Stromal and Epithelial Expression of Tumor Markers Hyaluronic Acid and HYAL1 Hyaluronidase in Prostate Cancer*

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Hyaluronic acid (HA), a glycosaminoglycan, regulates cell adhesion and migration. Hyaluronidase (HAase), an endoglycosidase, degrades HA into small angiogenic fragments. Using an enzyme-linked immunosorbent assay-like assay, we found increased HA levels (3–8-fold) in prostate cancer (CaP) tissues when compared with normal (NAP) and benign (BPH) tissues. The majority (~75–80%) of HA in prostate tissues was found to exist in the free form. Primary CaP fibroblast and epithelial cells secreted 3–8-fold more HA than respective NAP and BPH cultures. Only CaP epithelial cells and established CaP lines secreted HAase and the secretion increased with tumor grade and metastasis. The pH activity profile and optimum (4.2; range 4.0–4.3) of CaP HAase was identical to the HYAL1-type HAase present in human serum and urine. Full-length HYAL1 transcript and splice variants were detected in CaP cells by reverse transcriptase-polymerase chain reaction, cloning, and sequencing. Immunoblotting confirmed secretion of a ~60-kDa HYAL1-related protein by CaP cells. Immunohistochemistry showed minimal HA and HYAL1 staining in NAP and BPH tissues. However, a stromal and epithelial pattern of HA and HYAL1 expression was observed in CaP tissues. While high HA staining was observed in tumor-associated stroma, HYAL1 staining in tumor cells increased with tumor grade and metastasis. The gel-filtration column profiles of HA species in NAP, BPH, and CaP tissues were different. While the higher molecular mass and intermediate size HA was found in all tissues, the HA fragments were found only in CaP tissues. In particular, the high-grade CaP tissues, which showed both elevated HA and HYAL1 levels, contained angiogenic HA fragments. The stromal-epithelial HA and HYAL1 expression may promote angiogenesis in CaP and may serve as prognostic markers for CaP.

The majority of newly diagnosed prostate cancer (CaP) patients have clinically organ-confined disease. The limited knowledge about which CaP is aggressive and likely to progress, as well as when it will recur, severely impedes individualized selection of therapy and subsequent prediction of outcome (1). Routine biochemical (i.e. prostate-specific antigen levels) and surgical and pathologic parameters (i.e. Gleason sum, margin, and node status and seminal vesicle invasion) offer a glimpse of the biological potential of the tumor (2–7). However, many of the CaP patients (~50–60%) with clinically localized disease have prostate-specific antigen levels between 4 and 10 ng/ml and a biopsy Gleason score between 6 and 7, which limits the prognostic capability of these markers (1, 2, 6). The prognosis of CaP patients can be improved if molecules that associate with the biological potential of CaP are identified (7). We have recently shown that both tumor-associated hyaluronic acid (HA) and tumor-derived hyaluronidase (HAase) possibly play a role in tumor progression.

HA is a nonsulfated glycosaminoglycan made up of repeating disaccharide units, N-glucuronic acid and N-acetyl-D-glucosamine (8). HA is a component of extracellular matrix and is present in various tissues and tissue fluids. It performs several functions in normal physiology. Concentration of HA is elevated in several cancers including bladder, colon, breast, and lung and Wilms’ tumor (9–13). We have previously shown that the urinary HA levels are 2.5–6.5-fold elevated in bladder cancer patients and serve as a highly sensitive and specific marker for detecting bladder cancer, regardless of the tumor grade (14, 15). In tumor tissues, HA expands upon hydration and opens up spaces for tumor cell migration. Tumor cells migrate on HA-rich matrix that is mediated by cell surface HA receptors (e.g. CD44 and RHAMM; see Refs. 16–19). HA may also offer tumor cells some protection against immune surveillance and chemotherapeutic agents (20). Small fragments of HA (3–25 disaccharide units) are angiogenic (21). We have previously isolated such angiogenic HA fragments from the urine of high-grade bladder cancer patients and shown that these fragments induce endothelial cell proliferation (14). Furthermore, HA fragments of the same length also induce endothelial cell migration and lumen formation (22). Recent studies from our laboratory demonstrate that angiogenic HA fragments interact with RHAMM on the surface of human endothelial cells and induce the MAP kinase pathway (23, 24). Thus a regulated degradation of HA in tumor tissues may be important for both tumor metastasis and angiogenesis.

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‡ The abbreviations used are: CaP, prostate cancer; HA, hyaluronic acid; HAase, hyaluronidase; DAB, 3,3′-diaminobenzidine; CM, conditioned medium; SF-CM, serum-free conditioned medium; S-CM, serum containing conditioned medium; BSA, bovine serum albumin; HL fibroblast, human fetal lung fibroblast; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; bp, base pairs; kb, kilobase pair(s); NAP, normal prostate; BPH, benign prostatic hyperplasia.
HAases are a family of enzymes which degrade HA (25). Initially termed as a “spreading factor,” the presence of HAase is crucial to the spread of bacterial infections and toxins present in bee, snake, and other venoms (26–28). In human, 6 HAase genes have been identified (29–32). These genes cluster in two tightly linked triplets on human chromosomes 3p21.3 (HYAL1, HYAL2, and HYAL3) and 7q31.3 (HYAL4, PH20, and HYALP1) (32). Among these, HYAL1, HYAL2, and PH20 are relatively well studied at the protein level. HYAL1 gene encodes a HAase that is present in human serum, however, its cellular origin is unknown (31). HYAL2 gene encodes a lysosomal HAase (30). PH20 gene encodes the testicular-type HAase that shows a broad (pH 3.2–9.0) pH activity profile (29).

In establishing the association of HAase to tumor biology, we initially showed that HAase levels are elevated in CaP and these levels correlate with CaP progression (i.e. metastatic > high-grade ≫ low-grade > benign prostate hyperplasia (BPH)/normal) (33). In cell culture studies, we observed that, primary explant cultures of CaP cells secrete elevated levels of HAase. The elevated levels of HAase have now been demonstrated in metastatic breast tumors and in several carcinoma lines (34–40). However, the identity of the type of HAase expressed in most cancer tissues and cells is still unknown. In bladder cancer we observed that, elevated urinary HAase levels indicate the presence of G2 and G3 bladder cancer (41, 42). Recently, we purified the first tumor-derived HAase from the urine of bladder cancer patients and showed its similarity to HYAL1 (43). We also observed the expression of HYAL1 at the transcript and protein levels, in invasive bladder cancer cell lines, which secrete high levels of a HAase in their conditioned media. This HAase activity has a pH optimum in the range 4.1–4.3 (43).

In this study, using biochemical and molecular biology techniques, we have examined the expression of HA and HAase in prostate tissues and cell culture. Furthermore, we have been able to identify and characterize the type of HAase expressed in prostate cancer cells. In addition, we have localized these molecules in prostate tissues by immunohistochemistry. We also attempted to understand the function of the tumor-associated HA-HYAL1 system.

