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Inhibition of Hyaluronan Synthase-3 Decreases Subcutaneous Colon Cancer Growth by Increasing Apoptosis

Brian P. Teng^{1,#}, Melissa D. Heffler^{1,#,*}, Eric C. Lai¹, Ya-Li Zhao¹, Charles M. LeVea², Vita M. Golubovskaya¹, and Kelli M. Bullard Dunn¹

¹Department of Surgical Oncology, Roswell Park Cancer Institute and the University at Buffalo/State University of New York, Buffalo, NY, USA

²Department of Laboratory Medicine and Pathology, Roswell Park Cancer Institute and the University at Buffalo/State University of New York, Buffalo, NY, USA

Abstract

Hyaluronan (HA) and hyaluronan synthases (HAS) have been implicated in cancer growth and progression. We previously have shown that HAS3 and HA mediate tumor growth in SW620 colon cancer cells, but the mechanism remains poorly understood. In addition, the effect of HAS3 inhibition on tumor growth with other cells lines has not been explored. We therefore hypothesized that inhibition of HAS3 in highly tumorigenic HCT116 colon cancer cells would decrease tumor growth and that the underlying mechanism would involve altering proliferation and/or apoptosis. HAS3 expression was inhibited by transfection with siRNA; a scrambled sequence served as a control. Stable transfectants were injected into the flanks of nude mice and tumor growth followed for 30 days. Proliferation and apoptosis were then assessed in the harvested tumors. Results were compared using the Students' t-test and ANOVA where appropriate. siRNA transfection decreased HAS3 expression, protein production, and pericellular HA retention, and decreased *in vivo* tumor growth. Proliferation was unaffected in the HCT116 tumors, but increased slightly in the SW620 tumors. In contrast, HAS3 inhibition significantly increased apoptosis in all tumors. HAS3 inhibition decreases subcutaneous tumor growth by colon cancer cells and significantly increases apoptosis with less effect on proliferation. These data show that HAS3 and HA mediate colon cancer growth by inhibiting apoptosis.

Keywords

Colon carcinoma; hyaluronan; hyaluronan synthase; apoptosis proliferation; HCT116; SW620

INTRODUCTION

Hyaluronan (HA) is a high molecular weight cell surface glycosaminoglycan that increasingly has been implicated in cancer growth and progression. In human tissues, HA is synthesized by one of three hyaluronan synthases, HAS1, HAS2, or HAS3 [1]. Under normal conditions, HA and HAS are critical for processes requiring cellular proliferation and migration, such as embryogenesis and wound healing [2]. In carcinogenesis, however,

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*Address correspondence to this author at the Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263 USA; Tel: 716-845-8248; Fax: 716-845-1278; melissa.heffler@roswellpark.org.

#Equal contribution

CONFLICTS OF INTEREST

Dr. Vita Golubovskaya is a Co-Founder of CureFAKtor Pharmaceuticals.

these molecules appear to promote processes leading to tumor growth, invasion and metastasis [3–9]. HAS and HA therefore represent potential targets for studying the mechanisms underlying cancer growth and progression.

Colorectal cancer is a leading cause of cancer death in the United States. Nearly 150,000 new cases are diagnosed annually and approximately 50,000 patients succumb to this disease each year. While some patients with early stage colorectal cancer may be cured by surgical resection, many patients already have developed distant metastases at the time of diagnosis. In addition, among those patients who undergo curative resection, nearly one third will develop recurrent disease either locally, systemically, or both. Although the genetic changes associated with the development of colon cancer have been well described, the mechanisms by which colon cancer cells interact with and alter the surrounding extracellular matrix remain incompletely understood. Interestingly, several studies have suggested a correlation between the presence of HA and poor prognosis in colorectal cancer [10]. Moreover, although HA is not found in normal colonic epithelium, colonic adenocarcinomas stain intensely for HA [11], and intensity of staining correlates with clinical stage [10]. These observations suggest that HA and its biosynthetic enzymes, HAS, may play an important role in the progression of this disease, and as such may provide useful insight into the mechanism(s) of colorectal cancer progression.

