishes, and the day after were treated as described above. 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 performed in triplicate.

Measurement of Caspase-3 Cleavage and Activity. Caspase-3 cleavage was detected by Western blotting as described above using 40 μg of protein. Polyclonal antibody (New England Biolabs, Ipswich, MA) was used to detect full-length (M, 32,000 to 35,000) and large fragment of activated caspase-3 (M, 17,000 to 20,000) that results from cleavage after Asp175.

Caspase-3 activity in cell lysates was analyzed using caspase-3 colorimetric assay kit (New England Biolabs, Beverly, MA). Soluble cytosolic proteins were mixed with the caspase-3-specific substrate DEVD-pNA in a final volume of 100 μL and incubated at 37°C. Subsequently, substrate cleavage was monitored at 405 nm using microtiter plate reader (Becton Dickinson Labware, Lincoln Park, NJ). To confirm that substrate cleavage was due to caspase activity, extracts were incubated in the presence of 10 μmol/L of the caspase-3-specific inhibitor DEVD-CHO for 10 minutes at 37°C before the addition of substrate. The absorbance signal (in arbitrary units) of the DEVD-CHO-inhibited sample was subtracted from the absorbance signal of the uninhibited sample.

Assessment of In Vitro Tumor Growth. Approximately 1 x 10⁶ PC-3 cells were inoculated subcutaneously with 0.1 mL of Matrigel (Becton Dickinson Labware, Mississauga, Ontario, Canada) in the flank region of 6 to 8-week-old male athymic nude mice (Harlan Sprague Dawley, Inc., Indianapolis, IN) via a 27-gauge needle under halothane anesthesia. When PC-3 tumors reached 200 mm³, usually 3 to 4 weeks after injection, mice were randomly selected for treatment with Hsp27 ASO alone, scrambled ASO alone, Hsp27 ASO plus paclitaxel, scrambled ASO plus paclitaxel, or PBS alone. Each experimental group consisted in 10 mice. After randomization, 10 mg/kg Hsp27 or scrambled ASO was injected i.p. once daily for 42 days for ASO monotherapy groups and for 70 days in the ASO plus paclitaxel groups. A total of 0.5 mg with H&E (Vector Laboratories, Burlingame, CA). After ethanol rehydration, a cover glass was applied with Cytoseal, a xylene-based mounting media (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 serum. Photomicrographs were taken with 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 by Image Pro-Plus software (Media Cybernetics, Carlsbad, CA). Specimens 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 to 100%) was also indicated. All comparisons of staining intensities and percentages were made at x200 magnification.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay. The in vitro growth inhibitory effects of Hsp27 ASO or siRNA plus paclitaxel on PC-3 cells were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (38). Briefly, cells were seeded in each well of 12-well microtiter plates and allowed to attach overnight. Cells were then treated once daily with oligonucleotides at 30 nmol/L for 2 days and siRNA at 1 nmol/L concentration for 1 day. One or 2 days after ASO or siRNA treatment, respectively, cells were incubated with various concentrations of paclitaxel. Every 24 hours over a period of 4 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were carried out. Each assay was performed in triplicate.

Immunocytochemistry for In situ Apoptosis. PC-3 cells were plated in a 9.87-cm² labnek (Nunc, Roskilde, Denmark) and treated with oligonucleotides as described above. Two days after transfection, cells are harvested and fixed with methanol for 10 minutes. Slides were then transferred into a Coplin jar containing 50 mL of 50% formamide (v/v distilled H2O) in water bath at 56°C to 60°C for 20 minutes and then incubated for 10 minutes in 0.2% Triton. After PBS washing, endogenous peroxidase was quenched in 3% hydrogen peroxide for 5 minutes. Detection of cleaved apoptotic DNA fragments were performed using mouse monoclonal antibody MAB3299 (Chemicon International, Temecula, CA). MAB3299 that specifically reacts with single-stranded DNA, permitting detection of apoptotic cells and not necrotic cells (39).

