Tumorigenesis and Neoplastic Progression

Regulation of Hepatocyte Activator Inhibitor-1 Expression by Androgen and Oncogenic Transformation in the Prostate

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Hepatocyte activator inhibitor-1 (HAI-1) is a transmembrane serine protease inhibitor that regulates the conversion of latent to active hepatocyte growth factor (HGF). Studies supporting a role for the HGF pathway in prostate carcinogenesis prompted an analysis of HAI-1 expression in the prostate. Here we analyze the regulation of HAI-1 expression by androgen, oncogenic transformation, and cancer progression. Immunohistochemical analysis revealed that HAI-1 expression was restricted to prostate epithelium, where staining occurred primarily in basal and atrophic luminal epithelial cells. Compared to normal glands, HAI-1 expression was significantly increased in localized prostate cancer and was present in most prostate cancer metastases. HAI-1 protein expression levels were sensitive to androgen in normal epithelium but not in cancer. Although androgen did not increase HAI-1 protein expression levels in LNCaP cells, it decreased HAI-1 surface expression, consistent with previous data from our group (Martin DB, Gifford DR, Wright ME, Keller A, Yi E, Goodlett DR, Aebersold R, Nelson PS: Quantitative proteomic analysis of proteins released by neoplastic prostate epithelium. *Cancer Res* 2004, 64:347–355). HAI-1 overexpression in cancer was predictive of prostate-specific antigen recurrence (relative risk, 1.24). These results suggest that HAI-1 regulates the HGF Met axis on prostate epithelial cells and influences HGF mediated tu-

mor invasion and metastasis. (*Am J Pathol* 2005, 167:255–266)

Hepatocyte growth factor activator inhibitor-1 (HAI-1) is an integral membrane, Kunitz-type serine protease inhibitor, which is broadly expressed in epithelial cells of most human tissues.^{1–3} HAI-1 inhibits the enzymatic activity of the soluble hepatocyte growth factor activator (HGFA)⁴ and of matriptase,^{2,5–8} a type II transmembrane serine protease. However, HAI-1 has also been shown to function as a cell surface receptor for HGFA, sequestering active HGFA on the cell surface and increasing its concentration.⁹ HAI-1 is anchored in the plasma membrane via a 23 amino acid long C-terminal hydrophobic region and can be cleaved at the cell surface and released into the extracellular environment.¹⁰ This cleavage generates a 40-kd proteolytic fragment with an augmented inhibitory activity and a fragment of the same size is detected in the medium after treatment of prostate cancer cells with androgen.¹¹ Thus the activity of HAI-1 is regulated by its expression level and by the local proteolytic milieu of the cell or tissue.

Matriptase and components of the HGF pathway have been shown to influence several aspects of epithelial carcinogenesis. In addition to cleaving and activating latent hepatocyte growth factor/scatter factor (HGF/SF),⁸ matriptase also cleaves and activates urokinase (uPA) and protease-activated receptor 2 (PAR2). These proteases along with hepsin, a matriptase-related protease degrade the extracellular matrix and thereby regulate cell-cell and cell-matrix adhesion, promoting tumor invasion and metastasis.^{6,12–14} Hepatocyte growth factor/

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scatter factor (HGF/SF) is a mesenchymal cytokine that is secreted in its proform and requires proteolytic cleavage to gain activation.^{7,8,15,16} Two-chain HGF/SF binds to the Met cell surface receptor and induces its cytoplasmic kinase activity.¹⁷ The HGF/SF/Met system is a classic mechanism of mesenchymal-epithelial interactions, which triggers tumor cell invasion and metastasis and under certain conditions, tumor growth.¹⁸ The serine proteases, HGFA, matriptase, and uPA can convert latent HGF/SF into its active form.^{8,19,20}

Although matriptase, Met, and HGF have been studied in the context of prostate cancer, the evaluation of HAI-1 expression has not been reported. However, HAI-1 expression has been examined in carcinomas of the gastrointestinal tract, breast, and ovary. In most cancers, the expression of matriptase and HAI-1 is increased in cancerous compared to normal tissues.¹² Further, HAI-1 expression is elevated in regenerating mucosa associated with colitis in the gastrointestinal tract.²¹ In contrast, during oncogenic transformation of colonocytes, HAI-1 expression decreases,²² tilting the balance to an increase in HGFA activity in colon cancer cells. This leads to the production of active and prometastatic HGF/SF on the cell surface. In ovarian cancer, matriptase expression increases with tumor grade, while HAI-1 protein expression decreases.²³ High-grade ovarian cancers commonly express matriptase without concomitant HAI-1 expression, but there is no correlation between matriptase or HAI-1 expression and patient survival. Two separate studies analyze the expression and prognostic relevance of matriptase/HGFA and HAI-1/HAI-2 in breast cancer and clearly show that their expression is deregulated. Kang and colleagues²⁴ report that high levels of HAI-1, matriptase, and Met are associated with poor patient outcome in a cohort of 330 node-negative breast cancer patients with more than 30 years of follow-up. Although there was a significant association between Met, HGF/SF, and matriptase expression in breast carcinoma, the expression of HAI-1 was independent of the other three proteins, suggesting that HAI-1 expression is regulated via a mechanism different from matriptase and the HGF/SF/Met pathway.²⁴ Although HAI-1 expression was an important predictor of disease outcome in the study of Kang and colleagues,²⁴ in a study by Parr and colleagues,²⁵ decreased expression of HAI-2 and not of HAI-1 was significantly associated with late stage, poorly differentiated breast cancer. Because expression of HAI-1 and HAI-2 was not correlated in cell lines, their regulation of expression may differ among various cancer systems.²⁶