We previously have shown that HAS3 is upregulated in metastatic SW620 colon cancer cells when compared to cells derived from the primary tumor (SW480). We have further demonstrated that HAS3 and HA mediate processes that contribute to cancer growth and metastasis. Inhibition of HAS3 decreases anchorage independent growth [4], invasion through artificial basement membrane (Matrigel) [5], expression and activity of MMP-7 [12], and adhesion to laminin.[6] More recently, we have demonstrated that inhibition of HAS3 in SW620 cells also inhibits tumor growth *in vivo* [13]. These data further support a model of colon cancer growth in which HAS3 expression and HA production are critical for tumor progression. However, the mechanism(s) by which HAS3 and HA affect these processes are not well understood.

Our observation that inhibition of HAS3 decreased the growth of SW620 cells led us to hypothesize that HAS3 inhibition would have a similar effect in an unrelated colon cancer cell line. HCT116 is a human colon cancer cell line that is highly tumorigenic in mice and expresses high levels of HAS3 [14]. In the experiments described herein, we sought to determine if inhibition of HAS3 in HCT116 cells would decrease tumor growth in a mouse model. We further sought to investigate the mechanism underlying this effect and hypothesized that HAS3 affects tumor growth by altering proliferation and/or apoptosis.

MATERIALS AND METHODS

Cell Culture and Reagents

HCT116 cells were purchased from ATCC and maintained in McCoy's 5A medium + 10% fetal bovine serum (37°C, in 5% CO₂). Cells at passages 4 through 8 were used for all experiments.

Inhibition of HAS3 expression and HA production—HAS3 expression was inhibited by transfection with HAS3 siRNA. Custom HAS3 siRNA oligonucleotides were obtained from Ambion (Austin, TX) and utilized as previously described [13]. A scrambled sequence was used as a negative control. The sequences were inserted into pSuper puro plasmids (Oligoengine, Seattle, WA) following the manufacturer's protocol, then transfected using EugeneHD (Roche, Indianapolis, IN). Clonal selection was used to identify colonies that

expressed low levels of HAS3. Stable transfectants were maintained in medium supplemented with puromycin (0.25 ug/ml).

Determination of HAS Expression and HA Production

HAS3 isozyme expression was assessed by semi-quantitative RT-PCR as previously described [4]. Poly(A)⁺ RNA was isolated from subconfluent cells using the Oligotex mRNA isolation kit (Qiagen, Valencia, CA) and quantified by Ribogreen fluorescence (Molecular Probes, Eugene, OR). mRNA templates (50 ng) were reverse transcribed with an oligo(dT) primer using the Superscript II first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). A PCR oligonucleotide specific for HAS3 was utilized as previously described [3]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with every reaction (Invitrogen). Cycling conditions were as follows: 1 minute initial denaturation at 95°C; 30 cycles of 30 seconds denaturation at 95°C, 30 seconds annealing at 60°C, and 30 seconds of extension at 72°C; 5 minutes final extension at 72°C. Reaction products (5µl/reaction) were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and digitally photographed. To determine relative expression, digital images were integrated using Molecular Analyst software (NIH, Bethesda, MD) and band intensities normalized to the corresponding GAPDH band.

After confirming that siRNA inhibition of HAS3 decreased mRNA expression, Western blot analysis on whole cell lysates was used to assess protein production. Subconfluent cells were washed with PBS and incubated in lysis buffer (50mM Tris-HCl [pH 7.5], 150mM sodium chloride, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol with protease inhibitors [10µg/ml leupeptin, 10µg/ml PMSF, 1µg/ml aprotinin, 1mM sodium vanate and 5mM sodium fluoride]) for 30 minutes at 4°C. Protein samples (50ug) were separated by electrophoresis and transferred onto a Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) then blocked for one hour in 1% BSA + 0.01% Tween-20. Membranes were then incubated with primary anti-HAS3 (H-64) rabbit polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA), washed, then incubated with a peroxidase-conjugated donkey anti-rabbit secondary antibody (GE Healthcare UK Limited, Piscataway, NJ) + 3% BSA in Tris-buffered saline. Immunoblots were washed and developed with Western Blot Luminol Reagent (Santa Cruz Biotechnologies, Santa Cruz, CA). The membranes were then blocked and incubated overnight monoclonal anti-beta-actin mouse antibody to serve as a loading control (Sigma-Aldrich, St. Louis, MO), then incubated with peroxidase-conjugated sheep anti-mouse secondary antibody (GE Healthcare UK Limited, Piscataway, NJ), and developed as described.

