

Hyaluronan Facilitates Invasion of Colon Carcinoma Cells *in Vitro* via Interaction with CD44

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ABSTRACT

Hyaluronan (HA) and its biosynthetic enzymes, HA synthases (HAS1, 2, and 3) are thought to participate in cancer progression. We have shown previously that HA production and HAS3 expression are increased in metastatic colon carcinoma cells (SW620) when compared with cells isolated from a primary tumor (SW480). Because invasion of the extracellular matrix is a fundamental event in tumor growth and metastasis, we hypothesized that SW620 cells would show greater invasive capability than SW480 cells, that invasion is HA dependent, and that HA mediates invasion via interaction with a cell-surface receptor. Invasion into artificial basement membrane (Matrigel) was assessed *in vitro*. To assess HA functionality, HAS expression was inhibited in SW620 cells by transfection with antisense HAS constructs. Decreased HA secretion and retention in the transfectants were confirmed using competitive binding and particle exclusion assays. SW620 cells demonstrated greater invasion through Matrigel than did SW480 cells. Antisense transfection decreased Matrigel invasion by SW620 cells by >60%; addition of exogenous HA restored invasion. Because the cell-surface HA receptor CD44 has been implicated in cancer progression, HA-CD44 interaction was then inhibited by incubation with an anti-CD44 antibody. Anti-CD44 antibody impaired invasion into Matrigel by 95%. Taken together, these data suggest that pericellular HA is critical for colon carcinoma cell invasion and that this invasive capability is dependent on interaction with CD44.

INTRODUCTION

Hyaluronan (HA) is a high molecular weight cell-surface polysaccharide that has been implicated as a critical factor in invasion and metastasis of several malignancies including colon cancer (1–7). In normal tissues, HA provides an environment that facilitates the cellular proliferation and migration necessary for such processes as embryonic development and wound healing (8, 9). In cancer, however, HA may facilitate tumor progression by enhancing invasion, growth, angiogenesis, and metastasis (8, 10). HA is synthesized by three transmembrane HA synthases: HAS1, HAS2, and HAS3 (11–14). Differential expression of HAS isozymes has been shown to impact tumor growth in fibrosarcoma (15) and murine mammary carcinoma (16), and overexpression of HAS in prostate cancer cells increases tumor cell growth both *in vitro* and *in vivo* (17, 18). Our previous work has implicated HA and HAS as critical factors in the growth of colon carcinoma. Metastatic colon carcinoma cells (SW620) secrete and retain greater amounts of pericellular HA than cells from a primary tumor (SW480), and this increase results from up-regulation of HAS3. Inhibition of HAS3 impairs anchorage-inde-

pendent growth, a corollary of tumor growth *in vivo*. Addition of exogenous HA restores growth. These data are consistent with a model of tumor growth in which HAS3 expression, HA synthesis, and pericellular HA retention are crucial and suggest a role for HA and HAS in colon carcinoma progression (19).

To metastasize, cancer cells must not only grow, but must also invade the basement membrane and underlying collagen matrix, migrate into the lymphovascular system, adhere to endothelium and parenchyma of the target organ, and multiply to create a tumor. Invasion through the basement membrane represents one of the first steps in this metastatic process. Therefore, we hypothesized that metastatic SW620 cells would demonstrate greater invasive capability than SW480 cells and that this capability would be mediated by pericellular HA. *In vitro* models of tumor cell invasion use matrix components to simulate the extracellular environment. Matrigel, which is secreted by Engelbreth-Holm-Swarm sarcoma cells, is a complex mixture of basement membrane components. Matrigel is composed of laminin and type IV collagen, along with proteoglycans and a variety of growth factors, mimicking the naturally occurring basement membrane (20–24). Three-dimensional gels composed of Matrigel provide an easily manipulated system in which to investigate tumor cell invasion.

