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TARGETING HYALURONIDASE FOR CANCER THERAPY: ANTITUMOR ACTIVITY OF SULFATED HYALURONIC ACID IN PROSTATE CANCER CELLS

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Abstract

The tumor cell-derived hyaluronidase HYAL-1 degrades hyaluronic acid (HA) into pro-angiogenic fragments that support tumor progression. Although HYAL-1 is a critical determinant of tumor progression and a marker for cancer diagnosis and metastasis prediction, it has not been evaluated as a target for cancer therapy. Similarly, sulfated hyaluronic acid (sHA) has not been evaluated for biological activity, although it is a HAase inhibitor. In this study we show that sHA is a potent inhibitor of prostate cancer. sHA blocked the proliferation, motility and invasion of LNCaP, LNCaP-AI, DU145 and LAPC-4 prostate cancer cells, also inducing caspase 8-dependent apoptosis associated with downregulation of Bcl-2 and phospho-Bad. sHA inhibited Akt signaling including androgen receptor (AR) phosphorylation, AR-activity, NFκB activation and VEGF expression. These effects were traced to a blockade in complex formation between PI3K and HA receptors and to a transcriptional downregulation of HA receptors, CD44 and RHAMM, along with PI3K inhibition. Angiogenic HA fragments or overexpression of myristoylated-Akt or HA receptors blunted these effects of sHA, implicating a feedback loop between HA receptors and PI3K/Akt signaling in the mechanism of action. In an animal model, sHA strongly inhibited LNCaP-AI prostate tumor growth without causing weight loss or apparent serum-organ toxicity. Inhibition of tumor growth was accompanied by a significant decrease in tumor angiogenesis and an increase in apoptosis index. Taken together, our findings offer mechanistic insights into the tumor-associated HA-HAase system and a preclinical proof-of-concept of the safety and efficacy of sHA to control prostate cancer growth and progression.

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INTRODUCTION

Tumor-associated hyaluronic acid (HA) and hyaluronidase (HAase) system is known to promote tumor growth and metastasis (1). HA is a non-sulfated glycosaminoglycan that is elevated in tumor tissues (2–7). While HA synthesis is mediated by HA-synthases (HAS1, HAS2 and HAS3), cellular effects of HA are mediated through HA receptors, CD44 and RHAMM. HA-HA receptor interaction generates intracellular signaling which, in turn, promotes tumor growth, metastasis, angiogenesis, trafficking of tumor-associated macrophages and chemoresistance (8–14). Our recent work shows that 4-methylumbelliferone (4-MU), an HA synthesis inhibitor, has antitumor activity in prostate cancer cells (15).

The other component in the tumor-associated HA-HAase system is HYAL-1, a tumor cell-derived HAase. HYAL-1 at levels expressed in tumor cells and tissues promotes tumor growth, invasion and angiogenesis in prostate and bladder cancer models (16,17). Furthermore, HA synthase expression requires HYAL-1 to promote tumor growth and progression (18,19). HYAL-1 expression is potentially an independent predictor of metastasis (3,6,20,21). Although HYAL-1 is a molecular determinant of cancer growth and progression, so far no study has targeted it for cancer therapy.

Sulfated hyaluronic acid (sHA), generated by O-sulfation of HA was shown to inhibit both urinary and testicular HAases, sixty years ago (22). We have shown that sHA polymers such as sHA2.75, in which 75% of HA oligosaccharides contain 3-sulfate groups and 25% contain 2-sulfate groups, are potent inhibitors of HYAL-1 activity (23). sHA2.75 inhibits HAase activity through a mixed inhibition mechanism (i.e., competitive + uncompetitive) and it is 15-fold better as an uncompetitive inhibitor than as a competitive inhibitor. sHA polymers have been shown to affect proliferation of osteoblasts, gene expression in keratinocytes and astrocytes, and adhesion and motility in fibroblasts (24–26). However, antitumor activity of sHA compounds has not been explored. In this study we evaluated the antitumor activity of sHA and the molecular mechanism associated with such activity.

MATERIALS AND METHODS

Cell culture

Cell lines, LNCaP, DU145 and RWPE1 (immortalized normal adult prostate epithelial cells) were obtained from ATCC and cultured in RPMI1640 + 10% fetal bovine serum + gentamicin. LAPC-4 cells kindly provided by Dr. Charles Sawyer, (Memorial Sloan Kettering Cancer Center, New York) were maintained in Iscove's medium with 7.5 % FBS and 1 nM dihydrotestosterone. C4-2 and C4-2B cells were obtained from Viomed Laboratories (Minneapolis, MN) and cultured in T-Medium + 10% FBS + gentamicin. LNCaP-AI (LNAI) is a spontaneously derived androgen-independent subline of LNCaP. LNAI cells express androgen receptor (AR) and prostate-specific antigen (PSA), similar to LNCaP (27). The presence of dihydrotestosterone did not increase the growth of LNAI cells (Supplement Figure 1A). Cell lines were authenticated by Genetica® DNA Laboratories Inc (Cincinnati OH); further in-house characterization is presented in Supplement-Table 2. These authentications were performed during the course of this work.

Reagents

sHA was prepared from tributylamine salt of HA (mol wt. 320–490 kDa; Genzyme Corp. Cambridge, MA; 23). Antibodies, constructs and reagents used in this study are described in the Supplemental Information.

Cell proliferation and apoptosis

Prostate cancer cells ($1.5\text{--}2.0 \times 10^4$ cells/well) cultured in growth medium were exposed to sHA (0–40 $\mu\text{g/ml}$) and counted every 24 hours or after 48 to 72 hours. In some experiments, HA12K or HA 8K (average mol. wt. 12 kDa and 8 kDa respectively), caspase-8 inhibitor IETD-CHO (5 μM) or LY29400 was added to the wells at the time of sHA addition. For apoptosis assay, cells were treated with sHA and apoptosis was analyzed after 48 hours using the Cell Death ELISA Plus kit. Apoptosis index was calculated as O.D._{450 nm} per 20,000 cells. LNAI cells were also treated with sHA for 24 hours and cell cycle analysis was performed as described previously (16).