Flow Cytometric Analysis. Flow cytometric analysis of propidium iodide-stained nuclei was performed as described previously (40). Briefly, the PC-3 cells were plated in 75-cm² dishes, and the day after were treated as described above. 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 performed in triplicate.

Measurement of Caspase-3 Cleavage and Activity. Caspase-3 cleavage was detected by Western blotting as described above using 40 μg of protein. Polyclonal antibody (New England Biolabs, Ipswich, MA) was used to detect full-length (M, 32,000 to 35,000) and large fragment of activated caspase-3 (M, 17,000 to 20,000) that results from cleavage after Asp175.

Caspase-3 activity in cell lysates was analyzed using caspase-3 colorimetric assay kit (New England Biolabs, Beverly, MA). Soluble cytosolic proteins were mixed with the caspase-3-specific substrate DEVD-pNA in a final volume of 100 μL and incubated at 37°C. Subsequently, substrate cleavage was monitored at 405 nm using microtiter plate reader (Becton Dickinson Labware, Lincoln Park, NJ). To confirm that substrate cleavage was due to caspase activity, extracts were incubated in the presence of 10 μmol/L of the caspase-3-specific inhibitor DEVD-CHO for 10 minutes at 37°C before the addition of substrate. The absorbance signal (in arbitrary units) of the DEVD-CHO-inhibited sample was subtracted from the absorbance signal of the uninhibited sample.
micellar paclitaxel was administrated i.v. three times per week from days 7 to 14 and from days 21 to 28. Tumor volume measurements were performed once weekly and calculated by the formula length × width × depth × 0.5236 (9). Data points were expressed as average tumor volume levels ± SE. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and with appropriate institutional certification.

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

**RESULTS**

**Hsp27 Is Expressed at Higher Levels in Androgen-Independent Prostate Cancer Cell Lines.** Northern blot analysis revealed the presence of 0.95 Kb of mRNA hybridizing with the probe for human Hsp27 in hormone-sensitive LNCaP and AI PC-3 cell lines (Fig. 1A). The amount of RNA present in each sample was then normalized to the amount of 28S RNA present on the gel (Fig. 1B). On the basis of at least three independent preparations, the amount of Hsp27 mRNA in PC-3 cells *in vitro* was 2.3-fold higher than that in LNCaP cells (**, *P* ≤ 0.01). Hsp27 mRNA levels were also compared in PC3 and LNCaP xenografts, with similar patterns to the *in vitro* cell lines (Fig. 1, A and B).

**Hsp27 Immunoexpression Increases after Androgen Withdrawal in Human Prostate Specimens.** To determine whether Hsp27 expression is altered by androgen ablation or during androgen-independent progression, 232 prostate cancers spotted onto a TMA were immunostained for Hsp27 (Fig. 2). Hsp27 protein was present in the cytoplasm of epithelial cells. Hsp27 expression increased 4-fold after androgen ablation and was significantly higher at time points...
Fig. 3. Sequence-specific and dose-dependent inhibition of Hsp27 expression by Hsp27 ASO in PC-3 cells. A, PC-3 cells were treated daily with indicated concentrations of Hsp27 ASO or scrambled ASO controls for 2 days; total RNA was extracted from culture 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 densitometric analysis in PC-3 cells after treatment with various concentrations of Hsp27 ASO or scrambled control ASO. Points, means of triplicate analysis; bars, SE. ** and *** differ from scrambled control (P ≤ 0.01 and P ≤ 0.001, respectively) by Student’s t test. D and E, PC-3 cells were treated daily with various concentrations of Hsp27 ASO and its scrambled control ASO or Hsp27 siRNA or its siRNA-scrambled control. Two days after treatment, proteins were extracted from culture cells, and Hsp27 and vinculin protein levels were analyzed by Western blotting. Oligofectamine, oligofectamine treated cells only.

