Tumor-associated Hyaluronic Acid: A New Sensitive and Specific Urine Marker for Bladder Cancer

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ABSTRACT

Hyaluronic acid (HA), a glycosaminoglycan, is known to promote tumor cell adhesion and migration, and its small fragments stimulate angiogenesis. We compared levels of HA in the urine of normal individuals and patients with bladder cancer or other genitourinary conditions, using a sensitive ELISA-like assay. Among the 144 specimens analyzed, the urinary HA levels of bladder cancer patients with G1 (255 ± 41.7 ng/mg), G2 (291.8 ± 68.3 ng/mg) and G3 (428.4 ± 67 ng/mg) tumors are 4–9-fold elevated as compared to those of normal individuals (44.7 ± 6.2 ng/mg) and patients with other genitourinary conditions (69.5 ± 6.8 ng/mg; P < 0.001). Urinary HA measurement by the ELISA-like assay shows a sensitivity of 91.9% and specificity of 92.8% to detect bladder cancer. Therefore, urinary HA measurement is a simple, noninvasive yet highly sensitive and specific method for bladder cancer detection. The increase in urinary HA concentration is a direct correlate of the elevated tumor-associated HA levels, because the HA levels are also elevated (3–5-fold) in bladder tumor tissues (P < 0.001). The profiles of urinary HA species present in the urine of normal individuals and bladder cancer patients are different. Although only the intermediate-size HA species are found in the urine of normal and low-grade bladder cancer patients, the urine of high-grade bladder cancer patients contains both the high molecular mass and the small angiogenic HA fragments. These urinary HA fragments stimulate a mitogenic response (2.4-fold) in primary human microvessel endothelial cells, suggesting that the small HA fragments may regulate tumor angiogenesis by modulating endothelial cell functions.

INTRODUCTION

Carcinoma of the bladder is the most common cancer of the urinary tract, accounting for approximately 53,000 new cases and 11,700 deaths each year in the United States (1). TCCs3 account for the majority (~90%) of the bladder tumors (2–4). Squamous cell carcinomas and adenocarcinomas make up the rest. Bladder tumors, TCCs in particular, are heterogeneous in their ability to progress. For example, low-grade tumors (G1) rarely metastasize, whereas high-grade tumors (G3) have a ~50% chance of progression (5, 6). Another characteristic of bladder tumors is the high rate of recurrence. Hence, the bladder cancer patients are monitored closely following their initial treatment (6, 7). The methods for diagnosis, screening, and the follow-up of bladder cancer can be improved if molecular markers that specifically associate with this carcinoma are identified. These markers can then be used to develop specific diagnostic tests. Because proteoglycans and glycosaminoglycans, the components of the ECM, play an important role in normal bladder physiology (8, 9), some of them may be associated with bladder cancer and can serve as potential markers.

ECM and its degrading enzymes are known to control several steps in both tumor invasion and metastasis (10, 11). For example, certain ECM components and their degrading enzymes regulate tumor cell migration, adhesion, and invasion through basement membrane (11). ECM components also regulate tumor angiogenesis by modulating key endothelial cell functions, such as proliferation, migration, and lumen formation (12). For example, ECM components may sequester potent angiogenic factors, such as basic FGF, which upon release stimulate endothelial cell proliferation and migration (13). In addition, certain ECM components may themselves be angiogenic. For example, HA, and more specifically its small fragments, generated by the hyaluronidase digestion of HA, stimulate angiogenesis (14).

HA is a nonsulfated free glycosaminoglycan made of repeating disaccharide units, D-glucuronic acid, and N-acetyl-D-glucosamine (15). HA is present in body fluids, ECM, and connective tissues and is involved in the regulation of several normal physiological functions (15). HA also plays a role in several pathophysiological conditions, including cancer. For example, HA levels have been shown to be elevated in certain animal tumor models (e.g., rabbit V2 carcinoma; Ref. 16) and human cancers (e.g., lung, Wilms’ tumor, breast, and others; Ref. 16). In tumor tissues, HA expands upon hydration, opening spaces for tumor cell migration (16). Furthermore, tumor cells migrate on HA matrix by interacting through certain cell surface receptors (e.g., CD44; Ref. 17). HA also forms a halo around tumor cells that protects them against immune surveillance (18). More recently, small fragments of HA (~3–25 disaccharide units) have been shown to promote angiogenesis (14, 19). We have shown that an HA fragment of 10–15 disaccharide units (F1 fragment) stimulates proliferation of bovine aortic endothelial cells (20). A similarly sized fragment has also been shown to promote endothelial cell migration and tube formation (21). Because HA has been shown to be associated with tumor biology, we investigated its association with bladder cancer. In the following study, we measured the HA levels in the urine of normal individuals and bladder cancer patients and in the extracts prepared from normal bladder and tumor tissues. We also examined the profile of HA species present in the urine of normal individuals and bladder cancer patients. In addition, we determined whether the HA or HA fragments present in the urine affect the proliferation of human endothelial cells.

