Hyaluronic Acid Synthase-1 Expression Regulates Bladder Cancer Growth, Invasion, and Angiogenesis through CD44

Roozbeh Golshani,1 Luis Lopez,2 Veronica Estrella,2 Mario Kramer,2 Naoko Iida,2 and Vinata B. Lokeshwar1,2,3

Departments of 1Cell Biology and Anatomy and 2Urology and 3Sylvester Comprehensive Cancer Center, University of Miami Miller School of Medicine, Miami, Florida

Abstract
Hyaluronic acid (HA) promotes tumor metastasis and is an accurate diagnostic marker for bladder cancer. HA is synthesized by HA synthases HAS1, HAS2, or HAS3. We have previously shown that HAS1 expression in tumor tissues is a predictor of bladder cancer recurrence and treatment failure. In this study, we stably transfected HT1376 bladder cancer cells with HAS1-sense (HAS1-S), HAS1-antisense (HAS1-AS), or vector cDNA constructs. Whereas HAS1-S transfectants produced ~1.7-fold more HA than vector transfectants, HA production was reduced by ~70% in HAS1-AS transfectants. HAS1-AS transfectants grew 5-fold slower and were ~60% less invasive than vector and HAS1-S transfectants. HAS1-AS transfectants were blocked in G2-M phase of the cell cycle due to down-regulation of cyclin B1, cdc25c, and cyclin-dependent kinase 1 levels. These transfectants were also 5- to 10-fold more apoptotic due to the activation of the Fas-Fas ligand-mediated extrinsic pathway. HAS1-AS transfectants showed a ~4-fold decrease in ErbB2 phosphorylation and down-regulation of CD44 variant isoforms (CD44-v3, CD44-v6, and CD44-E) both at the protein and mRNA levels. However, no decrease in RHAMM levels was observed. The decrease in CD44-v mRNA levels was not due to increased mRNA degradation. Whereas CD44 small interfering RNA (siRNA) transfection decreased cell growth and induced apoptosis in HT1376 cells, HA addition modestly increased CD44 expression and cell growth in HAS1-AS transfectants, which could be blocked by CD44 siRNA. In xenograft studies, HAS1-AS tumors grew 3- to 5-fold slower and had ~4-fold lower microvessel density. These results show that HAS1 regulates bladder cancer growth and progression by modulating HA synthesis and HA receptor levels. [Cancer Res 2008;68(2):483–91]

Introduction
Hyaluronic acid (HA) and its degrading enzyme, hyaluronidase (HAase), are intrinsically involved in tumor growth and metastasis. HA is a nonsulfated glycosaminoglycan made up of repeating disaccharide units D-glucuronic acid and N-acetyl-D-glucosamine (1). HAase is an endoglycosidase that breaks HA into fragments, some of which are angiogenic (2). HA levels are elevated in many carcinomas (3–10). We have shown that HA levels are elevated in the urine of bladder cancer patients and, together with urinary HAase levels, serve as an accurate diagnostic marker (11–13). Urinary HA and HAase levels correlate with levels in tissues (14). We have also shown that tumor-derived HAase, HYAL1, either alone or together with HA, serves as an independent prognostic indicator for prostate cancer progression (15, 16).

HA regulates cell adhesion, migration, and proliferation by interacting with receptors such as CD44 and RHAMM (17–19). It has been shown that pericellular HA produced by tumor cells binds CD44 and induces a lipid raft-associated, signaling complex containing phosphorylated ErbB2 (p-ErbB2), CD44, ezrin, phosphoinositide 3-kinase, and chaperone molecules Hsp90 and cdc37. This complex is apparently necessary for promoting survival activities of tumor cells (20, 21). Moreover, endogenous HA produced by breast cancer cells induces a complex between CD44 and RHAMM, which then recruits extracellular signal-regulated kinase 1/2 in the complex and stimulates its kinase activity (22). HA may also aid tumor cells in overcoming the contact inhibition of growth and avoiding immune surveillance (23, 24).

