Identification of Bladder Tumor-derived Hyaluronidase: Its Similarity to HYAL1

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ABSTRACT

The glycosaminoglycan hyaluronic acid (HA) and its degrading enzyme, hyaluronidase, are intricately associated with tumor metastasis and angiogenesis. HA promotes tumor cell adhesion and migration, whereas its small fragments stimulate angiogenesis. Such small HA fragments are generated from the degradation of HA by hyaluronidase. We have previously shown (V. B. Lokeshwar et al., Cancer Res., 57: 773–777, 1997) that the HA levels are elevated in the urine and tumor tissues of bladder cancer patients regardless of the tumor grade (G). The hyaluronidase levels were found to be elevated in the urine and tumor tissues of G2 and G3 bladder cancer patients. Furthermore, angiogenic HA fragments were isolated from the urine of G2/G3 bladder cancer patients, which stimulated endothelial cell proliferation, a key event in angiogenesis. In this study, we characterized the bladder tumor-derived hyaluronidase. Analysis of hyaluronidase activity in the culture-conditioned media (CM) of 11 bladder cancer cell lines, using an ELISA-like assay and a substrate (HA)-gel technique, showed that the invasive bladder cancer cell lines secrete elevated levels of a M, −60,000 hyaluronidase. Reverse transcription-polymerase chain reaction, cloning, and sequence analyses revealed the expression of an HYAL1 transcript in bladder cancer lines. HYAL1 encodes for a hyaluronidase that is present in serum. Immunoblot analysis using an anti-HYAL1 peptide IgG confirmed the presence of a M, −60,000 HYAL1-related protein in the CM of bladder cancer cell lines, in the urine specimens from G2 and G3 bladder cancer patients, and in the partially purified preparations of bladder tumor-derived hyaluronidase. No HYAL1-related protein was detected in urine specimens from normal individuals, G1 bladder cancer patients, and patients with a history of bladder cancer but no disease at the time of testing. The bladder tumor-derived hyaluronidase present in CM and partially purified preparations was found to have maximum activity at a pH range of 4.1–4.3. The identification of bladder tumor-derived hyaluronidase should help in elucidating its role in bladder tumor progression.

INTRODUCTION

The malignant spread of primary tumors is the main cause of cancer-related deaths. The complex cascade of events that leads to metastasis includes: (a) detachment of tumor cells from the tumor mass; (b) overcoming the barrier of extracellular matrix and basement membrane; (c) entry into the blood circulation; and (d) regrowth at the secondary site (1). Another key process that is complementary to metastasis is neovascularization or angiogenesis. The nurturing of tumor mass at both the primary and the secondary metastatic sites as well as the enabling of tumor cells to gain entry into the circulation depend on angiogenesis (2). Extracellular matrix and matrix-degrading enzymes perform critical functions in both metastasis and angiogenesis. For example, several matrix components including HA3 promote metastasis by supporting tumor cell migration (3). On the other hand, many matrix-degrading enzymes, secreted by tumor cells themselves, degrade the basement membrane and help tumor cells to escape into the circulation (4). Interestingly, extracellular matrix components also sequester potent angiogenic factors that, on release, stimulate endothelial cell proliferation and migration (5). Some matrix components, such as HA, directly participate in the angiogenic process (6).

HA is a nonsulfated glycosaminoglycan, made of repeating disaccharide units, D-glucuronic acid, and N-acetyl-D-glucosamine (7). It is the only glycosaminoglycan that is not attached to any protein core. HA is present in body fluids, tissues, and extracellular matrix. It performs several functions in normal physiology such as keeping tissues hydrated, maintaining osmotic balance, and supporting cartilage integrity (7). It also interacts with specific cell surface receptors (e.g., CD44, RHAMM, hyaluronectin, and so forth) and, through these interactions, regulates cell adhesion, migration, and proliferation (8–10). The concentration of HA is elevated in several carcinomas (e.g., lung, breast, colon, Wilms’ tumor, and so forth; Refs. 11–15). We have recently shown that, in bladder cancer, the HA levels are elevated 3- to 5-fold in tumor tissues. More importantly, we showed that the HA concentration is elevated in the urine of bladder cancer patients and serves as a diagnostic marker for detecting bladder tumor regardless of its grade (16). In tumor tissues, HA actively supports tumor cell migration and adhesion and may also protect them from immune surveillance (17–19). In addition, we recently demonstrated the presence of angiogenic HA fragments (i.e., 10–15 disaccharide units in length) in the urine of G2 and G3 bladder cancer patients (16). These HA fragments stimulated the proliferation of primary human microvessel endothelial cells, which is a key event in angiogenesis (6, 16). Such small fragments are generated from the degradation of HA by hyaluronidase (20, 21). We recently demonstrated the presence of hyaluronidase in prostate and bladder tumors (22, 23).