Immunocytochemistry was then used to detect HAS3 protein expression in cultured cells. Circular cover slips were placed in each well of a 24-well plate, and then seeded with 1×10^5 cells per well. After 24 hours, the cells were washed and fixed in 0.5ml of 3.7% formaldehyde. After washing, the plates were placed on ice with 0.5ml of permeabilizing solution, incubated for 3 minutes, and then washed. Cells were then blocked for 20 minutes at room temperature with 10 µL of 25% normal goat serum. Cells were then incubated with primary anti-HAS3 antibody (1:200; Invitrogen, Carlsbad, CA) for 30 minutes, washed, then incubated with Alexa Fluor 594 goat anti-rabbit secondary antibody (1:200) for 30 minutes under dark conditions. Cells were counterstained with a FITC conjugated phalloidin (1:25; Molecular Probes, Eugene, OR) to label actin. The coverslips were then transferred to a slide and examined using a Zeiss Observer.A1 microscope at 100X magnification.

To determine if inhibition of HAS3 resulted in a decrease in pericellular HA matrix retention, a particle exclusion assay (PEA) was performed.[12] Cells were cultured in 48 well plates, then treated with either sodium acetate in phenol red-free MEM+0.1% BSA or Streptomyces hyaluronidase to degrade HA (20 min. at 37° C). Medium was removed and

the cells were incubated with 2mg/ml aggrecan in MEM/0.1% BSA for 90 min. at 37° C. The aggrecan solution was removed and 1×10^8 glutaraldehyde-fixed sheep red blood cells (RBCs; Accurate Chemical and Scientific Corp., Westbury, NY) in PBS/1% BSA was added, allowed to settle for 15 minutes, and viewed and photographed using phase-contrast microscopy at 400X magnification. The HA matrix appeared as a halo surrounding each cell from which the RBCs were excluded. The absence of the halo around cells pretreated with hyaluronidase demonstrated that the halos consisted of HA. To quantify matrix retention, outlines of matrices and cellular boundaries from 20 randomly selected representative cells were traced and relative areas calculated using NIH ImageJ software (Bethesda, MD). HA matrix thickness was reported as the ratio of matrix area to cell area (matrix: cell ratio = 1 indicates no matrix).

Colon Cancer Cell Growth *In Vivo*

Male BALB/c nude mice (National Cancer Institute, Bethesda, MD) were housed in laminar air-flow cabinets and all procedures were performed aseptically in a laminar air-flow hood with approval of and according to Animal Care and Use Committee guidelines.

Tumors cells (7×10^6) were suspended in serum free medium, and injected subcutaneously into the flanks of the mice (one injection per mouse; HAS3 silenced group N = 24, HAS3 scrambled group N= 16). Tumor diameters were measured with digital calipers and volumes calculated. The animals were euthanized by CO₂ inhalation after 30 days. Tumors were dissected free of surrounding tissues, measured, and weighed.

To assess whether proliferation and/or apoptosis were affected by HAS3 inhibition, immunohistochemistry was used to detect either ki-67 (proliferation) or caspase-3 (apoptosis). Tumors from the HCT116 cell line (N= 24 in the HAS3 silenced group, N= 16 in the HAS3 scrambled group) and the previously harvested tumors from the SW620 cell line (N= 20 in the HAS3 silenced group, N=17 in the HAS3 scrambled group) were studied [13]. Tumor tissue was fixed in 10% buffered formalin and processed using routine pathologic techniques. Slides were deparaffinized in Xylene and rehydrated using graded alcohols. Endogenous peroxidase was quenched with aqueous 3% H₂O₂ for 10 minutes. Antigen retrieval was performed in the microwave for 10 min. in pH 6.0 citrate buffer. Slides were then loaded on the DAKO autostainer and the following program used for staining: Casein 0.03% in PBS/T block for 30 minutes, followed by incubation with primary antibody for one hour (ki-67 Lab Vision rabbit monoclonal 1μg/ml or caspase-3 Cell Signaling #9661 rabbit polyclonal 0.33μg/ml). An isotype-matched control (0.33 μg/ml rabbit IgG) was used as a negative control. A wash was followed by incubation with goat anti-rabbit biotinylated secondary antibody and ABC reagent (Vector Universal kit). DAB chromagen was applied for 5 minutes and the slides counterstained with hematoxylin. Assessment was performed by a pathologist who was blinded to the study condition (CL). Proliferation was assessed in the ki-67 stained slides by a standard pathologic grading system for staining intensity; slides were graded from 1 (low) to 4 (high). Apoptotic index was calculated based upon caspase-3 staining; apoptotic index was defined as the ratio of positively stained cells (apoptotic) to total cells.