HA has been implicated in cancer cell invasion of the extracellular matrix (25, 26). In the studies presented herein, we demonstrate that metastatic colon carcinoma cells that secrete and retain large amounts of pericellular HA (SW620) invade Matrigel more avidly than cells from a primary tumor (SW480) that secrete and retain less HA. Because the HA synthases have also been implicated in cancer progression (15–18), HAS expression was then inhibited in SW620 cells by transfection with antisense HAS constructs to determine whether inhibition of HAS would impact invasive capability. Transfection with antisense HAS2 (asHAS2), asHAS3, or both, asHAS2/3, decreased pericellular HA secretion and retention. Stable antisense transfectants demonstrated limited invasion into Matrigel, and addition of exogenous HA restored invasion, suggesting that pericellular HA enhances cellular invasion. These data suggest that HAS expression and production of a pericellular HA matrix are crucial for colon carcinoma cell invasion of the basement membrane.

The mechanism by which pericellular HA affects invasion and metastasis remains undefined. Cell-surface receptors are likely to participate in these processes, and several HA receptors have been described. HA is known to bind to CD44 (27), receptor for HA-mediated motility (27, 28), lymphatic vessel endothelial receptor 1 (29), layilin (30), toll-like receptor-4 (31), HA receptor for endocytosis (32), and stabilin (33, 34). Of these, CD44 has been implicated most often in tumor growth (27, 35). We demonstrated that SW620 cells express CD44 and therefore hypothesized that CD44-HA interaction mediates invasion. To test this, HA binding was inhibited by incubating SW620 cells with a CD44 blocking antibody. Incubation with anti-CD44 decreased Matrigel invasion in a dose-dependent manner. Taken together, these data are consistent with a model in which basement membrane invasion by colon carcinoma cells is

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dependent on the production of a pericellular HA matrix, and invasive capability is mediated via HA-CD44 interaction.

MATERIALS AND METHODS

Cell Culture and Reagents. SW480 and SW620 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM +10% FBS (37°C, 5% CO₂). Cells used for all experiments ranged from passage 25 to 30.

Plasmid Construction. Antisense HAS2 and HAS3 constructs were synthesized as described previously (17, 18). HAS2 or HAS3 cDNA was used as the template to amplify the first 300 bases of each isoform sequence, which was then subcloned in the antisense orientation into pIRES2-EGFP (Clontech, Palo Alto, CA). For double antisense HAS inhibition, antisense HAS2 was expressed as a bicistronic message with green fluorescent protein (pIRES2-EGFP vector) and antisense HAS3 with hygromycin resistance (pIRES-Hyg2).

Transfection of Colon Cancer Cells. Cells were transfected using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Each cell line was cotransfected with two plasmids, including pIRES2-EGFP and pIRES-Hyg2 for the vector control, pIRES2-EGFP-HAS2 or pIRES2-EGFP-HAS3 with pIRES-Hyg2 for HAS2 and HAS3 single antisense cells, respectively, and pIRES2-EGFP-HAS2 and pIRES-Hyg2-HAS3 for HAS2 and HAS3 double antisense cells. Cells were selected through multiple rounds of fluorescence-activated cell sorting and antibiotic selection in 1.0 mg/ml G418 (Invitrogen) and 0.8 mg/ml hygromycin (Sigma, St. Louis, MO) until the final cell populations were 55%–98% positive for green fluorescent protein expression. Subsequently, stable transfectants were maintained in medium supplemented with 1.0 mg/ml G418 and 0.8 mg/ml hygromycin (17, 18).

Cell Viability. To assess cellular viability, stable transfectants and untransfected SW620 cells were seeded in a 24-well plate (10,000 cells/well) and incubated at 37°C. Viability was assessed every 24 h for 7 days. The cells were released with trypsin-EDTA and viability was determined with trypan blue using a ViCell Counter (Beckman Coulter, Fullerton, CA).