Fig. 4. Effect of treatment with Hsp27 ASO and siRNA plus or minus paclitaxel on PC-3 cell growth in vitro. PC-3 cells were treated daily with 30 nmol/L Hsp27 and scrambled ASO for 2 days or 1 nmol/L Hsp27 or scrambled siRNA for 1 day. After ASO or siRNA treatment, the medium was replaced with medium containing FBS as described previously. A, After 24 hours of incubation, cell viability was determined daily during 4 days by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. B, Two days after siRNA treatment, cells were incubated with different concentrations of paclitaxel. * P ≤ 0.05; ** P ≤ 0.01; and *** P ≤ 0.001 differ from scrambled control by Student’s t test.

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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 androgen independent were 0.66, 1.29, 1.76, 1.99, and 2.08, respectively (Fig. 2B). Androgen-independent tumor tissue demonstrated highly positive Hsp27 staining in all specimens (Fig. 2A).

**Sequence-Specific and Dose-Dependent Inhibition of Hsp27 Expression by ASO and siRNA.** To study the functional role of hormone therapy-induced increases in Hsp27, ASO- or siRNA-induced inhibition of Hsp27 expression was determined by Northern and Western blot analysis. As shown in Fig. 3, A and B, treatment of PC-3 cells with ASO significantly reduced Hsp27 mRNA levels by up to 75% in a dose-dependent manner (**, P ≤ 0.01; ***, P ≤ 0.001), whereas Hsp27 mRNA expression was not significantly suppressed by scrambled oligonucleotide. Similar dose-dependent inhibition was observed using siRNA between 10⁻⁴ to 1 nmol/L (Fig. 3C). Significant inhibition of Hsp27 protein levels was also observed after ASO (Fig. 3D) or siRNA treatment (Fig. 3E).

**Hsp27 ASO and siRNA Inhibits PC-3 Cell Growth and Enhances Chemotherapy In vitro.** To determine whether the reduction of Hsp27 expression affects PC-3 cell growth on paclitaxel chemosensitivity in vitro, cells were treated for 2 days with 30 nmol/L Hsp27 ASO or scrambled control or 1 day with 1 nmol/L Hsp27 siRNA or scrambled control. At the end of the treatment, PC-3 cells were incubated with different concentrations of paclitaxel. Growth rates of PC-3 cells were examined daily for 4 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 4). Fig. 4A shows an 87% (***, P ≤ 0.001) reduction in PC-3 cell growth 4 days after treatment with Hsp27 ASO 30 nmol/L alone compared with scrambled control. Similar growth inhibition was found with 1 nmol/L Hsp27 siRNA (data not shown). Significant single-agent activity makes detection of additive chemosensitivity difficult. However, Fig. 4B shows that 1 nmol/L siRNA can enhance paclitaxel sensitivity by up to 29% in a dose-dependent manner.

**Hsp27 ASO and siRNA Treatment Induces Apoptosis in PC-3 Cells In vitro.** Treatment of PC-3 cells with 30 nmol/L Hsp27 ASO for 2 days induced morphologic changes characteristic of apoptosis.

![Flow cytometry](image)
Hsp27 INHIBITION INCREASES APOPTOSIS IN PC-3

Similar increases in apoptotic bodies and sub-G$_0$-G$_1$ fraction were associated with ASO-induced Hsp27 silencing. Data were confirmed by measuring caspase-3 activity (Fig. 6). Very low signal was present in the nontreated cells. Induction of apoptosis by Hsp27 ASO was also demonstrated by flow cytometry. After the same treatment schedule described above, the fraction of cells undergoing apoptosis (sub-G$_1$-G$_0$ fraction) was significantly higher after treatment with 30 nmol/L Hsp27 ASO compared with those treated with scrambled control oligonucleotide (Fig. 5, B and C). Very low signal was present in the nontreated cells. Induction of apoptosis by Hsp27 ASO was also demonstrated by flow cytometry. After the same treatment schedule described above, the fraction of cells undergoing apoptosis (sub-G$_1$-G$_0$ fraction) was significantly higher after treatment with 30 nmol/L Hsp27 ASO compared with those treated with scrambled control oligonucleotide (33.8 versus 9.04%; **; $P \leq 0.01$; Fig. 5D). Similar increases in apoptotic bodies and sub-G$_1$-G$_0$ fraction were also found using Hsp27 siRNA 1 nmol/L (data not shown), which clearly demonstrate an increased apoptotic rate associated with Hsp27 silencing.