In tumor tissues, elevated HA levels are contributed by both the tumor-associated stroma and tumor cells (3–16). HA synthesis occurs at the plasma membrane by a transmembrane HA synthase (HAS). There are three HAS isoforms, HAS1, HAS2, and HAS3, which synthesize HA at different catalytic rates, resulting in different size polymers (25–27). For example, HAS3 is catalytically more active and synthesizes smaller HA polymers (1 × 105 to 1 × 106 Da) than HAS1 and HAS2 (2 × 105 to 2 × 106 Da).

It has been shown that HAS1 and HAS2 expression increases after the malignant transformation of a rat fibroblast line, by viral oncogenes, and is involved in promoting tumor growth (25). Silencing of HAS2 expression decreases cell growth due to cell cycle arrest and inhibits cell migration (28). Coexpression of HAS2 with HYAL1 significantly increases tumor growth than either enzyme alone (29). In xenograft models, HAS2 and HAS3 overexpression has been shown to increase tumor growth and invasion (28, 30–32).

At the present time, not much information is available on HAS1 function in tumor cell growth, invasion, and angiogenesis either in vitro or in vivo. Nonetheless, when compared with HAS2 and HAS3, HAS1 expression is elevated in multiple myeloma patients (33, 34). The expression of a HAS1 splice variant (HAS1-vb) has been shown to correlate with survival in multiple myeloma patients (35). HAS1 expression in tumor tissues also serves as a prognostic indicator in many carcinomas (6, 34–37). We have shown that, in bladder cancer, HAS1 mRNA and protein expression is elevated in bladder tumor cells and tissues and correlates with a positive inference on the HA urine test. More importantly, HAS1 expression correlated with bladder tumor recurrence and response to treatment (38).

We recently showed that HYAL1 expression is a molecular determinant of bladder and prostate cancer growth, invasion, and angiogenesis (39, 40) and that the HA-HAase system seems to promote tumor growth and progression. Therefore, in this study, we investigated HAS1 functions in bladder cancer.
Materials and Methods

Generation of HAS1 transfectants. HAS1-sense (HAS1-S), HAS1-antisense (HAS1-AS), and the vector transfectants were generated by transfecting HT1376 bladder cancer cell line with HAS1-S, HAS1-AS, and vector cDNA constructs as described previously (38). The transfectants were selected and maintained in growth medium (RPMI 1640 + 10% fetal bovine serum + gentamicin) containing 3.5 μg/mL blasticidin. 253J-Lung bladder cancer cell line was kindly provided by Dr. Colin Dinney (The University of Texas M. D. Anderson Cancer Center, Houston, TX).

Analysis of HA, HAase activity, and HAS1 expression. HA and HAase levels present in the serum-free conditioned medium (RPMI 1640 + insulin, transferrin and selenium supplement + gentamicin) of transfectants were measured by the HA and HAase ELISA-like assays (13) and normalized to cell number. Cell lysates of HT1376 transfectants were subjected to immunoblot analysis using a rabbit polyclonal anti-HAS1 IgG or an anti-v5 monoclonal antibody (Invitrogen) as described previously (38, 41).

Cell proliferation, cell cycle, and apoptosis assays. In cell proliferation assay, transfectants plated on 24-well plates in growth medium + basicfined were counted every 24 h for 96 h. In actively growing cultures, cell cycle phase distribution was estimated by propidium iodide staining of DNA followed by flow cytometry (39–41). For the apoptosis assay, 96-h cultures of transfectants were analyzed using the Cell Death ELISA Plus kit (Roche Diagnostics). In some cell growth and apoptosis experiments, human umbilical cord HA (0–50 μg/mL; MBL) was added.

Matrigel invasion assay. Transfectants (3 × 10^5) cells were plated in the upper chamber of a Matrigel-coated Transwell (12-μm pore) plate in serum-free medium. The bottom chamber contained the growth medium. After 48 h, invasion of cells in the bottom chamber was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (39–41).