**EXPERIMENTAL PROCEDURES**

**Tissue Specimens**—Normal prostate (NAP) tissues from adults (21–50 years) were obtained from organ donors. Neoplastic and BPH tissues were obtained from patients undergoing open prostatectomy. The tissue specimens were split and the mirror segment was fixed in formalin, embedded in paraffin, and sectioned; then hematoxylin and eosin staining evaluated the histologic grades of these tumors. In this study, we have included data from only those specimens, which were histologically confirmed as normal, benign, and malignant.

**Tissue Extracts**—Fresh or frozen (−0.5–1 g) specimens were suspended in ice-cold homogenization buffer (5 mM Hepes pH 7.2, 1 mM phenylmethylsulfonfluoride) and homogenized for 30 s in a tissue homogenizer. The tissue extracts were clarified by centrifugation at 40,000 g for 30 min (14, 33). The supernatants were designated as “Hepes extracts.” The tissue pellets were re-extracted in 50 mM sodium acetate (pH 5.8), 6 M guanidine HCl, and 1 mM phenylmethylsulfonfluoride. Following clarification by centrifugation, the supernatants were designated as “guanidine extracts.” Both Hepes and guanidine extracts were assayed for HA and protein concentration.

**Primary Fibroblast and Epithelial Cultures**—Primary cultures from prostate tissues were set up as described previously (33). For culturing fibroblasts, collagenase-digested tissue fragments were cultured in RPMI 1640 + 10% fetal bovine serum medium. The fibroblast growth in cultures was controlled by anti-keratin labeling. When the fibroblast cultures became ~80% confluent, the cultures were washed extensively in PBS and incubated in serum-free RPMI 1640 containing insulin, transferrin, and selenium (ITS solution, Life Technologies, Inc., Gaithersburg, MD). The serum-free conditioned medium (SF-CM) was collected after 2–3 days.

The prostate epithelial explant cultures were set up in a prostate epithelial cell growth medium, PrEGM (Prostate Epithelial Growth Medium, BioWhitaker/Clonetics, San Diego, CA) as described before (33). PrEGM is a serum-free growth medium. The epithelial cell growth in cultures was controlled by anti-keratin labeling (33). The SF-CM from primary cultures was collected at second passage, 3 days after subculturing, and concentrated (10-fold).

**Tissue Culture**—Prostate cancer cell lines DU145 and LNCaP, bladder cancer line HT1376, and human embryonic lung fibroblast (HL fibroblast; passage 11) were obtained from the American Type Culture Collection (Rockville, MD). The prostate cancer line PC3-ML was a gift from Dr. M. E. Stearns, Medical College of Pennsylvania, Philadelphia, PA. The bladder cancer cell line 253J-Lung was kindly provided by Dr. Colin Dinney, M.D., Anderson Cancer Center, University of Texas, Houston, TX. All of these cell lines were cultured in RPMI 1640 + 10% fetal bovine serum and gentamicin. At ~60% confluence, the cultures were washed three times in PBS and incubated in serum-free RPMI + ITS. The SF-CM from these cultures was collected after 2–3 days.

Additionally, CaP fibroblast and HL fibroblast were grown in the culture medium (i.e. RPMI 1640 + 10% fetal bovine serum and gentamicin) to 80–90% confluence and the conditioned medium was collected. This medium was designated as S-CM, since it contained 10% fetal bovine serum. The S-CM were collected from fibroblast cultures to examine HAase activity, such S-CM have been used previously to demonstrate HAase activity in fibroblast cultures at pH 3.7 (44). In these experiments, these levels correlate with CaP progression (43).

**Measurement of HAase Levels**—HAase levels present in tissue extracts and SF-CMS-CM were measured using an ELISA-like assay originally developed by Fosang et al. (45), with modifications (14, 15). Briefly, 96-well microtiter plates were coated with 25 µg/ml human umbilical cord HA (ICN Biomedicals, Costa Mesa, CA). The HA-coated wells were incubated with various amounts of tissue extracts or SF-CM (unconcentrated) from different cell types, in the presence of a biotinylated HA-binding protein. The HA-binding protein was isolated from bovine nasal cartilage according to the method described by Tengblad (46), which utilizes HA affinity chromatography and trypsinization to isolate the HA binding part of the proteoglycan monomer. The purified HA-binding protein was biotinylated using N-hydroxysuccinimido biotin (Sigma). The amount of biotinylated HA-binding protein bound to the microtiter wells was determined using an avidin-biotin detection system (Vector Laboratories, Inc., Burlingame, CA). The amount of HA present in each sample (ng/ml) was determined using a standard graph. We routinely normalize the amount of HA in biological fluids (e.g. urine) or in culture CM to total protein. Normalization of HA levels in biological fluids such as urine to total protein eliminates the influence of the hydration status of an individual on HA levels (15). For each sample, 3 different analyses, each in duplicate, were tested. The results are expressed as mean ± S.E.

**Measurement of HAase Levels**—HAase levels present in tissue extracts and SF-CMS-CM were measured using an ELISA-like assay similar to that developed by Stern and Stern (47), with modifications (15, 41). Briefly, 96-well microtiter wells were coated with 200 µg/ml human umbilical cord HA. The HA-coated wells were incubated with various amounts of tissue extracts or SF-CM (unconcentrated) in 0.1 M sodium formate, 0.15 M NaCl, pH 4.2, 0.2 mg/ml bovine serum albumin (BSA; ELISA-grade; Sigma). The HA remaining on the wells after incubation was determined using the same biotinylated HA-binding protein that is used in the HA-ELISA-like assay, and an avidin-biotin detection system. In the avidin-biotin detection system, we do not include anti-keratin sulfate monoclonal antibody to enhance the signal and routinely normalize the amount of HAase activity (milliunits/ml) in any sample (CM, in this case) to total protein (mg/ml). We also routinely normalize the amount of HAase in biological fluids (e.g. urine) to eliminate the influence of the hydration status of an individual on HAase levels. This is especially important when determining urinary HAase levels of patients with hematuria (i.e. blood in urine; Ref. 15).

The pH activity profile of HAase present in various CM was determined as follows: 1) pooled serum from 3 normal adults (0.5 µl); 2) human urine (2.0 µl) collected from 4 normal individuals (3 adults: 2 females and 1 male: age 25–40 years and 1 child: 7 years); 3) CM (4 µl, 10-fold concentrated) from DU145 (SF-CM), CaP fibroblasts (established from a Gleason 7 CaP, SF-CM and S-CM), and HL fibroblast cultures (SF-CM and S-CM). The indicated amounts of various samples were tested in HAase assay buffer at different pH values (2.5–7.0). Between pH 3.5 and 5.0, the HAase activity was tested in buffers differing by 0.1 pH unit (i.e. pH 3.5, 3.6, 3.7 . . . 5.0). The control wells received the buffers of specified pH, identical to those added to the sample wells. In addition, 10-fold concentrated RPMI + ITS (SF-medium control) and RPMI + 10% fetal bovine serum + gentamicin (S-medium control) were also tested at different pH values.
These media served as controls for SF-CM and S-CM collected from different cell types. The results are expressed as (control – sample) A450; the control represents buffer only.