Hsp27 ASO and siRNA Treatment Increase Caspase-3 Cleavage and Activity. Hsp27 has been reported to interact with caspase-3, inhibiting caspase-3 activation (41). Fig. 6A shows the presence of active cleaved caspase-3 fragments (17–20 kDa) only in PC-3 cells treated with Hsp27 ASO 30 nmol/L. Caspase-3 cleavage after Hsp27 ASO treatment was also seen by immunostaining with an antibody that recognizes only cleaved caspase-3 (data not shown). Similar increases in caspase-3 cleavage were also found using Hsp27 siRNA 1 nmol/L (data not shown). This data were confirmed by measuring caspase-3 activity (Fig. 6B). Caspase-3 activation associated with ASO-induced Hsp27 silencing was also demonstrated using a caspase-3 colorimetric assay (Bio-mol), with a 3.8-fold increase caspase-3 activity in PC-3 cells after Hsp27 ASO treatment 30 nmol/L.

**Hsp27 ASO Treatment Inhibits PC-3 Tumor Progression and Enhances Chemotherapy In vivo.** We next evaluated the effects of Hsp27 ASO treatment on the growth of PC-3 tumors in vivo (Fig. 7). Male nude mice bearing PC-3 tumors (200 mm$^3$) were randomly selected for Hsp27 ASO versus scrambled ASO and 10 mg/kg ASO were administered once daily by i.p. injection for 91 days. From days 7 to 14 and 21 to 28, 0.5 mg of micellar paclitaxel were administrated i.v. once daily. Mean tumor volume was similar in all groups before therapy. Fig. 7A shows that Hsp27 ASO monotherapy significantly reduced PC-3 tumor volume by ~50% from days 28 to 42 (**, $P \leq 0.01$). Moreover, treatment with Hsp27 ASO, compared with scrambled control, significantly enhanced the apoptotic effects of paclitaxel in vivo, reducing mean PC-3 tumor volume by ~70% by 13 weeks after initiation of treatment (*, $P \leq 0.01$; Fig. 7B). Under the experimental conditions described above, no adverse effects were observed. Apoptotic rates, detected by single-stranded DNA nuclear staining (MAB 3299; Chemicon) and scored by Image Pro-Plus, increased by 72% (from 0.22 to 0.36%) in mice treated with Hsp27 ASO ($P \leq 0.05$) compared with scrambled control oligonucleotide. No differences in staining for the proliferative marker Ki-67 (Clone MIB-1, M 7240l; DAKO, Mississauga, Ontario, Canada) were observed, indicating that differences in rates of tumor progression resulted from increased apoptotic rates in Hsp27 ASO-treated mice.
DISCUSSION

Androgen withdrawal results in prostatic epithelial cell death associated with rapid increase in transcription of multiple genes normally suppressed by testosterone (42, 43). Although many patients with prostate cancer respond initially to androgen ablation, some cells survive androgen withdrawal by adjusting their apoptotic rheostat through expression of various survival factors. The development of androgen resistance represents a major obstacle to effective control of disseminated disease. Before we can have a significant impact on survival with the development of new therapeutic strategies, an improved understanding of the molecular pathogenesis of androgen-independent progression is needed. It is now generally accepted that the vectorial balance between proliferation and apoptosis determines rates of tumor growth and tumor progression. Overexpression of antiapoptotic genes such as bcl-2, clusterin, and insulin-like growth factor binding protein 2 is associated with hormone-refractory prostate cancer and chemoresistance (8–11), indicating that several distinct mechanisms can be induced to regulate programmed cell death.