Hyaluronidase, which is also termed “spreading factor,” is crucial for the spread of bacterial infections and of toxins present in bee, snake, and other venoms (24–28). Many early studies generated contradictory data on the increase in hyaluronidase levels in tumor tissues as compared with those in normal tissues (29, 30). However, recent studies from our laboratory and from others have established the association of elevated levels of hyaluronidase with cancer progression (22, 23, 31–35). For example, we initially showed that the elevated levels of hyaluronidase correlate with prostate cancer progression (22). It has now been shown that the hyaluronidase levels are also elevated in metastatic breast tumors and in several carcinoma lines (31–35). More recently, we showed that hyaluronidase levels are elevated in the urine of G2 and G3 bladder cancer patients and serve as markers for detecting these types of tumors (23). Our studies on bladder cancer lines also showed that the secretion of hyaluronidase correlates with the invasive potential of these tumor cells (36). Taken together, these observations suggest that hyaluronidase present in

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3 The abbreviations used are: HA, hyaluronic acid; CM, conditioned medium/media; ITS, insulin, transferrin, and selenium; G, grade; RT, reverse transcription; MAP, multiple antigenic peptide.
tumor tissues facilitates tumor “spread.” In addition, by generating angiogenic HA fragments, as in the case of G2/G3 bladder tumors, hyaluronidase may help in tumor angiogenesis.

The identification, isolation, and purification of a tumor-derived hyaluronidase have been difficult because of its small quantity (but high specific activity). At present three human hyaluronidase genes have been identified (37–39). The HYAL1 gene encodes a hyaluronidase that is present in human serum. This hyaluronidase has a pH optimum of 3.8, and its cellular origin is unknown (39, 40). The gene HYAL2 encodes a lysosomal hyaluronidase that has a pH optimum of ~3.0. This enzyme is expressed in many cell types and is known to degrade HA into large fragments consisting of ~100 sugar residues (38). The third gene is PH20, which encodes a testicular type of hyaluronidase that is located on both the plasma membrane and the inner acrosomal membrane of the sperm (41–43). Recently, some soluble isoforms of this enzyme have been identified (42, 43). Both the membrane-bound and soluble isoforms of PH20 display a broad pH activity profile (between pH 3.2 and 9.0; Refs. 42, 43). Among three hyaluronidase genes, a PH20 transcript has been shown to be expressed in melanoma, colon, glioma, and glioblastoma cell lines (31). However, these cancer cell lines were not examined for the expression of PH20 protein, and the hyaluronidase activity secreted by them was not characterized. Interestingly, in the same study, the cell lines that secrete hyaluronidase activity were also found to induce neovascularization.

In this study, using several biochemical and molecular biology techniques, we attempted to identify the bladder tumor-derived hyaluronidase that is expressed in several bladder cancer cell lines and G2/G3 patient urine.

MATERIALS AND METHODS

Bladder Cancer Cell Lines and Culture. All of the bladder cancer cell lines used in this study were established from human transitional cell carcinoma of the bladder. Transitional cell carcinomas account for ~90% of bladder tumors (44). The bladder cancer cell lines RT4, T24, J82, HT5637, HT1197, HT1376, and UMC-3 were obtained from the American Type Culture Collection (Rockville, MD). The cell lines 253J-Parent, 253J-Lung, and 253J-BV were kindly provided by Dr. Colin Dinney (M. D. Anderson Cancer Center, University of Texas, Houston, TX). The cell lines used in this study differ in their invasive potential (45–48). All of the lines were grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum and gentamicin. For analyzing hyaluronidase activity, ~60% confluent cultures from each cell line were incubated in serum-free RPMI 1640 containing ITS. The ITS-culture CM from various cultures were collected after 48 h and concentrated 10-fold before analysis.