Substrate (HA)-Gel Assay—A method described by Gutenhoner et al. (48) was used to detect the presence of HAase activity in various samples (48). Aliquots (1.5 µl) of SF-CM or S-CM were applied to 0.7% agarose-DU145, CaP fibroblast, and HLC fibroblast cultures. These CM and S-medium control were concentrated – 10-fold (100 µl). A 20-µl aliquot of each concentrated SF-CM, S-CM, S-medium control, human serum (1.5 µl), 20 µl of 10-fold concentrated normal human urine and ELISA-grade BSA (10 µg) were separated on an 8.5% SDS-polyacrylamide gel containing 0.1% SDS, 0.1% Triton X-100. Four such gels were prepared and simultaneously electrophoresed. Following electrophoresis, the gels were soaked in 3% Triton X-100, to renature the HAase present in various samples. Each gel was then incubated in HAase assay buffer of pH 3.0, 3.7, 4.2, or 4.5 without BSA. Following incubation at 37 °C for 16 h, the gels were stained sequentially with 0.5% Alicant Blue and 0.15% Coomassie Blue solutions and then destained.

RT-PCR Cloning and Sequence Analyses—Total RNA was extracted from CaP cell lines, a Gleson 7 CaP primary epithelial explant culture and bladder cancer lines, HT1376 and 253J-Lung using a RNA extraction kit (Qiagen, Valencia, CA). Total RNA (1 µg) was subjected to first strand cDNA synthesis using the Superscript™ preamplification system and oligo(dT) primers (Life Technologies, Inc., Gaithersburg, MD). The cDNA was amplified using three different HYAL1-specific primer pairs. The primers were designed based on the HYAL1 cDNA sequence deposited in the GenBank™ data base (accession number HSU03056). The sequences of the first pair of primer were the following: (a) HYAL1-L1 (the sequence between nucleotides 214 and 233), 5'-CTGGTGGAAGGAGACAGGAG-3'; (b) HYAL1-R1 (the reverse complementary sequence between nucleotides 564 and 583), 5'-GGGAGGAGCT-GAGAAGCAG-3'. The second primer pair was designed to amplify the entire coding region of HYAL1. The sequence of the second primer pair was the following: (a) HYAL1-L2 (the sequence between nucleotides 594 and 613), 5'-TTGTCTCCGACGAGTCCGCTT-3'; (b) HYAL1-R2 (the reverse complementary sequence between nucleotides 1906 and 1925), 5'-ATCACACATTCCGCTTCCG-3'. The sequence of the third primer pair was designed to amplify both the long and short forms of HYAL1 transcript. The primer sequences were the following: (a) HYAL1-L3, this sequence is between nucleotides 27,274 and 27,294 in a human cosmid clone LUC13 from sp2p1.3 (GenBank™ accession number AC002455). The cDNA clones, GenBank™ accession numbers AF173154 (spliced form) and HSU03056, which contain the entire HYAL1 coding sequence, lack the nucleotide base ‘C’ present at the 5’ end of the HYAL1 primer, and the 3’ end of the HYAL1 primer, is 5’-CTCCCTCAAGAGTCTT-GTT-3’. The PCR conditions for HYAL1-L1/R1 primer pair were the following: (a) initial melting at 94 °C for 5 min; (b) 35 cycles of 94 °C for 1 min, 62 °C for 30 s, and 72 °C for 1 min; (c) 72 °C for 10 min. For PCR analysis Tag polymerase (Promega Corp., Madison, WI) was used. The PCR conditions for HYAL1-L2/R2 and HYAL1-L3/R3 primers were the following: (a) 95 °C for 10 min (hot start); (b) 10 cycles of 94 °C for 30 s (70–60 °C for 30 s, i.e. annealing temperature dropping by 1 °C at each cycle, 72 °C for 1 min; (c) 25 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min; (d) 72 °C for 7 min, final extension. The PCR mixture contained 5% dimethyl sulfoxide and Ampli-Taq Gold™ (PerkinElmer Life Sciences, Wellesley, MA).

Immunoblot Analysis—Prostate epithelial cell culture SF-CM were separated on an 8.5% SDS-polyacrylamide gel, under nonreducing conditions, and then blotted onto a polyvinylidene difluoride membrane. The blotted membrane was stained with 0.15% Coomassie Blue in 30% methanol for 1 min and then destained to visualize and compare total protein profile in each lane as described previously (23). This method rules out the possibility that any differences observed in the intensity of the HYAL1 band among various samples is simply due to differences in sample loading and protein transfer (20). Following visualization of the total protein profile, the blot was completely destained, rehydrated, and blocked with 3% BSA in 20 mM Tris- HCl, 0.15 M NaCl, and 0.05% Tween 20. The blot was probed with 5 µg/ml anti-HYAL1 antibody at 4 °C for 16 h. The anti-HYAL1 antibody was purified as the IgG fraction using protein G-Sepharose, according to the manufacturer's protocol (Amer sham Pharmacia Biotech). The blot was then washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:7500 dilution; Sigma), at room temperature for 2 h. The blot was then washed and developed using an alkaline phosphatase color detection system, involving nitro blue tetrazolium and 5-bromo-4-chloro-3-indoly phospho substrates (Bio-Rad). To determine the specificity of the immuno blot analysis, in some experiments, the HYAL1-(321–338)-MAP peptide (50 µg/ml) was included during the anti-HYAL1 antibody incubation. This MAP peptide was used as an antigen to generate the anti-HYAL1 antibody (43). We have previously characterized this antibody for its specificity and ability to detect partially purified HYAL1 protein, and HYAL1 protein present in complex biological fluids (e.g. urine, plasma) (43).

Immunohistochemistry—Immunohistochemical localization of HA and HAase was carried out in 57 prostate tissues (NAP, n = 5; BPH, n = 7, Gleason 5, n = 7; Gleason 6, n = 11; Gleason 7, n = 16; and Gleason ≥ 8, n = 11) and one each of locally extended and metastatic CaP lesions, using a method described previously.2 The Gleason scoring is a standard method of grading CaP tissues. Paraffin-embedded blocks were cut into 3-µm thick sections and placed on positively charged slides. The specimens were deparaffinized, rehydrated, and subjected to antigen retrieval, as described before. For each specimens two slides were prepared, one for HA and the other for HYAL1 staining, as described below. HA Staining—One set of the slides was incubated with 2 µg/ml biotinylated HA-binding protein for 30 min. The second set of the slides was incubated with 2 µg/ml biotinylated HA-binding protein used in this experiment was the same as that used in the ELISA-like assay for HA level measurement. The specificity of HA staining was established by incubating tissue specimens with 100 million/ml of Streptomyces HAase (ICN Biomedicals, Costa Mesa, CA) at 37 °C for 3 h, prior to incubation with biotinylated HA-binding protein. Following incubation with HA-binding protein, the slides were washed in PBS and processed as described below.

