Heat Shock Protein Expression Independently Predicts Clinical Outcome in Prostate Cancer

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ABSTRACT

Heat shock proteins (hsp) occupy a central role in the regulation of intracellular homeostasis, and differential expression of individual hsp occurs in a broad range of neoplastic processes. This study was performed to test the hypothesis that the particular patterns by which individual hsp become specifically modulated in human prostate cancers are correlated with behavioral phenotype and hence may be of value in determining the most appropriate clinical management of individual patients. Monoclonal antibodies specific for each hsp protein were used to assess expression of hsp27, hsp60, and hsp70 in formalin-fixed, paraffin wax-embedded, archival tissue specimens of early prostatic adenocarcinomas (pT1–N0M0) removed at radical prostatectomy (n = 25) and in advanced cancers (n = 95) identified at transurethral resection of prostate (TURP). These findings were compared with similar data from control prostate (n = 10) removed at primary cystectomy for urinary bladder neoplasia not involving the prostate and also at TURP for benign prostatic hyperplasia (n = 50). Western blotting of whole cell lysates derived from established human prostatic epithelial cell lines PNT2, LNCaP, DU145, and PC3 was compared with expression of hsp by the primary human tissues. This study found that early in situ neoplastic transformation of normal prostatic epithelium was consistently associated with loss of hsp27 expression and that the level of hsp27 expression by individual prostate cancers was correlated with their Gleason grade. In advanced cancers, hsp27 expression was invariably associated with poor clinical outcome (P = 0.0001). Data from cell lines supported the primary tissue findings, with elevated hsp27 expression only in aggressive malignant cell lines and androgen-insensitive cell lines. Expression of hsp60 was significantly increased in both early and advanced prostate cancer when compared with nonneoplastic prostatic epithelium (P < 0.00001), as well as in malignant prostate cancer cell lines. Expression of hsp70 was unaltered in early prostate cancers when compared with nonneoplastic prostatic epithelium but showed a diminished expression in morphologically advanced cancers (P = 0.0029). No consistent correlation was found between levels of hsp60 or hsp70 expression and phenotypic behavior of individual primary prostatic cancers. Thus, patterns of hsp27 expression have been confirmed to be specifically and consistently modulated in both early and advanced human prostate cancers. Whereas absence of hsp27 is a reliable objective marker of early prostatic neoplasia, reexpression of this protein by an individual invasive prostate carcinoma invariably heralds poor clinical prognosis. Because this protein has been shown to alter the balance between proliferation and apoptosis, understanding the mechanism(s) by which individual hsp regulate intracellular homeostasis may assist in explaining some key processes that occur during evolution of human prostate cancers. We suggest that hsp27 expression provides novel diagnostic and prognostic information on individual patient survival which, if obtained at the time of primary diagnosis, would assist in determining tumor-specific management strategies. Development of techniques to therapeutically modulate hsp27 expression raises the possibility of novel targeted approaches to regulate this homeostatic mechanism, thus allowing better control over tumor cell proliferation and hence patient survival.

INTRODUCTION

hsp27 are highly conserved throughout evolution. They mediate and modulate a diverse range of intracellular activities which, according to their relative levels, fulfill protective functions (1, 2), ensure metabolic homeostasis (3, 4), and participate in a diverse range of pathogenic processes (5, 6). Some are normal cellular proteins expressed under nonstressed conditions and in a cell cycle-dependent manner (7, 8), whereas others are chaperonins of nascent proteins (9). In neoplasia, hsp have been implicated in multidrug resistance (10), in regulation of apoptosis (11), and as modulators of p53 function (12).