Immunoblot analysis. Cell lysates (4 × 10^6) cells were immunoblotted using anti-cyclin B1, anti-cyclin-dependent kinase 1 (cdk1), anti-cdc25c, anti-chk1, anti-wee1, anti–active caspase-3, anti–cleaved poly(ADP-ribose) polymerase (PARP; Asp214), anti–active caspase-9, anti-Fas, anti–Fas ligand anti–cyclin B1, anti–cyclin-dependent kinase 1 (cdk1), anti-cdc25c, anti-chk1, anti-wee1, anti–active caspase-3, anti–cleaved poly(ADP-ribose) polymerase (PARP; Asp214), anti–active caspase-9, anti-Fas, anti–Fas ligand (Fas-L), anti–Fas-associated death domain (FADD), anti–caspase-8, and anti-BID antibodies as described previously (41). Cell lysates were also immunoblotted using anti-RHAMM (Novocastra), pan-CDC4 (Santa Cruz Biotechnology), CD44 variant CD44-v3 (Alexis Biochemicals), CD44-v6, and CD44-v10 (Chemicon), and c-ErbB2 and p-ErbB2 (Lab Vision) mouse monoclonal antibodies.

Semiquantitative reverse transcription-PCR and cloning. CD44 variant isoforms expressed in HT1376 transfectants were detected by reverse transcription-PCR (RT-PCR) and TOPO-TA cloning (Invitrogen) as described previously (42, 43) using the primers that map in exon 5 (forward primer) and exon 15 (reverse complementary primer); CD44, GCATCCAG-GAGGTATAC (forward) and ATCCATGAAGATGTGATTGGGC (reverse).

Real-time RT-PCR. Real-time RT-PCR to measure HAS2 and HAS3 mRNA levels was carried out as described previously (38) using the following primers: HAS2, 5′-GAGACAGGTACCGTTCTTC-3′ (forward), 5′-TCC-CTATTCTATGACGAA-3′ (reverse), and FAM 5′-ATGCCTGCTATCAAGGATCCACAG-3′ BHQ1 (probe); HAS3, 5′-CCCTCTCTCCCTCCTTATATTGCT-3′ (forward), 5′-AATGCCCCACAGGTGAGGA-3′ (reverse), and FAM 5′-AAGGAGCCTAAGGTTACACCAAC-3′ BHQ1 (probe). For the measurement of CD44 standard and CD44 variant mRNA levels, real-time PCR was performed using the iQ SYBRGreen Supermix (Bio-Rad) and the following primers: CD44 standard–specific primers, 5′-CTGCTACCCCATCCACAG-3′ (forward) and 5′-TGTTCTCTGCTCTCTGTTGAC-3′ (reverse). For normalization, β-actin real-time PCR was carried out on the same plates (38). Normalized mRNA levels for each transcript were calculated as (1/2^−ΔCt (test mRNA) − ΔCt (β-actin mRNA)).

Small interfering RNA transfection. Small interfering RNA (siRNA) transfection using the ON-TARGETplus SMARTpool siRNA against HAS1, CD44, or Fas and ON-TARGETplus siCONTROL Nontargeting siRNA was carried out as described previously (41).

Pericellular matrix (coat) assay. HA-dependent pericellular matrices (coats) around HAS1 transfectants were visualized using a particle exclusion assay involving formaldehyde-fixed human erythrocytes as described before (40). Results were expressed as % cells with pericellular matrix ± SD. Differences among transfectants with respect to pericellular matrix were determined using Tukey-Kramer multiple comparison test.

CD44 and RHAMM cell surface localization by flow cytometry. Cell surface labeling of HT1376 and 253J-Lung cells for CD44 (all isoforms) and RHAMM was carried out using mouse anti-pan-CD44 and mouse anti–CD168 (i.e., RHAMM) antibodies as described before (42).

Tumor xenografts. Transfectants (2 × 10^6) cells were s.c. implanted on the dorsal flank of 5-week-old mice (five animals per clone). Time for the tumors to become palpable was noted. Tumor size was measured twice weekly and tumor volume was calculated by approximating the tumor to an ellipsoid (39–41). At necropsy, tumors were weighed and Tukey-Kramer multiple comparison test was used to compare the differences in tumor growth rate and tumor weight.

Localization of HYAL1-v1 and microvessel density determination. HAS1, HA, and microvessels were localized in tumor specimens by immunohistochemistry using HAS1 IgG (1:1,000 dilution), HA (1 μg/mL), and a rat anti-mouse CD34 IgG (3.1 μg/mL) as described previously (39–41). Microvessel density (MVD) was determined as described previously (39–41).