Histologic Analysis & HA Staining

Tumors were fixed in 10% buffered formalin, paraffin embedded, sectioned at 4μm intervals, and stained with hematoxylin and eosin for routine histologic analysis. To assess the presence of HA in the tumors, sections were stained with colloidal iron, which binds HA, as previously described [15]. To confirm the specificity of staining, a second set of slides were pretreated with hyaluronidase to digest HA. Briefly, slides were incubated at 37°C for 1 hour in either buffer solution (pH 6.0; 0.1 M monobasic potassium phosphate,

and 0.1M dibasic sodium phosphate) or hyaluronidase digestion solution (4 mg/ml testicular hyaluronidase + buffer solution; Sigma Aldrich, St. Louis, MS). Slides were rinsed in tap water for 5 minutes and then in 12% acetic acid for 3 minutes, then incubated in colloidal iron solution (20mL Mullers Colloidal Iron solution, 15mL ddH₂O, 5mL glacial acetic acid; Poly Scientific R&D Corp., Bay Shore, NY) for 1 hour. Samples were then rinsed four times in 12% acetic acid solution, and placed in hydrochloric acid/potassium ferrocyanide solution (50mL 5% potassium ferrocyanide, 50ml 5% hydrochloric acid) for 20 minutes. The difference in intensity of staining between non-digested and hyaluronidase digested sections represented HA within the tumors.

All experiments were performed at least in triplicate. Statistical analysis was performed using GraphPad Prism version 5.00. The results were compared using Student's t-test and ANOVA where appropriate.

RESULTS

siRNA Transfection Inhibits HAS3 Expression

Our previous work demonstrated that HAS3 inhibition decreased cellular growth *in vitro* in two closely related colon cancer cell lines, SW480 (isolated from a primary tumor) and SW620 (isolated from a lymph node metastasis in the same patient).[4] Subsequently, we have shown that inhibition of HAS3 in SW620 cells decreases subcutaneous tumor growth in mice [13]. However, this effect has not yet been shown in colon cancer cells that are unrelated to SW480 and SW620, nor has a mechanism been fully described. We therefore set out to determine if HAS3 inhibition in highly tumorigenic HCT116 cells would decrease growth and affect proliferation and/or apoptosis. We silenced HAS3 using an siRNA transfection strategy. Unmanipulated (control) HCT-116 cells expressed high levels of HAS3 mRNA (data not shown). Similarly, control cells transfected with a scrambled sequence of the HAS3 siRNA expressed high levels of HAS3. Inhibition of HAS3 in these cells decreased HAS3 band intensity by 70% Fig. (1). Western blot analysis confirmed that the decrease in HAS3 mRNA translated into a decrease in HAS3 protein in whole cell lysates Fig. (2A) and decreased HAS3 immunocytochemical staining in cultured cells Fig. (2B).

siRNA Transfection Inhibits HA Production

A particle exclusion assay was used to confirm that inhibition of HAS3 expression resulted in decreased pericellular HA retention. The HAS3 silenced cells had smaller pericellular HA matrices than did the HAS3 scrambled cells mean matrix to cell ratio 1.160+/-0.1022 vs. 1.409+/-0.2516, $p < 0.0001$; Fig. (3). These data confirmed that our siRNA HAS3 silencing strategy successfully inhibited HAS3 expression and HA production.

Inhibition of HAS3 Expression Decreases *In Vivo* Tumor Growth

After stable transfection with either HAS3 siRNA or the HAS3 scrambled sequence, cells were injected into the flanks of nude mice. Individual mouse weights were not recorded, but there was no qualitative difference in size between the mice bearing the HAS3 silenced tumors and controls. The HAS3 silenced cells produced significantly smaller tumors than did the HAS3 scrambled cells. By day 10, there was a statistically significant difference in tumor volume between the two cell lines Fig. (4A). Final mean tumor mass was significantly lower in the HAS3 silenced group compared to the HAS3 scrambled group 0.62+/-0.37 g vs. 0.93+/-0.53 g, $p = 0.014$; Fig. (4B), as was mean tumor volume 588+/-276 mm³ vs. 1199+/-543 mm³ $p = 0.006$; Fig. (4C). Qualitatively, HAS3 silenced tumors stained less intensely for HA than did HAS3 scrambled tumors Fig. (5). No attempt was made to quantify differences in HA staining.