hsp27 is constitutively expressed at low levels in the cytosol of most human cells (13). After induction, the protein becomes phosphorylated while simultaneously translocated from the cytoplasm to within or around the nucleus (14–16). Phosphorylation is a key regulator of hsp27 function occurring at serine residues 78 and 82 (17) through interaction with a specific kinase, but it may be activated by several different signal transduction mechanisms. The presence of the protein contributes to cell survival after diverse stress insults (14, 16, 18). In murine L929 (19) and in human HT-29 and CaCo2 colorectal cancer cell lines (20), hsp27 inhibits apoptosis induced by a variety of different stimuli. It has been proposed that hsp27 modulates reactive oxygen species via a glutathione-dependent pathway (21), thereby protecting intracellular proteins and explaining, in part, the protective effect of hsp27 against chemotherapeutic agents (22, 23).

hsp60 is abundant in most mammalian cells under normal conditions (24), where its major functions are protein chaperoning and protein folding (25). Both processes are coregulated by hsp10 (26). Whereas aberrant expression of hsp60 has been associated with autoimmune disease, hsp60 has a role together with hsp70 in antigen presentation in malignant diseases (27), with enhanced hsp60 expression reported in breast carcinoma (28) and myeloid leukemia (29).

hsp70 regulates a wide range of protein-associated activities (30–33). Expression of hsp70 is enhanced after transformation by oncogenes (34, 35). Elevated levels of hsp70 protect cells from apoptotic death induced by TNF-α and TNF-β (36). hsp70 interacts with p53 to stabilize mutant but not the wild-type protein (37). Conversely, wild-type (but not mutant) p53 down-regulates hsp70 expression (38).

Our recent studies demonstrated that expression of other homeostatic regulator proteins, including PKC isoenzymes (39), Ca2+-binding proteins (40), and ion channels (41, 42), are differentially modulated in prostate cancers. When enhanced, these proteins promote the metastatic phenotype, and their expression predicts poor clinical outcome (39). Because some members of the PKC and hsp families are...
integral components of the same intracellular information cascades, together promoting cell migration, inhibiting proliferation, and down-regulating apoptosis, it was predicted that expression of some hsps would also contribute to the behavioral phenotype of individual prostate cancers. Therefore, this study was undertaken to test the hypothesis that hsps are differentially expressed during induction and progression of prostatic neoplasia and that particular hsps, together with other homeostatic regulatory proteins, determine the clinical outcome of individual prostate cancers. Identification of such proteins at the time of diagnosis could provide new prognostic information with respect to the behavioral or responsive phenotype of prostate cancers, thus influencing appropriate clinical management for individual patients. Depending on their critical roles in maintaining cellular integrity and function, this spectrum of homeostatic proteins may also become a target for novel forms of tumor-specific therapeutic intervention (43).

MATERIALS AND METHODS

Tissues. All tissue samples were retrieved from the archives of the Department of Pathology, Royal Liverpool University Hospital (Liverpool, United Kingdom).

Control Tissues. Prostates removed from men (n = 10; mean age, 52.4 years) undergoing primary prostaticctomy for invasive transitional cell carcinoma of the urinary bladder were used to control the immunohistochemical findings on the radical prostatectomy tissues. All of these patients were asymptomatic for lower urinary tract symptoms. Prostate samples removed at TURP (n = 50; mean age, 70.2 years; range, 54–84 years) were used to control the findings in prostatic carcinoma specimens obtained at TURP and comprising 10 incidentally identified carcinomas and 85 advanced carcinomas, as detailed below. All control specimens were reviewed microscopically to confirm the absence of incidental PIN and carcinoma of either prostatic or bladder origin.

Prostate Carcinoma Tissue. Three groups of tissues were examined: (a) radical prostatectomy specimens obtained from 25 consecutive and otherwise unselected patients with organ-confined disease (pT1N0M0); (b) specimens from 10 patients with incidentally diagnosed early invasive prostate cancer (pT1N0M0) at TURP for lower urinary tract symptoms; and (c) transurethral resection specimens from 85 patients with advanced prostate cancer (pT3a, pT3b, T4N1M0) who had undergone BSCO at least 3 months previously. Patients were clinically staged by digital rectal examination, serum PSA, bone scan, and chest X-ray. Patients undergoing radical prostatectomy were also assessed by transrectal ultrasound scanning and with abdominal computed tomography scan or, after 1994, transrectal magnetic resonance imaging. Patient demographic details and tumor characteristics are detailed in Table 1.