Results

Analysis of HAS1 and HA expression in HT1376 transfectants. We chose HT1376 cells as a model to test HAS1 functions because real-time RT-PCR showed that in HT1376 cells HAS1 expression was the highest (normalized HAS1 mRNA levels: 5.4 ± 0.8) followed by HAS3 (1.59 ± 0.31) and HAS2 (0.81 ± 0.32). We analyzed 25 to 30 stable clones of each transfectant type (i.e., vector, HAS1-S, and HAS1-AS) for HA production and HAS1 expression. The mean HA levels (ng/10^5 cells) in vector (103 ± 15.6) and HAS1-S (156 ± 20) clones were three to five times higher than in HAS1-AS (38.7 ± 14.3) clones. The data on two (vector and HAS1-S) or three (HAS1-AS) representative clones are shown here. As shown in Fig. 1A-a, HAS1-S transfectants produce 1.6-fold more HA than vector transfectants and there is 70% reduction in the amount of HA produced by HAS1-AS transfectants. HAS1 immuno- blotting reveals that HAS1 expression in HAS1-S and HAS1-AS transfectants mirrors HA production by these cells (Fig. 1A-b). The presence of v5 epitope-tagged protein only in HAS1-S transfectants confirms that the increased HAS1 expression in HAS1-S transfectants is due to the expression of recombinant HAS1-v5 fusion protein.

In a breast cancer cell model, silencing of HAS2 expression led to increased expression of HAS1 and HYAL1 (28). However, real-time RT-PCR analyses showed that HAS2 and HAS3 expression was not significantly different (P > 0.05) among vector (0.41 ± 0.19; 2.2 ± 0.26), HAS1-S (0.37 ± 0.18; 1.2 ± 0.21), and HAS1-AS (0.62 ± 0.33; 1.1 ± 0.3) transfectants. HYAL1 expression and HAase activity were also not significantly different among various HAS1 transfectants (data not shown).

Effect of HAS1 expression on cell proliferation, cell cycle, and apoptosis. The growth rate of vector and HAS1-S transfectants is comparable (doubling time, ~26–28 h; Fig. 1B-a). However, the HAS1-S transfectants grow 4- to 5-fold slower than vector clones (doubling time, ~120 h). Similar growth kinetics were obtained for the ~25 clones examined in each category, and this measurement was done in conjunction with HA level measurement (data not shown). To confirm that the observed decrease in cell proliferation among HAS1-AS transfectants was due to decreased HA production, we examined whether the addition of exogenous HA could rescue HAS1-AS
transfectants. As shown in Fig. 1B-b, addition of HA modestly increases the growth of HAS1-AS transfectant (clone 2) in a dose-dependent manner.

Cell cycle analysis shows that the decreased growth rate of HAS1-AS transfectants is at least partially due to cell cycle arrest in the G2-M phase. As shown in Fig. 1B-c, when compared with vector and HAS1-S transfectants, there is a ~300% increase in the number of HAS1-AS transfectants in the G2-M phase of the cell cycle, with a corresponding decrease in the S phase ($P < 0.001$, Tukey test). Because HA is involved in cell adhesion, we determined whether a decrease in HA production causes HAS1-AS cells to undergo apoptosis due to perturbation in cell adhesion.

As shown in Fig. 1C-a, HAS1-AS transfectants are 5- to 10-fold more apoptotic when compared with vector and HAS1-S clones ($P < 0.001$, Tukey test). Addition of exogenous HA caused a dose-dependent but modest decrease in the apoptotic activity of HAS1-AS cells (maximum decrease, ~45%; $P < 0.05$; Fig. 1C-b).

We also down-regulated HAS1 expression in 253J-Lung bladder cancer cells by transiently transfecting them with HAS1 siRNA. siRNA transfection down-regulated HAS1 expression by >80% (Fig. 1D-a). Down-regulation of HAS1 expression caused a 3.5-fold decrease in cell growth and a 3-fold increase in apoptosis (Fig. 1D-b). Therefore, the effect of HAS1 depletion on cell growth and apoptosis is not a peculiarity of the HT1376 cell line.