Inhibition of HAS3 Expression Increases Apoptosis

Inhibition of tumor growth may result from decreased proliferation, increased apoptosis, or some combination of these two processes, among others. We therefore sought to determine if either of these processes was affected by HAS3 inhibition in both the HCT116 derived tumors and in the previously harvested SW620 derived tumors. In the HCT116 derived tumors, proliferation was unaffected by HAS3 silencing Fig. (6A); however, in the SW620 derived tumors, HAS3 silencing resulted in a slight increase in proliferation Fig. (6B). Apoptosis, on the other hand, was more dramatically affected. HAS3 silencing increased apoptotic indices in the HCT116 derived tumors by approximately 25% and increased apoptotic indices in the SW620 derived tumors by greater than 50% Fig. (6C). Taken together, these data suggest that inhibition of HAS3 decreases colon cancer growth *in vivo* primarily by increasing apoptosis.

DISCUSSION

HA and its biosynthetic enzymes, hyaluronan synthases (HAS) are ubiquitous in normal human tissues and play critical roles in tissues undergoing remodeling and rapid cellular turnover. In cancer, however, these properties may promote cancer cell growth, invasion and metastasis. HA increasingly has been implicated in the growth and progression of a variety of malignancies including colon cancer.[10] Similarly, differential expression of HAS isozymes also affects tumor growth in several cancers [3, 16, 17]. Our previous work has demonstrated that HAS3 in particular may be critical for colon cancer progression. We found that HAS3 was upregulated in metastatic SW620 cells compared to primary SW480 cells, and that HA and HAS3 mediate anchorage independent growth, invasion of artificial basement membrane, and adhesion to laminin [4–6]. We subsequently demonstrated that inhibition of HAS3 in SW620 cells reliably decreased subcutaneous tumor growth in a mouse model [12]. These observations suggested that HA and HAS3 mediate important processes that promote colon cancer cell growth, and as such, may represent new diagnostic and/or therapeutic targets. The mechanism by which HA and HAS3 promote tumor growth, however, is incompletely understood.

HCT116 is a highly tumorigenic human colon cancer cell line that expresses high levels of HAS3. Ghatak, *et al.*, have shown that HA mediates HCT116 anchorage independent growth *in vitro* [18], therefore we sought to determine if inhibition of HAS3 in these cells would decrease tumor growth *in vivo*. Moreover, we sought to elucidate the mechanism underlying HA and HAS3 mediated growth in these tumors. Increased tumor growth may result from increased cellular proliferation or decreased apoptosis, among other processes. Our previous work suggested that HAS3 inhibition did not affect either viability or proliferation *in vitro* [4]; therefore, in addition to proliferation, we studied apoptosis by comparing caspase-3 staining. Inhibition of HAS3 in HCT116 cells successfully inhibited HAS3 expression and HA production, and resulted in a decrease in subcutaneous tumor growth by nearly 50%. Moreover, the HAS3 silenced tumors demonstrated a marked increase in apoptotic index without any appreciable effect on proliferation.

Like the HCT116 derived tumors, the previously harvested SW620 tumors also demonstrated a significant increase in apoptosis in the HAS3 silenced tumors, but the magnitude of change was much greater in the SW620 cell line. In addition, in contrast to our previous *in vitro* work, the SW620 derived tumors demonstrated a slight, but statistically significant, increase in proliferation. Although these results seem contradictory, the balance between proliferation and apoptosis is likely to determine the overall effect on tumor growth. It is possible, therefore, that the greater than 50% increase in apoptotic index in these tumors was abrogated to some degree by the increase in proliferation. Nevertheless, the overall effect of HAS3 inhibition was a decrease in tumor weight and volume[13],

suggesting that the proapoptotic effect was of greater biologic importance than the proliferative effect. Finally, because apoptotic indices were very low in all tumors (5% or less), small changes in the percent of apoptotic cells may have resulted in significant biologic differences. These data suggest that HAS3 and HA mediate tumor growth *in vivo* in two unrelated colon cancer cell lines and do so primarily by inhibiting apoptosis. Nevertheless, the association between apoptosis and proliferation is likely to be complex, and our ongoing work is focused on better delineating the relationship between HAS3, HA and these processes.