Patients were reviewed at 3-month intervals for the first year and at 6-month intervals thereafter. Serum PSA was measured at review. Other investigations were directed by the clinical findings in individual cases.

Of the radical prostatectomy group (n = 25), at the time of last follow-up, one patient had died of recurrent prostatic carcinoma at day 1123 after surgery, and two patients had clinical evidence of recurrent tumor recorded at 578 and 1665 days after surgery. A fourth patient recorded an elevated serum PSA (7.2 µg/liter) and was considered to have chemical evidence of recurrence (PSA began to rise 1004 days after surgery). Three patients died of unrelated noncancer causes with no evidence of recurrent carcinoma and were deemed to be disease free.

Four patients with incidentally diagnosed prostate cancer (n = 10) died of unrelated conditions, whereas six remain alive and well with no evidence of disease progression. None of these patients required hormonal manipulation. Of the 85 patients with advanced prostate cancer 44 (51.7%) died of prostate cancer, 2 (2.4%) patients had evidence of either chemical or clinical progression, 16 (18.8%) died of other causes without evidence of disease progression, and 23 (27.1%) patients remain alive and show no evidence of disease progression.

Gleason grading of all prostatic carcinomas included in this study was performed by two pathologists according to conventional criteria (44). Antibodies and Antisera. Murine monoclonal antibodies to hsp27 and hsp70 were purchased from Novocastra (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom). The antibody against hsp60 was purchased from Stress Gen Biotechnologies Corp. (York, United Kingdom). Biotinylated antimouse immunoglobulin raised in sheep was purchased from Amersham Life Science (Little Chalfont, United Kingdom). Microwave antigen retrieval was performed at 850 W for 15 min in 10 mM EDTA solution (pH 7.0), and samples were then allowed to cool to room temperature over a 15-min period. All antibodies were diluted in TBS containing 5% (w/v) BSA to the concentration shown in Table 2.

Table 2 Dilution, pretreatment, and positive controls for primary antibodies

<table>
<thead>
<tr>
<th>Immunohistochemistry</th>
<th>Western blotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution</td>
<td>Pretreatment</td>
</tr>
<tr>
<td>hsp27</td>
<td>1:20</td>
</tr>
<tr>
<td>hsp60</td>
<td>1:400</td>
</tr>
<tr>
<td>hsp70</td>
<td>1:40</td>
</tr>
<tr>
<td>Actin</td>
<td>n/a</td>
</tr>
</tbody>
</table>

* MW, microwave; n/a, not applicable.

Table 3 Expression of hsp27 in prostatic epithelium

<table>
<thead>
<tr>
<th>N</th>
<th>Negative</th>
<th>Weak</th>
<th>Strong</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control normal tissues</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>Radical prostatectomy</td>
<td>25</td>
<td>21</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>TURP-BPH</td>
<td>50</td>
<td>0</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>TURP-early CaP</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>1</td>
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<tr>
<td>TURP-advanced CaP</td>
<td>85</td>
<td>47</td>
<td>18</td>
<td>20</td>
</tr>
</tbody>
</table>

* Significance relative to appropriate control tissues.