Figure 1. Examination of HA production, HAS1 expression, and growth characteristics of HAS1 transfectants. A-a, measurement of HA levels by HA ELISA-like assay. Columns, mean of three separate experiments; bars, SE. A-b, analysis of HAS1 expression. Cell lysates from the transfectants were subjected to anti-HAS, anti-v5, or anti-actin immunoblotting. GFP, green fluorescent protein. B-a, determination of the proliferation rate of HT1376 transfectants. Points, mean; bars, SD. B-b, effect of HA on the proliferation of HAS1-AS transfectant. Columns, mean; bars, SD. B-c, cell cycle analysis of HT1376 transfectants. The bar graphs (i-iii) show the percentage of cells in G0-G1, S, and G2-M phases of the cell cycle. Columns, mean; bars, SD. C-a, apoptotic activity measured using the Cell Death ELISA Plus assay. Columns, mean; bars, SD. C-b, effect of HA on the apoptosis of HAS1-AS cells. D-a, immunoblot analysis of HAS1 in 253J-Lung cells transfected with control or CD44 siRNA. D-b, analysis of cell growth and apoptosis in 253J-Lung cells following control and CD44 siRNA transfection. Columns, mean; bars, SD.
Next, we examined the expression of G2-M regulators (i.e., cdc25c, cdk1, cyclin B1, chk1, and wee1) in various transfectants by immunoblotting. As shown in Fig. 2A, there is a -2-fold decrease in cyclin B1, cdk1, and cdc25c expression in HAS1 transfectants when compared with the vector and HAS1-S transfectants. However, no change in the expression of negative regulators of the G2-M phase (i.e., wee1 and chk1) was observed in any transfectants. These results show that HAS1-AS transfectants are arrested in the cell cycle due to a down-regulation of some of the positive regulators of the G2-M phase.

Because HAS1-AS transfectants were highly apoptotic, we examined PARP cleavage and caspase-3 and caspase-9 activation. As shown in Fig. 2B, cleaved PARP and activated caspase-3 and caspase-9 are detected in HAS1-AS transfectants but not in vector and HAS1-S transfectants. Because HA production is severely reduced in HAS1-AS transfectants, we reasoned that the cells may be undergoing apoptosis due to loss of adhesion and may involve the receptor-mediated (Fas/Fas-L) or the extrinsic pathway. This pathway involves the formation of the death-inducing signaling complex and FADD-mediated activation of caspase-8, which in turn causes BID cleavage and caspase-3 activation, leading to the abnormal cleavage of PARP (44).

As shown in Fig. 2B, caspase-8 activation (i.e., cleaved caspase-8), BID cleavage, up-regulation of FADD levels, and Fas expression are observed in all HAS1-AS clones when compared with vector and HAS1-S clones. There is also a small increase in Fas-L expression of HAS1-AS transfectants.

We next examined whether blocking Fas expression by Fas siRNA will reduce apoptosis in HAS1-AS transfectants (41). As shown in Fig. 2C, transient transfection of Fas siRNA decreases apoptosis in HAS1-AS transfectants by -2-fold. Immunoblot analysis was used to confirm that Fas siRNA decreased Fas expression in these HAS1-AS clones (data not shown). These results show that blocking HAS1 expression most likely induces apoptosis via the extrinsic pathway.

**Effect of HAS1 expression on HA-dependent pericellular matrix formation.** We tested the presence of pericellular matrices around HAS1 transfectants using the particle exclusion assay (40, 44). As shown in Fig. 3A, vector and HAS1-AS clones do not exhibit pericellular matrices, as the erythrocytes closely abut the surface of each cell and in some cases cover the cells. Figure 3B shows that the percent of cells with pericellular membrane in vector and HAS1-AS transfectants is similar. The lack of pericellular HA matrix around vector clones is likely because HT1376 cells express high levels of HYAL1 (40). Contrarily, HAS1-S cells exhibit a pericellular matrix, as the erythrocytes do not abut the cell membrane. Figure 3B shows that there is a 5-fold increase in the number of cells with pericellular matrix in HAS1-S cells (P < 0.001). However, not all HAS1-S cells have the pericellular coat. This is because HAS1-S transfectants also express HYAL1 at levels similar to those produced by vector and HAS1-AS transfectants, and this should result in pericellular HA degradation.