Particle Exclusion Assay. Pericellular HA matrices were visualized using a particle exclusion assay as described previously (19, 36, 37). To determine whether the addition of exogenous HA was capable of reconstituting the pericellular HA matrix, cells (SW620, vector control, and aS_{HAS2/3} cells) were incubated with 10 µg/ml HA in MEM/0.1% BSA for 30 min at 37°C and the assay repeated. In the particle exclusion assay, the HA matrix appeared as a halo surrounding each cell when viewed using phase contrast microscopy. The absence of the halo around cells pretreated with *Streptomyces* hyaluronidase (CalBiochem, San Diego, CA) demonstrated that the halos were created by HA. Representative cells were photographed at ×400 magnification. To quantify matrix retention, outlines of matrices (haloes) and cellular boundaries from 20 randomly selected individual cells of each type were traced, and relative areas were calculated using NIH Image software (Bethesda, MD). Because cell size varied, HA matrix thickness was reported as the ratio of matrix area to cell area for each cell type; a ratio of one indicates complete absence of a pericellular matrix.

Quantitation of HA Secretion. HA concentrations in cell culture supernatants were determined using a competitive binding assay as described previously (19, 38). The mean HA concentration for each sample was calculated and normalized to the cell number (ng HA/10⁴ cells). Six wells per cell line were assayed.

Invasion Assay. Invasion assays were performed according to the method described by Albini *et al.* (21), using 12-mm Transwell chambers (Costar, Cambridge, MA) with porous filters (pore size 12 µm). Matrigel was diluted in serum-free medium (4 mg/ml), added to the filters (200 µl/well), and allowed to gel (4 h, 37°C). Cells (20,000 cells/well) were then seeded onto the reconstituted membrane of the upper compartment of each chamber. The lower compartment was filled with 1.0 ml of medium containing 10 µg/ml type I collagen (bovine dermal collagen; Cohesion, Palo Alto, CA) as an adhesive substrate. The chambers were then incubated for 48 h at 37°C. The filters were then removed and stained with Diff-Quick solution. Cells that migrated to the lower surface of the filter were considered to have invaded through the overlying matrix and were counted using light microscopy (×200 magnification; 30 random fields/filter). Invasion was expressed as percentage of seeded

cells. To determine whether exogenous HA would increase invasion, Matrigel was prepared with or without HA (1 mg/ml; 54 kD or 220 kD HA; Lifecore Biomedical, Chaska, MN) or the control molecule chondroitin sulfate A (1 mg/ml; bovine trachea; Sigma). The purity of the HA was determined by the manufacturer, and no contaminants, proteins, microbes, or endotoxin were detected (aerobic plate count <10 cfu/g; protein < 0.01%; endotoxin < 0.04 EU/ml). Invasion was assessed as described above.

Detection of CD44. CD44 expression by SW620 cells, SW480 cells, and stable transfectants was characterized by reverse transcription-PCR. Polyadenylated RNA was isolated from subconfluent cells using the Oligotex mRNA isolation kit (Qiagen, Valencia, CA) and quantified by Ribogreen fluorescence (Molecular Probes, Eugene, OR). Each mRNA template (50 ng) was reverse transcribed with an oligodeoxythymidylic acid primer using the Superscript II first-strand cDNA synthesis kit (Invitrogen; Ref. 19). Oligonucleotides specific for CD44 were designed from the sequences database.⁴ Glyceraldehyde-3-phosphate dehydrogenase was amplified with every reaction to standardize conditions. Cycling conditions were as follows: initial denaturation for 2 min at 94°C; 30 cycles of 30-s denaturation at 94°C, 30-s annealing at 60°C, and 30 s of polymerization at 72°C; and 5 min final extension at 72°C. Reaction products were fractionated on a 2% agarose gel and stained with ethidium bromide.