HA and HAS have been implicated in both cellular proliferation and in apoptosis, and effects have been variably reported. Although we have demonstrated that inhibition of HAS3 results in increased apoptosis, the effects of HA and HAS inhibition on this process are inconsistent. HA previously has been shown to inhibit apoptosis in human chondrocytes [19], epithelial corneal cells [20], prostate cancer cells [21], and breast cancer cells [22]. However, inhibition of HAS in melanoma cells by 4-Methylumbelliferone (4-MU) affects proliferation, but not apoptosis. [23] In contrast, Lokeshwar and colleagues report that 4-MU increases apoptosis in prostate cancer cells [21]. Proliferation data are also variable. Our data suggests that while HAS3 silencing does not affect proliferation in HCT116 cells, it does increase proliferation in SW620 cells. Torre, *et al.*, have similarly shown that exogenous HA increases proliferation and prevents chemotherapy-induced cell death in head and neck cancer cells [24]. In contrast, inhibition of HAS2 and/or HAS3 by either shRNA or 4-MU decreases proliferation in esophageal squamous cell carcinoma cells [25]. In addition, the contribution of other HAS isozymes is variable. HAS1, for example, seems to play a primary role in embryogenesis, but is not expressed in either SW620 or HCT116 colon cancer cells. Similarly, HAS2, though present, is found in colon cancer cells to a lesser extent than HAS3, and although HAS2 inhibition impedes *in vitro* cancer growth, this effect is less significant than that seen with HAS3 inhibition [5]. Clearly, the cellular effects of HA and HAS are complex and the effect on cancer growth and progression will likely depend upon the balance between proapoptotic and proliferative forces.

The downstream mechanism(s) by which HA and HAS3 affect apoptosis are not well defined, however interaction with any of several cell surface HA receptors is likely to play a role. In addition to being retained at the cell surface by the HAS isozymes, HA binds to and activates several protein receptors. HA binds to CD44 [26], the receptor for hyaluronan mediated motility (RHAMM) [27], lymphatic vessel endothelial receptor 1 [28], layilin [29], toll-like receptors 2 and 4 [30], the HA receptor for endocytosis (HARE) [31], and stabilin [32]. Of these, CD44 and RHAMM are the best studied and best described in cancer. CD44 has long been recognized in human cancers. Overexpression of CD44 and several of its splice variants has been detected in colorectal adenomas, carcinomas, and in advanced colon cancer [33, 34]. Increasingly, RHAMM also has been implicated in cancer growth and progression, and overexpression of RHAMM predicts poor prognosis in colon and rectal cancers [35–37]. Finally, co-expression of both CD44 and RHAMM recently has been reported to predict poor prognosis in diffuse large B-cell lymphomas and there is increasing evidence that these two receptors may function synergistically [38]. These lines of evidence suggest that HA interaction with CD44, RHAMM, or both is likely to mediate the tumorigenic effects of this molecule.

We previously have shown that HA mediates invasion through artificial basement membrane (Matrigel) *via* interaction with CD44 in SW620 cells [5]. Similarly, HCT116 cells are known to express CD44 and disruption of the HA-CD44 interaction inhibits cell growth [18]. CD44 activation is linked to downstream signaling cascades including the RAF/MEK/MAPK, PI3K/AKT, Rho kinase, and FAK pathways that are known to affect apoptosis, cell survival and proliferation [24–26]. HA-CD44 interaction has been shown to

decrease apoptosis in breast tumor cells [22] and human myeloid cells [39]. Misra and colleagues have further demonstrated that HA-CD44 interactions mediate cell survival and proliferation *via* cyclooxygenase-2 activation and that inhibition of COX-2 activity induces caspase-3 activity [40]. Interestingly, inhibition of CD44 expression in HT-29 colon cancer cells has been shown to increase susceptibility to etoposide-induced apoptosis, further supporting the contention that HA-CD44 interaction may decrease apoptosis [41]. Therefore, it is possible that HA-CD44 interaction is responsible for inhibiting apoptosis in HCT116 and SW620 colon cancer cells. Our previous work suggested that HA-CD44 interaction was critical for invasion, and experiments are underway to assess whether this interaction underlies tumor growth *in vivo*.