We have recently shown that HAI-1 processing is regulated by androgen in the LNCaP prostate cancer cell line.¹¹ Using a quantitative proteomic analysis of protein released into the culture medium, the abundance of proteolytically cleaved HAI-1 increased after androgen stimulation, while the total amount of HAI-1 protein in the whole cell lysate remained constant. In addition, protein levels of matriptase in the cell lysate increased after androgen stimulation. Thus, androgen stimulation results in a coordinated increase in matriptase expression and HAI-1 inhibitory activity,¹¹ a combination of events that

could enhance prostate tumorigenicity. The current study was designed to determine whether alterations in HAI-1 expression occur as a result of oncogenic transformation in the prostate, if HAI-1 protein expression is regulated by androgen *in vivo*, and if HAI-1 could serve as a prognostic marker for patients with localized prostate cancer.

Materials and Methods

Antibodies, Cell Lines, and Cell Culture

The HAI-1 goat polyclonal antibody was purchased from R&D Systems, Minneapolis, MN; c-Met antiserum was obtained from Santa Cruz, Santa Cruz, CA; and cytokeratin, CK5/6, from DAKO, Carpinteria, CA. LNCaP and PC-3 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum.

Tissue Microarray (TMA) Cohorts

TMA1: Benign and Cancerous Prostate Tissues

Tissues from 69 archival prostatectomies containing Gleason pattern 3 or 4 were obtained under an institutional review board-approved protocol and patient identifiers were removed before the release of tissue blocks from the pathology department. Six cores of tissue per patient were obtained from areas of cancer and normal in the same block according to preservation of p27 staining positivity in normal glands. The fixation-sensitive marker, p27, was used to identify those areas that are properly fixed in an attempt to improve the quality of tissue on the TMA.²⁸

TMA2: Neoadjuvant Hormonal Therapy (NHT) Prostate Tissues

Tissues from patients treated with NHT are from Vancouver General Hospital and were described previously.²⁹ Briefly for this study, a total of 166 specimens were evaluated on the TMA. Specimens and treatment information were obtained under an institutional review board-approved protocol from hormone-naïve patients, and after treatment with NHT for <3 months, 3 to 6 months, and 6 to 8 months. Prostate-specific antigen (PSA) levels at the time of surgery were used to confirm treatment groups. Three samples per tumor specimen were arrayed.

TMA3: Outcomes Cohort

Tissue were obtained from 640 patients who underwent a radical prostatectomy at Baylor College of Medicine. Clinical data of patient follow-up were retrieved from the prostate SPORC patient database. Clinical characteristics of patients: age of patients ranged from 37 to 80 years with a mean of 62 years and a median of 63 years. The patients were followed postoperatively for an average of 42.08 ± 33.2 months (mean $\pm$ SD; median, 45.2 months; maximum, 167.74 months). Preoperative PSA

(pre-PSA) level was available in 603 PCa cases and ranged from 0.3 to 100 ng/ml with a median of 7.2 ng/ml and a SD of 10.99 ng/ml. Approximately 30% percent of the patients had a pre-PSA level >10.5 ng/ml. Approximately 7% had a Gleason score less than 6 and 85% had a Gleason score of 6 or 7, whereas 8% had a higher Gleason score.⁷⁻⁹ Lymph node metastasis was found in 40 (6.4%) patients, and biochemical recurrence was seen in 120 patients (19.3%). Extracapsular extension was found in 44.5%, margins were positive in 15.3%, and seminal vesicle invasion had occurred in 12.4% of the patients.

TMA4: Metastasis Cohort

Tissues were obtained from the rapid autopsy program at the University of Washington. Patients and families are consented and tissues from multiple organ sites are obtained under an institutional review board-approved protocol, following a systematic tissue collection scheme. Slides were analyzed for the presence of metastatic cancer and three cores with cancer were transferred from each block onto the TMA. Tissues from 10 patients are displayed on the TMA, including multiple metastatic sites from each patient.

Tissue Microarray Construction and Sectioning

The TMAs were constructed using either a manual tissue arrayer or the ATA-27 tissue arrayer (both from Beecher Instruments, Silver Spring, MD). The index tumor, defined as the largest and/or highest Gleason score tumor was identified on the slide and areas representative of the highest Gleason grade were circled. Biopsies of 0.6-mm-diameter triplicate cores were obtained from donor blocks and embedded into a single recipient block. Five- μ m sections were cut with a microtome by use of an adhesive-coated tape sectioning system (Instrumedics, Hackensack, NJ) to support the adhesion of the array elements. The final outcome array set consisted of 15 blocks with 9 cores for every 1 of the 640 patients for a total of ~6000 cores (large outcomes array).