Motility and invasion assays

Matrigel™ invasion and motility assays were carried out as described previously (15–17) except that sHA was added in both chambers of the Transwell (Supplemental Information).

Immunoblot analyses, time course and VEGF ELISA

Prostate cancer cells (40,000 – 50,000 cells/6-well plate) were exposed to sHA (0–10 $\mu\text{g/ml}$) for 48 hours. For time course experiments, 8–12 hour cultures of LNAI cells were exposed to sHA (5 $\mu\text{g/ml}$) for 3, 6, 12, 24 hours. In some wells, 50 $\mu\text{g/ml}$ HA12K was added at the time of sHA addition. Cell lysates (~20,000 cell equivalent) were analyzed by immunoblotting using specific antibodies; β -actin and PPIA4 were used as loading controls. For VEGF ELISA, serum-free conditioned media (16,17) from control and sHA-treated LNAI cells were assayed for VEGF levels by using an ELISA kit.

HAase activity assay

Serum-free conditioned media of LNAI and DU145 cells were subjected to HAase ELISA-like assay in the presence of sHA (0–40 $\mu\text{g/ml}$). The HAase activity was normalized to units $\times 10^{-4}/10^6$ cells, as described before (23).

Immunoprecipitation and PI3-kinase activity assay

LNAI and DU145 cells treated with sHA were immunoprecipitated using a rabbit anti-p85 PI3-kinase subunit antibody or rabbit IgG. The immunoprecipitates were subjected to either immunoblotting using a mouse anti-CD44, anti- RHAMM or anti-p85 PI3-kinase antibody or subjected to PI3-kinase activity assay (Supplemental information).

Transient transfection assays

LNAI and DU145 cells were transiently transfected with Myr-HA-Akt1 plasmid (myr-Akt), pcDNA3.1-RHAMM or pcDNA3.1-CD44 (standard form, CD44s) expression plasmids or vector. Alternatively, cells were transfected with control siRNA or CD44 and/or RHAMM siRNA (50 nM each) (15). Twenty-four hours following transfection, the cells were exposed to sHA and analyzed after 48 hours for proliferation, apoptosis, protein and gene expression (Supplemental Information). For NF- κ B- and PSA-reporter assays, vector and myr-Akt transfectants were transiently transfected with pNF κ B-luc or PSAe1p/Luc plasmid (28).

Quantitative RT-PCR (Q-PCR) assays

Transient transfectants or prostate cancer cells treated with sHA +/- HA12K were subjected to Q-PCR using the iQ SYBR Green Supermix and the primers described in Table 1 (Supplemental Information). mRNA levels were normalized to peptidylprolyl isomerase A (cyclophilin A or PPIA4) mRNA levels (15).

Tumor xenografts

LNAI cell suspension (2×10^6 cells/0.1 ml) was mixed 1:1 with Matrigel and implanted subcutaneously on the dorsal flank of 5–6 month old athymic mice. In the first experiment, there were 4 mice in each group, vehicle (phosphate buffered saline), sHA-25 mg/kg, sHA-50 mg/kg. sHA was injected intraperitoneally twice weekly. The treatment began on the day of injection. Animals in the control group were euthanatized on day 29, and on day 40 in the sHA 25 mg/kg group. In the sHA 50/kg group, 3 mice were euthanatized on day 40 and the remaining on day 64 after stopping the treatment on day 50. Tumor volume was measured twice weekly. In a second experiment, there were 5 animals in the control and 10 animals in the sHA 50 mg/kg group. In the sHA-50 mg/kg group, 5 animals were euthanatized on day 42 and 5 mice were left untreated from day 53 to day 70. Tumors or the Matrigel™ sac (if visible) were fixed for immunohistochemistry (to localize microvessels or TUNEL positive cells; 15–17) and histopathology (performed at Charles River Laboratories, (Wilmington, MA). Microvessel density (MVD) and TUNEL assays are described in the Supplemental Section. Serum chemistry analysis and histopathological evaluation of kidney, lung and liver were performed by the Division of Comparative Pathology, University of Miami.

Determination serum of sHA levels

Fourteen-week old athymic mice (av. weight ~30 g) were injected intraperitoneally with sHA (86.7 mg/kg). At various time intervals, mice were euthanatized and serum was assayed for uronate levels (total glycosaminoglycan) by a modified uronic carbazole assay established by Bitter and Mürir (29). Serum sulfated glycosaminoglycan levels were measured using Farndale's dimethylmethylene blue assay (29). We have previously demonstrated the detection of sHA by Farndale assay (23).

RESULTS

sHA inhibited HAase activity and cell proliferation in prostate cancer cells

We have previously shown that LNCaP and DU145 cells express 5–10-fold more HAase activity when compared to PC3-ML cells and that HYAL-1 is the only HAase expressed in prostate cancer cells (7). Furthermore, consistent with our previous results, sHA inhibited the HAase activity secreted in the conditioned media of LNAI and DU145 cells, in a dose-dependent manner (Supplement Fig. 1B). sHA did not affect HYAL-1 expression, as determined by Q-PCR (data not shown). As shown in Fig. 1A, sHA inhibited the growth of all prostate cancer cell lines, but not of prostate epithelial cells (RWPE-1). The IC_{50} for LNCaP, LNAI and LAPC-4 cells was 5–10 μ g/ml, whereas for DU145 and C4-2 cells it was 20–40 μ g/ml. At $\geq IC_{50}$, the differences in cell numbers between untreated and sHA-treated samples were statistically significant ($P \leq 0.005$; un-paired t-test). Time course experiment showed that sHA inhibited the growth of LNAI cells at each time point (Supplement Fig. 1C). To determine whether the anti-proliferative effect of sHA was due to the inhibition of HAase activity, we treated LNAI and DU145 cells with sHA in the presence of angiogenic HA fragments, which are generated due to the degradation of HA by HAase. As shown in Fig. 1B, in LNAI cells, HA12K partially reversed the growth inhibition by sHA (64% inhibition at 5 μ g/ml sHA; 36% inhibition at 5 μ g/ml sHA + HA12K). This partial reversal was independent of the average mol. wt. of HA fragments, because HA8K showed a similar effect on the growth inhibition by sHA in DU145 cells (Fig. 1C).