HYAL1 Staining—The second set of slides was incubated with 3.7 µg/ml anti-HYAL1 antibody (IgG fraction) at 4 °C for 16 h to localize HYAL1 in prostate tissues. The concentration of the anti-HYAL1 antibody used in these experiments was that concentration at which the preimmune IgG displayed no staining of tissues (i.e. no nonspecific staining). To further determine the specificity of tissue staining observed by anti-HYAL1 antibody, in some cases, 50 µg/ml HA ELISA MAP peptide-(321–338) was included during incubation of tissue specimens with anti-HYAL1 antibody. Following incubation with anti-HYAL1 antibody, the slides were washed in PBS and incubated with a linking solution containing biotinylated goat anti-rabbit IgG (Dako LSAB kit, Dako Laboratories, Carpinteria, CA) at room temperature for 30 min. The biotinylated HA-binding protein used in this experiment was the same as that used in the ELISA-like assay for HA level measurement. The slides were then counterstained with hematoxylin and mounted. Three independent readers evaluated the slides. Two independent readers (D. R. and V. B. L.) graded the slides in a blinded fashion. These readings were confirmed by study pathologist (M. N.). The slides were graded with respect to the staining intensity as, 0 (no staining), +1 (weak), +2 (moderate), and +3 (high).

Gel-filtration Chromatography—The size profiles of HA species present in the Hapes and guandine extracts of NAP, BPH, and low-grade and high-grade CaP tissues (n = 2) were determined. Six milligrams of total protein in each extract were applied to a Sephadex G-50 column (1.5 × 120 cm) equilibrated with PBS. The column was eluted at 7 ml/h, and 3.6-ml fractions were collected. The column fractions were assayed using the ELISA-like assay for HA, as described above. The protein profile of the column was determined by measuring absorbance at 280 nm. The column was calibrated using human umbilical cord HA, and HA fragments (F1 (10–15 disaccharide units), F2 (2–3 disaccharide units), and F3 (≥ 2 disaccharide units)) (14).

RESULTS

Measurement of HA Levels in Prostate Tissues

The concentration of HA in prostate tissues has not been determined previously. We used the HA ELISA-like assay to measure HA levels (µg/mg) in the extracts of NAP, BPH, and CaP tissues. In tissues, HA may exist in the free form or as bound to the link molecules (50, 51). Therefore, we extracted

2 Hautmann, S. H., Lokeswar, V. B., Schroeder, G. L., Civantos, F., Duncan, R. C., Gann, R., Friedrich, M. G., and Soloway, M. S. (2001) J. Urol., in press.
HA present in prostate tissues sequentially, in a Heps buffer (Heps extract; free HA) and in a 6 M guanidine/ HCl buffer (guanidine extract: HA bound to link molecules). The differences in the amount of HA bound to link molecules in each extract was determined by the ELISA-like assay for HA, as described under “Experimental Procedures.” The results are expressed as mean ± S.E. A, Heps extract; B, guanidine extract.

FIG. 1. Determination of HA levels in prostate tissue extracts. The NAP, BPH, and low- and high-grade CaP tissues (n = 5/category) were sequentially extracted in a Heps buffer (Heps extract) and in a guanidine buffer (guanidine extract). The HA concentration in each extract was determined by the ELISA-like assay for HA as described under “Experimental Procedures.” The results are expressed as mean ± S.E. A, Heps extract; B, guanidine extract.

Identification of HAase Secreted by CaP Cells

**RT-PCR Analysis**—We have previously shown that CaP cells secrete a HAase of apparent Mr ~ 55,000 in their CM (33). However, the type of HAase secreted by CaP cells has not been identified. Since we have previously detected HYAL1-type HAase expression in invasive bladder cancer cells, we studied the expression of HYAL1 in CaP cells by RT-PCR analysis using a HYAL1-specific primer pair that was used to amplify HYAL1 PCR product from bladder cancer cells (43). As shown in Fig. 3A, an expected 370-bp PCR amplification product is visible in RNA preparations from a Gleason 7 CaP epithelial explant culture, and three established CaP cell lines, LNCaP, PC3-ML, and DU145. The 370-bp amplified PCR product from Gleason 7 CaP explant culture and DU145 cells was cloned and sequenced. The sequence of this product matched 100% with the known HYAL1 sequence (GenBank™ accession number HSU703056). As expected, no PCR product is amplified in the negative control lane (Fig. 3A).

To further confirm the expression of HYAL1 transcript in CaP cells, we performed RT-PCR analysis on total RNA from CaP tissues, using a HYAL1-specific primer pair that should amplify the entire 1.3-kb coding region in HYAL1 cDNA.
shown in Fig. 3B, the expected 1.3-kb PCR product is amplified from DU145, LNCaP, PC3-ML, and Gleason 7 CaP explant samples. The bladder cancer lines HT1376 and 253J-Lung that express HYAL1 were used as positive controls. The same 1.3-kb PCR product is amplified from the RNA of both of these bladder cancer cells. The specificity of the 1.3-kb PCR product is further confirmed from the negative PCR control (Fig. 3B). The cDNA cloning and sequencing of this 1.3-kb PCR product from both the CaP cells and bladder cancer cells confirmed that the sequence of this product matches 100% with the known HYAL1 sequence (data not shown).

Based on the HYAL1 cDNA sequences deposited in the GenBank™, the 5′-untranslated region between nucleotides 104 and 588 in the HYAL1 transcript (GenBank™ accession number HSU03056) appears to be alternatively spliced, giving rise to two HYAL1 transcripts. To examine which one of the two transcripts are expressed in CaP cells, we performed RT-PCR analysis using a HYAL1-specific primer pair that lies outside the boundary of the alternatively spliced region. Using this primer pair we expected a 267-bp PCR amplification product from the spliced HYAL1 transcript and a 752-bp PCR amplification product from the unspliced HYAL1 transcript, respectively. As shown in Fig. 3C, both 260- and 750-bp PCR products are amplified from Gleason 7 CaP explant culture, LNCaP, PC3-ML, and DU145 RNAs, suggesting that CaP cells express both the spliced and unspliced HYAL1 transcripts. Furthermore, the bladder cancer lines, HT1376 and 253J-Lung that were used as a positive controls, show the expression of the same two PCR products that are amplified from the spliced (260-bp product) and the unspliced HYAL1 transcripts (750-bp product) (Fig. 3C, lane 5). The sequence of the shorter product revealed that it contains 267 bp and it lacks the region between nucleotides 104 and 588, that is present in the longer 752-bp PCR product (data not shown). As expected, the negative control shows no amplification (Fig. 3C). These results demonstrate that CaP cells express both the spliced and unspliced HYAL1 transcripts.

Detection of HYAL1-related Protein in CaP Cells—To examine whether an HYAL1-related protein is expressed in CaP cells, we performed immunoblot analysis on the CM of primary prostate epithelial explant cultures, using a rabbit anti-HYAL1 antibody, as described previously (43). In this experiment, 2 primary cultures from each category (i.e. NAP, BPH, etc.) were analyzed. Since identical results were obtained in both experiments, results of one experiment are shown in Fig. 4. As shown in Fig. 4, CM from NAP, BPH, and Gleason 5 CaP explant cultures do not show any cross-reactivity with the anti-HYAL1 antibody. However, the CM of Gleason 7 CaP explant culture, and that of LNCaP, PC3-ML, and DU145 show the presence of a ~60-kDa protein that cross-reacts with the anti-HYAL1 antibody. The densitometric scanning showed that the expression of this HYAL1-related protein is 7.5 to >10-fold higher than that in NAP, BPH, and Gleason 5 CaP explant cultures. The detection of the 60-kDa HYAL-related protein can be blocked when HYAL1-(321–338)-MAP peptide is included during anti-HYAL1 antibody incubation (data not shown).