To determine whether CD44 protein was present in the cells, Western blot analysis was performed on whole cell lysates. Subconfluent cells were released with 3 mM EDTA/PBS. One × 10⁷ cells were then lysed by incubation in lysis buffer (25 mM Tris-HCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, and protease inhibitors leupeptin 1 µg/ml, phenylmethylsulfonyl fluoride 30 mM, and trypsin inhibitor 10 µg/ml) for 60 min on ice. Protein samples (20 µg) were separated by electrophoresis and transferred onto Immobilon-P membrane (Millipore). The membrane was blocked overnight at room temperature with 5% milk in Tris-buffered saline. Membranes were then incubated for 1 h with primary antibody (anti-CD44s antibody and rat antimouse antibody IM7; BD Biosciences, San Diego, CA), washed in Tris-buffered saline, then incubated with a peroxidase-conjugated goat antirabbit secondary antibody (Jackson Immunoresearch, West Grove, PA) + 3% BSA in Tris-buffered saline, washed, and developed with a chemiluminescence detection kit (Amersham).

CD44 Receptor Blocking. The anti-CD44 antibody used in these experiments, rat antimouse antibody IM7 (BD Biosciences), has been shown to cross-react with human CD44 (38, 39) and to block HA-CD44 interaction (40). To confirm that this anti-CD44 antibody inhibited HA-CD44 interaction, adhesion to HA-coated wells was assessed in the presence of the blocking anti-CD44 antibody *versus* an isotype matched antibody (IgG 2b; Cymbus Biotechnology, Hants, United Kingdom) or a nonblocking anti-CD44 antibody (monoclonal antihuman CD44; R&D Systems, Minneapolis, MN). We coated 96-well plates (Costar) with 100 µl of a 100 µg/ml solution of HA (220 kD) and incubated overnight at 37°C. The plates were then gently washed and incubated with 200 µl of 2% BSA in PBS (2 h; 37°C). Cells were incubated with 10 µg/ml of blocking anti-CD44 antibody, nonblocking antiCD44 antibody, IgG, or no antibody for 30 min at 4°C. Cells were added to the HA-coated plates (100,000 cells/well) and incubated for 1 h at 37°C. The plates were then washed five times with 100 µl DMEM and 20 µl of CellTiter 96 Aqueous One Solution added (Promega, Madison, WI). After 3 h, the absorbance was read at 490 nm using a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). A standard curve was determined for each cell line and number of cells per experimental well determined.

To assess whether cellular invasion was dependent on HA-CD44 interaction, HA binding to CD44 was inhibited by preincubating cells with the blocking anti-CD44 antibody, nonblocking anti-CD44 antibody, or isotype-matched control IgG at concentrations of 0, 5, 10, and 20 µg/ml (30 min at 4°C) before seeding on Matrigel. Invasion assays were then repeated as described above. To determine whether anti-CD44 antibody was capable of inhibiting invasion in the presence of HA supplementation, invasion assays were repeated with and without HA supplementation of the Matrigel (1 mg/ml; 54kD HA), in the presence of blocking anti-CD44 antibody, nonblocking anti-CD44 antibody, or IgG at concentrations of 20 µg/ml.

⁴J. McCarthy, personal communication.

Table 1 Inhibition of HAS2 and/or HAS3 expression in SW620 cells by antisense transfection reduces HA^a retention and secretion

Pericellular HA matrices were detected using a particle exclusion assay as described in "Materials and Methods" (matrix:cell ratio = 1 is equivalent to no matrix). HA secretion was detected in cell culture supernatants using a competitive binding assay as described in "Materials and Methods" and is expressed as percentage of secretion compared with Vector cells (Vector secretion of HA = 100%). Data are presented as the mean of three experiments \pm SE.

Cell line	Percent cells with HA matrices	Matrix:cell ratio	Percent HA secretion
Vector	75 \pm 8	2.4 \pm 0.2	100
asHAS2	57 \pm 7 ($P = 0.0353$ versus Vector)	1.6 \pm 0.4 ($P < 0.0001$ versus Vector)	33.1 \pm 2.5 ($P < 0.0001$ versus Vector)
asHAS3	46 \pm 9 ($P = 0.0595$ versus Vector)	1.6 \pm 0.1 ($P < 0.0001$ versus Vector)	29.2 \pm 2.2 ($P < 0.0001$ versus Vector)
asHAS2/3	53 \pm 10 ($P = 0.1500$ versus Vector)	1.5 \pm 0.2 ($P < 0.0001$ versus Vector)	31.7 \pm 1.8 ($P < 0.0001$ versus Vector)

^aHA, hyaluronan.