CaP, prostate cancer.
Immunohistochemistry. Sections of each specimen were cut at 4 μm onto poly-L-lysine-coated glass slides and dewaxed with xylene followed by rehydration through graded ethanols. Endogenous peroxidase activity was blocked by immersion in a 3% (w/v) solution of H2O2 in methanol for 15 min. Sections were then rinsed in tap water followed by distilled water. After equilibration in fresh TBS comprising 0.05M Tris (pH 7.6) containing 0.12 M NaCl, sections were incubated with primary antibodies for 60 min at room temperature, washed twice with TBS, and incubated with biotinylated antimouse immunoglobulin for 45 min at room temperature. Thereafter, sections were washed twice before application of horseradish peroxidase-labeled streptavidin-biotin complex (Dako Ltd., High Wycombe, United Kingdom) for 30 min at room temperature. After rinsing, sections were immersed in a solution of 3,3'-diaminobenzidine tetrahydrochloride (Dako Ltd.) diluted to 250 μg/ml in water containing 0.03% (v/v) hydrogen peroxide for 7 min to reveal sites of antibody binding. Nuclei were counterstained with Gill’s hematoxylin before mounting slides in DPX. For the two negative controls used in each experiment, either the primary or the secondary antibody was replaced with 5% BSA. All sections were independently scored by two investigators (C. S. F. and P. A. C.).

Analysis of hsp Expression. Specimens were considered positive only when at least 5% of the contained epithelial cells (either normal or malignant) unequivocally expressed hsp staining. The 5% cutoff was chosen because it conforms to the international European Organization for Research and Treatment of Cancer-Gynaecological Cancer Cooperative Group recommendations (45). Furthermore, this cutoff was used as the criterion to distinguish positive and negative immunohistochemical staining in our previous studies of prostate cancer including MHC expression (46) and PKC isoenzyme expression by this identical group of prostatic neoplasms (39), thus ensuring consistency of criteria between studies. For each tissue section, staining was assessed as negative, weakly positive or only focally positive (low-level expression), or strongly positive (high-level expression) and scored as 0, 1, or 2, respectively. For positive sections, the assessments made were: (a) cellular distribution of each hsp in benign and malignant human prostatic epithelium; and (b) the relationship between expression of hsp and tumor grade.

Cell Lines. Expression of hsps by human transformed benign prostate cell line PNT2 (43), androgen-sensitive prostate cancer cell line LNCaP (44), and androgen-resistant cell lines DU145 (45) and PC3 (46) was analyzed. Cells were grown as monolayers in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies, Inc.), 1 mM glutamine, 100 IU/ml penicillin G, and 100 IU/ml streptomycin in an atmosphere of 5% CO2 in air at 100% humidity and 37°C. Media were changed on alternate days.

Fig. 1. a, hsp27 expression in a prostatic duct containing strongly stained residual nonneoplastic luminal epithelial cells and basal cells. Regions of high-grade PIN and carcinoma in situ are unstained. The tissue surrounding the duct comprises weakly stained smooth muscle cells together with foci of invasive carcinoma (⁎) that are not stained. ×150. b, hsp27 expressed by poorly differentiated (Gleason pattern 4) invasive prostate cancer in which the protein is distributed as fine granules throughout the cytoplasm of all malignant cells. ×250. c, hsp60 expressed as coarse granules throughout the cytoplasm of malignant cells. In contrast, expression of this protein in nonneoplastic prostatic epithelium is qualitatively distinct and appears as fine, pale granules. ×250. d, hsp70 is expressed heterogeneously in the invasive malignant cells, although it is significantly up-regulated when compared with the adjacent nonmalignant epithelium. ×250. e, hsp70 strongly but heterogeneously expressed by Gleason pattern 5 invasive prostate cancer. No relationship was identified between morphological grade and intensity of protein expressed by individual prostate cancers. ×250.
Fig. 3. hsp27 expression predicts outcome in advanced prostate cancers (n = 85; \( \chi^2 = 20.78; \) log-rank \( P = 0.0001 \)).