To confirm that the detection of the 60-kDa HYAL1-related protein in CaP CM correlates with the presence of HAase activity, the same CM were analyzed by the HAase ELISA-like assay. As shown in Table I, NAP (1.7 ± 0.5), BPH (2.2 ± 0.8), and Gleason 5 CaP (3.9 ± 0.4) cultures secrete very little HAase activity (milliunits/mg) in their CM. These results are consistent with our previous observation (33). Furthermore, as expected, the Gleason 7 prostate explant culture (36.9 ± 9.5), LNCaP (84.2 ± 13.8), PC3-ML (16.1 ± 3.4), and DU145 (206.5 ± 11.4) cells secrete high levels of HAase activity in their CM (Table I). One of the reasons why PC3-ML cells appear to secrete lower amounts of HAase activity than LNCaP and DU145 cells, despite expressing comparable amount of HYAL1 protein, may be that the high levels of HA secreted by PC3-ML cells in their CM interferes with the HAase ELISA-like assay (15).

Using the HAase ELISA-like assay we also investigated the secretion of HAase activity in the culture CM of fibroblast cultures set up from Gleason 5 and Gleason 7 specimens. In these cultures no HAase activity was detected (see below).

Determination of pH Activity Profile of HAase

We have previously shown that the HAase activity expressed in bladder cancer cells, which is HYAL1-related, has a pH optimum range between 4.1 and 4.3. However, the HAase activity present in human serum and normal human urine, that is also attributed to HYAL1, has been shown to have a pH optimum at 3.7–3.8 (53–56). Furthermore, HYAL1 purified from human plasma and urine, as well as, recombinant HYAL1 have been shown to have a pH optimum at 3.7 (53, 54). In addition, fibroblast cultures (human dermal, and fetal, as well as, fibrosarcoma) were shown to secrete a HAase that is active at pH 3.7 but not at pH 4.5 (44). Among these observations, some were based on the “in-gel HAase activity” detected by substrate (HA)-gel assay performed at pH 3.7 (44, 56). To understand the differences in pH activity profiles of HYAL1-related HAase reported in different laboratories, we compared
Furthermore, BSA is not known to have any HAase activity with the HAase ELISA-like assay results presented in Fig. 5. The absence of clear bands at pH 3.0 in various samples is not consistent of such tremendous HAase activity (as judged by the intensity of "clear bands" that would appear to arise due to the presence of BSA as a carrier protein). In addition, no HAase activity was detected at pH 4.2 in human serum, urine, and DU145 SF-CM, which is consistent with the data on the HAase ELISA-like assay, at pH 3.7 the intensity of the clear band is decreased by ~50% in CaP fibroblast S-CM, HL fibroblast S-S-Medium, S-medium control, and BSA samples, almost all disappear at pH 4.2 (Fig. 6C). It should be noted that no HAase activity is detected at pH 4.2 in CaP fibroblast and HL fibroblast SF-CM (Fig. 6C). At pH 4.5, the HAase activity in human serum, urine, and DU145 SF-CM is decreased by ~50% from that at pH 4.2, nonetheless, it is still significant (Fig. 6D). No clear bands are detected in CaP fibroblast S-CM, HL fibroblast S-S-Medium, S-medium control, and BSA samples at pH 4.5 (Fig. 6D).

These results demonstrate the following. 1) The HAase activity present in human serum, urine, and DU145 SF-CM has a similar pH optimum (4.1–4.2), when assayed using both the HAase ELISA-like assay and substrate (HA)-gel assay. 2) CaP fibroblast and HL fibroblast cultures do not secrete any HAase activity in SF-CM, as detected by the ELISA-like assay and the substrate (HA)-gel assay. The clear band that is present in S-CM from these cells, at pH 3.0 and 3.7, may be an artifact since the S-medium control and BSA alone also show the presence of the same clear band with comparable intensity. This clear band disappears, whereas, the intensity of the true HAase activity bands in serum, urine, and DU145 SF-CM samples

| Cell culture CM     | HAase levels | milliunits/mg |
|---------------------|--------------|---------------|
| NAP                 | 1.7 ± 0.5    |               |
| BPH                 | 2.2 ± 0.8    |               |
| Gleason sum 5 CaP   | 3.9 ± 0.4    |               |
| Gleason sum 7 CaP   | 36.9 ± 9.5   |               |
| LNCaP               | 84.2 ± 13.8  |               |
| PC3-ML              | 16.1 ± 3.4   |               |
| DU145               | 206.5 ± 11.4 |               |

The pH activity profiles of HAase expressed in various sources using both the HAase ELISA-like assay and substrate (HA)-gel assay.

**HAase ELISA-like Assay**—We measured the pH activity profile of HAase expressed in DU145 CM, human serum, and normal human urine. We also measured whether HAase activity can be detected in the CaP fibroblast and HL fibroblast culture CM. The CaP and HL fibroblast CM were either serum-free (SF-CM) or contained 10% fetal bovine serum (regular growth medium; S-CM). The S-CM was included in the analysis, since Stair-Nawy et al. (44) have shown that HAase activity is detected in fibroblast S-CM at pH 3.7 but not at pH 4.5. As shown in Fig. 5, the HAase activity secreted in DU145 SF-CM has a pH optimum at 4.2 (range 4.0–4.3). In comparison to the optimum activity at pH 4.2, the enzyme is 55% active at pH 3.7 and 50% active at pH 4.5 (Fig. 5). The HAase expressed in human serum, shows optimum activity at pH 4.0 and 4.1. The enzyme is 85% active both, at pH 3.7 and pH 4.5 (Fig. 5). The HAase activity present in normal human urine has a pH optimum at pH 4.1 and 4.2. The enzyme is 72% active at pH 3.7 and 69% at pH 4.5 (Fig. 5). The results demonstrate that the HAase activity expressed in CaP cells has a pH optimum similar to that in human serum and urine, and it is at pH 4.1 to 4.2. The HAase in all of these three sources is similarly active pH 3.7 and 4.5.

The SF-CM or S-CM, both either uncentrated or concentrated, from CaP fibroblast and HL fibroblast cultures do not show any HAase activity at any pH between 2.5 and 7.0. The SF- and S-medium controls have no HAase activity at any pH tested (Fig. 5). These results demonstrate that the CaP fibroblast and HL fibroblast cultures do not secrete any HAase activity, as determined by the HAase ELISA-like assay.