Urine Specimens. For the purpose of purifying hyaluronidase activity, approximately 7 liters of urine was pooled from 13 bladder cancer patients who had G2 or G3 disease. For analyzing individual samples, urine was collected from age-matched (65–83 years old) normal individuals, patients with bladder cancer (G1, G2, or G3), and patients with a history of bladder cancer but no disease at the time of testing. Both the pooled and individual urine specimens were stored at ~20°C until further analysis.

RT-PCR, Cloning, and Sequence Analyses. Total RNA was extracted from bladder cancer lines using the standard guanidinium-HCl extraction procedure (49). RNA (2 μg) was subjected to first-strand cDNA synthesis using avian myeloblastosis virus reverse transcriptase and oligo-dT primers, as per the manufacturer’s instructions (Promega Corp., Madison, WI). The cDNA was amplified by PCR using either the HYAL1 or PH20 specific primers. The HYAL1 primers were as follows: (a) left primer (sequence between nucleotides 208 and 227 in the HYAL1 cDNA): 5'-CGTCTTCTATTAGAAGACGTTT-GGA-3' and (b) right primer (reverse complementary sequence between nucleotides 558 and 577 in the HYAL1 cDNA): 5'-GGAGGGCAGCTTGCAG-GGAGG-3' (GenBank accession number U96289; Refs. 39, 40). The PH20 primers were: (a) left primer (sequence between nucleotides 908 and 927 in the PH20 cDNA): 5'-CTTAGTCTTCAGAGGGCCAC-3' and (b) right primer (reverse complementary sequence between nucleotides 1516 and 1536 in PH20 cDNA): 5'-TACACCTCTTGCTCTTTGG-3' (GenBank accession number S67979 and Ref. 37). The PCR conditions were as follows: (a) initial melting at 94°C for 5 min; (b) 35 cycles of 94°C for 1 min; (c) 62°C for 30 s; (d) 72°C for 1 min; and (e) the final step at 72°C for 10 min. The PCR products were analyzed on a 1.2% agarose gel and visualized by etidium bromide staining. The PCR product was cloned into PCR TOPO vector, using the TOPO kit and the protocol supplied by the manufacturer (Invitrogen, Carlsbad, CA). Both strands of the cloned cDNA insert were sequenced in the University of Miami’s DNA core facility, using an automated DNA sequencer and T7 and SP6 universal primers.

Preparation of Anti-HYAL1 Peptide Antibody. An 18-amino-acid-long peptide comprising the sequence NH2-WYSWENTKVSCQAIKE-COOH was expressed in the Protein Core Facility at University of Florida (Gainsville, FL). This sequence corresponds to amino acids 321–338 in the HYAL1 sequence (40). This sequence shares only four amino acids (positions 321, 328, 332, and 333) with the PH 20 sequence and five amino acids (positions 321, 327, 333, 334, and 337) with the HYAL2 sequence (37, 38). The HYAL1 peptide was synthesized as a MAP and injected into a New Zealand rabbit to generate anti-HYAL1 peptide antiserum. The IgG fraction was purified from the antiserum using a Protein-G column (Pharmacia, Piscataway, CA).

ELISA-like Assay for Hyaluronidase Activity Determination. Microtiter plates (96-well) were coated with 200 μg/ml human umbilical cord HA (ICN Biochemicals, Costamesa, CA). The HA-coated wells were incubated with serial dilutions of bladder cancer cell CM or partially purified urinary hyaluronidase in a hyaluronidase assay buffer [0.1 m sodium formate (pH 4.3), 0.1 m NaCl, and 0.2 mg/ml BSA]. After incubation at 37°C for 16–18 h, the HA remaining on each well was quantified using a biotinylated HA-binding protein and an avidin-biotin detection system (22, 23). In each assay, the maximum absorbance (control) was obtained by incubating the HA-coated wells with buffer alone. The hyaluronidase activity present in each sample (milliunits/ml) was estimated using a standard graph and was normalized to total protein, as described previously (22, 23, 36). The data are presented as mean hyaluronidase activity ± SE. In some experiments, CM from 253J-Lung cells (0.5 ml) was preincubated with anti-HYAL1 peptide IgG-conjugated Sepharose beads (50 μl; 1 mg IgG coupled to 1 ml of swollen CNBr-activated Sepharose beads, Pharmacia) or control IgG-conjugated Sepharose beads. The preincubation was carried out at 4°C for 8 h, in a buffer containing 10 mM sodium phosphate (pH 7.4), 0.15 m NaCl, 0.1% BSA, 0.003% Brij 35, and 1 mM benzamidine-HCl. After incubation, the mixtures were centrifuged, the supernatants were concentrated 10-fold, and various aliquots (2.5–10 μl) were assayed for hyaluronidase activity using the ELISA-like assay. The results were expressed as (Control – Sample) A405. To determine the pH activity profile of the bladder tumor-derived hyaluronidase, the HA-coated wells were incubated with aliquots of culture CM or the partially purified preparation of urinary hyaluronidase in formate-NaCl buffer at different pH values (2.0–8.5). The results are expressed as (Control-Sample) A405.