sHA induced apoptosis in prostate cancer cells

To examine why sHA inhibits cell growth, we performed cell cycle analysis. As shown in Supplement Fig. 1D, sHA induced ~20% increase G0-G1 phase with a corresponding

decrease in G2-M and S-phases. However, the cytotoxic effect of sHA was more likely mediated by its ability to induce apoptotic cell death; at IC₅₀, the increase in apoptosis was 200% in DU145 and C4-2 and 500% in LNAI cells, respectively (Fig. 1D). Since apoptosis induction was substantial we investigated its mechanism. In LNAI and LAPC-4 cells, sHA induced the activation of proapoptotic effectors (caspase-3, caspase-9, and caspase-8; 2–5-fold), PARP cleavage and upregulation of death receptor signaling complex proteins (Fas, Fas-L, DR4, DR5, FADD and bid cleavage) in a dose-dependent manner (Fig. 2A). The upregulation of proapoptotic effectors and death receptors was observed as early as 6–12 hours after the exposure of LNAI cells to sHA (Fig. 2B). In both LNAI and LAPC-4 cells, sHA also downregulated bcl-2 and phosphorylated-Bad levels, without significantly affecting Bcl-XL, bax and total Bad levels. Except for Fas, DR4, p-Bad and DR5 levels, the addition of HA12K during sHA incubation, either did not prevent (caspase 3, caspase 8, bcl-2) or partially prevented (PARP, Fas-L, p-15 Bid) the effect of sHA on apoptosis effectors. When LNAI cells were incubated with sHA in the presence of a cell permeable caspase-8 inhibitor, IETD-CHO, both growth inhibition and sHA-induced apoptosis were significantly attenuated (Supplement Fig. 1E), suggesting the involvement of the extrinsic pathway in sHA- induced apoptosis.

sHA inhibits chemotactic motility and invasion

Since HYAL-1 promotes tumor invasion and metastasis (18–21), we investigated whether sHA inhibits chemotactic motility and invasive potential of prostate cancer cells. As shown in Fig. 2C and D, sHA caused $\geq 75\%$ inhibition of chemotactic motility in both LNAI and DU145 cells ($P \leq 0.0001$; un-paired t-test). HA12K caused 150% increase in the motility of LNAI cells, and reduced the sHA induced inhibition of motility by 80%. Similarly, sHA inhibited the invasive activity of LNAI and DU145 cells by 40–50% ($P < 0.001$; unpaired t-test); in LNAI cells, HA12K reduced this inhibition by $> 80\%$. Therefore, sHA inhibits chemotactic motility and invasion plausibly by inhibiting HAase activity.

sHA inhibits Akt signaling

Since Bad is phosphorylated by Akt at Ser136 and sHA downregulated phosphorylated Bad levels, we investigated whether sHA inhibits Akt activation. As shown in Fig. 3A, sHA downregulated phosphorylated-Akt levels (> 3 -fold decrease at 5–10 $\mu\text{g/ml}$) in both LNAI and LAPC-4 cells and this decrease was partially prevented by HA12K. Akt activates NF κ B by phosphorylating IKK α , which in turn, phosphorylates IKB, targeting it for degradation. As shown in Fig. 3A, sHA decreased phosphorylated IKB levels in both LNAI and LAPC-4 cells and the addition of HA12K prevented this decrease. As expected sHA inhibited NF κ B promoter luciferase reporter activity (Fig. 3B). At 7.5 $\mu\text{g/ml}$ sHA caused $\sim 100\%$ inhibition of NF κ B reporter activity (35.6 ± 10.6 versus 0.67 ± 0.32 ; $P < 0.0001$). NF κ B activation induces VEGF expression (31). As shown in Fig. 3B, sHA decreased VEGF levels secreted in the conditioned medium of LNAI cells in a dose dependent manner.

The PI3K/Akt signaling pathway regulates AR activity by phosphorylating AR at Ser210/213 and Ser791/790 (32–34). sHA inhibited AR phosphorylation on Ser 210/213 in both LNAI and LAPC-4 cells; however the addition of HA12K did not prevent the decrease in phosphorylated AR levels (Fig. 3A). The time course of the decrease in phosphorylated – Akt, IKB and AR levels followed the same pattern; a decrease was observed in as early as 3 hours and the levels decreased $> 80\%$ after 12 hour incubation (Fig. 2B).

sHA transcriptionally downregulates HA receptor and VEGF expression

Angiogenic HA fragments induce CD44 expression and cellular signaling through both CD44 and RHAMM (35–37). sHA treatment down regulated RHAMM levels in a dose dependent manner in both LNAI and LAPC-4 cells (Fig. 3A). RHAMM downregulation was

observed within 6 hours of sHA treatment (Fig. 2B) and it was not effectively prevented by HA12K (Fig. 3A). sHA also down regulated RHAMM and VEGF mRNA levels by 3-fold and 100-fold, respectively in LNAI cells (Fig. 3B). Addition of HA12K partially (RHAMM) or completely (VEGF) prevented the observed decrease in these transcripts (Fig. 3C). In LAPC-4 cells, sHA downregulated RHAMM and VEGF transcript levels by 18.5- and 25.7-fold respectively (Fig. 3D).