Immunohistochemistry

TMA slides, containing 5- μ m-thick tissue cores were deparaffinized and rehydrated. Slides were treated with 0.3% hydrogen peroxide for 10 minutes at room temperature and antigen retrieval for HAI-1 and CK5/6 was performed for 20 minutes in a Black and Decker vegetable steamer in 10 mmol/L citrate buffer (pH 6.0). For Met and AR, 1 mmol/L ethylenediamine tetraacetic acid (EDTA), pH 8.0, was used for 30 and 20 minutes, respectively. Slides were cooled for 20 minutes. Blocking occurred with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 10 minutes, and incubation with primary antibodies was performed for 1 hour at room temperature. HAI-1 (affinity-purified goat anti-human antiserum; R&D Systems Inc.) was diluted 1:50, Met 1:300, AR 1:200, and CK5/6 1:50. Slides were

washed in PBS/BSA/1% Triton X-100. Biotinylated secondary antibody was used at 1:200 for 30 minutes. Bound antibodies were visualized with the Vector Elite ABC reagent (Vector Laboratories, Burlingame, CA), used according to the manufacturer's instructions, 0.05% diaminobenzidine enhanced with ferric chloride for 7 minutes, followed by counterstaining with Mayer's hematoxylin, and coverslipped.

Immunohistochemistry Scoring

The basic and NHT TMAs were scored as described previously.³⁰ Briefly, stained slides were imaged using the BLISS system (Bachus Laboratories). Cores were visualized with a Web-based image display at $\times 20$ magnification. To assist with diagnostic interpretation in NHT tissues, hematoxylin and eosin- and CK5/6-stained replicate array slides were used. In untreated tissue cores, Gleason patterns were assigned to areas of cancer, if they consisted of more than three glands. Normal tissue was evaluated for the presence of atrophy and atrophy was analyzed in a separate category for expression levels of HAI-1.

A scoring system incorporating staining intensity parameters and percentage of cell reactivity was used for determining the overall HAI-1 expression in each of four morphological categories, normal (secretory) epithelium, atrophy, PIN, and invasive carcinoma. Each tissue core was used as an independent sample and multiple histological entities of cancer and normal could co-exist in the same core. Four categories of staining were defined: no stain, faint, moderate, and intense. The percentage of cells falling into each of these categories was estimated by one (basic TMA) or two (NHT TMA) study pathologists and used to compute a single score as follows: score = $0 \times (\text{percent with no stain}) + 1 \times (\text{percent with faint stain}) + 2 \times (\text{percent with moderate stain}) + 3 \times (\text{percent with intense stain})$. The minimal score was 0 and the maximal score was 300. When two tissue types (eg, PIN, cancer) were present in the same TMA score, a separate score was calculated for each tissue type. Outcome TMAs were analyzed at Baylor College of Medicine using a categorical scoring scheme with scores from 1 (no staining) to 9 (intense staining).

cDNA Microarrays and Data Analysis

Prostate surgical specimens were obtained with informed consent from 27 patients at the University of Washington Medical Center and were selected based on their grade and absence of any treatment before prostatectomy. Five thousand epithelial cells from normal or cancerous glands were captured by laser-capture microdissection (LCM) using the Arcturus PixCell II (Arcturus, Mountain View, CA). Only one Gleason pattern was included in each laser-captured cancer sample. A total of 32 different Gleason patterns were captured from the 27 patients. Isolated RNA was amplified in two rounds (MessageAmp aRNA kit; Ambion, Austin, TX) and 2- μ g aliquots of amplified RNA were indirectly labeled by amino-allyl reverse

transcription and hybridized to the human prostate expression data base (PEDB) and human 18K microarrays as described previously.³¹ Individual Gleason samples were co-hybridized patient-matched normals in duplicate (dye-swaps.) Fluorescent array images were collected for both Cy3 and Cy5 using a GenePix 4000B fluorescent scanner (Axon Instruments, Foster City, CA). The image intensity data were gridded and extracted using GenePix Pro 4.1 software and spots of poor quality determined by visual inspection were removed from further analysis. Normalization of the Cy3 and Cy5 fluorescent signal on each array was performed using Silicon Genetics GeneSpring 6.2 software (Silicon Genetics, Redwood City, CA.) Data from the two duplicate cDNAs spots on each PEDB chip were combined and the average ratios were used for comparative analyses. To compare the overall expression patterns of all cancer samples divided by their patient-matched normal, $\log_2$ ratio measurements were statistically analyzed using the statistical analysis of microarrays (SAM) procedure³² (<http://www-stat.stanford.edu/~tibs/SAM/>).