Substrate(HA)-Gel Assay. Urine specimens, partially purified preparations of hyaluronidase, and CM from various transitional cell carcinoma lines were separated on an 8.5% SDS-polyacrylamide gel containing 0.085 mg/ml human umbilical cord HA (ICN Biochemicals). After SDS-PAGE, the proteins separated in the gel were renatured by incubating the gel in 3% Triton X-100. The gel was then incubated in the hyaluronidase assay buffer at 37°C for 16–18 h to allow HA digestion. The presence of hyaluronidase in the gel was visualized by using an Alcian Blue and Coomassie Blue double-staining procedure, as described previously (22, 23).

Partial Purification of Hyaluronidase Activity. Pooled urine (300 ml) from G2 and G3 bladder cancer patients was dialyzed against 0.1 mM ammonium bicarbonate buffer (pH 8.5), containing 1 mM benzamidine-HCl at 4°C for 16 h. After incubation, the urine was centrifuged and incubated with PBA60 phenyl boronate gel (Millipore Corp., Bredford, MA) at 4°C for 2 h. After incubation, the gel was packed in a column, and the column was washed sequentially, with 300 ml each of buffer A [50 mM Hepes, 10 mM EDTA, and 1 mM benzamidine-HCl (pH 8.5)] and buffer B [50 mM Hepes, 120 mM NaCl, and 1 mm benzamidine-HCl (pH 8.5)]. The hyaluronidase activity was eluted from the column using buffer C [20 mM Tris-HCl, 120 mM NaCl, 1 mm benzamidine-HCl, and 0.003% Brij 35 (pH 8.5)]. The fractions containing hyaluronidase activity were pooled, concentrated, buffer-exchanged with 10 mM sodium phosphate (pH 7.4), 25 mM NaCl, 1 mM
alkaline phosphatase-developing system. Anti-HYAL1 peptide IgG at 4°C for 16 h. The blots were developed using an alkaline phosphatase system. The purity of the preparation was analyzed by 8.5% SDS-PAGE followed by silver staining.

**Immunoblot Analysis.** Bladder cancer cell CM, various urine specimens, partially purified hyaluronidase preparations were separated by 8.5% SDS-PAGE under nonreducing or reducing conditions, and then blotted on to a polyvinylidene difluoride membrane. The blots were probed with 5 μg/ml of anti-HYAL1 peptide IgG. As shown above, these cells secrete little hyaluronidase activity in their CM (Figs. 1 and 2). However, the CM of invasive bladder cancer cell lines correlates with the secretion of hyaluronidase activity by these cells.

**RESULTS**

**Analysis of Hyaluronidase Activity in Bladder Cancer Cell CM.** To investigate whether bladder cancer cells secrete hyaluronidase, we measured hyaluronidase activity in the serum-free CM of 11 human transitional cell carcinoma-of-the-bladder cell lines using an ELISA-like assay. As shown in Fig. 1, the non invasive lines such as RT4 (0.23 ± 0.13 milliunit/mg), T24 (0.36 ± 0.14 milliunit/mg), J82 (0.44 ± 0.15 milliunit/mg), HT 5637 (0.38 ± 0.09 milliunit/mg), and 253J-Parent (1.7 ± 0.7 milliunits/mg) secrete very little hyaluronidase activity in their CM. However, the invasive lines such as UMUC-3 (6.1 ± 1.05 milliunits/mg), TCCSUP (6.9 ± 0.4 milliunits/mg), HT1197 (9.2 ± 1.5 milliunits/mg), 253J-Lung (20.1 ± 1.2 milliunits/mg), 253J-BV (16.4 ± 1.4 milliunits/mg), and HT1376 (32 ± 2.4 milliunits/mg) secrete 10- to 100-fold elevated levels of hyaluronidase activity (Fig. 1).