**Effect of HAS1 expression on invasion.** The invasive activity of vector transfectant clones (31.2 ± 3.4%) was normalized as 100%. As shown in Fig. 3C, HAS1-AS transfectants are 50% to 90% less invasive than the vector transfectants. Contrarily, HAS1-S transfectants are 33% to 40% more invasive than vector transfectants. Incorporation of HA in Matrigel did not influence the invasive
properties of various HT1376 transfectants. Therefore, blocking HAS1 expression, and thereby blocking HA production, significantly reduces the invasive activity of bladder cancer cells.

Effect of HAS1 on HA receptor expression. It has been reported that, in Hs5787 breast cancer cells, blocking HAS2 expression causes a slight decrease in CD44 protein levels (28). We therefore examined the expression of HA receptors CD44 and RHAMM in HAS1 transfectants. As shown in Fig. 4A-a, anti-pan-CD44 immunoblotting reveals high expression of three high molecular mass (\(~150–220\) kDa) proteins and somewhat lower expression of \(~90\)-kDa CD44 standard protein in vector and HAS1-S transfectants. However, very little CD44-related expression is observed in HAS1-AS transfectants. Immunoblotting using CD44 variant–specific antibodies shows that the three high molecular mass CD44-related proteins are variant isoforms, CD44-v3, CD44-v6, and CD44E (contains variant exons 8–10), respectively, and the expression of each of these isoforms was significantly reduced in HAS1-AS transfectants (Fig. 4A-b). In contrast, RHAMM expression is not decreased in HAS1-AS transfectants (Fig. 4A-c).

CD44, but not RHAMM, down-regulation was also observed in HAS1 siRNA-treated 253J-Lung cells (data not shown). It has been shown that the cell surface HA-CD44 interaction induces a complex formation between CD44 and ErbB2, leading to ErbB2 activation (20, 21). We therefore examined the levels of p-ErbB2 and total ErbB2 in HAS1 transfectants. As shown in Fig. 4A-d, there is over 4-fold decrease in p-ErbB2 levels in HAS1-AS transfectants when compared with vector and HAS1-S transfectants, with no change in total ErbB2 levels.

In contrast to CD44, RHAMM lacks a transmembrane domain, and therefore, its expression could be intracellular and extracellular. In bladder tumor tissues, RHAMM expression seems to be intracellular (45). Flow cytometry analyses show low cell surface expression of RHAMM but high CD44 expression in HT1376 cells (median peak: anti-RHAMM, 2.22; anti-CD44, 76.8; control IgG, 0.51; Fig. 4B-a). In 253J-Lung cells, there is little RHAMM expression on the cell surface (median peak: anti-RHAMM, 1.03; anti-CD44, 111.5; control IgG, 0.51; Fig. 4B-b).

Next, we down-regulated CD44 expression in HT1376 cells by siRNA (Fig. 4C-a). Unlike the effect of HAS1 on CD44 expression, CD44 down-regulation does not have any effect on HAS1 expression (Fig. 4C-a). However, it results in a \(~2\)-fold decrease in cell growth and a \(~1.7\)-fold increase in apoptosis, suggesting that CD44 is necessary for growth and inhibition of apoptosis (Fig. 4C-b).

Because HA addition modestly rescued the HAS1-AS phenotype, we determined whether HA addition to HAS1-AS transfectants increases CD44 levels. As shown in Fig. 4D-a, exposure of HAS1-AS cells to HA increases CD44 levels. To determine whether the increased CD44 levels due to HA addition are responsible for the partial rescue of the HAS1-AS phenotype, we down-regulated CD44 in HAS1-AS cells by siRNA in the presence or absence of HA (Fig. 4D-b). As shown in Fig. 4D-c, blocking of CD44 expression further decreases the growth of HAS1-AS transfectants (compare Fig. 1B-a and Fig. 4D-c). Furthermore, although HA addition modestly increases the growth of HAS1-AS cells, it fails to increase the growth of HAS1-AS transfectants blocked in CD44 expression (Fig. 4D-c). Because in these experiments cell growth was severely inhibited, apoptosis experiments could not be performed.