HCT116 cells also express RHAMM [42] and we have detected RHAMM mRNA in SW620 cells (unpublished data). Like CD44, this receptor has been linked to a variety of cellular processes including apoptosis and proliferation. Recently, RHAMM has been shown to influence cell motility and mitosis by activating the MEK1/ERK1 and 2 pathway [43–45]. RHAMM also activates FAK and supports proliferation and migration of esophageal cancer cells [25]. Therefore, it is equally possible that the effect of HA on apoptosis is mediated *via* RHAMM activation with or without CD44 activation.

In addition to interactions with CD44 and RHAMM, the biologic effects of HA and HAS3 depend upon a variety of factors. For example, the differential effect of HA on different tumor types may also be influenced by the length of the HA polymer. We have shown that 54kD exogenous HA restores anchorage independent growth of colon cancer cells *in vitro* while 220kD HA does not [4, 18]. In addition, although an increase in HA production in a variety of cancer cell lines has been shown to increase growth, small HA oligosaccharides inhibit cancer cell growth [18]. Recently, Alaniz and colleagues demonstrated that low molecular weight HA inhibits tumor cell proliferation by decreasing proliferation [46]. In contrast, high molecular weight HA appears to decrease apoptosis in a human epithelial cell line [20]. Sheehan, *et al.*, have shown that high molecular weight HA induces apoptosis and decreases proliferation in macrophages, a result similar to our observation in the HCT116 cells [47]. Because HAS isozymes synthesize and retain different length polymers [48], it is possible that our selective inhibition of HAS3 resulted in a decrease in a specific HA polymer that inhibits apoptosis. Further investigation will be necessary to determine the mechanism underlying this activity.

Similarly, whether HA is freely secreted or retained at the cell surface may impact its biological activity. Inhibition of cellular production consistently has been shown to decrease tumor cell growth by our group and others, however the addition of exogenous HA has a more variable effect. While exogenous HA increases anchorage independent growth by colon cancer cells *in vitro*, the addition of HA in the form of a hyaluronan carboxymethylcellulose membrane decreased intraperitoneal tumor growth *in vivo* [49]. Interestingly, the combination of hyaluronan and irinotecan has been shown to increase progression free survival in patients with metastatic colorectal cancer in a randomized phase II trial [50]. Whether these differential effects result from differences in receptor binding, polymer length and/or degradation, or some other process remains to be determined.

In summary, we have shown that inhibition of HAS3 in HCT116 human colon cancer cells decreases HA production and significantly decreases tumor growth *in vivo*. We have further demonstrated that HAS3 inhibition in both HCT116 derived tumors and SW620 derived tumors increases apoptosis. These data support a model of tumor progression in which HA and HAS3 mediate colon cancer growth and metastasis by inhibiting apoptosis. As such, HAS3 and HA may provide potential targets for diagnosis and treatment of patients with colorectal cancer.

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Fig. (1).

HAS3 inhibition by siRNA transfection decreased HAS3 expression in HCT116 cells. **A.** HAS3 expression was detected in transfected cells by semi-quantitative RT-PCR. Poly(A)+ RNA was isolated from subconfluent cells and quantified by Ribogreen fluorescence. Each mRNA template (50 ng) was reverse transcribed with an oligo(dT) primer using the Superscript II first strand cDNA synthesis kit. A PCR oligonucleotide specific for HAS3 was used as previously described and amplified using the cycling conditions described in "Materials and Methods" [3]. GAPDH was amplified with every reaction to standardize conditions. **B.** HAS3 band intensity was decreased by approximately 70% by HAS3 silencing. Band intensity was calculated by comparing the HAS3 bands to the corresponding GAPDH band.

Fig. (2).