Statistical Analysis. Data were compared using Student's *t* test (two-tailed); $P < 0.05$ was considered statistically significant. All of the experiments were repeated in triplicate.

RESULTS

Transfection of SW620 Cells with Antisense HAS Constructs.

HA production and HAS expression have been implicated in cancer progression (5–7, 19, 41). Because HA production and HAS3 expression are increased in the metastatic SW620 cells (19), we sought to determine whether cellular invasion would be altered by inhibition of HAS in these cells. We have shown previously that SW620 cells express both HAS2 and HAS3 (19). Therefore, we used an antisense inhibition strategy in which SW620 cells were transfected with constructs for asHAS2, asHAS3, and asHAS2/3. Successful transfection was verified by detection of enhanced green fluorescent protein and selection based upon antibiotic resistance. Initial transfection efficiency was 25–35%; after selection, the final population of enhanced green fluorescent protein-positive cells was 55–98%. Cellular viability (determined by trypan blue exclusion) was equivalent in all of the cell lines (>70% viable cells).

Pericellular HA Secretion and Retention. We have shown previously that SW480 cells retain and secrete significantly less pericellular HA than SW620 cells and that transfection with an antisense HAS3 construct is capable of decreasing HA matrix production (19). To confirm that inhibition of HAS2 and/or HAS3 expression resulted in decreased HA production in these experiments, pericellular HA matrix retention was assessed in stable transfectants using a particle exclusion assay. Transfection of SW620 cells with antisense HAS2 and/or HAS3 inhibited pericellular HA matrix retention (Table 1). These data suggested that inhibition of either HAS2 or HAS3 is sufficient to decrease cell surface HA matrix assembly. Preincubation with HA reconstituted pericellular matrices in the antisense transfectants (matrix:cell ratio in asHAS2/3 cells with HA supplementation was 1.6 \pm 0.1 versus 1.3 \pm 0.04 in asHAS2/3 cells without HA supplementation; $P = 0.0005$) but did not affect the matrix in SW620 cells (matrix:cell ratio in SW620 cells with HA supplementation was 2.1 \pm 0.1 versus 2.0 \pm 0.1 in SW620 cells without HA supplementation; $P =$ not significant) or vector control cells (matrix:cell ratio in vector control cells with HA supplementation = 1.9 \pm 0.2 versus 1.8 \pm 0.1 in vector control cells without HA supplementation; $P =$ not significant).

HA secretion was then quantified using a competitive binding assay. Inhibition of HAS by transfection with antisense HAS2 and/or HAS3 decreased HA secretion by \sim 70% (Table 1). These data confirmed that our antisense strategy successfully created transfectants that retained and secreted less HA than SW620 cells or SW620 cells transfected with vector alone.

Matrigel Invasion. Reconstituted basement membrane (Matrigel) has been used to model the naturally occurring basement membrane to assess cellular invasion (22–26). Therefore, we hypothesized that metastatic SW620 cells would demonstrate greater invasion into Matrigel than SW480 cells. Invasion was 23% greater in the SW620 cells

compared with the SW480 cells, confirming that the metastatic cells are more invasive than cells isolated from a primary tumor (Fig. 1).

Invasive capability was then assessed in the stably transfected cell lines. Antisense transfectants demonstrated significantly decreased Matrigel invasion when compared with either SW620 cells or vector control cells (Fig. 1). Inhibition was greatest in cells transfected with asHAS3, although the difference in invasion did not reach statistical significance. To confirm that a deficit in the sustained presence of extracellular HA was the major cause of inhibited invasion in the antisense transfectants, invasion was then assessed in the presence or absence of HA supplementation of the Matrigel. Chondroitin sulfate A, which is similar to HA in polyanionic properties, was used as a control molecule. Addition of HA restored invasive capability in the all of the antisense transfectants (Fig. 2). HA also slightly increased invasion by SW620 and SW480 cells, although this increase was not statistically significant. Exogenous HA had minimal effect on vector control cells. Chondroitin sulfate A did not affect invasion of any cell line, confirming that the effect on antisense HAS-inhibited cells was caused by HA. These data suggest that pericellular HA is crucial for invasion of the basement membrane by colon carcinoma cells and that inhibition of HAS can inhibit basement membrane penetration.