Mechanism of CD44 down-regulation. Semiquantitative RT-PCR to amplify CD44 standard and CD44 variant transcripts in various transfectants followed by DNA sequencing shows that CD44 v3-v10 (CD44-v3), CD44 v6-v10 (CD44-v6), and CD44 v8-v10 (CD44-E) variants and CD44 standard transcripts are expressed in

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**Figure 3.** Examination of pericellular coat and invasion activity. A, pericellular matrices surrounding vector, HAS1-S#1, and HAS1-AS#1. B, quantification of the % cells with pericellular matrices. Columns, mean; bars, SD. C, analysis of invasive activity. Columns, mean of triplicate determinations in two independent experiments; bars, SD.
vector and HAS1-S transfectants (Fig. 5A). However, a weak expression of only CD44-E and CD44 standard isoforms is detected in HAS1-AS (clones 1 and 2) transfectants. Real-time RT-PCR analysis shows that CD44 variant mRNA levels are 4- to 8-fold higher in vector and HAS1-S transfectants than in HAS1-AS transfectants (Fig. 5B-a). There are no significant differences among HAS1-AS, HAS1-S, and vector transfectants with respect to CD44 standard mRNA levels, which are 30- to 80-fold lower than the CD44 variant mRNA levels (Fig. 5B-b).

The decreased CD44 variant mRNA levels in HAS1-AS transfectants are not due to increased rate of mRNA degradation because the rate of CD44 variant mRNA degradation in vector, HAS1-S, and HAS1-AS transfectants, determined in the presence of actinomycin D, is very similar (Fig. 5B-c). HAS1 expression did not affect RHAMM expression, as the real-time RT-PCR showed that RHAMM mRNA levels are similar in vector (2.81 ± 0.95), HAS1-S (3.33 ± 0.83), and HAS1-AS (3.1 ± 0.14) transfectants.

Effect of HAS1 expression on tumor xenografts. As shown in Fig. 6A-a, there was a 3- to 4-fold delay in the generation of palpable s.c. tumors in the animals injected with HAS1-AS transfectants when compared with the animals injected with the vector and HAS1-S clones (palpable tumors: 7–10 days; P < 0.001). The 3- to 7-fold decrease in the weight of HAS1-AS tumors when compared with vector and HAS1-S tumors is also statistically significant (P < 0.001, Tukey-Kramer test; Fig. 6A-b). These results show that blocking HAS1 expression in HT1376 bladder cancer cells decreases tumor growth.

Immunohistochemistry was performed to determine whether tumor cells in vector, HAS1-S, and HAS1-AS tumors retain their phenotype with respect to HAS1 and HA expression. As shown in Fig. 6B, there is high HAS1 and HA expression in tumor cells in the vector and HAS1-S specimen. MVD is higher in vector and HAS1-S tumor specimens when compared with HAS1-AS specimens (Fig. 6B). Quantification of microvessels in various specimens shows that the MVD in vector and HAS1-S specimens is 2- to 6-fold higher than that in HAS1-AS specimens (Fig. 6C). These results show that, by regulating HA expression, HAS1 affects tumor growth and angiogenesis.

Discussion

In this study, we show that, consistent with the role of HA in regulating cell adhesion, migration, and proliferation, modulation
of HAS1 expression significantly affects bladder tumor cell growth and invasion. Unlike HAS2, where increased HAS1 and HYAL1 expression compensates for the loss of HAS2 expression (28), HAS1 expression did not result in a compensatory increase in other HAS or HYAL1 genes. This suggests that regulation of the expression of one HAS gene by other HASs may be restricted to HAS2 and/or it is cell type specific. The slow growth rate of HAS1-AS transfectants is partially due to cell cycle arrest in the G2-M phase and can be modestly rescued by HA addition. It is noteworthy that blocking the expression of HYAL1 in bladder and prostate tumor cells also causes a G2-M arrest, which can be partially rescued by the addition of angiogenic HA fragments (42). This suggests that the tumor cell-associated HA-HAase system is involved in cell cycle progression at the G2-M phase.