A. HAS3 inhibition decreased HAS3 protein production in HCT116 cells. Both HCT116 control and HAS3 scrambled cell lysates demonstrated intense HAS3 protein bands, whereas the HAS3 band was undetectable in the HAS3 silenced lysates. HAS3 protein was detected by Western blot analysis. Subconfluent cells were and incubated in lysis buffer for 60 minutes on ice. Protein samples (20ug) were separated by electrophoresis and transferred Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked overnight, incubated with primary antibody, washed, incubated with a peroxidase-conjugated secondary antibody, washed, and developed with a chemiluminescent detection kit. **B.** HAS3 inhibition decreased HAS3 protein detection by

immunocytochemistry in cell culture. HAS3 (red fluorescence) was easily detectable in both the HCT116 control cells and the HAS3 scrambled cells, but was minimally detectable in the HAS3 silenced cells. Beta-actin control (green fluorescence) was similar among all cell lines. Cells in 24-well plates were fixed and then permeabilized, blocked, incubated with primary antibody, washed, incubated with secondary antibody and FITC conjugated phalloidin, then viewed using a Zeiss Observer. A1 microscope at 100× magnification.

Fig. (3).

Inhibition of HAS3 decreased pericellular HA retention detected by a particle exclusion assay. Matrix thickness (matrix to cell ratio) was smaller in the HAS3 silenced cells than in the HAS3 scrambled or HCT116 control cells (* $p < 0.0001$). Cells were cultured under normal conditions, then incubated with aggrecan to bind the pericellular HA matrix. The aggrecan was removed and the cells were overlayed with glutaraldehyde-fixed sheep RBCs. Pericellular haloes from which the red blood cells were excluded represented HA matrices. The absence of this halo in cells pretreated with hyaluronidase proved that the coats were comprised of HA (data not shown). To quantify matrix retention, outlines of matrices and cellular boundaries were traced and relative areas compared to generate a matrix to cell ratio (matrix:cell ratio = 1 indicates no matrix).

Fig. (4).

Inhibition of HAS3 decreased subcutaneous tumor growth in mice. Tumors cells (7×10^6) were injected subcutaneously into the flanks of the nude mice (HAS3 silenced N = 24, HAS3 scrambled N= 16). Tumor diameters were measured with digital calipers and volumes calculated. After sacrifice, the tumors were measured and weighed. **A.** After 10 days, tumor volumes were significantly decreased in the HAS3 silenced tumors and this difference persisted for the length of the experiment (* $p < 0.05$). **B.** Tumor mass was decreased by approximately 30% ($p = 0.0136$), and **C.** tumor volume was decreased by greater than 50% ($p = 0.0063$).

Fig. (5).

Tumors grown from HAS3 silenced cells stain less intensely with colloidal iron than do tumors grown with HAS3 scrambled (control) cells. Digestion with hyaluronidase decreased intensity of staining in both sets of tumors, but the qualitative difference was greater in the HAS3 scrambled tumors compared to the HAS3 silenced tumors. This observation confirms that HAS3 inhibition resulted in decreased tumoral HA *in vivo*. (A) HAS3 silenced tumor stained with colloidal iron (blue pigment represents HA); (B) HAS3 silenced tumor pretreated with hyaluronidase then stained with colloidal iron; (C) HAS3 scrambled stained with colloidal iron; (D) HAS3 scrambled tumor pretreated with hyaluronidase then stained with colloidal iron.

Fig. (6).

Inhibition of HAS3 in both HCT 116 and SW620 derived tumors increased apoptosis. Apoptosis was assessed using caspase-3 immunostaining and proliferation assessed using ki-67 immunostaining. Slides were deparaffinized, endogenous peroxidase quenched, and antigen retrieval performed by microwave heating. Slides were then incubated with the appropriate primary antibody, washed, incubated with a biotinylated secondary antibody, and developed using an ABC reagent and DAB chromagen. An isotype matched primary antibody was used as a control. **A.** HCT116 derived tumors display greater caspase-3 staining (brown pigment) in the HAS3 silenced tumors than in the HAS3 scrambled tumors. Ki-67 staining (brown pigment) is similar in both groups. **B.** Proliferation was quantified by

assessing ki-67 staining intensity (low = 1; high = 4). HAS3 inhibition had no effect on proliferation in the HCT116 derived tumors, but slightly increased proliferation in the SW620 derived tumors ($p=0.0255$). **C.** Apoptotic index was calculated by comparing total apoptotic cells to total cells. Apoptotic index was significantly increased by HAS3 inhibition in both sets of tumors (* $p<0.0001$; ** $p=0.0252$). Isotype matched controls for both ki-67 and caspase-3 staining showed no staining (data not shown).