Androgen Treatment and Fluorescence-Activated Cell Sorting Analysis

LNCaP and PC3 cells were grown to 40% confluency in RPMI 1640 media supplemented with 10% fetal bovine serum. They were subsequently washed three times in PBS and grown in serum-free RPMI 1640 for 48 hours at which point half of the flasks of each cell line were supplemented with 10 nmol/L R1881. After 24 hours of R1881 stimulation cells were prepared for cell surface labeling of HAI-1 as follows: the cells were washed twice in trypsin-free PBS/EDTA and then incubated for 10 minutes at 37°C in the same PBS/EDTA. Once lifted off of the growing surface, dispersed cells were transferred to a 50-ml tube. After 5 minutes of centrifugation at 700 rpm they were resuspended in a solution of PBS/EDTA and 5% BSA. Cells were counted, diluted, and aliquoted into 100- μ l fractions that contained 5×10^5 cells. Each sample was then incubated on ice for 1 hour with a 1:50 dilution of anti-HAI-1 or control goat IgG. After repeated washing each cell pellet was resuspended in ice-cold PBS/EDTA with 1% BSA. Cells were then labeled with a 1:1000 dilution of rabbit anti-goat Alexa 488 in the dark, on ice, for 30 minutes. After extensive washing in ice-cold PBS/EDTA with 1% BSA the cells were resuspended and fixed in 1 ml of 2% paraformaldehyde/PBS and subjected to flow cytometric analysis. Cells were analyzed on a FACScan Calibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) and collected data were postprocessed using the associated CellQuest software package.

Statistical Analysis

Box Plots

Box plots were obtained by entering cell type (normal-secretory, atrophy, PIN, cancer) and treatment-specific

HAI-1 expression scores for each core into the statistical package R Version 1.9.0., which contains a function for creating boxplots.³³

Comparison of HAI-1 Expression Levels across Cell Types

The slope parameter and its associated *P* value describe the association between the cell type (normal-secretory, atrophy, PIN, cancer) and HAI-1 expression. To test the association between cell type (normal-secretory, atrophy, PIN, cancer) and score, we used generalized estimating equations. This technique allows us to correctly estimate the relationship between score and cell type while accounting for the correlation that arises among observations from the same patient. The models fit using generalized estimating equations were simple regression models of the form: HAI-1 score = intercept + slope* (cell type) or HAI-1 score = intercept + slope* (treatment).

HAI-1 Expression and Correlation with PSA Recurrence

To determine the correlation between the HAI-1 staining intensity and PSA recurrence, we used a Cox proportional hazards model fit using the statistical package R Version 1.9.0.

Interobserver Correlation

To assess the reliability of HAI-1 expression scoring, two independent pathologists scored the same tissue microarray. We then calculated the Pearson correlation of the tissue diagnoses (cell-type designation: present or absent) within each category (normal-secretory, atrophy, PIN, cancer) to evaluate the interobserver variability between the two pathologists. Using cores where the cell type designation matched, we calculated the Pearson correlation between two pathologists of HAI-1 expression scores for each cell type.

Results

HAI-1 Protein Is Expressed in Basal and Intermediate Cells of Prostate Epithelium

In the normal prostate epithelium, a continuous layer of basal epithelial cells underlies a luminal, columnar, secretory cell layer. Basal cells differentiate into secretory cells transitioning through in intermediate/rapidly proliferating compartment.³⁴ Intermediate cells are abundant in atrophic or regenerative glands where they form the luminal cell layer.^{35,36} There are several histological categories of atrophy, including postatrophic hyperplasia, in which cell proliferation is increased and which may thus represent a regenerative process.³⁷ Cytokeratin 5 (CK5) and the Met receptor tyrosine kinase are highly expressed in basal and in suprabasal, intermediate pros-

Figure 1. Expression of HAI-1 in basal/intermediate cells of normal prostate epithelium. Consecutive sections through a normal gland show an area of partial atrophy, containing luminal intermediate cells (**long arrow** in all four panels). The difference in the appearance of the atrophic region in panels **A** to **C** compared to **D** is due to the tangential sectioning in the first three panels. However, the absence of an apical cytoplasmic compartment and the diminished expression of the androgen receptor (AR) clearly identify the cells in panel **D** as intermediate cells. In panel **A**, HAI-1 staining is appreciated in the cytoplasm of atrophic cells by a dark brown color in the slide (**long arrow**) and is markedly decreased in the cytoplasm of differentiated cells, which stain slightly brownish in the original slide (**arrowhead**). In panel **B**, CK5 staining occurs in the full thickness of the epithelium in the atrophic region consistent with its expression of basal and intermediate cells (**long arrow**). In panel **C**, the same cluster of CK5 positive cells also expresses the Met receptor, another marker of intermediate cell differentiation (**long arrow**). In panel **D**, AR expression is appreciated as a brown colored nuclear staining in the original slide. The brown color is markedly decreased in the cells that are of cuboidal shape and thus atrophic (**long arrow**), compared to adjacent secretory epithelial cells (**arrowhead**). HAI-1 is co-expressed with Met and CK5 in basal and in intermediate cells within the atrophic epithelium (**long arrow**) and its expression is decreased in secretory epithelial cells (**arrowheads**). Magnification in all panels is X400.

tate epithelial cells.^{36,38} Although CK5 is not expressed in secretory cells, Met staining occasionally appears in secretory cells at the basolateral plasma membrane. To visualize and distinguish the basal, intermediate, and secretory cell compartments, we stained consecutive sections from radical prostatectomy specimens with CK5 (Figure 1B), Met (Figure 1C), and androgen receptor (AR) antibodies (Figure 1D). Cells that stain CK5+/Met+ and AR- and that adhere to the basement membrane are identified as basal epithelial cells. CK5+/Met+ and weakly AR-positive cells situated above a basal cell layer represent intermediate cells (Figure 1, long arrows) and CK5-, weakly Met, and strongly AR staining cells are identified as secretory cells. In comparison with CK5, Met, and AR staining, HAI-1 protein in a parallel tissue

section is expressed in the basal and intermediate cells of the normal prostate epithelium (Figure 1A).