HA polymer size has been shown to impact the biological affect on colon carcinoma cells. For this reason, both 54 kD and 220 kD HA were added to Matrigel to determine whether size affected invasion. 54 kD HA dramatically increased invasion by all of the antisense transfectants. We found that 220 kD HA also increased invasion, but results were more variable. (Fig. 3).

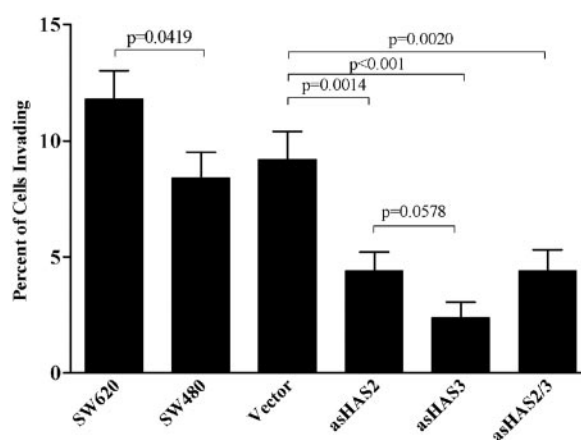


Fig. 1. SW620 cells are more invasive than SW480 cells. Inhibition of HAS2 and/or HAS3 expression in SW620 cells decreases Matrigel invasion. SW620 cells transfected with antisense HAS2 (asHAS2), HAS3 (asHAS3), or double antisense HAS2/HAS3 (asHAS2/3) constructs demonstrated >60% less invasion than SW620 cells or vector control cells. Invasion was assessed in Matrigel-coated transwell chambers as described in "Materials and Methods." Briefly, cells were seeded onto the Matrigel-coated membrane of the upper compartment of each chamber, and the lower compartment was filled with medium containing type I collagen as an adhesive substrate. The chambers were incubated for 48 h at 37°C. The filters were then removed and stained. Cells that migrated to the lower surface of the filter were considered to have invaded through the overlying matrix. Data are presented as the mean of three experiments; bars, \pm SE.

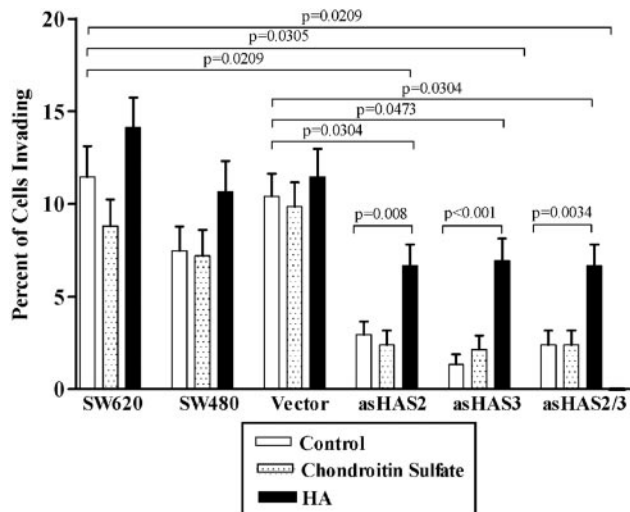


Fig. 2. Addition of exogenous hyaluronan (HA) restored invasion in the antisense transfectants. To determine whether exogenous HA would increase invasion, Matrigel was prepared with or without 54 kD HA supplementation. Addition of HA to Matrigel more than doubled invasion of the antisense transfectants. Addition of HA had little effect on SW620, SW480, and vector control cells. Addition of chondroitin sulfate A had no effect on invasion. Invasion was assessed as described in "Materials and Methods." Data are presented as the mean of three experiments; bars, \pm SE.