We next used a substrate (HA)-gel technique to determine the relative molecular mass of the hyaluronidase expressed in bladder cancer cell lines. Serum-free CM (4.4 μg protein) from 11 bladder cancer lines were analyzed on an 8.5% substrate (HA)-gel. As shown in Fig. 2, A and B, the cell lines RT4, T24, J82, HT5637, and 253J-Parent, which secrete little hyaluronidase activity, did not show the presence of any hyaluronidase protein in their CM. However, a band of Mr ~60,000 was observed in the CM of TCCSUP, 253J-Lung, 253J-BV, HT1376, UMUC-3, and HT1197 (Fig. 2, A and B). This suggested that a single hyaluronidase species is present in the CM of invasive bladder cancer cell lines.

**Identification of Hyaluronidase Expressed in Bladder Cancer Cell Lines.** To investigate which hyaluronidase gene is expressed in bladder cancer cell lines, we performed RT-PCR analysis on total RNA extracted from 253J-Lung, HT1376, and RT4 cells. For RT-PCR analysis, primers that are specific for PH20 and HYAL1 cDNA sequences were used. As shown in Fig. 3A, both 253J-Lung and HT1376 cell lines, which secrete high levels of hyaluronidase in their CM, amplified a 370-bp DNA product when HYAL1-specific primers were used (Fig. 3A, Lanes 1 and 2). The length of this PCR product was the same as that expected, based on the positions of the left and right primers in the HYAL1 sequence (40). Interestingly, RT4 cells, which secrete little hyaluronidase activity in their CM, showed no PCR amplification product when HYAL1 primers were used (Fig. 3A, Lane 3). None of the three bladder cancer cell lines amplified any cDNA product when primers specific for the PH20 cDNA sequence were used (Fig. 3A, Lanes 4–6).

To confirm that the sequence of the 370-bp PCR-amplified product from 253J-Lung and HT1376 RNA is related to the HYAL1 sequence, the 370 PCR product generated from 253J-Lung and HT1376 cells was cloned and sequenced. As shown in Fig. 3B, the sequence of the 370-bp product matches that of the HYAL1 in all of the positions but one, between nucleotides 208 and 577 (39, 40). These results indicate that an HYAL1 transcript is expressed in bladder cancer cell lines. Furthermore, the expression of HYAL1 mRNA by bladder cancer cell lines correlates with the secretion of hyaluronidase activity by these lines.

**Detection of HYAL1-related Protein in Bladder Cancer Cells.** To examine whether an HYAL1-related protein is present in the CM of bladder cancer cell lines, we generated a rabbit polyclonal IgG against a peptide sequence (HYAL-1 321–338) present in the HYAL1 sequence. As shown above, these cells secrete little hyaluronidase activity in their CM (Figs. 1 and 2). However, the CM of 253J-Lung, 253J-BV, TCCSUP, UMUC-3, HT1197, and HT1376, which contain high levels of hyaluronidase activity, show the presence of a Mr ~60,000 protein, that cross-reacts with the anti-HYAL1
peptide IgG. Interestingly, among these CM, the intensity of the Mr; 60,000 band correlates with the amount of hyaluronidase activity present in each CM. For example, the intensity of the Mr; 60,000 band, detected in the CM of 253J-Lung, 253J-BV, and HT1376 cells, is 3- to 4-fold higher than that detected in the CM of UMUC-3, HT1197, and TCCSUP cells. As shown above, the CM of 253J-Lung, 253J-BV, and HT1376 cells contain 3- to 7-fold more hyaluronidase activity than that present in the CM of UMUC-3, HT1197, and TCCSUP cells.