**Substrate (HA)-gel Assay**—We next analyzed HAase activity in various samples using a substrate (HA)-gel assay (48). The in-gel HAase activity was assayed in HAase assay buffer adjusted to pH 3.0, 3.7, 4.2, and 4.5. As shown in Fig. 6A, at pH 3.0 “clear bands” (that would appear to arise due to the presence of a HAase activity that is digesting the HA in the gel) are present in electrophoresed human serum, urine, DU145 SF-CM, CaP fibroblast S-CM, HL fibroblast S-CM, S-medium control, and BSA samples appear to have faster electrophoretic mobility. However, in the uncentrated samples, the mobility of the clear bands is closer to ~60 kDa, the approximate molecular weight of BSA (data not shown). The presence of such tremendous HAase activity (as judged by the intensity of clear bands) at pH 3.0 in various samples is not consistent with the HAase ELISA-like assay results presented in Fig. 5. Furthermore, BSA is not known to have any HAase activity and the HAase assay buffer used in the ELISA-like assay contains BSA as a carrier protein. In addition, no HAase activity is detected in S-medium control at any pH tested between 2.5 and 7.0 (Fig. 5). At pH 3.0, no HAase activity is detected in SF-CM of CaP fibroblast and HL fibroblast cultures, even after 10-fold concentration (Fig. 6A).

At pH 3.7, the intensity of the clear band is higher in serum (3.5-fold) and DU145 SF-SM-CM samples. The urine sample alone does not show an increase in the intensity of the clear band (3.8-fold), but a new band appears. The presence of two HAase species in urine has been reported previously (41, 54). The molecular weight of the HAase species in DU145 SF-CM is between the two urinary HAase species (Fig. 6B). Contrary to an increase in the intensity of clear bands observed in human serum, urine, and DU145 SF-CM, which is consistent with the data on the HAase ELISA-like assay, at pH 3.7 the intensity of the clear band is decreased by ~50% in CaP fibroblast S-CM, HL fibroblast S-CM, S-medium control, and BSA sample lanes, when compared with that at pH 3.0 (Fig. 6B). At pH 4.2, consistent with the HAase ELISA-like assay results, the intensity of “clear HAase activity bands” in human serum (1.7-fold), urine (1.8-fold), and DU145 SF-CM (1.4-fold) samples, is increased further when compared with that at pH 3.7 (Fig. 6C).

However, the clear band detected at pH 3.0 and 3.7, in CaP fibroblast S-CM, HL fibroblast S-CM, S-medium, and BSA samples, almost all disappears at pH 4.2 (Fig. 6C). It should be noted that no HAase activity is detected at pH 4.2 in CaP fibroblast and HL fibroblast SF-CM (Fig. 6C). At pH 4.5, the HAase activity in human serum, urine, and DU145 SF-CM is decreased by ~50% from that at pH 4.2, nonetheless, it is still significant (Fig. 6D). No clear bands are detected in CaP fibroblast S-CM, HL fibroblast S-CM, S-medium, and BSA samples at pH 4.5 (Fig. 6D).

**TABLE I**

Measurement of HAase activity in prostate epithelial cell cultures

| Cell culture CM     | HAase levels | milliunits/mg |
|---------------------|--------------|---------------|
| NAP                 | 1.7 ± 0.5    |               |
| BPH                 | 2.2 ± 0.8    |               |
| Gleason sum 5 CaP   | 3.9 ± 0.4    |               |
| Gleason sum 7 CaP   | 36.9 ± 9.5   |               |
| LNCaP               | 84.2 ± 13.8  |               |
| PC3-ML              | 16.1 ± 3.4   |               |
| DU145               | 206.5 ± 11.4 |               |
increases at pH 4.2. 3) CaP fibroblast and HL fibroblast do not secrete any HAase activity that is active between pH 2.5 and 7.0.

**Immunohistochemical Localization of HA and HAase in Prostate Tissues**

To investigate the distribution of elevated HA and HAase (i.e. HYAL1) in CaP tissues, we utilized a biotinylated HA-binding protein and the anti-HYAL1 antibody to localize HA and HAase in prostate tissues. The biotinylated HA-binding protein has been utilized previously to localize HA in tumor tissues (10, 12).

**HA Localization—** Deparaffinized archival NAP (*n* = 5), BPH (*n* = 7), and CaP (*n* = 45) tissues were sequentially incubated with the biotinylated HA-binding protein, streptavidin peroxidase, and DAB substrate to localize HA. As shown in Fig. 7A, little HA staining is observed in the NAP specimen. Out of the five NAP tissues tested, 4 showed no HA staining (0) and 1 specimen showed +1 staining intensity (Table II). The BPH specimen, shown in Fig. 7B, stains with +1 intensity and the staining is focal. Furthermore, all of the staining is present in the stromal component and none is observed in epithelial cells. Out of the 7 BPH specimens that were stained, +1 and +2 staining intensity was seen in 5 and 2 specimens, respectively. As shown in Fig. 7, panels C-F, HA staining in CaP specimens is significantly higher (intensity +2 to +3) and diffuse, regardless of whether the specimen is from low-grade (i.e. Gleason sums 5 and 6) or high-grade (i.e. Gleason sums 7, 8 and 9) CaP. All of the HA staining in CaP specimens is in the stroma and none in the tumor epithelial cells. Furthermore, the stroma surrounding normal prostate glands present in the CaP specimens shows only 0 to +1 staining (data not shown). Among the seven Gleason sum 5 specimens that were stained, +2 and +3 staining intensity was seen in 4 and 2 specimens, respectively, and no staining (i.e. 0 intensity) was seen in 1 specimen (Table II). Similarly, among Gleason 6 specimens (*n* = 11) that were stained, 1, 2, 6, and 2 specimens showed 0, +1, +2, and +3 intensity, respectively. All of the Gleason sum 7 (*n* = 16) and ≥8 (*n* = 11) specimens showed +2 and +3 HA staining intensity (Table II). These results demonstrate that in prostate tissues HA is localized in the stromal compartment and is significantly elevated in the tumor-associated stroma.

**HAase Localization—** The same paraffin-embedded archival prostate specimens that were used to localize HA were also used to localize HYAL1-type HAase. As shown in Fig. 8, no staining for HYAL1 is observed in NAP, BPH, and Gleason sum 5 tissues (Fig. 8, panels A-C). Out of the five NAP specimens that were stained, none showed HYAL1 staining (Table III). Among the 7 BPH specimens that were stained, 4 showed no staining, whereas, 3 specimens showed +1 staining intensity. Out of the 7 Gleason 5 specimens that were stained, 6 showed no staining and 1 specimen showed +2 staining intensity, respectively (Table III). Among the 7 BPH specimens that were stained, 4 showed no staining, whereas, 3 specimens showed +1 staining intensity. Out of the 7 Gleason 5 specimens that were stained, 6 showed no staining and 1 specimen showed +2 staining intensity, respectively (Table III). As shown in Fig. 8, panel D, the Gleason sum 6 specimen shows +1 intensity for HYAL1 staining and the staining is exclusively localized in tumor epithelial cells. Among 11 Gleason sum 6 tissues that were stained, 4 specimens showed +1 and 7 showed +2 intensity for HYAL1 staining (Table III). In all of these Gleason 6 specimens, the
HA was localized in prostate tissues using a biotinylated HA-binding protein and a streptavidin peroxidase DAB-chromogen detection system as described under "Experimental Procedures." A, NAP; B, BPH; C, Gleason sum 5 CaP; D, Gleason sum 6 CaP; E, Gleason sum 7 CaP; and F, Gleason sum 9 CaP, specimens.