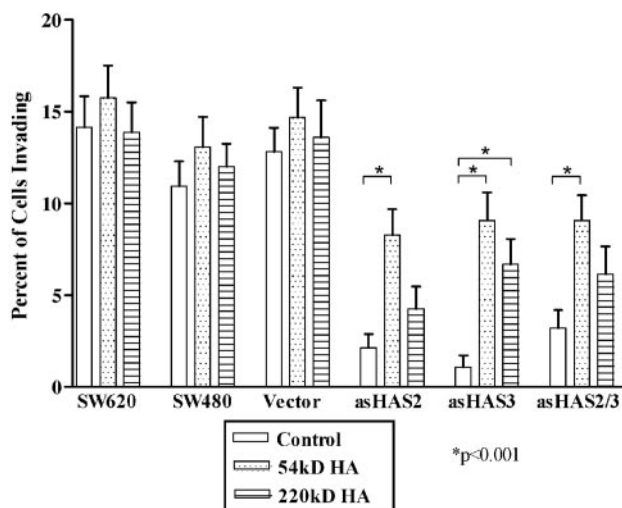


Fig. 3. We found that 54 kD and 220 kD hyaluronan (HA) are capable of increasing invasion in antisense transfectants. To determine whether the size of the HA polymer would affect invasion, Matrigel was supplemented with either low molecular weight HA (54 kD; 1 mg/ml) or high molecular weight HA (220 kD; 1 mg/ml) and invasion assays performed as described in "Materials and Methods." Invasion was significantly increased by 54 kD HA. Invasion was also increased by 220 kD HA, but results were more variable. Data are presented as the mean of three experiments; bars, \pm SE.

Effect of CD44 Inhibition on Matrigel Invasion. CD44-HA interactions appear to play important roles in tumor growth, invasion, and metastasis (42–44). We hypothesized that cell-surface CD44 might facilitate Matrigel invasion by SW620 cells. The presence of CD44 has been variably reported in SW620 cells (45). Therefore, we characterized both CD44 mRNA expression and CD44 protein in SW620 cells, SW480 cells, and stable transfectants. CD44 mRNA was detected in all of the cell lines by reverse transcription-PCR. Similarly, CD44 protein was present in cell lysates from all of the cell lines (Fig. 4).

Because SW620 cells possess CD44, we hypothesized that HA-CD44 interaction mediates invasion in these cells and that inhibition of HA binding would prevent invasion. The ability of an anti-CD44 antibody to block HA-CD44 binding was confirmed using an adhe-

sion assay. Preincubation with the blocking anti-CD44 antibody inhibited SW620 cell adhesion to HA by \sim 50% (Fig. 5). Incubation with a nonblocking CD44 antibody or IgG did not inhibit adhesion. To determine whether inhibition of HA-CD44 interaction would inhibit invasion, SW620 cells were incubated with anti-CD44 antibody before seeding on Matrigel plates. The blocking anti-CD44 antibody inhibited invasion up to 95% in a dose-dependent manner (Fig. 6A), suggesting that HA-CD44 interaction is required for maximal invasion of the Matrigel matrix. Incubation with either a nonblocking anti-CD44 antibody or IgG did not inhibit invasion. The ability of the anti-CD44 antibody to inhibit HA-mediated invasion was then additionally assessed in Matrigel supplemented with HA. The anti-CD44 blocking antibody (20 μ g/ml concentration) decreased invasion by antisense transfectants into Matrigel HA by $>$ 90% (Fig. 6B). The nonblocking anti-CD44 antibody and IgG had no effect. These data are consistent with a model of colon carcinoma cell progression in which interaction between the pericellular HA matrix and cell-surface CD44 are crucial to facilitating basement membrane invasion.