With the exception of a derivative CL1, LNCaP cell line and its derivatives (e.g., C4-2, C4-2B), and LAPC-4 cells do not express CD44 because of promoter hypermethylation (38–45). We also did not observe CD44 expression (standard (CD44s) and variant (CD44v) isoform(s)) in these cells (Supplement Table 2). Two publications have reported CD44 expression in LNCaP and C4-2 cells (46,47), including the expression of a CD44 variant, CD44-v9 (46). However, no PCR product was amplified from LNCaP, LNAI, LAPC-4, C4-2 and C42B cells using the same PCR primer pair that was used to amplify CD44-v9 (46,48). A 632 base PCR product (CD44-Epithelial isoform) was amplified from PC3-ML and DU145 cells which included exon v9 (exon 13), v10 (exon 14) and common exons 15 through 17 (Supplement Fig. 2A). The expression of CD44s transcript was 26- and 49-fold higher than CD44v transcript in DU145 and PC3-ML cells, respectively (Supplement Table 2). In DU145 cells, sHA caused 5.6- and 2.5-fold decrease in CD44 (data shown for CD44s) and RHAMM mRNA levels, respectively, and a 4-fold decrease in VEGF levels (Fig 3D).

sHA inhibits PI3-kinase activity and complex formation between HA-receptors and PI3-kinase

sHA-mediated inhibition of Akt phosphorylation suggested that sHA might either be inhibiting the activity of PI3 kinase or accelerating dephosphorylation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) by PTEN. Since sHA downregulated Akt phosphorylation in PTEN-positive (DU145) and PTEN-negative (LNAI, LAPC-4) cells, we investigated whether sHA inhibited PI3-kinase activity. As shown in Supplement Fig. 2B and C, there was no difference in the amount of PI3-kinase (p85 subunit) immunoprecipitated from the control and sHA treated LNAI and DU145 cells; however, the PI3-kinase activity was significantly inhibited in sHA treated samples. Furthermore, while both CD44 and RHAMM were co-immunoprecipitated with the p85 subunit in untreated samples, their amount was significantly reduced in immunoprecipitates from sHA treated samples. Treatment of LNAI cells with LY29400, a PI3-kinase inhibitor, and sHA synergistically inhibited cell growth (Supplement Fig. 2D). The combination index (CI) calculated by Chou-Talalay analysis (Calculusyn, Biosoft Inc, UK) was 0.017, suggesting strong synergy. These results showed that sHA decreased Akt signaling by inhibiting PI3-kinase activity.

Combined effect of HA receptor downregulation and sHA treatment on prostate cancer cells

Since both HA and angiogenic HA fragments signal through HA receptors, we examined whether downregulation of CD44 and/or RHAMM mimics sHA effects. In DU145 cells sHA downregulated RHAMM and CD44 protein (Fig. 4A) and mRNA expression (~ 6-fold; Supplement Fig. 3). RHAMM and CD44 siRNAs downregulated RHAMM and CD44 protein expression, respectively (RHAMM, CD44 and CD44+RHAMM panels, Fig. 4A). Addition of sHA did not further decrease HA receptors levels plausibly because the siRNAs decreased the levels of respective transcripts by > 30-fold (Supplement Fig. 3). RHAMM protein and mRNA were not detectable when LNAI cells were treated with both sHA and RHAMM siRNA (Fig. 4A and Supplement Fig. 3).

In DU145 cells, downregulation of RHAMM or CD44 decreased phosphorylated Akt levels; however, sHA was more effective. sHA and CD44+RHAMM siRNAs caused > 5-fold decrease in phosphorylated Akt levels. In LNAI cells, both sHA and RHAMM siRNA caused > 50% decrease in phosphorylated Akt levels, and their combination caused > 80% decrease (Fig. 4A).

Both sHA and HA receptor siRNA treatments decreased cell growth. However, the combination of sHA and CD44+RHAMM siRNAs caused a 5.1-fold inhibition of cell growth and a 4.4-fold increase in apoptosis in DU145 cells (Fig. 4B; $P < 0.0001$; unpaired t-test). In LNAI cells, sHA and RHAMM siRNA combination inhibited LNAI cell growth by 5-fold and increased apoptosis by ~ 9-fold (Fig. 4C; $P < 0.0001$; unpaired t-test). Chou-Talalay analysis showed a synergistic effect of sHA and HA receptor siRNA treatments on growth inhibition. The CI for each combination to inhibit the growth of DU145 cells was as follows: sHA + CD44 siRNA: 0.143; sHA + RHAMM siRNA: 0.102; sHA + (CD44 + RHAMM) siRNA: 0.051. In LNAI cells, the CI for sHA + RHAMM siRNA was 0.082. In DU145 cells, HA receptor downregulation or sHA caused a 5–10-fold decrease in VEGF mRNA levels and when both were combined VEGF mRNA levels were undetectable. In LNAI cells both sHA and RHAMM siRNA, completely downregulated VEGF mRNA levels (Fig. 4D). Conversely, overexpression of CD44, RHAMM or CD44+RHAMM in LNAI cells, attenuated sHA-induced growth inhibition; growth inhibition in transfectants at 5 μ g/ml: vector: 64.4%; RHAMM: 20%; CD44: 12%; CD44+RHAMM: 20% (Supplement Fig. 4C). Similar results were observed with respect to VEGF downregulation by sHA (data not shown).

Effect of constitutive Akt activation on sHA-induced cellular effects

Since Akt activation was downregulated by both sHA and HA-receptor siRNA treatments, we investigated various effects of sHA in LNAI cells transfected with myristoylated-Akt plasmid (myr-Akt). As shown in Fig. 5A, myr-Akt transfection, increased total Akt and phosphorylated-Akt levels by >10-fold. sHA treatment did not downregulate phosphorylated-Akt, phosphorylated-IkB, phosphorylated-AR and RHAMM levels in myr-Akt transfectants. Myr-Akt expression also attenuated the effect of sHA on cell proliferation, apoptosis, caspase-8 activation and Fas-L upregulation (Fig. 5A, B). Myr-Akt expression caused a 4-fold increase in NFkB-reporter activity and prevented the sHA-mediated decrease in NFkB transcriptional activity (Fig. 5C). Similarly, myr-Akt reversed the effect of sHA on prostate specific antigen (PSA) promoter activity and downregulation of VEGF transcript levels (Fig. 5C, D).