<table>
<thead>
<tr>
<th>Covariate</th>
<th>Coefficient</th>
<th>SE</th>
<th>( P )</th>
<th>Relative risk</th>
<th>95% Confidence limits for relative risk</th>
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<td>hsp27</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong</td>
<td>0.915</td>
<td>0.037</td>
<td>0.0492</td>
<td>1.00</td>
<td>1.00–1.519</td>
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<tr>
<td>Weak</td>
<td>0.322</td>
<td>0.047</td>
<td>0.0143</td>
<td>1.48</td>
<td>1.23–2.718</td>
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<tr>
<td>Gleason</td>
<td>1.390</td>
<td>0.060</td>
<td>0.0023</td>
<td>3.38</td>
<td>1.76–6.479</td>
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<tr>
<td>GS-10</td>
<td>0.348</td>
<td>0.077</td>
<td>0.0496</td>
<td>1.41</td>
<td>1.14–1.76</td>
</tr>
<tr>
<td>GS-7</td>
<td>0.049</td>
<td>0.007</td>
<td>1.65</td>
<td>0.37</td>
<td>0.12–1.11</td>
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<tr>
<td>Stage</td>
<td>0.031</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( T_1 )-CaP</td>
<td>0.031</td>
<td>0.010</td>
<td>0.0001</td>
<td>3.38</td>
<td>1.76–6.479</td>
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<tr>
<td>( T_1 )-MiP</td>
<td>0.034</td>
<td>0.010</td>
<td>0.0005</td>
<td>3.65</td>
<td>2.04–6.59</td>
</tr>
<tr>
<td>( T_1 )-MiP</td>
<td>0.034</td>
<td>0.010</td>
<td>0.0005</td>
<td>3.65</td>
<td>2.04–6.59</td>
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</tbody>
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**RESULTS**

**Immunohistochemistry**

**hsp27 Expression.** All 60 control nonneoplastic tissues comprising 10 normal prostates and 50 TURP-BPH specimens were immunoreactive for hsp27 with the distributions shown in Table 3. Staining was characterized by cytoplasmatic and finely granular, basal cells as well as luminal epithelial cells were stained. Expression of hsp27 in the 50 BPH tissues occurred with a frequency similar to that seen in the 10 normal controls, so that no qualitative or quantitative differences in staining were identified when these tissues were compared. With respect to early malignancy (T1–2), as defined in the 25 radical prostatectomy specimens, there was a highly significant (\( P < 0.0001 \)) decrease in expression of hsp27, such that the neoplastic components in 21 of the 25 specimens (84%) did not express this protein. However, expression of hsp27 by adjacent nonneoplastic epithelial components was indistinguishable from that found in the control normal and BPH tissues. A similar pattern was seen in the 10 incidental prostate cancers diagnosed on TURP. Whenever PIN was identified according to morphological criteria, staining was heterogeneous with respect to hsp27 expression (Fig. 1a). Of the 85 invasive prostate cancers (pT1–3 N0–1 M0), 38 (45%) were positive (\( P < 0.0001 \)) for hsp27, with hsp27 protein expressed as fine granules distributed throughout the cytoplasm of the malignant cells in different regions of their invasive components (Fig. 1b). A strong correlation was demonstrated between hsp27 expression by each of the 85 invasive carcinomas and its corresponding Gleason grade (\( P < 0.04 \); Fig. 2). However, of greater importance was the more significant correlation between hsp27 expression and tumor behavior (\( P < 0.0001 \)) confirmed on univariate analysis (Fig. 3). This relationship became an even more powerful predictor of poor clinical outcome with Cox regression analysis (\( P = 0.014 \); Table 4).

**hsp60 Expression.** Control nonneoplastic tissues varied from negative to strong with respect to hsp60 expression within the epithelial compartment (Table 5). Although the distribution of this protein was

<table>
<thead>
<tr>
<th>( N )</th>
<th>Negative</th>
<th>Weak</th>
<th>Strong</th>
<th>95% CI</th>
</tr>
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<tr>
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<td>4</td>
<td>3</td>
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<td>Radical prostatectomy</td>
<td>25</td>
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<td>TURP-BPH</td>
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<td>0</td>
<td>6</td>
</tr>
<tr>
<td>TURP-early CaP</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>TURP-advanced CaP</td>
<td>85</td>
<td>0</td>
<td>12</td>
<td>73</td>
</tr>
</tbody>
</table>

\( ^a \) Significance relative to appropriate control tissues.