To further investigate whether the major amount of hyaluronidase activity secreted in the CM of bladder cancer lines is related to HYAL1, we examined whether the hyaluronidase activity present in the CM of bladder cancer cell lines binds to anti-HYAL1 peptide IgG immunoaffinity beads. As shown in Fig. 5, the anti-HYAL1 peptide IgG immunoaffinity beads remove >90% of hyaluronidase activity from the CM. These results demonstrate that the hyaluronidase activity present in the CM of bladder cancer cell lines is related to HYAL1.

Partial Purification and Identification of Hyaluronidase from G2 and G3 Bladder Cancer Patient Urine. We have previously shown the presence of two distinct hyaluronidase species of Mr; 65,000 and 55,000, that are present in the urine of G2 and G3 bladder cancer patients (23). However, we recently observed that only one major hyaluronidase species of Mr; ~60,000 is present in the pooled urine from G2 and G3 bladder cancer patients as well as individual urine specimens. A second band at Mr; ~55,000 is observed, however, it displays less hyaluronidase activity (data not shown). The hyaluronidase activity present in the urine of G2 and G3 bladder cancer patients was partially purified using sequential PBA60 phenyl boronate and hydroxyapatite chromatography steps. As shown in Fig. 6a, a major protein of Mr; ~60,000 is visible in the partially purified preparation of hyaluronidase when analyzed by SDS-PAGE and silver staining. The presence of hyaluronidase activity during sequential purification steps was monitored by the ELISA-like assay (data not shown) and substrate (HA)-gel zymography. As shown in Fig. 6b, pooled urine, proteins eluted from the PBA60 phenyl boronate column, and the flow-through fraction from the hydroxyapatite show the presence of a hyaluronidase activity band of Mr; ~60,000.

We next determined whether an HYAL1-related protein is present in the urine of G2/G3 bladder cancer patients and in the partially purified preparations of urinary hyaluronidase. We performed immu...
Fig. 6. Analysis of hyaluronidase activity in bladder cancer patient urine. A, partial purification of hyaluronidase activity. Pooled urine from G2/G3 bladder cancer patients was chromatographed sequentially on PBA60 phenyl boronate and hydroxyapatite columns, as described in “Materials and Methods.” The proteins eluted from each column were analyzed by 8.5% SDS-PAGE followed by silver staining. Lane 1, pooled dialyzed urine; Lane 2, PBA60 phenyl boronate column-eluted material; Lane 3, flow-through fraction of the hydroxyapatite column. B, analysis of hyaluronidase activity during purification. Aliquots from various steps during purification were analyzed by 8.5% SDS substrate (HA)-gel for detecting the presence of hyaluronidase as described in “Materials and Methods.” Lane 1, pooled dialyzed urine; Lane 2, PBA60 phenyl boronate column-eluted material; Lane 3, flow-through fraction of the hydroxyapatite column. C: Immunoblot analysis of urine specimens and partially purified urinary hyaluronidase using anti-HYAL1 peptide IgG. Urine specimens (40 μg protein) from normal individuals (Lanes 1 and 2), a G1 bladder cancer patient (Lane 3), a patient with a history of G3 bladder cancer but no disease at the time of urine collection (Lane 4), a G2 bladder cancer patient (Lane 5), and a G3 bladder cancer patient (Lane 6) as well as material eluted from the PBA60 phenyl boronate column (Lane 7) and hydroxyapatite-flow through fraction (Lane 8) were subjected to immunoblot analysis using anti-HYAL1 peptide IgG. The immunoblot analysis was carried out as described in “Materials and Methods.”

nobil blot analysis on urine specimens (~40 μg protein) from normal individuals; patients with G1, G2, or G3 bladder cancer; and a patient with a history of G3 bladder cancer but no disease at the time of urine collection. As shown in Fig. 6C, the urine specimens from normal individuals and patients with G1 bladder cancer or a history of bladder cancer do not cross-react with the anti-HYAL1 peptide IgG. However, the urine specimens from G2 and G3 bladder cancer patients show the presence of a Mr ~60,000 protein that cross-reacts with the HYAL1 IgG. Interestingly, a Mr ~60,000 protein that cross-reacts with the anti-HYAL1 peptide IgG copurifies with the hyaluronidase activity during the sequential purification scheme (Fig. 6C). Taken together, these observations show that the urine of G2 and G3 bladder cancer patients contains a Mr ~60,000 HYAL1-related protein that constitutes the major, if not all of the, hyaluronidase activity present in the urine of G2 and G3 bladder cancer patients. Furthermore, this protein can be partially purified using a sequential column chromatography scheme.