Effect of sHA on tumor growth and angiogenesis

Effect of sHA treatment on LNAI xenografts is shown in Fig. 6A. sHA significantly inhibited tumor growth at 25 and 50 mg/kg doses. On day 29 the average tumor volumes in the vehicle group ($1191.1 \pm 299.5 \text{ mm}^3$) were significantly higher than in sHA 25 mg/kg ($176.1 \pm 105 \text{ mm}^3$) and sHA 50 mg/kg ($0.143 \pm 0.01 \text{ mm}^3$); $P < 0.001$ (Tukey's multiple comparison test). The tumors in the vehicle group were vascular and hemorrhagic (Fig. 6B). The experiment was terminated on day 40 in the sHA 25 mg/kg group (mean tumor volume = $385 \pm 263 \text{ mm}^3$). Only one animal developed a palpable tumor by day 40 in the sHA 50 mg/kg group; 8 out of 14 animals were euthanized after 39–42 days. In the remaining six animals, treatment was stopped for one animal on day 50 until day 63 and this animal did not develop tumor. For five animals, treatment was stopped from day 53 until day 70. As shown in Fig 6A, the mean tumor volume at day 70 was $249 \pm 192 \text{ mm}^3$; only 2 animals developed a tumor. There was no decrease in the weight of sHA treated animals and the animals gained weight (Supplement Fig. 4). Evaluation of fixed kidney, liver and lung

tissues revealed no organ toxicity in sHA-treated animals (Supplement Figure 5B), which was further confirmed by serum chemistry analysis (Supplement Table 3).

The serum half life of sHA was determined by measuring serum uronate (total glycosaminoglycan) and sulfated glycosaminoglycan levels. As shown in Fig. 6C, following sHA administration, there was an increase in both serum uronate and sulfated glycosaminoglycan levels; peak levels were achieved at 6 hours following intraperitoneal injection. The serum half life of sHA in circulation was ~ 24 hours.

Tumor histology showed that tumors in the vehicle group formed larger masses and had clear evidence of formation of fibrovascular stroma (Fig. 6B). Contrarily, the Matrigel sac removed from the injection site in the sHA 50 mg/kg group (day 39), consisted primarily of groups of tumor cells still embedded in the Matrigel without clear evidence of neovascularization. Tumor cells in the sHA-treated group were degenerate, some with small dark (pyknotic) or fragmented (karyorrhectic) nuclei. Tumor cells present in the Matrigel sac in sHA 50 mg/kg group were apoptotic (~ 100% TUNEL positive cells), whereas, 5–10% of cells in the vehicle treated-group were TUNEL positive (Fig. 6D). A tumor specimen from the sHA-treated group had lower MVD than the vehicle-treated group (Fig. 6D). MVD (mean $\pm$ S.D.) from sHA-treated group (1.8 ± 1.5) was ~ 10-fold lower than in the vehicle treated group (22.5 ± 6.5 ; $P < 0.0001$; unpaired t-test).

DISCUSSION

Although HYAL-1 is a critical determinant of tumor growth and metastasis and is an accurate prognostic marker for cancer metastasis, no study so far has targeted HYAL-1 or any other HAase for cancer therapy. The present study not only targets HYAL-1 using a non-toxic, HAase inhibitor – sHA, but also delineates the molecular mechanism through which the HA-HAase system might mediate tumor growth and progression. sHA is not a specific inhibitor of HYAL-1 HAase or for prostate cancer cells, rather it inhibits the activity of different HAases by a mixed inhibition mechanism. sHA is also 5-fold more potent in inhibiting HYAL-1 activity than the activity of testicular HAase (7). We have previously shown that unlike acidic HAases (e.g., HYAL-1), HAases which are active at pH ≥ 5.0 (e.g., testicular), are more resistant to different classes of HAase inhibitors (7). Since HYAL-1 and testicular HAases share about 40% amino acid identity, only the comparison between the crystal structures of sHA bound to HYAL-1 and to testicular HAase can reveal why sHA is more potent in inhibiting HYAL-1 activity; the crystal structure of HYAL-1 has not been deciphered.

The salient features of our study are: 1. sHA inhibits tumor growth mainly by inducing apoptosis via the extrinsic pathway; 2. sHA is effective in both androgen-dependent and androgen-independent prostate cancer cells; 3. Antitumor, anti-angiogenic and anti-invasive effects of sHA are primarily mediated by the inhibition of PI3-Kinase/Akt signaling and transcriptional downregulation of HA receptors; 4. Plausibly, a feedback loop between Akt signaling and HA receptors controls prostate cancer cell growth, invasion/motility, androgen receptor activity and VEGF production. 5. In xenografts models, sHA is highly effective in inhibiting tumor growth. > 60% of animals did not form tumors even when the treatment was stopped after a certain period; 6. sHA is a potent anti-angiogenic agent and causes transcriptional downregulation of VEGF. With low toxicity, high efficacy and an easy assay to measure circulating levels, sHA is potentially a promising anticancer agent.

The tumor-associated HA-HAase system plausibly promotes cell survival, proliferation, motility and invasion and up-regulates HA receptor expression by stimulating the PI3-kinase-Akt pathway. By inhibiting the signaling complex between PI3-kinase and HA

receptors, sHA inhibits Akt-signaling and related events (Supplement Fig. 6). Since the overexpression of myr-Akt restores the downregulation of HA receptors caused by sHA, inhibition of Akt signaling appears to be responsible for this downregulation. Furthermore, since HA receptor downregulation decreases phosphorylated Akt levels and the overexpression of HA receptors attenuates sHA-mediated inhibition of cell growth, it appears that there is a feedback loop between PI3-kinase/Akt activation and HA receptor expression and the inhibition of this feedback loop is primarily responsible for the antitumor effects of sHA.

Downregulation of HA receptors by sHA is of key importance in shutting down the HA-HAase system. This is because, although, the inhibition of HYAL-1 activity will inhibit the generation of angiogenic HA fragment-mediated signaling, in the absence of HAase activity, pericellular HA still can generate intracellular signaling through HA receptors. However, since sHA downregulates both HA receptors, signaling through the HA-HAase system will be inhibited regardless of the presence of pericellular HA. Indeed more sHA is needed to inhibit cell growth, invasion, motility and gene expression in DU145 cells, which express both CD44 and RHAMM, when compared to LNAI and LAPC-4 cells which express only RHAMM. Therefore, sHA plausibly affects two interrelated events - inhibition of HAase activity and downregulation of HA receptors.

sHA causes inhibition of Akt phosphorylation as early as 3 hours and it precedes the downregulation of HA receptors and AR phosphorylation. Overexpression of myr-Akt reverses the biological effects of sHA including the inhibition of NFκB reporter activity, AR phosphorylation and PSA promoter activity. This suggests that downregulation of PI3-kinase/Akt signaling by sHA is the initial event which triggers the inhibition of the feedback loop between Akt and HA receptors leading to induction of apoptosis (49) and the inhibition of VEGF expression, cell motility and invasion. Inhibition of PI3-kinase/Akt as the initial event may also be the reason why we observed a substantial increase in apoptosis but a modest cell cycle arrest in sHA treated cells; the latter would be expected due to the downregulation of RHAMM by sHA (50).