\( ^b \) CaP, prostate cancer.

**Statistical Analyses.** Statistical values of significance were determined using the \( \chi^2 \) test to compare nominal data. Correlations were assessed using the Kruskal-Wallis test for nonparametric data. Survival advantage was calculated by Kaplan-Meier analysis. Cox proportional hazard regression analysis was used to assess the relationship of each of the clinicopathological factors with disease-free survival in the advanced prostate cancers previously treated with BSCO. Age, hsp27 staining, preoperative PSA; Gleason grade, and clinical stage were included as covariates for the first analysis. Stepwise procedure selected pathological stage, Gleason grade, and hsp27 as significant prognostic factors (race was not included because all patients were Caucasians and indigenous to Liverpool-Merseyside). Statistical significance was set at \( P < 0.05 \). All calculations were performed using Stat View statistical software.
also cytoplasmic, the appearance of the stained protein was coarsely granular, in contrast to that seen for hsp27 and hsp70. An appreciable amount of epithelium in all BPH tissues expressed hsp60. Whereas this expression was heterogeneous, varying in intensity according to location, its presence was not simply related to some obvious factor, such as adjacent inflammation, but appeared to be endogenous to the particular region of epithelium. Similarly, the in situ neoplastic and invasive malignant components in all of the radical prostatectomy and TURP specimens variably expressed hsp60 protein. Characteristically, the appearance of the coarse granular cytoplasmic deposits of protein in the cancer cells appeared more numerous and intense than those in the nonneoplastic component of the corresponding specimen (Fig. 1c). In regions of high-grade PIN, expression of hsp60 was elevated when compared with BPH and with normal control tissues. In 24 of 25 (96%) radical prostatectomies and 83 of 95 (87%) morphologically advanced invasive prostate cancers, hsp60 expression was strong when compared with that of nonneoplastic tissues (P < 0.0001). With respect to semiquantitative assessment of the level of hsp60 expression and Gleason grade, no association was apparent in either the early or the advanced cancers.

**hsp70 Expression.** This protein was expressed to a variable extent in all 10 control normal tissues examined (Table 6). Characteristically, staining was uniformly cytoplasmic and varied from diffusely nongranular to finely granular. In 6 of the 50 (12%) BPH tissues, no staining of the epithelium was identified. Neither the in situ neoplasia nor the invasive malignant component in 4 of 25 (16%) radical prostatectomies and 30 of 95 (32%) advanced invasive carcinomas expressed hsp70. This finding was of only moderate statistical significance (P < 0.005) with respect to the control normal tissues. Immunochemical expression of this protein by invasive prostate cancer cells (Fig. 1d) was generally less intense, although of the same character (i.e., uniform and finely granular) as that in the nonmalignant epithelium. No association was found between intensity of staining and Gleason grade in either the early or the advanced cancers, so that some Gleason grade 5 carcinomas expressed this protein very strongly (Fig. 1e).

**Western Blotting Cell Lines**

Prostatic epithelial cell lines revealed marked differences in hsp expression between the transformed but nonmalignant cell line PNT2, the androgen-responsive cell line LNCaP, and the androgen-resistant lines DU145 and PC3 (Table 7). hsp27 was not detectable in transformed cell line PNT2 but was weakly expressed in LNCaP. Expression of hsp27 was stronger in androgen-independent cell lines DU145 and PC3 (Fig. 4), a finding that correlated with data from tissue specimens in which enhanced expression occurred in less differentiated tumors. The finding that expression of hsp60 was barely detectable in PNT2 but appreciably enhanced in all three malignant cell lines, particularly LNCaP, is consistent with data from tissue specimens in which increased expression occurred in invasive cancers but with no identifiable difference in relative expression between stage or grade of individual cancers. Expression of hsp70 was found only in the LNCaP cell line.