**Determination of the pH Activity Profile of Bladder Tumor-derived Hyaluronidase.** The pH profile of hyaluronidase activity present in the CM of 253J-Lung cell line and in the partially purified preparation of hyaluronidase was determined using the ELISA-like assay. As shown in Fig. 7, the partially purified preparation of the G2/G3 bladder cancer patient urinary hyaluronidase shows a pH optimum within the pH range, 4.1–4.3. The enzyme is 80% active at pH 4.5, 30% at pH 4.9 and ~20% at pH 5.0. The pH profile of the hyaluronidase activity present in the CM of 253J-Lung CM is also very similar to that of the urinary hyaluronidase activity and shows the pH optimum within the range pH 4.1–4.3.

**DISCUSSION**

In this study, we identified the type of hyaluronidase that is expressed in bladder cancer. The association of HA, the substrate for hyaluronidase, with cancer is well established (11, 12). HA is known to promote tumor metastasis by enhancing tumor cell migration (17, 18). It also increases the resistance of cancer cells to immune attack (50). In tumor tissues, the elevated levels of HA are synthesized by the activated tumor stroma (11–13). Although, the association of hyaluronidase with tumor biology is relatively new, the published reports from our laboratory and of others show that, indeed, the secretion of hyaluronidase in tumor tissues (or in urine) correlates with tumor grade and the metastatic potential of the tumor (22, 23, 31–36). Thus, hyaluronidase may act as a spread factor in a variety of cancers.
The presence of elevated hyaluronidase levels in tumor tissues seems to be the contribution of tumor epithelial cells. In this study, several invasive/metastatic bladder cancer cell lines were found to secrete hyaluronidase in their CM (Fig. 1). We have previously shown (36) that the primary cultures of tumor epithelial cells derived from G3 bladder tumors secrete 7-fold more hyaluronidase activity than that secreted by the normal bladder epithelial cells. Other types of tumor cells (e.g., prostate, melanoma, and so forth) also secrete hyaluronidase, and, in our previous study, the levels were correlated with the invasive potential of the prostate cancer cells (22, 31, 34).

Although, the concept that tumor cells secrete hyaluronidase is gaining grounds, the type of hyaluronidase secreted by the tumor cells is largely unknown. In this study, using both biochemical and molecular biology approaches, we established that an HYAL1-related hyaluronidase might, in fact, be the bladder tumor-derived hyaluronidase (Figs. 2–4). These results show that an HYAL1-related hyaluronidase may be the bladder tumor-derived hyaluronidase. Our reports resulted here are different from those reported by Liu et al. (31). They observed the expression of PH20 mRNA in several melanoma, glioblastoma, glioma, and colon carcinoma cell lines. In contrast, we did not observe PH20 mRNA expression in any of the three bladder cancer cell lines that we tested in this study (Fig. 3). Victor et al. (35) recently reported that the hyaluronidase activity secreted by several primary tumor cell cultures is not related to PH20. Thus, both HYAL1- and PH20-type hyaluronidases may be expressed in tumor tissues, but the actual type of hyaluronidase expressed may depend on the origin of the tumor tissue.

We have previously shown that the urinary hyaluronidase levels of G2/G3 bladder cancer patients are 3- to 7-fold elevated as compared with those present in the urine of normal individuals, patients with G1 bladder cancer, and patients with a history of bladder cancer (23). In this study, immunoblot analysis using the anti-HYAL1 peptide IgG, showed the presence of a Mr ~60,000 HYAL1-related protein only in the urine of G2 and G3 bladder cancer patients. This suggests that the hyaluronidase activity present in the urine of G2/G3 bladder cancer patients is related to HYAL1. Furthermore, the increased urinary hyaluronidase levels in these bladder cancer patients result from the secretion of high levels of an HYAL1-type hyaluronidase by invasive bladder cancer cells. The tumor cell-derived hyaluronidase is very likely released into the urine while it is being stored in the bladder. It was recently shown that all of the hyaluronidase activity present in normal human urine is also of the HYAL1 type (51). However, the cellular origins of normal urinary and serum hyaluronidases, both encoded by the HYAL1 genes, are unknown (39, 40, 51). It is likely that the anti-HYAL1 peptide IgG failed to detect any HYAL1 in normal urine because it is present in normal urine in low quantity. In a previous study where we measured the urinary levels of 513 individuals, the urinary hyaluronidase activity of normal individuals was found to be 7- to 8-fold less than that present in the urine of G2/G3 bladder cancer patients. Recently, Csöka et al. used 20 liters of normal human urine to purify HYAL1 (39). In the present study, 300 ml of urine from G2/G3 bladder cancer patients was sufficient to partially purify the hyaluronidase and to conduct further analysis (Fig. 6).