The present study shows that in prostate cancer models, sHA has potent antitumor activity with desirable toxicity profile and ease of detection in serum. In fact, > 60% of the animals remained tumor-free even when the treatment was terminated after a certain period, and there was no detectable treatment-related toxicity. Serum sulfated and total glycosaminoglycan (uronate) levels provide an inexpensive surrogate marker for determining sHA levels in circulation. In addition to prostate cancer, this study will have a broad impact on cancer biology, therapeutics and the mechanistic understanding of the tumor-associated HA-HAase system; specifically from the standpoint of tumors that express HYAL-1 (e.g., bladder, breast, prostate) and the HA-receptor-Akt-dependent pathways activated in these tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

CI	combination index
HA	hyaluronic acid
HAase	hyaluronidase
LNAI	LNCaP-AI
sHA	sulfated hyaluronic acid
myr-Akt	myristoylated Akt; sulfated hyaluronic acid
Q-PCR	Quantitative reverse transcription PCR

References

1. Simpson MA, Lokeshwar VB. Hyaluronan and hyaluronidase in genitourinary tumors. *Front Biosci.* 2008; 13:5664–80. [PubMed: 18508614]
2. Tammi RH, Kultti A, Kosma VM, Pirinen R, Auvinen P, Tammi MI. Hyaluronan in human tumors: pathobiological and prognostic messages from cell-associated and stromal hyaluronan. *Semin Cancer Biol.* 2008; 18:288–95. [PubMed: 18468453]
3. Ekici S, Cerwinka WH, Duncan R, Gomez P, Civantos F, Soloway MS, et al. Comparison of the prognostic potential of hyaluronic acid, hyaluronidase (HYAL-1), CD44v6 and microvessel density for prostate cancer. *Int J Cancer.* 2004; 112:121–9. [PubMed: 15305383]
4. Posey JT, Soloway MS, Ekici S, Sofer M, Civantos F, Duncan RC, et al. Evaluation of the prognostic potential of hyaluronic acid and hyaluronidase (HYAL1) for prostate cancer. *Cancer Res.* 2003; 63:2638–44. [PubMed: 12750291]
5. Aaltomaa S, Lipponen P, Tammi R, Tammi M, Viitanen J, Kankkunen JP, et al. Strong stromal Hyaluronan expression is associated with PSA Recurrence in local prostate cancer. *Urol Int.* 2002; 69:266–72. [PubMed: 12444281]
6. Gomez CS, Gomez P, Knapp J, Jorda M, Soloway MS, Lokeshwar VB. Hyaluronic acid and HYAL-1 in prostate biopsy specimens: predictors of biochemical recurrence. *J Urol.* 2009; 182:1350–6. [PubMed: 19683287]
7. Lokeshwar VB, Rubinowicz D, Schroeder GL, Forgacs E, Minna JD, Block NL, et al. Stromal and epithelial expression of tumor markers hyaluronic acid and HYAL1 hyaluronidase in prostate cancer. *J Biol Chem.* 2001; 276:11922–32. [PubMed: 11278412]
8. Toole BP, Slomiany MG. Hyaluronan: a constitutive regulator of chemoresistance and malignancy in cancer cells. *Semin Cancer Biol.* 2008; 18:244–50. [PubMed: 18534864]
9. Toole BP. Hyaluronan: from extracellular glue to pericellular cue. *Nat Rev Cancer.* 2004; 4:528–39. [PubMed: 15229478]
10. Kobayashi N, Miyoshi S, Mikami T, Koyama H, Kitazawa M, Takeoka M, et al. Hyaluronan deficiency in tumor stroma impairs macrophage trafficking and tumor neovascularization. *Cancer Res.* 2010; 70:7073–83. [PubMed: 20823158]
11. Itano N, Zhuo L, Kimata K. Impact of the hyaluronan-rich tumor microenvironment on cancer initiation and progression. *Cancer Sci.* 2008; 99:1720–5. [PubMed: 18564137]
12. Maxwell CA, McCarthy J, Turley E. Cell-surface and mitotic-spindle RHAMM: moonlighting or dual oncogenic functions? *J Cell Sci.* 2008; 121:925–32. [PubMed: 18354082]
13. Orian-Rousseau V. CD44, a therapeutic target for metastasising tumours. *Eur J Cancer.* 2010; 46:1271–7. [PubMed: 20303742]
14. Toole BP. Hyaluronan-CD44 Interactions in Cancer: Paradoxes and Possibilities. *Clin Cancer Res.* 2009; 15:7462–7468. [PubMed: 20008845]
15. Lokeshwar VB, Lopez LE, Munoz D, Chi A, Shirodkar SP, Lokeshwar SD, et al. Antitumor activity of hyaluronic acid synthesis inhibitor 4-methylumbelliferone in prostate cancer cells. *Cancer Res.* 2010; 70:2613–23. [PubMed: 20332231]