**Table II**

**Distribution of HA staining intensity in prostate tissues**

To localize HA, prostate tissue specimens were stained with a biotinylated HA-binding protein. The stained specimens were scored as 0, +1, +2, and +3 for staining intensity, where 0 intensity indicates no staining, and +3 intensity indicates the highest staining.

| Tissue                  | 0 | +1 | +2 | +3 |
|-------------------------|---|----|----|----|
| NAP (n = 5)             | 4 | 1  | 2  |    |
| BPH (n = 7)             |   | 5  | 2  |    |
| Gleason sum 5 CaP (n = 7)|1 | 4  | 2  |    |
| Gleason sum 6 CaP (n = 11)|1| 2  | 6  | 2  |
| Gleason sum 7 CaP (n = 16)|1| 9  | 7  |    |
| Gleason sum $\geq$ 8 CaP (n = 11)|1| 9  | 2  |    |

normal prostate glands showed no staining for HYAL1 (data not shown). The staining intensity for HYAL1 further increased (+2 to +3) in CaP specimens with Gleason sum 7 and $\geq$8. As shown in Fig. 8E, the tumor epithelial cells in Gleason 7 specimen show +3 staining intensity for HYAL1. Among the 16 Gleason 7 CaP specimens examined, 2, 1, 10, and 3 specimens showed 0, +1, +2, and +3 staining intensity, respectively. Fig. 8F shows HYAL1 staining in a Gleason 9 CaP specimen. All of the tumor cells in this specimen are stained with +3 intensity and this is further confirmed in the magnified view of the specimen (Fig. 8, panel F, inset).

**Determination of Tissue HA Profiles**

The stromal-epithelial pattern of HA and HYAL1 expression and increased concentration of HYAL1 in higher Gleason sum CaP tissues, raises the question whether the tumor cell-derived HYAL1 might degrade stroma-associated HA. If this were the case, angiogenic HA fragments might be present in CaP tissues. To address this issue, we examined the profiles of HA species present in the Hepes (free HA) and guanidine (HA

**Localization of HA and HYAL1 in Locally Extended and Metastatic CaP Lesions**—To determine whether the intensity and the patterns of HA and HYAL1 staining are the same in a primary CaP lesion and in the clinically progressed lesions, we localized both molecules in a Gleason 9 specimen, in seminal vesicles invaded with CaP (locally extended lesion), and in a lymph node that is positive for CaP (i.e., metastatic lesion). These three specimens were obtained from the same patient. As shown in Fig. 9, +3 intensity is seen for HA (panel A) and HYAL1 (panel B) in the primary CaP lesion. HA is localized in stromal components, whereas, HYAL1 is localized in tumor epithelial cells. In seminal vesicles invaded with CaP, the stroma surrounding the seminal vesicle that is invaded by CaP shows +3 staining intensity for HA (Fig. 9, panel C). Interestingly, tumor cells in this locally extended CaP lesion, as well as, the seminal vesicle itself stain with +3 intensity for HYAL1 (Fig. 9, panel D). However, any clinical significance of this observation cannot be evaluated at the present time since the staining intensity of HYAL1 in normal seminal vesicles is unknown. We are currently working on obtaining some normal seminal vesicle tissues to clarify this issue. In the lymph node, the same pattern of HA and HYAL1 staining is observed. The stroma in the lymph node lesion stains with +3 intensity for HA and the tumor cells stain with +3 intensity for HYAL1.

These results demonstrate that both the divergent HA and HYAL1 staining pattern and the intensity of staining seen in primary CaP lesion are duplicated in the locally extended and metastatic CaP lesions.
bound to link molecules) extracts of NAP, BPH, and low-grade and high-grade CaP tissues, using gel-filtration chromatography. Two tissues were tested per category in separate experiments. As shown in Fig. 10A, which represents the profiles of HA species present in the Hepes extracts, the NAP and BPH tissues contain one major HA species that corresponds to the high molecular mass HA. A minor intermediate size HA species (peak II) is observed in the BPH sample. On the contrary, HA species of different sizes are present in CaP tissues, which represent peaks I to IV. Although, the high molecular mass HA appears to be a major species, a significant amount of HA species present in low-grade CaP tissue sample elute as peaks II and III. The high-grade CaP sample predominantly contains high molecular mass (peak I) and intermediate size (peak II) HA species. However, ~25% of the HA species elute at peak III, which represents the angiogenic HA fragments. The column profile of total protein present in the Hepes extracts of various samples was similar (Fig. 10A and data not shown).

The profiles of HA species present in guanidine extracts of various prostate tissue samples is very similar, with the majority of HA eluting as high molecular mass HA (Fig. 10B). A small amount of intermediate size HA (peak II) is also present in high-grade CaP sample (Fig. 10B). These results demonstrate that in CaP tissues, the size distribution of HA existing in the free and bound forms is different. Predominantly, the free HA is degraded into smaller species of different sizes, presumably due to the action of HAase. In high-grade CaP tissues, tumor-associated free HA may be degraded by HYAL1 into angiogenic HA fragments.

**DISCUSSION**

In this study we have been able to demonstrate the expression of both HA and HAase in CaP. We also identified which tumor components (i.e. stroma and tumor cells) contribute to the elevated levels of these molecules in CaP. Tumor-associated HA is known to enhance tumor metastasis, and increased HA levels are observed in several human cancers (9–14). The molecular biological, biochemical, and immunohistochemical studies presented here reveal the expression of HYAL1-type HAase in CaP cells. Some of the earlier findings have demonstrated that HAase secretion correlates with the invasive/metastatic potential of tumor epithelial cells (40–42). The fact that HYAL1-type HAase is also expressed in invasive bladder tumor cells and in the urine of high-grade bladder cancer patients (43) suggests that HYAL1-type HAase may be expressed by invasive tumors of various tissue origins.

Unlike the HA expression, secretion of the HYAL1-type HAase correlates with CaP progression (Figs. 4 and 8 and Table I). The molecular biological, biochemical, and immunohistochemical studies presented here reveal the expression of HYAL1-type HAase in CaP cells. Some of the earlier findings have demonstrated that HAase secretion correlates with the invasive/metastatic potential of tumor epithelial cells (40–42). The fact that HYAL1-type HAase is also expressed in invasive bladder tumor cells and in the urine of high-grade bladder cancer patients (43) suggests that HYAL1-type HAase may be expressed by invasive tumors of various tissue origins.

The pH activity profile and substrate (HA)-gel assay studies

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**TABLE III**

**Distribution of HYAL1 staining intensity in prostate tissues**

| Tissue                  | 0 | +1 | +2 | +3 |
|-------------------------|---|----|----|----|
| NAP (n = 5)             | 5 | 4  | 2  | 1  |
| BPH (n = 7)             | 3 | 4  | 1  | 1  |
| Gleason sum 5 CaP (n = 7)| 6 | 4  | 1  | 1  |
| Gleason sum 6 CaP (n = 11)| 4 | 7  | 3  | 0  |
| Gleason sum 7 CaP (n = 16)| 2 | 1  | 10 | 3  |
| Gleason sum ≥ 8 CaP (n = 11)| 5 | 6  | 3  | 1  |

**Fig. 9. Localization of HA and HYAL1 in primary, locally extended, and metastatic CaP lesions.** Primary, locally extended (seminal vesicles invaded with CaP) and metastatic (i.e. involved lymph node) CaP specimens, obtained from a single patient were stained for HA (panels A, C, and E) and HYAL1 (panels B, D, and F), as described under “Experimental Procedures.” Panels A and B, primary Gleason sum 9 CaP specimen. Panels C and D, seminal vesicles invaded with CaP. Panels E and F, involved lymph node.