The high apoptotic activity in HAS1-AS transfectants can be reversed only partially by the addition of exogenous HA. This indicates that many functions of tumor cell-associated HA cannot be replaced by the addition of soluble HA in the medium. Our results are consistent with the results of Li et al. (28), who also reported that addition of HA in HAS2 siRNA-treated cells partially restored cyclin B expression but did not increase cell proliferation or migration. The slower growth of HAS2/HAS3 antisense transfectants of PC3LN4 prostate cancer cells is also not restored when they are mixed with HA. However, when these cells are mixed with HA and implanted in mice, the tumor growth rate was restored to that of the wild-type PC3LN4 cells (46). It is possible that the architecture of HA matrix surrounding the tumor cells is critical for how HA affects growth, invasion, and migration.

HA is necessary for cell adhesion, and therefore, reduction in HA production by tumor cells may very likely induce anoikis. Our results support this notion, as the down-regulation of HAS1 and reduction in HA synthesis induce Fas and FADD expression, leading to the activation of the extrinsic pathway. It has been shown that stimulation of CD44 decreases Fas expression and CD44 isoforms interfere with Fas signaling (47, 48). Our study shows that decreasing HA production by blocking HAS1 expression down-regulates the expression of CD44 variant isoforms. Therefore, down-regulation of CD44 variant isoforms may very likely be responsible for Fas up-regulation and Fas-mediated apoptosis.

Our study is the first to report that silencing of a HAS gene (i.e., HAS1) significantly down-regulates CD44 variant isoforms at the mRNA and protein levels. At present, it remains unclear whether HAS1 similarly regulates CD44 standard transcript levels because, in HT1376 cells, CD44 standard transcript levels are 30- to 80-fold less than that of the CD44 variant transcripts. Because HAS1 down-regulation does not affect RHAMM expression, it suggests that the effect of HAS1 on CD44 expression is not common for all HA receptors.

The down-regulation of CD44 due to decreased HA synthesis seems to be involved in the decreased growth and increased apoptosis observed in HAS1-AS transfectants. Because CD44 down-regulation alone decreases cell growth and HA addition to HAS1-AS cultures, blocked in CD44 expression, fails to increase the cell growth, these suggest that CD44-HA interaction is important for bladder cancer cell growth and attenuation of apoptosis. It remains to be determined whether the interaction between all or specific CD44 variants and HA is important for bladder tumor cell growth and inhibition of apoptosis.

Our data show that HAS1 is involved in promoting tumor growth, invasion, and angiogenesis. HT1376 cells express both HYAL1 and HA, and in these cells, HYAL1 expression is necessary for tumor growth and progression (39–41). Simpson (29) reported
that concurrent expression of HAS2 and HYAL1 has higher tumorigenic potential than either molecule alone. Overexpression of HAS3 slows the growth of 22Rv1 prostate cancer cells. Furthermore, HAS3-overexpressing tumors are less angiogenic and the growth-inhibitory effects of HAS3 overexpression can be reversed by stable expression of HYAL1 (49). Taken together, these findings show that the tumor-associated HA-HYAL1 system is important in tumor growth and progression. HAS1 expression correlates with invasion, disease progression, tumor recurrence, and poor survival in a variety of carcinomas, including bladder (6, 35–38). The results presented in this study show that HAS1 is a positive regulator of tumor growth progression, and therefore, it may be an accurate diagnostic and prognostic tumor marker and a possible therapeutic target.

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Figure 6. Characterization of the HAS1 transfectant tumors. A, vector and HAS1 transfectants were injected s.c. in athymic mice and tumor volume was measured twice weekly once the tumor became palpable. a, tumor volume. Points, mean; bars, SD. b, tumor weight at 35 d. Columns, mean tumor weight (g); bars, SD. B, localization of HAS1, HA, and microvessels by immunohistochemistry. a, d, and e, vector clone 1; b, e, and h, HAS1-S clone 1; c, f, and j, HAS1-AS clone 1. Magnification, ×400. C, microvessels were counted in 10 random high-power fields representing the highest MVD per tumor specimen. Columns, mean; bars, SD.

References

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Roozbeh Golshani, Luis Lopez, Veronica Estrella, et al.


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