16. Lokeshwar VB, Cerwinka WH, Lokeshwar BL. HYAL1 hyaluronidase: a molecular determinant of bladder tumor growth and invasion. *Cancer Res.* 2005; 65:2243–50. [PubMed: 15781637]
17. Lokeshwar VB, Cerwinka WH, Isoyama T, Lokeshwar BL. HYAL1 hyaluronidase in prostate cancer: a tumor promoter and suppressor. *Cancer Res.* 2005; 65:7782–9. [PubMed: 16140946]
18. Bharadwaj AG, Kovar JL, Loughman E, Elowsky C, Oakley GG, Simpson MA. Spontaneous metastasis of prostate cancer is promoted by excess hyaluronan synthesis and processing. *Am J Pathol.* 2009; 174:1027–36. [PubMed: 19218337]
19. Kovar JL, Johnson MA, Volcheck WM, Chen J, Simpson MA. Hyaluronidase expression induces prostate tumor metastasis in an orthotopic mouse model. *Am J Pathol.* 2006; 169:1415–26. [PubMed: 17003496]
20. Kramer MW, Escudero DO, Lokeshwar SD, Golshani R, Ekwenna OO, Acosta K, et al. Association of hyaluronic acid family members (HAS1, HAS2, and HYAL-1) with bladder cancer diagnosis and prognosis. *Cancer.* 2010 Oct 19. [Epub ahead of print].
21. Poola I, Abraham J, Marshalleck JJ, Yue Q, Lokeshwar VB, Bonney G, et al. Molecular risk assessment for breast cancer development in patients with ductal hyperplasias. *Clin Cancer Res.* 2008; 15:1274–80. [PubMed: 18281563]
22. Balazs EA, Hogberg B, Laurent TC. The biological activity of hyaluron sulfuric acid. *Acta Physiol Scand.* 1951; 23:168–78. [PubMed: 14868516]
23. Isoyama T, Thwaites D, Selzer MG, Carey RI, Barbucci R, Lokeshwar VB. Differential selectivity of hyaluronidase inhibitors toward acidic and basic hyaluronidases. *Glycobiology.* 2006; 16:11–21. [PubMed: 16166602]
24. Kunze R, Rösler M, Möller S, Schnabelrauch M, Riemer T, Hempel U, et al. Sulfated hyaluronan derivatives reduce the proliferation rate of primary rat calvarial osteoblasts. *Glycoconj J.* 2010; 27(1):151–8. [PubMed: 19941065]
25. Ahmed S, Tsuchiya T, Nagahata-Ishiguro M, Sawada R, Banu N, Nagira T. Enhancing action by sulfated hyaluronan on connexin-26, -32, and -43 gene expressions during the culture of normal human astrocytes. *J Biomed Mater Res A.* 2009; 90:713–9. [PubMed: 18570339]
26. Nagira T, Nagahata-Ishiguro M, Tsuchiya T. Effects of sulfated hyaluronan on keratinocyte differentiation and Wnt and Notch gene expression. *Biomaterials.* 2007; 28:844–50. [PubMed: 17084447]
27. Gomez LA, de Las Pozas A, Perez-Stable C. Sequential combination of flavopiridol and docetaxel reduces the levels of X-linked inhibitor of apoptosis and AKT proteins and stimulates apoptosis in human LNCaP prostate cancer cells. *Mol Cancer Ther.* 2006; 5:1216–26. [PubMed: 16731754]
28. Perez-Stable CM, de las Pozas A, Roos BA. A role for GATA transcription factors in the androgen regulation of the Prostate-Specific Antigen gene enhancer. *Mol Cell Endocrin.* 2000; 167:43–53.
29. Wei DC, Politano VA, Selzer MG, Lokeshwar VB. The association of elevated urinary total to sulfated glycosaminoglycan ratio and high molecular mass hyaluronic acid with interstitial cystitis. *J Urol.* 2000; 163:1577–83. [PubMed: 10751891]
30. Lokeshwar VB, Lokeshwar BL, Pham HT, Block NL. Association of elevated levels of hyaluronidase, a matrix-degrading enzyme, with prostate cancer progression. *Cancer Res.* 1996; 56:651–7. [PubMed: 8564986]
31. Novotny NM, Markel TA, Crisostomo PR, Meldrum DR. Differential IL-6 and VEGF secretion in adult and neonatal mesenchymal stem cells: role of NFkB. *Cytokine.* 2008; 43:215–9. [PubMed: 18621544]
32. Lin HK, Hu YC, Yang L, Altuwaijri S, Chen YT, Kang HY, et al. Suppression versus induction of androgen receptor functions by the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer LNCaP cells with different passage numbers. *J Biol Chem.* 2003; 278:50902–7. [PubMed: 14555644]
33. Li Y, Wang Z, Kong D, Li R, Sarkar SH, Sarkar FH. Regulation of Akt/FOXO3a/GSK-3beta/AR signaling network by isoflavone in prostate cancer cells. *J Biol Chem.* 2008; 283:27707–16. [PubMed: 18687691]
34. Liu X, Choi RY, Jawad SM, Arnold JT. Androgen-induced PSA expression requires not only activation of AR but also endogenous IGF-I or IGF-I/PI3K/Akt signaling in human prostate cancer epithelial cells. *Prostate.* 2010 [Epub ahead of print].