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3 V. B. Lokeshwar and D. Rubinowicz, unpublished results.
4 V. B. Lokeshwar and D. Rubinowicz, unpublished results.
The HAase activity expressed in CaP cells is distinctly different from that of the known membrane-bound and soluble forms of PH20 (61, 66). The CaP HAase is inactive at pH > 5.0. This is noteworthy, since expression of PH20 transcripts has been detected in CaP tissues by RT-PCR analysis (63). However, except for LNCaP cells, we did not detect PH20 mRNA expression in other CaP cells. Our results on the pH activity profile are consistent with the results of Podyma et al. (37) who tested optimum pH for HAase activity secreted by human lung carcinoma H460 cells. Using buffers differing in 0.5 pH intervals, they demonstrated that the HAase expressed by H460 cells has the same pH optimum as human serum, both at pH 4.0.

Our results demonstrate that CaP fibroblasts do not secrete any HAase that has activity between pH 2.5 and 7.0. Similar results are observed for HL fibroblasts. The substrate (HA)-gel assay results demonstrate that this technique might generate artifacts that may be mistaken for the true HAase activity at pH 3.7 and lower. However, at pH 4.2 this artifact disappears, while the true HAase remains active. The artifact may become problematic, if one is analyzing HAase activity in S-CM or biological fluids that contain proteins such as the serum albumin (Fig. 6). It is possible that certain serum proteins may precipitate at acidic pH (≤ 4.0).

At present, we do not know whether HYAL1-type HAase is the only HAase expressed in CaP cells. Together with prostate cancer, the expression of PH20 mRNA has been demonstrated in melanoma, glioblastoma, glioma, and colonic carcinoma cell lines, as well as in invasive breast carcinomas (34, 40, 63). However, the pH optimum (3.8 to 4.0) of the HAase activity present in the extracts of breast primary tumors, and regional metastases is similar to the pH optimum of HAase present in human serum, and CaP SF-CM, observed in this study and it is different from that of the known pH20 isoforms (35, 61, 62). Therefore, it may be necessary to demonstrate the expression of PH20 protein, PH20-related HAase activity along with PH20 mRNA expression in various tumors. In CaP cells, the detection of HYAL1-related protein by anti-HYAL1 peptide IgG is HYAL1 specific. This is because, the HYAL1 peptide sequence against which this antibody was generated, shares only 4, 5, and 3 amino acids with PH20, HYAL2, and HYAL3 sequences, respectively. Furthermore, the shared amino acids between the HYAL1 sequence and other HAases do not occur in a continuous order.

Due to the widespread acceptance of prostate-specific antigen as a biochemical screening marker, it is perceived that there is limited need for another tumor marker for CaP. However, the majority of men with clinically localized CaP have very similar prostate-specific antigen values (i.e. 4–10 ng/ml) and biopsy Gleason score (i.e. between 5 and 7) (1, 2, 6). Thus, based upon these two parameters alone, it is difficult to identify which patients have aggressive disease. Certain molecules that regulate CaP growth and metastasis have shown promise as prognostic markers (64–70). Our data presented here show that both HA and HAase (i.e. HYAL1) associate with the biology of CaP and show a distinct stromal epithelial pattern of expression in CaP tissues. It is possible that either one or both of these molecules function as prognostic indicators for CaP.

The physiological consequence of elevated HA and HAase levels in tumors may be stimulation of angiogenesis, due to the generation of small HA fragments. Detection of angiogenic HA fragments in CaP tissues supports this notion. We have previously shown that angiogenic HA fragments bind to RHAMM-type HA receptor and subsequently induce the MAP kinase pathway which stimulates endothelial cell proliferation (23). Given the facts that HYAL1 is ~80% active at pH 4.5, and the

![Figure 10: Examination of tissue HA profile](image-url)

The sizes of HA species present in Hepes extracts (A) and guanidine extracts (B) of NAP, BPH, Gleason 5 CaP (low-grade) and Gleason 7 CaP (high-grade) tissues were determined by Sephadex G-50 gel-filtration chromatography as described under “Experimental Procedures.” Six milligrams of total protein from each extract were applied to the column. The column fractions were assayed for total protein (A280 nm) and HA (ng/ml). The total protein profile of extracts from different tissues was very similar. The protein profile of high-grade CaP extracts is shown in A and B. The column was calibrated using high molecular mass HA and HA fragments (F1, F2, and F3) as described under “Experimental Procedures.” Peaks I–IV represent HA species present in tissue extracts.

demonstrate that the HAase activity secreted by CaP cells is similar to the HAase activity present in human serum and urine (Figs. 5 and 6). The pH optimum of the HAase activity from these sources appears to be at ~4.2 (range 4.0–4.3). The enzyme is also 50–85% active at pH 4.5. We had previously reported that the pH optimum of the HAase activity secreted by CaP cells is 4.6 (33). However, in that study we did not examine the pH profile in the 0.1 pH unit intervals and had not compared the HAase activity between different sources in a side-by-side fashion. Nonetheless, it is important to note that the HAase activity secreted in human urine and serum, that is attributed to HYAL1, is active at pH 4.5 and to the same degree as seen at pH 3.7. These results are different from those reported previously (53–56). The HYAL1-related HAase activity in human serum and urine has been shown to have a pH optimum at 3.7 (53–56). However, in these studies the pH activity profile using the ELISA-like assay was measured at 0.5 pH intervals and some of these results are also based on substrate (HA)-gel assay performed at pH 3.7 (53, 54, 56).

The results presented in Fig. 6 demonstrate that the substrate (HA)-gel assay may give rise to an artifact that can be mistaken for “true HAase” activity, if the assay is performed at pH < 4.0 and the sample contains other proteins such as BSA (or human serum albumin). Such artifacts may have been seen previously, when the HAase activity was assayed in serum-containing media (59, 60). Given the observation that substrate (HA)-gel assay gives rise to artifacts, and yet, it is a good assay to determine the molecular weight of the active HAase species, it may be important to: 1) assay the HAase activity by both the ELISA-like assay and substrate (HA)-gel assay; 2) perform the substrate (HA)-gel assay at pH > 4.0; 3) assay the HAase activity in serum-free culture CM. However, it is noteworthy that some commercially available ITS solutions (e.g. Sigma) contain BSA as a carrier protein, and therefore, it is important to include an appropriate medium control while assaying the samples.
interstitial environment in malignant tumors is acidic (49), the tumor-associated HA-HYALI system may aid in angiogenesis. The localization of HA and HAase in CaP tissues, documentation of the divergent pattern of their production, and identification of at least one of the prostate tumor cell-derived HAase should help the determination of the clinical relevance of these two markers in CaP.

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