MATERIALS AND METHODS

Urine Specimens. Voided (clean-catch) urine specimens were collected from 144 individuals under a protocol approved by the Institutional Review Board of University of Miami. The samples were divided into three groups. Group 1: normal (healthy) age-matched (30–70 years) individuals (n = 25). Group 2: patients with other GU conditions (n = 45), such as BPH (n = 8), prostate cancer (n = 7), kidney stones (n = 5), cystitis (n = 12), urinary tract infections (n = 8), prostatitis (n = 2), epididymitis (n = 1), and renal trauma (n = 2). Group 3: bladder cancer patients with G1 (n = 17, stage Ta), G2 (n = 14, stages Ta–T2), or G3 (n = 43) tumors. The G3 subcategory of bladder cancer patients included 34 individuals with G3 tumors (stages T1-T4) and 9 with CIS. CIS is a subclass of high-grade bladder tumors that are flat and superficial (confined to the urothelium). All specimens were collected and stored at ~20°C until assayed.

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6 The abbreviations used are: TCC, transitional cell carcinoma; HA, hyaluronic acid; ECM, extracellular matrix; HUVEC-L, human lung microvessel endothelial cell; GU, genitourinary; CIS, carcinoma in situ; BPH, benign prostate hyperplasia; EGM, endothelial growth medium; EBM, endothelial basal medium; FGF, fibroblast growth factor.
**Tissue Specimens.** Normal bladder tissues from adults (21-50 years) were obtained from organ donors according to relevant state and federal regulations. Neoplastic bladder tissues were obtained from patients (41-72 years) undergoing cystectomy or transurethral resection of the bladder tumor. Group 1: normal adult bladder tissues (n = 6). Group 2: low-grade TCC (G1 tumor; n = 6). Group 3: high-grade TCC (G2 tumor; n = 2; G3 tumor; n = 6). To evaluate the grade, each tumor specimen was split, and the mirror image segment was fixed in formalin, embedded in paraffin, sectioned, and analyzed histologically.

**Tissue Extracts.** Tissue specimens (~0.5-1 g) were homogenized in 5 mM HEPES buffer, pH 7.2, containing 1 mM benzamidine-HCl. The homogenates were clarified by centrifugation at 40,000 x g for 30 min, and the clear extracts were assayed.

**ELISA-like Assay for HA Level Determination.** The concentration of HA in urine specimens and tissue extracts was determined by an ELISA-like assay described by Fosang et al. (22), with the following modifications. Ninety-six-well microtiter plates coated with human umbilical cord HA (25 μg/ml) were incubated with serial dilutions of urine specimens, tissue extracts, or human umbilical cord HA (Sigma Chemical Co., St. Louis, MO), in PBS + 0.05% Tween 20 (PBS-Tween), and a biotinylated bovine nasal cartilage HA-binding protein (1 μg/ml). Following incubation at room temperature for 16 h, the wells were washed in PBS-Tween. The HA-binding protein bound to these wells was quantitated using an avidin-biotin detection system and 2,2'-azino-bis(3-ethyl-benzthiazolin-6-sulfonic acid) substrate (Vector Laboratories, Burlingame, CA). A standard graph was prepared by plotting absorbance (405 nm) versus human umbilical cord HA concentrations (ng/ml). Using this graph, the HA concentration in each dilution of either the urine specimen or tissue extract was calculated. From several such determinations, the mean HA concentration in each sample was determined and then normalized to urinary protein concentration. The total protein concentration in each clinical sample was determined by Bio-Rad protein detection kit (Bio-Rad Laboratories Inc., Hercules, CA).