Hsps comprise several different families of proteins that are induced in response to a wide variety of physiologic and environmental insults (44). One such protein that becomes highly induced during the stress response is a M, 27,000 protein, termed Hsp27, the expression of which correlates with increased survival in response to cytotoxic stimuli. The cytoprotective effects of Hsp27 may result from its role as molecular chaperone or through direct interference with caspase activation, modulation of oxidative stress, and regulation of the cytoskeleton (45, 46). Several previous studies identify Hsp27 as an independent survival factor in prostate cancer (28, 47, 48). The aims of this study were to characterize the changes in Hsp27 expression after androgen withdrawal and during androgen-independent progression and to assess its functional significance role in hormone-refractory prostate cancer using ASO or siRNA loss-of-function analyses.

Our data confirm that Hsp27 levels are higher in androgen-independent PC-3 compared with androgen-sensitive LNCaP cells, supporting earlier reports by Cornford et al. (12). The pattern of change in Hsp27 expression in human prostate cancer after androgen withdrawal parallels that seen in LNCaP tumors after castration. TMA of 232 prostate cancer specimens showed increased Hsp27 level after androgen withdrawal, with uniformly high expression in all cases of hormone-refractory prostate cancer. Previous reports have linked Hsp27 expression to reduced survival in breast cancer and prostate cancer (12, 32). Bubendorf et al. (27) also noted that Hsp27 was one of the most overexpressed genes in hormone-refractory prostate cancer xenografts. Moreover, Hsp27 is overexpressed in several other hormone-sensitive organs and human tumors and correlates with increased resistance to various cytotoxic chemotherapeutic agents (22, 25, 49).

To assess the role of Hsp27 as a chemoresistance factor in hormone-refractory prostate cancer, we chose androgen-independent PC-3 cells to study the effect of Hsp27 down-regulation. Antisense ASO and siRNA are one strategy to specifically silence gene expression. Hsp27 ASO and siRNA used in this study reduced Hsp27 levels by 75% and significantly decreased (~90%) cell growth in vitro. Pretreatment of PC-3 cells with Hsp27 ASO enhanced apoptosis via caspase-3 activation, supporting recent data showing that Hsp27 functions as a negative regulator of cytochrome c-dependent activation of procaspase-3 (41). Recently, Concannon et al. (45, 46) also reported that Hsp27 inhibits cytochrome c-mediated caspase activation by sequestering both procaspase-3 and cytochrome c. Consistent with these in vitro data, systemic administration of Hsp27 ASO monotherapy suppressed PC-3 tumor growth in vivo and also significantly enhanced paclitaxel activity in vitro and in vivo. These results are consistent with recent reports that Hsp27 overexpression confers resistance to doxorubicin in MDA breast cancer cells (23).

Collectively, the findings of this study support the hypothesis that Hsp27 up-regulation after apoptotic triggers represents an adaptive cell survival mechanism and provides proof of principle that Hsp27 is an inhibitor of apoptosis in human prostate cancer plus a rational target for biotherapy.

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REFERENCES


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22-24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on "The Effects of Radiation on Aqueous Solutions," which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is "Physical Measurements for Radiobiology" and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray's lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, "The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration," November, 1952, page 891; substitute for the last paragraph:

The data in Table 8 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose "cleavage products" exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if \( M = +0.27 \) and \( L = -0.16 \) and the normal differential is 65 per cent M and 35 per cent L, then

\[
0.65 \left( +0.27 \right) + 0.35 \left( -0.16 \right) = +0.12 ,
\]

a figure identical to the observed +0.12 for normal leukocytes.
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