To evaluate the expression of HAI-1 in a range of normal prostate epithelia, we used a tissue microarray. On this array we scored HAI-1 staining intensity in 207 (three per patient) tissue cores from 69 patients. In some cores, membrane staining of HAI-1 appeared as the predominant staining patterns, while in most cores HAI-1 expression occurred primarily in the cytoplasm. Secretory epithelium was identified by the presence of luminal cells with an apical cytoplasmic compartment of at least two times the nuclear diameter (Figure 2, A and B). Consistent with the expression of HAI-1 in intermediate prostate epithelial cells, atrophic/regenerative glands stained more intensely (median, 150) than glands with

Figure 2. HAI-1 expression in secretory and atrophic epithelium. **A** and **B**: TMA cores with normal glands with secretory (◆) and atrophic (►) epithelium. HAI-1 expression is significantly increased in atrophic compared to secretory epithelium. **C**: Quantification of HAI-1 expression in TMA cores containing benign epithelium. Boxplots show the mean HAI-1 expression in secretory or atrophic epithelium and the 25th and 75th percentile of the score distribution. The whiskers shown above and below the boxes represent the largest and smallest observed scores that are less than 1.5 box lengths from the end of the box. Circles represent cores with scores more than 1.5 box lengths from the end of the box. The difference in the mean expression levels of secretory versus atrophic epithelium, indicated by **, has a *P* value of ≤ 0.01 . Original magnifications, $\times 200$.

secretory differentiation (median, 40) (Figure 2C). This difference in expression is significant ($P \leq 0.01$).

HAI-1 Protein Expression Is Regulated by Androgen in Benign Prostate Epithelium

HAI-1 expression was restricted to the prostate epithelium and no HAI-1 protein was detectable in the prostate stroma by immunohistochemistry. To investigate whether HAI-1 protein expression is regulated by androgen in normal prostate epithelium *in vivo*, we compared patients who received no treatment before radical prostatectomy and patients who were treated for increasing periods of time with androgen-ablative therapy before radical prostatectomy. Androgen-ablative treatment caused atrophy and basal cell hyperplasia in the normal prostate epithelium. HAI-1 expression decreased significantly after 3 months of androgen-ablative therapy in atrophic epithelium ($P_{<3\text{ m}, >3\text{ m}} = 0.03$). In Figure 3, A and B, HAI-1 expression in atrophic epithelium is demonstrated, next to cancer. In both cases the cancer stains positive, however adjacent atrophic epithelium is only positive in the untreated case. Comparing untreated patients with patients who received androgen-ablative therapy reveals that there is a significant decrease of HAI-1 expression starting after 3 months of androgen-ablative treatment. Although there was no difference between no treatment and less than 3 months treatment, HAI-1 expression was significantly reduced in the 3 to 6 months and greater than 6 months treatment groups (Figure 3C). Secretory epithelium was negative in all cases ($P_{<3\text{ m}, >3\text{ m}} = 0.249$). Thus, under prolonged low-circulating androgen concentrations, HAI-1 expression declines in basal and intermediate cells of atrophic epithelium. This clearly shows that in nonmalignant epithelial cells, HAI-1 protein expression is regulated by androgen.

HAI-1 Protein Is Overexpressed in Prostate Cancer

The abundance of transcripts encoding proteins of the HGF/SF/Met activation system was compared between normal and cancerous prostate epithelium using cDNA microarray analysis. RNA was extracted from 30 cases of matched laser-microdissected normal and neoplastic prostate epithelium, amplified, and hybridized to microarrays comprised of $\sim 21,000$ cDNAs. A one-sample *t*-test comparing cancer to normal ratios identified 4483 cDNAs with significant differential expression ($\text{FDR} \leq 0.1\%$). There was a significant decrease in Met and urokinase (uPA) RNA expression in tumor compared to normal prostate epithelium with an average fold-change for Met of -1.6 and for uPA of -1.5 . A simple explanation for this result is the high expression of Met and uPA in normal basal epithelial cells, which are absent in prostate cancer. There was no significant expression difference between normal and cancer for HGF/SF, HAI-1, HAI-2, matriptase, or the uPA receptor (Figure 4A). In addition, there was no significant difference in RNA expression change across Gleason grades 3, 4, and 5 ($\text{FDR} \geq 3.47\%$).