**DISCUSSION**

This study has confirmed distinct and consistent differences in the profiles of hsp27 by individual prostatic carcinomas. In particular, expression of hsp27 has been shown to correlate closely with the phenotypic behavior of individual prostate cancers. Together with PKC-β (39), Ca<sup>2+</sup>-binding proteins (40), and voltage-gated ion channels (41, 42), we have now confirmed that enhanced expression of hsp27 may be an important contributor in determining the metastatic capability of human prostatic carcinoma cells.

In the normal prostate, hsp27 was expressed in both luminal and basal epithelial cells, particularly the latter. However, it was not identified in early dysplastic lesions of intact tissues, including PIN, or in transformed cell line PNT2. Whereas failure to express hsp27 in the PNT2 cell line might be related to transformation, its expression was much stronger in androgen-independent cell lines (Fig. 4), which correlates with findings in the intact tissue specimens, where enhanced expression occurred in less differentiated tumors. The observation that early dysplastic lesions, including PIN, invariably failed to express hsp27 is a finding now being evaluated as an objective assessor of dysplastic transformation. In a range of human tissues, hsp27 is a marker of functional estrogen receptors such that in both breast and endometrial carcinoma, hsp27 overexpression has been correlated with differentiation (47). In prostate cancer cell lines, estradiol-induced growth inhibition may be mediated through hsp27 by transforming growth factor β1 (48).

Expression of hsp60 was barely detectable in PNT2 cells but appreciably enhanced in all three malignant cell lines, and consistent with our findings in tissue specimens where increased expression occurred in the cancers, but was unrelated to the stage or grade of each individual cancer. In limited studies of normal tissues, expression levels of hsp60 correlate directly with the requirement to regenerate mitochondria after cell proliferation or after high mitochondrial activity.

Expression of hsp27 was not found in the intact tissues and was found only in the LNCaP cell line. Its presence protects cells from diverse types of stress including heat, TNF-α, and ceramide but not ionizing radiation (49). It is suggested that this group of hsp27 should be classified, together with Bcl-2, as inhibitors of apoptosis. Activation of the latent DNA binding function of human p53 protein by hsp27 represents a unique reaction in which a hsp interacts with a native protein to affect its function (50). Induction of latent hsp70 results in its binding to a divergent group of proteins including members of the PKC family (51), the expression of which has been shown to correlate with the behavioral phenotype of prostate cancer (39).

Despite the expected variability in expression of individual hsp27 by differing intracellular homeostatic functions, the current data demonstrate that hsp27 expression is highly correlated with the clinical outcome of individual prostate cancers. However, our observations are at variance with an early report by Storm et al. (52), who found no

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**Table 7 Expression of hsps by prostatic epithelial cell lines by Western blotting**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PNT2</th>
<th>LNCaP</th>
<th>DU145</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsp27</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>hsp70</td>
<td></td>
<td></td>
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</table>

*−*, negative; +, barely detectable; ++, moderate; ++++, strong.

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**Fig. 4. Western blot of hsp27 expression by prostate cancer cell lines. A total of 100 µg of protein were analyzed by electrophoresis per lane. Western blot of the ubiquitous protein actin was used to confirm that equal amounts of protein were loaded to each lane.**
expression of hsp27 in either normal or neoplastic prostatic epithelium. This is surprising because hsp27 is ubiquitous in normal tissues, and its enhanced expression had already been correlated with poor prognosis in human breast cancer (53, 54). In contrast, and with findings similar to those we now report, Thomas et al. (55) confirmed strong immunohistochemical staining for hsp27 in benign prostatic epithelium, with heterogeneous expression being described in both PIN and invasive prostate cancer. In the latter, reduction in staining reflected increasing Gleason score and invasiveness. A smaller but more detailed study of seven cases of prostate cancer found that whereas differential expression of hsp27 occurred in nonneoplastic basal and luminal epithelial cells, this protein was generally absent in regions of established malignancy (56). Nevertheless, after thermal stress of prostatic carcinoma tissues in vivo, hsp27 expression could be up-regulated in a manner similar to that we described recently in human normal epidermis (57).