**Preparation of HA Fragments.** We have previously described the procedure used to prepare HA fragments (20). Briefly, human umbilical cord HA (~500 mg) was digested with 20,000 units of testicular hyaluronidase (Sigma Chemical Co.) at 37°C for different time intervals. The HA fragments generated were separated on a Sephadex G-50 column (1.5 x 120 cm). Ten-ml fractions were collected and assayed for the uronic acid content (23). The fractions were combined to give three preparations, F1, F2, and F3. The number of reducing ends in each fraction was determined by the Dygert assay (24). Because each linear polysaccharide of HA or its fragment contains a single reducing end, the chain length of each fragment was calculated from the number of reducing ends per mol of uronic acid. The size range of oligosaccharides in each fraction was also determined by incorporating 1H-labeled HA (prepared as described previously; Ref. 25) during HA digestion and analyzing the fragments by gel electrophoresis and fluorography.

**Isolation of HA and HA Fragments from Patient Urine.** Urine specimens from normal subjects (n = 4) and patients with low-grade TCC (G1 tumor; n = 4) or high-grade TCC (G2 tumor; n = 2; G3 tumor; n = 3) were concentrated 10-fold and dialyzed extensively against PBS. Approximately 2 ml of each of the dialyzed specimens (~20 mg protein) were applied to a Sepharose 6 CL-B column (1.5 x 120 cm; Pharmacia, Piscataway, CA) equilibrated with PBS. The column was eluted in PBS at 7 ml/h, and 3.5 ml fractions were collected. The fractions were assayed for HA by the ELISA-like assay as described above. Because the standard globular protein markers and linear polysaccharides, such as HA and HA fragments, have different shapes, the column was calibrated using human umbilical vein HA (~2 x 105 Da) and the HA fragments F1, F2, and F3. Alternatively, to test the effect of HA and HA fragments isolated from urine on endothelial cell proliferation, the specimens were precipitated with trichloroacetic acid (5%, w/v) at 4°C for 4 h. The precipitation step was included to denature and remove any protein growth factors (e.g., basic FGF) present in the urine. The urine specimens treated with trichloroacetic acid were centrifuged; the supernatants were dialyzed against water, lyophilized, resuspended, in PBS and then chromatographed on the Sepharose 6 CL-B column.

**Malignant Assay.** Growing primary cultures of HMVEC-Ls, in their second or third passage, were obtained from Clonetics Corp. (San Diego, CA) and grown on 48-well culture plates in EGM (Clonetics Corp., San Diego, CA). At 80% confluence, the cultures were preincubated in a serum-free and additive (bovine pituitary extract, EGF, and hydrocortisone)-free basal medium (EBM) for 12 h at 37°C. Following preincubation, the cells were incubated for 18 h in fresh EBM containing HA or HA fragments of known lengths or peak HA fractions (1-V) isolated from patient urine. Following incubation, [3H]thymidine (1 μCi/ml) was added to these cultures and the assay was terminated after a 2-h incubation as described previously (26). The results presented are mean ± SD from triplicate determinations.

**RESULTS**

Determination of HA Concentration in Urine Specimens. An ELISA-like assay, involving the use of a biotinylated HA binding protein was used to determine the HA concentration in urine specimens. Because urinary HA levels (ng) were found to be influenced by the hydration status and urine output, these levels were normalized to urinary protein content (mg). The urinary HA levels were normalized to total protein rather than to creatinine because the normalization to protein was found to be less influenced by hematuria, a condition commonly found in bladder cancer patients (3). We compared the urinary HA levels between normal individuals (n = 25) and those with bladder cancer [e.g., G1, G2, and G3 (including CIS); n = 74]. As controls, the enzyme levels of patients with GU conditions other than bladder cancer [e.g., BPH, prostate cancer, kidney stones, bacterial infections, cystitis, prostatitis, renal trauma, and epididymitis; n = 45] were also measured in this study. As shown in Fig. 1A, the distribution of urinary HA levels among normal individuals and patients with other GU conditions is very similar, and the HA levels of most of the individuals included in these two groups are <100 ng/mg. However, the urinary HA levels are uniformly elevated in bladder cancer patients, regardless of the tumor grade (e.g., G1, G2, and G3), and for most patients, these levels are >100 ng/mg (Fig. 1A).