The pH activity profile of the bladder tumor-derived hyaluronidase is slightly different from those of the recombinant HYAL1, and the HYAL1 isolated from normal human serum and urine (Fig. 7; Refs. 40, 51). The pH optimum of the bladder tumor-derived hyaluronidase activity is in the range of pH 4.1–4.3. The enzyme is nearly 80% active at pH 4.5. However, the recombinant HYAL1 and the HYAL1 isolated from normal serum and urine have a pH optimum at 3.8, and these are inactive at pH ≥ 4.5 (40, 51). It is possible that the bladder cancer-associated hyaluronidase and that present in normal human serum and urine are different isomers of HYAL1 which differ from each other in posttranslational modifications of the same HYAL1 polypeptide. It has been previously shown that the hyaluronidase secreted by human carcinoma cells differs from the human serum hyaluronidase, with respect to glycosylation and the glycosylation (i.e., N-glycosylation) is necessary for the enzyme activity (34).

A second possibility is that in addition to HYAL1, another type of hyaluronidase also contributes to the total hyaluronidase activity secreted by bladder tumor cells. Because the PH20 message was not detected by RT-PCR analysis in the three bladder cancer cell lines that were tested, the probability of the expression of a known PH20-type hyaluronidase in bladder cancer is low. In addition, the pH activity profiles of partially purified urinary hyaluronidase and the hyaluronidase present in bladder cancer cell CM are very different from those of the known membrane-bound and the soluble isofoms of PH20 (43). Thus, HYAL1 may itself represent a family of closely related isoforms that differ in their pH activity profiles and glycosylation.

The identification of HYAL1 as the major hyaluronidase secreted by bladder tumor cells raises the following question: How accurately can the measurement of urinary hyaluronidase levels predict the presence of G2 and G3 bladder cancer? Because human serum contains high levels of HYAL1, hematuria—caused by any unrelated factors—would falsely indicate the presence of G2/G3 bladder cancer (52). However, we have always normalized the urinary hyaluronidase levels (milliunits/ml) to total protein content (mg/ml), which eliminates the influence of hematuria on the inference obtained from urinary hyaluronidase measurement (hyaluronidase test; Ref. 23).

The secretion of hyaluronidase by tumor cells has been shown to induce tumor angiogenesis in vivo (31). HA is known to cause avascularity (53), and, thus, a tumor-derived hyaluronidase—by digests the tumor-associated HA—may allow the growth of new blood vessels. In addition, certain growth factors that are bound to proteoglycans and HA may be released when a tumor-derived hyaluronidase digests the extracellular matrix, rich in HA. The presence of angiogenic HA fragments in the urine of G2/G3 bladder cancer patients suggests that the bladder tumor-associated HA and the hyaluronidase system may also participate directly in tumor angiogenesis (16, 20, 21, 54). The identification of a bladder tumor-derived hyaluronidase may help suggest new avenues to inhibit critical steps in HA- and hyaluronidase-mediated bladder tumor progression.

ACKNOWLEDGMENTS

We thank Dr. Mark S. Soloway for providing clinical specimens and critically reviewing the manuscript. We gratefully acknowledge the help of Dr. Norman L. Block for critically reviewing the manuscript. We thank Dr. Jung San Huang, Department of Biochemistry and Molecular Biology, St. Louis University, St. Louis, MO for helpful suggestions during the course of this project. We also thank Drs. Bal L. Lokeshwar and Keith Zuker for their help during this project, and Cynthia Soloway for excellent editorial assistance.

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