Our observations strongly support previous studies by Thor et al. (58), Chammess et al. (53), and Tandon et al. (54), who reported that hsp27 overexpression was correlated with reduced overall survival of patients with breast cancer. Those findings were extended by Conroy et al. (59), who showed that production of anti-hsp27 autoantibodies also correlated with improved survival in breast cancer patients. The current study confirms and extends the novel and innovative work of Bubendorf et al. (60), who showed, using DNA microarray technology, that the gene encoding hsp27 was among the most consistently overexpressed genes in hormone-refractory prostate cancer xenografts when compared with the parental, hormone-sensitive strain of the same cancer. To validate their findings in intact prostate cancers, immunohistochemical analysis of human tissue microarrays confirmed overexpression of hsp27 in 31% of the hormone-refractory tumors, a finding in agreement with our observation of increased expression in the more malignant tumors. Surprisingly, and in contrast to our data and the findings of previous investigators (55, 56), none of the benign prostatic tissues expressed this protein. This is an important discrepancy that may be explained, in part, by possible differences in epitope specificity of the various monoclonal antibodies used in the different studies. Apparent disparity in the immunohistochemical observations is unlikely to be due to errors in tissue sampling for the microarray technology because this potential error has already been identified and fully addressed by the authors, with high concordance demonstrated in their previous work (61, 62). In our current study, we were able to validate the identity of the protein identified immunohistochemically by Western blotting the cell lines using the same monoclonal antibody used for the immunohistochemistry.

Our previous analysis of PKC isoform expression by this identical group of early prostate cancers revealed a correlation between strong PKC-β expression, aggressive behavioral phenotype, and poor clinical outcome (39), which is similar to that now demonstrated for hsp27. This finding is not surprising because hsp27 and PKC-β are not only regulators of metabolic homeostasis but are also components of the same intracellular information cascades. Activation of PKC results in phosphorylation of hsp27, which, in turn, determines expression or activity of androgen and/or estrogen receptors. Because nonneoplastic control epithelia normally express hsp27, it was surprising to find that strong expression of hsp27 or PKC-β by invasive prostate cancers correlated with poor clinical prognosis. One hypothesis to account for this observation is that the PKC-β-hsp27 pathway becomes promiscuous in malignant cells, thus modulating one or more proteins that are differentially expressed between nonneoplastic and malignant epithelia. Whereas the PKC-β-hsp27 axis is both powerful and important to intracellular homeostasis, it is unlikely to be the sole control of the metastatic malignant phenotype of prostate cancer cells. To be successfully metastatic, prostate cancer cells abrogate cell surface expression of MHC class I and class II molecules (46, 63) as well as p170, the volume-regulated Cl− channel (41). Conversely, neoeexpression of voltage-gated Na+ and K+ channels and enhanced expression of Ca2+-binding protein p9Ka (40) appear to be prerequisites for development of the malignant phenotype (43). To date, no functional relationship has been reported between modulation of these proteins and expression of either PKC-β or hsp27 in prostate cancer.

In summary, this examination of a large number of well-characterized human prostate tissues (60 benign and 120 malignant tissues) has confirmed that enhanced or maintained expression of hsp27 protein is an independent and accurate predictor of poor clinical outcome for individual prostate cancers. Two corollaries arise from these observations: (a) hsp27 and PKC-β should be further investigated as powerful objective discriminants to predict likely clinical behavior and possibly to determine appropriate therapeutic strategies for individual patients with prostate cancer; and (b) hsp27 (together with or independently of PKC-β) may be useful as a target for developing novel therapeutic approaches aimed at controlling the aggressive malignant phenotype of early but locally invasive prostate cancers expressing these proteins.

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Heat Shock Protein Expression Independently Predicts Clinical Outcome in Prostate Cancer

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