The comparison of the mean urinary HA levels among various groups is shown in Fig. 1B. The mean urinary HA levels among normal individuals (44.7 ± 6.2 ng/mg) and those with other GU conditions (69.5 ± 6.8 ng/mg) do not vary significantly. The statistical analysis of these data by Dunn’s multiple comparison test shows that the differences observed in the mean urinary HA levels among normal individuals and patients with other GU conditions are not
The data on urinary HA levels were further analyzed to determine the specificity and sensitivity of the ELISA-like assay for detecting bladder cancer. As shown in Table 2, the overall specificity of this assay, using 100 ng/mg as a minimum cutoff limit, is 92.8%. At the same cutoff limit, the sensitivity of this assay to detect bladder cancer is 91.9%. The analysis shows that the false positive and the false negative outcomes from this assay are 7.2 and 8.1%, respectively.

Comparison of HA Concentrations in Bladder Tissues. To determine whether the increase in urinary HA levels is due to secretion of tumor-associated HA into the urine, we examined HA levels in the tissue extracts prepared from normal bladder, low-grade TCC (G1 tumor), and high-grade TCC (G2 and G3 tumors) using the ELISA-like assay. As shown in Fig. 2A, the HA levels are elevated in bladder tumor tissues, regardless of the tumor grade, as compared to those in normal bladder tissues. The mean HA levels present in the low-grade TCC tissues (5.8 ± 2.1 μg/mg) and high-grade TCC tissues (9.3 ± 3.3 μg/mg) are 3- and 5-fold higher, respectively, than those present in the normal bladder tissues (1.8 ± 0.4 μg/mg). As shown in Table 3, the differences in tissue HA levels among normal bladder specimens and bladder tumors (low-grade or high-grade TCC) are statistically significant (P < 0.001). However, the differences in the HA levels present in the low-grade and high-grade TCC tissues are not statistically significant (P > 0.05; Table 3). These results show that there is a direct correlation between elevated urinary HA levels and increased tumor-associated HA.

Determination of Urinary HA Profile. It has been shown that small fragments of HA (3-25 disaccharide units) generated by the hyaluronidase digestion of HA, are angiogenic in vivo (14). To determine whether such HA fragments are present in urine, we examined the profiles of HA species that are present in the urine of normal individuals and patients with low-grade or high-grade TCC, using gel-filtration chromatography. The sizes of urinary HA species were determined by calibrating the column with high molecular mass HA (~2 × 10^5 Da) and HA fragments of known lengths [F1 (10–15 disaccharide units), F2 (2–3 disaccharide units), and F3 (~2 disaccharide units); Ref. 20]. The F1 fragment has been shown to modulate various functions of bovine aortic endothelial cells (20, 21). As shown in Fig. 3, the urine of normal individuals contains a small amount of HA, and its size is intermediate between the high molecular mass HA and the F1 fragment. The urine of low-grade TCC patients contains a small amount of high molecular mass HA and a broad second peak of intermediate-size HA (Fig. 3). The second peak appears to contain some amount of F1 fragment (Fig. 3). The HA profile of the high-grade TCC patient urine shows a complicated pattern. The profile consists of two large peaks, corresponding to the high molecular mass HA and the F1 fragment. These two peaks are separated by a peak of the intermediate size HA (Fig. 3). In addition, the high-grade TCC patient urine contains two small HA peaks that correspond approximately to the F2 and F3 HA fragments (Fig. 3). These results show that although HA concentration is increased in all bladder cancer patients, the HA profile is different among the low-grade and high-grade TCC patients.

Effect of HA and HA Fragments on the Proliferation of Human Microvessel Endothelial Cells. Using a [3H]thymidine incorporation assay, we examined the effect of high molecular mass HA and HA fragments (F1, F2, and F3), either generated in vitro or isolated from the urine of high-grade TCC patients, on the proliferation of the primary cultures of HMVEC-Ls. As shown in Fig. 4A, the high molecular mass HA and the F1 fragment induce a mitogenic response in HMVEC-Ls in a dose-dependent manner and cause a maximum increase of 1.5- and 2.3-fold, respectively, at 2 μg/ml concentration. The
The low-grade and high-grade TCCs indicate the groups of individuals with Gl and G2 + G3 tumors, respectively.

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Table 3 Dunn's multiple comparison test for comparing mean HA levels among normal and bladder tumor tissue extracts

The data presented in Fig. 2 were analyzed by the Dunn's multiple comparison test. The low-grade and high-grade TCCs indicate the groups of individuals with Gl and G2 + G3 tumors, respectively.