In contrast to RNA expression, protein expression of HAI-1 was significantly greater in cancerous versus normal prostate epithelium (Figure 4B). HAI-1 was expressed in 128 of 131 cores (97.7%) with cancer from 58 individuals. In 160 cores containing secretory epithelium, the median HAI-1 score was 40, while in 131 cores with cancer, the median score was 130. Thus HAI-1 expression was significantly increased in cancer compared with normal secretory epithelium ($P < 0.001$). When secretory and atrophic epithelia were combined, HAI-1 remained significantly increased in prostate cancer compared to benign epithelium ($P < 0.001$). However, HAI-1 expression in cancer (med = 130) was comparable to expres-

Figure 3. Androgen deprivation decreases HAI-1 expression in normal prostate epithelium. **A:** HAI-1 expression in atrophic prostate epithelium of an untreated patient). **B:** HAI-1 expression in atrophic epithelium in a patient treated with androgen-ablative therapy (Lupron) for 6 months before prostatectomy. The atrophic epithelium is negative for HAI-1, whereas cancer cells express HAI-1. **C:** Comparison of HAI-1 expression in treated and untreated normal prostate tissues. The first boxplot represents untreated tissue, while the other boxes represent increasing length of treatment. Boxplots show the mean HAI-1 expression in untreated or treated epithelium and are described in detail in Figure 2. The mean HAI-1 scores of patients who received no or less than 3 months treatment are significantly greater than HAI-1 scores of patients treated for more than 3 months. Original magnifications, $\times 200$.

sion in atrophic epithelium (med = 150) (compare Figures 1C and 4B, graph). Because the differentiation state of cancer, based on cytokeratin expression, resembles that of secretory epithelium, the comparison of cancer with secretory epithelium is more valid than with atrophic epithelium. Notably, comparisons of HAI-1 expression in cancer adjacent to normal secretory epithelium in the same core revealed increased HAI-1 expression in cancer in 39 of 46 cores. Because HAI-1 staining in cancer was mostly cytoplasmic, the cell surface expression was difficult to evaluate. The increase in steady-state HAI-1 protein abundance in cancer compared to normal suggests that oncogenic transformation of prostate epithelial cells increases HAI-1 protein translation or reduces its degradation.

Androgen Deprivation Does Not Alter HAI-1 Protein Expression in Prostate Cancer

Previous experiments have demonstrated that androgens modulate the secretion or release of HAI-1 *in vitro*.¹¹ To extend these findings, we sought to determine whether androgens influence HAI-1 expression *in vivo*. The same TMA that was analyzed for androgen-regulated HAI-1 expression in normal tissues was evaluated for changes in HAI-1 protein expression in prostate cancer after an-

drogen ablation. In 174 cores that contained prostate cancer, the median HAI-1 expression score was 100 in androgen-intact ($n = 49$) and 120 in androgen-deprived ($n = 125$) tissues ($P = 0.167$), indicating that HAI-1 expression did not change after androgen deprivation (data not shown). Even after androgen ablation for longer than 6 months, the expression of HAI-1 protein remained unchanged. The interobserver correlation for the four diagnostic groups (secretory, atrophic, PIN, and cancer) and for the scoring of staining intensities was greater than 0.69 in all categories. Remarkably, the overexpression of HAI-1 protein in cancer compared to normal was consistent between the two TMAs (TMA1 and TMA2), which display tissues of patients from different cohorts, different years and protocols of tissue collection and processing, and different institutions and surgeons. However, TMA2 containing the androgen-deprived tissues was less intensely stained overall and the mean HAI-1 staining intensity in atrophic epithelium of untreated cases in TMA2 was 30, whereas it was 150 in TMA1.

Androgen Stimulates the Release of HAI-1 from the Cell Surface

Although androgen deprivation did not induce a measurable change in HAI-1 protein levels *in vivo*, androgen

Figure 4. Expression of HAI-1 RNA and protein in human prostate cancer tissues and of HAI-1 protein in human prostate cancer cell lines. **A:** Array data: genes associated with the HGF/SF activation axis and listed in the first column were identified in DNA arrays from 27 individuals and analyzed for their differential expression in prostate cancer versus matched normal control tissues. For four patients more than one Gleason grade was present in the cancer and each Gleason pattern was analyzed on a separate array. Gene expression ratios of cancer versus normal are depicted on a pseudocolor scale, a red color indicating overexpression in cancer compared to normal. A supervised analysis using the SAM method revealed underexpression (negative fold change) or overexpression (positive fold change) in cancer compared to normal. The fold change in gene expression between normal and cancer from the same individual is indicated in the adjacent column. A false discovery rate (FDR) of ≤ 0.05 was considered a significant difference between cancer and normal. Gene expression changes between low- and high-grade cancer were evaluated using the SAM method. However, in all cases the FDR was ≥ 0.05 and thus there was no significant difference in the expression of these genes across Gleason grades. **B:** HAI-1 protein expression in tissue microarrays. Increased HAI-1 staining in cancer (Ca) compared to normal (N). Boxplots are described in detail in Figure 2 and show the mean HAI-1 expression in normal secretory (normal), normal plus atrophic (NI + A) epithelium, and cancer (CaP). $**P \leq 0.001$ for the difference of normal or NI + A versus CaP.

stimulation clearly reduced cell surface expression of HAI-1 in cell culture experiments. HAI-1 expression was reduced after androgen stimulation of androgen-sensitive LNCaP cells, but not androgen-insensitive PC-3 cells (Figure 5). This finding is consistent with the cleavage of HAI-1 from the cell surface and not an accelerated secretion of intercellular HAI-1. We previously demonstrated that HAI-1 protein accumulates in the conditioned medium of LNCaP cells after androgen stimulation and that the size of the two HAI-1 bands on Western blot suggested proteolytic cleavage.¹¹