35. Campo GM, Avenoso A, Campo S, D'Ascola A, Nastasi G, Calatroni A. Small hyaluronan oligosaccharides induce inflammation by engaging both toll-like-4 and CD44 receptors in human chondrocytes. *Biochem Pharmacol.* 2010; 80:480–90. [PubMed: 20435021]
36. Ohno-Nakahara M, Honda K, Tanimoto K, Tanaka N, Doi T, Suzuki A, et al. Induction of CD44 and MMP expression by hyaluronidase treatment of articular chondrocytes. *J Biochem.* 2004; 135:567–75. [PubMed: 15173194]
37. Lokeshwar VB, Selzer MG. Differences in hyaluronic acid-mediated functions and signaling in arterial, microvessel, and vein-derived human endothelial cells. *J Biol Chem.* 2000; 275:27641–9. [PubMed: 10882722]
38. Freedland SJ, Seligson DB, Liu AY, Pantuck AJ, Paik SH, Horvath S, et al. Loss of CD10 (neutral endopeptidase) is a frequent and early event in human prostate cancer. *Prostate.* 2003; 55:71–80. [PubMed: 12640663]
39. Harrison GM, Davies G, Martin TA, Mason MD, Jiang WG. The influence of CD44v3-v10 on adhesion, invasion and MMP-14 expression in prostate cancer cells. *Oncol Rep.* 2006; 15:199–206. [PubMed: 16328056]
40. Liu AY, Brubaker KD, Goo YA, Quinn JE, Kral S, Sorensen CM, et al. Lineage relationship between LNCaP and LNCaP-derived prostate cancer cell lines. *Prostate.* 2004; 60:98–108. [PubMed: 15162376]
41. Lou W, Krill D, Dhir R, Becich MJ, Dong JT, Frierson HF Jr, et al. Methylation of the CD44 metastasis suppressor gene in human prostate cancer. *Cancer Res.* 1999; 59:2329–31. [PubMed: 10344738]
42. Dhir R, Gau JT, Krill D, Bastacky S, Bahnson RR, Cooper DL, et al. CD44 expression in benign and neoplastic human prostates. *Mol Diagn.* 1997; 2:197–204. [PubMed: 10462610]
43. Horiguchi A, Zheng R, Shen R, Nanus DM. Inactivation of the NF2 tumor suppressor protein merlin in DU145 prostate cancer cells. *Prostate.* 2008; 68:975–84. [PubMed: 18361411]
44. Verkaik NS, Trapman J, Romijn JC, Van der Kwast TH, Van Steenbrugge GJ. Down-regulation of CD44 expression in human prostatic carcinoma cell lines is correlated with DNA hypermethylation. *Int J Cancer.* 1999; 80:439–43. [PubMed: 9935187]
45. van Bokhoven A, Varella-Garcia M, Korch C, Johannes WU, Smith EE, Miller HL, et al. Molecular characterization of human prostate carcinoma cell lines. *Prostate.* 2003; 57:205–25. [PubMed: 14518029]
46. Ghatak S, Hascall VC, Markwald RR, Misra S. Stromal hyaluronan interaction with epithelial CD44 variants promotes prostate cancer invasiveness by augmenting expression and function of hepatocyte growth factor and androgen receptor. *J Biol Chem.* 2010; 285:19821–32. [PubMed: 20200161]
47. Müller I, Wischniewski F, Pantel K, Schwarzenbach H. Promoter- and cell-specific epigenetic regulation of CD44, Cyclin D2, GLIPR1 and PTEN by methyl-CpG binding proteins and histone modifications. *BMC Cancer.* 2010; 10:297. [PubMed: 20565761]
48. van Weering DH, Baas PD, Bos JL. A PCR-based method for the analysis of human CD44 splice products. *PCR Methods Appl.* 1993; 3:100–6. [PubMed: 7505677]
49. Suhara T, Kim HS, Kirshenbaum LA, Walsh K. Suppression of Akt signaling induces Fas ligand expression: involvement of caspase and Jun kinase activation in Akt-mediated Fas ligand regulation. *Mol Cell Biol.* 2002; 22:680–91. [PubMed: 11756562]
50. Maxwell CA, McCarthy J, Turley E. Cell-surface and mitotic-spindle RHAMM: moonlighting or dual oncogenic functions? *J Cell Sci.* 2008; 121:925–32. [PubMed: 18354082]

Figure 1. Effect of sHA on cell proliferation

A: Cell counting data at 72 hours following treatment with sHA. **B and C:** LNAI (B) and DU145 (C) cells were treated with sHA plus 50- $\mu\text{g/ml}$ of HA8k or HA12k and counted after 72 hours. Data: Mean $\pm$ S.D. **D.** Measurement of apoptosis in cells treated with sHA for 48 h. Data: Mean $\pm$ S.D.

Figure 2. Effect of sHA on apoptosis, invasion and chemotactic motility

A: LNAI and LAPC-4 cells were treated with sHA in the presence (LNAI cells, middle panel) or absence of HA12K for 48 hours and subjected to immunoblot analyses for apoptosis-related proteins. **B:** LNAI cells were treated with sHA (5-μg/ml) for the indicated time periods and subjected to immunoblot analyses. **C and D:** Determination of invasive activity and chemotactic motility of LNAI (C) and DU145 (D) cells treated with sHA and/or HA12K. Data: Mean ± S.D.

Figure 4. Effect of sHA and CD44-RHAMM siRNA treatment on LNAI and DU145 cells
 DU145 and LNAI cells were transfected with CD44 and/or RHAMM siRNA followed by sHA treatment. **A:** Immunoblot analysis of transfectants for HA receptors, Akt and phosphorylated Akt. **B & C:** 48 hour following sHA treatment, the DU145 (B) and LNAI (C) transfectants were counted in duplicate (left panel) or subjected to the measurement of apoptosis in triplicate (right panel). Data: Mean $\pm$ S.D. **D.** Measurement of VEGF mRNA levels by Q-PCR. Data: Mean $\pm$ S.D.

Figure 5. Effect of myr-Akt expression on sHA induced effects in LNAI cells

LNAI cells were transfected with either vector or myr-Akt plasmid. Twenty-four hour following transfection, cells were treated with sHA for 48 hours. **A:** Immunoblot analysis of vector and myr-Akt transfectants. **B:** Cell proliferation and apoptosis: Following transfection and sHA treatment the cells were counted or subjected to apoptosis measurement. **C:** NF κ B-reporter and PSA-promoter activities: Vector and myr-Akt transfectants of LNAI cells were transfected with pNF- κ B-luc or PSAe1p/Luc plasmids and treated with sHA. The firefly luciferase and Renilla luciferase activities were assayed after 24 hours. **D:** Measurement of VEGF mRNA levels, by Q-PCR in vector and myr-Akt transfectants following sHA treatment.

Figure 6. Effect of sHA on LNAI tumor xenografts

A: Athymic mice were implanted subcutaneously with LNAI cells and treated with vehicle or sHA (25 mg/kg or 50 mg/kg), twice weekly. In the sHA 50 mg/kg group, for 5 mice treatment was stopped from day 53 to day 70 and for one mouse from day 50 to day 63. **B:** Tumor pictures at day 29 for three treatment groups. **C:** Serum total- (uronate) and sulfated-glycosaminoglycan level measurement. Data: Mean±S.D. **D:** Hematoxylin-eosin staining, TUNEL assay and MVD determination by immunohistochemistry on paraffin fixed specimens (vehicle, day 29; sHA 50 mg/kg 40 days). For all panels magnification is 400X. In the hematoxylin-eosin stained sHA 50 mg/kg specimen, arrow shows Matrigel fibers and nuclear debris.