DISCUSSION

Heterogeneity in progression and frequent recurrence demand early detection and close follow-up of bladder cancer patients (3). The data presented here show that urinary HA levels are significantly elevated (4—9-fold; Fig. 1) in all bladder cancer patients and suggest the usefulness of urinary HA levels as a marker for bladder cancer.

The increase in the urinary HA levels of bladder cancer patients appears to be a direct correlate of the high concentrations of HA in tumor tissues (Fig. 2). The tumor-associated HA is most likely secreted into the urine because the tumor is directly in contact with urine. At present it is unknown which cell types (e.g., tumor epithelial cells, stromal fibroblasts, or endothelial cells) in the bladder tumor are responsible for the synthesis of elevated HA. However, the increase in tumor-associated HA may be a result of tumor-stroma interaction. For example, Knudson et al. (27) have shown that an invasive bladder carcinoma line HCV-29T induces cultured fibroblasts to synthesize HA.

The profiles of HA species present in the urine of normal individuals and bladder cancer patients show interesting differences. Normal urine contains the intermediate-size HA, whereas the urine of high-grade TCC patients contains both the high molecular mass HA (~2 x 10^6 Da) and HA fragments, including those that are shown to be angiogenic (e.g., 10—15 disaccharide units, 6—8 kDa). Such a pattern has not been observed previously (28, 29). The urine of Wilms' tumor patients and normal children, for example, has been shown to contain the intermediate-size HA (10—100 kDa; Ref. 28). However, the sera of children with certain other rare renal tumors contain only the angiogenic HA fragments (29). Therefore, it is possible that the sizes of HA species and their relative distribution may be different in tumors of different origins (28, 29).

The pattern of HA species present in urine samples from patients with varying grades of bladder cancer may also be different. This is because our data show that the urine of low-grade TCC patients contains the intermediate-size HA (including some angiogenic HA fragments), whereas the urine of high-grade TCC patients contains both the high molecular mass HA and HA fragments of different lengths (Fig. 3). Currently, we are investigating the usefulness of a urinary HA profile to monitor prognosis.

The presence of both the high molecular mass HA and HA fragments in the urine of high-grade TCC patients suggests that the high molecular mass HA is synthesized in tumor tissues and is then degraded by a
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bladder tumor-associated hyaluronidase to generate small fragments. Our results presented in the accompanying paper (30) appear to confirm this notion. Thus, in high-grade tumor tissues, the high molecular mass HA may support tumor invasion/metastasis by promoting tumor cell migration and adhesion and offering the cells protection against immune surveillance. Also, the small HA fragments may stimulate neovascularization by promoting endothelial cell functions (16).

The current mode of detecting bladder cancer relies on the clinical presentation of patients with hematuria or other symptoms (3). The standard work-up for these patients involves cystoscopy, which is invasive and expensive (3). Urine cytology is a useful adjunct to cystoscopy, but its sensitivity is low in detecting low-grade tumors (31). A few other markers, such as DNA ploidy, p53 mutations, microsatellite DNA, basic FGF levels, autocrine motility factor receptor, and so forth, have been shown to be associated with bladder cancer (32-37). However, most of these have not yet been used clinically as diagnostic markers.

Currently, some noninvasive urine tests (e.g., home hematuria screening, BTA, and NMP22) have become available for bladder cancer testing (38–40). The home hematuria screening test is sensitive but not specific because several benign GU conditions also cause hematuria (38, 41). The BTA test has been shown in a multicenter trial to have a sensitivity of 40–50% to detect recurrent bladder tumors (39). In another multicenter study involving 90 patients, Soloway et al. (40) have shown that the NMP22 test has an overall sensitivity of 70% to detect bladder tumor recurrence. In our study of 144 specimens, the measurement of urinary HA levels by the ELISA-like assay shows a sensitivity of 91.9% and a specificity of 92.8% to detect bladder cancer regardless of its grade and stage.

The measurement of urinary HA levels is technically simple because it is an ELISA-like assay. For HA detection, this assay requires only an HA-binding protein, which can be purified in large quantities using a well-established procedure (42). The ELISA-like assay for the measurement of urinary HA levels is a simple, noninvasive yet highly sensitive and specific test that may be used clinically for bladder cancer detection.

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