HAI-1 Protein Expression Levels Are Associated with Prostate Cancer Progression

Previous studies have demonstrated correlations between HAI-1 expression and aggressive behavior in several tumor types. We next questioned whether HAI-1 expression levels in prostate cancer tissues predicted

Figure 5. Androgen-regulation of HAI-1 in prostate cancer cell lines. Androgen stimulates release of HAI-1 from the cell surface. LNCaP or PC-3 prostate cancer cells were deprived of androgen by culture in charcoal-stripped serum. Cells were stimulated with 10 nmol/L R1881 for 48 hours. HAI-1 cell-surface expression was measured by fluorescence-activated cell sorting. Numbers above the bracket indicate fraction of cells showing positive HAI-1 staining, after subtraction of the background signal obtained with a normal goat Ig control.

Table 1. HAI-1 Expression and Risk for PSA Recurrence ($n = 299$)

Variable	Variable type	Mean (25th quantile, 75th quantile)	RR for PSA recurrence	P (relative risk)
HAI-1	Continuous	3.80 (3.00, 5.13)	1.24	< 0.001
Preop-PSA	Continuous	11.53 (5.00, 13.30)	2.03	< 0.001
Gleason sum	Continuous	6.71 (6.00, 7.00)	2.88	< 0.001
SVI	Binary	Percent positive 17.39%	3.47	< 0.001
Margin status	Binary	16.39%	3.75	< 0.001

HAI-1 scores from 299 of 614 patients were evaluated for association with PSA recurrence. The means for preoperative PSA (pre-op PSA), Gleason sum, and the proportions of patients with a positive surgical margin (margin status) were not significantly different between the 614 patients and subset 299 patients. However, the difference in the proportions of seminal vesical invasion (SVI) is borderline significant according to Fisher's exact test ($n = 299$: 17.4%; $n = 614$: 12.5%; $P = 0.054$). The estimated relative risk (RR) and P value for each variable are calculated using a Cox proportional hazards model.

cancer progression as measured by PSA recurrence after prostatectomy. Tissue arrays of 614 patients with clinical follow-up for more than 3 years after radical prostatectomy were analyzed for HAI-1 expression and 299 patients had interpretable HAI-1 staining in cancer. The association of HAI-1 staining intensity and PSA recurrence was assessed using a Cox proportional hazards model. The model included the log of the PSA level before radical prostatectomy, seminal vesical invasion status, margin status, Gleason sum, and HAI-1 expression score. There was a significant association between HAI-1 expression and increased risk of PSA recurrence. HAI-1 expression was independent of other clinical variables, including Gleason grade. The relative risk for recurrence was 1.24 for HAI-1 expression and >2.0 for several clinical and histopathological variables (Table 1). Because of this weak association with patient outcome, it is unlikely that HAI-1 by itself would be of clinical utility as a marker of prostate cancer progression.

HAI-1 Expression in Prostate Cancer Metastases

Because HAI-1 expression was associated with unfavorable prognosis indicating increased risk for prostate cancer recurrence, we analyzed the expression of HAI-1 in prostate cancer metastases. HAI-1 protein expression was maintained after tumor growth at distant sites. However, there was no measurable difference in HAI-1 expression between localized and metastatic prostate cancer, and HAI-1 expression did not differ between metastasis involving different sites such as lymph node, bone, and soft tissues (Table 2).

Table 2. HAI-1 Expression in Prostate Cancer Metastases ($n = 49$)

HAI-1 Staining	Tissue Type		
	Bone	Lymph node	Soft tissue
Negative (0)	0 (0%)	0 (0%)	3 (17%)
Positive (1-3)	16 (100%)	15 (100%)	15 (83%)

A tissue microarray of prostate cancer metastases was stained with anti HAI-1 and HAI-1 expression was evaluated for each of 49 informative cores. Numbers indicate the total number of cores in each metastatic site. In parentheses, the fraction of positively stained cores is specified. The soft tissue group contains metastases from all sites other than bone and lymph node.

Discussion

In this study we have shown that the expression of HAI-1 is significantly dysregulated in prostate neoplasia *in vivo*. As observed in other organ systems, HAI-1 expression in the prostate is restricted to the epithelium. Within the epithelium, HAI-1 is expressed in basal and atrophic luminal cells, which include a subpopulation of intermediate epithelial cells. Because basal and intermediate cells are more abundant in atrophy compared to fully differentiated epithelium, HAI-1 expression in atrophy appears high. Oncogenic transformation stimulates HAI-1 expression to levels indistinguishable from atrophic epithelium and androgen may further increase the activity of HAI-1 through proteolytic cleavage at the cell surface in LNCaP cells.^{8,11} Interestingly, the Met receptor shows the same distribution of expression as HAI-1 in the normal epithelium. Therefore, through co-expression on the same cell type, HAI-1 may modulate Met receptor kinase activity by regulating the proteolytic activation of the Met receptor ligand, HGF/SF. Together these data suggest that the regulation of the HGF/SF/Met axis through HAI-1 expression may occur during prostatic development, atrophy, regeneration, and localized or metastatic prostate cancer. Because the epithelial cell expression of Met is high in these conditions, the generation of active HGF/SF could affect branching morphogenesis, epithelial differentiation, tumor cell invasion, and metastasis.³⁶ HAI-1 joins a group of proteins such as *BclII*, CD44, CD138-syndecan-1, and Met, that are normally expressed in basal, but not in secretory cells of the prostate epithelium, and are overexpressed in a subset of prostate cancers.^{30,39} It is currently unclear whether these proteins are co-expressed in the same cancers and thus may identify a group of prostate cancers with basal cell characteristics.

Although in prostate cancer the expression of HAI-1 protein was significantly higher than in normal prostate epithelium, this difference was not apparent at the RNA expression level. Thus, it is possible that HAI-1 protein expression in cancer is regulated translationally or post-translationally. Under these circumstances we would not expect RNA levels to be different. Alternatively it is conceivable that similar levels of RNA expression in normal and cancer result from the differences in cell types that exist in normal and cancerous epithelium. Even though there was a marked difference in HAI-1 protein expres-

sion comparing normal secretory epithelial cells and cancer cells, this difference was diminished comparing basal epithelial cells and cancer cells. Because basal and secretory epithelial cells are not separated during the laser capture microdissection of normal glands, the basal epithelial cells may be the main contributors of HAI-1 RNA in the normal samples. Therefore, the basal cells will mask RNA expression differences between normal and cancerous epithelium.

Based on current knowledge of HAI-1 function, the increased expression of HAI-1 in prostate cancer is puzzling and counterintuitive. Why would expression of a protease inhibitor be increased in cancer and why would this increased expression level be associated with a small, but statistically significant increased incidence of PSA recurrence? In support of our results, a positive correlation between HAI-1 and adverse prognosis was identified previously in breast cancer.²⁴ A similar observation exists for the well-characterized plasminogen activator inhibitor, PAI-1.⁴⁰ As an explanation for the inverse correlation of PAI-1 and disease-free survival, the pleiotropic effects of PAI-1 have been emphasized. A similar scenario may underlie the observed HAI-1 expression pattern in this study: HAI-1 may participate in activating as well as in inhibiting the cell surface-associated serine proteases, matriptase and HGFA. However, this hypothesis is based on *in vitro* data and requires *in vivo* validation, which may be possible through a recently published antibody that specifically binds to the active form of matriptase.²⁵

Functionally altered HAI-1 expression could have important effects on interactions between epithelium and constituents of the microenvironment. The HGF/SF/Met ligand-receptor system represents a classic mechanism for mesenchymal-epithelial crosstalk, and its role in prostate cancer development has recently been demonstrated in a genetically altered mouse model.⁴¹ Cuhna and colleagues⁴² have demonstrated the important inductive influence of stroma on normal prostate morphogenesis; a process that depends on an intact androgen signaling pathway in the stroma. To date, the HGF/SF/Met axis has not been associated with androgen effects. HGF/SF is secreted in its latent form exclusively from prostate stromal cells²⁷ and may reach the epithelium in an inactive conformation. On the surface of epithelial cells, single-chain HGF/SF can be activated through cleavage by the cell surface-associated serine proteases. Matriptase is specifically regulated by HAI-1 and HAI-2. Although matriptase is considered a critical activator of HGF/SF, it is not the only one. Matriptase knockout mice do not display the embryonic lethal phenotype of the HGF/SF or Met knockout.^{43–45} Because the early lethality of HGF/SF and Met knockout mice results from the role of Met in angiogenesis and liver development, the lack of resemblance in phenotypes of the matriptase and HGF/SF knockout mice is not surprising. In addition, HGF/SF knockout mice display a defect in myosite migration and organization, caused by the disruption of autocrine Met activation in mesenchymal cells. Together these and the current study suggest that differences in

proteolytic activation of HGF/SF may exist between mesenchymal, endothelial, and epithelial cells. Further studies are required to determine whether the androgen-mediated release of HAI-1 from epithelium influences the effects of mesenchymally-derived HGF in the context of normal prostate development or carcinogenesis.

Although androgenic effects on prostate stromal cells are the prime regulators of stromal-epithelial interactions in the prostate, this study illustrates an androgen-dependent regulatory mechanism for growth factor activation by prostate epithelial cells. Thus, androgenic stimulation of prostate epithelial cells could modulate the proteolytic environment on the cell surface and the conversion of latent to active stromally-derived growth factors.

We present *in vivo* evidence that androgen regulates HAI-1 protein expression in atrophic epithelium and that HAI-1 protein expression significantly increases with oncogenic transformation of prostate epithelium. In addition, HAI-1 expression is predictive of PSA recurrence in a high-risk group of patients with prostate cancer.

Note Added in Proof

Since submission of this manuscript, it has been demonstrated that HAI-1 is an inhibitor of the cell surface protease, hepsin, which is strongly implemented in the aggressive behavior of prostate cancer cells.⁴⁶ In addition, hepsin has been added to the group of proteases that can cleave HGF/SF and convert it from a latent to an active growth factor.^{47–49}

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