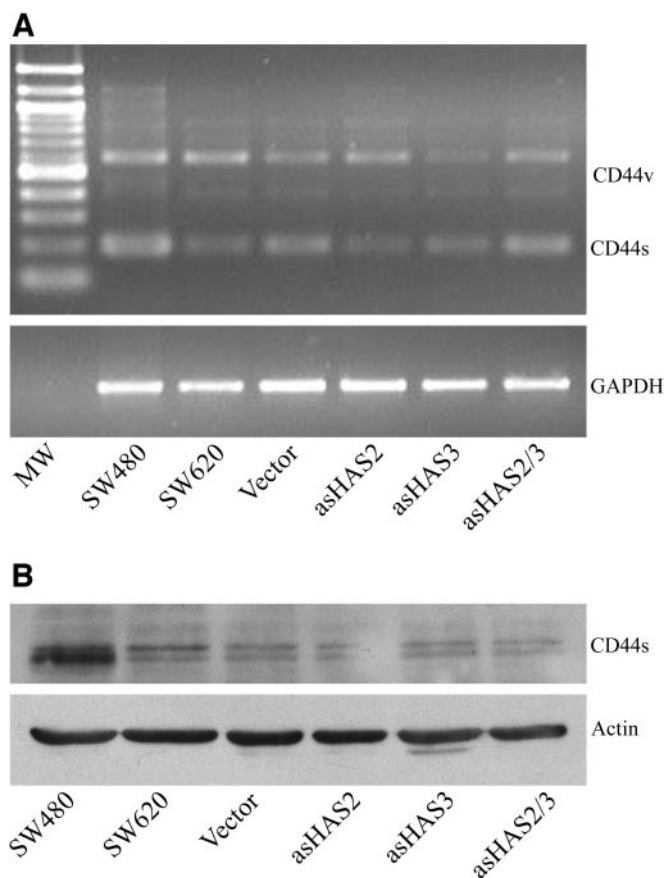


Fig. 4. A, CD44 expression was detected in colon carcinoma cells using reverse transcription-PCR as described in "Materials and Methods." Polyadenylated RNA was isolated from subconfluent cells and quantified by Ribogreen fluorescence as described in "Materials and Methods." Each mRNA template (50 ng) was reverse transcribed with an oligodeoxythymidylic acid primer. Oligonucleotides specific for CD44 were designed from the sequences database and amplified using cycling conditions described in "Materials and Methods." Glyceraldehyde-3-phosphate dehydrogenase was amplified with every reaction to standardize conditions. Expected CD44 reaction product size was 130 bp (CD44s). Higher bands represent splice variants (CD44v). B, CD44 protein was detected in colon carcinoma cells using Western blot analysis as described in "Materials and Methods." One $\times 10^7$ cells were lysed by incubation in lysis buffer for 60 min on ice. Protein samples (20 μ g) were separated by electrophoresis and transferred onto Immobilon-P membrane (Millipore). The membrane was blocked overnight, then incubated for 1 h with primary antibody, washed, then incubated with a peroxidase-conjugated goat anti-rabbit secondary antibody, washed, and developed with a chemiluminescence detection kit. Actin served as a loading control.

DISCUSSION

HA has been detected in a variety of malignancies (1, 3, 4) and implicated in cancer progression (5–7, 41). In colon cancer, the presence of HA may correlate with malignant transformation and disease stage (2, 46). Our previous work has demonstrated that HA production and HAS expression are increased in metastatic SW620 cells and are crucial for cellular growth, suggesting that HA and HAS may be important factors influencing colon cancer progression (19). These data may be particularly significant, because few molecular markers have proven to correlate well with stage and prognosis in colon cancer (47–49).

In addition to primary tumor growth, HA has been implicated in invasion and metastasis. HA increases adhesion and motility of breast carcinoma cells (50) and facilitates Matrigel invasion by glioma (25, 26), astrocytoma (51), and chondrosarcoma cells (52). In this study, metastatic colon carcinoma cells (SW620), which secrete and retain large amounts of HA, demonstrated greater invasion through Matrigel than did SW480 cells, which were derived from a primary tumor and produce less HA. Inhibition of HA secretion and retention in SW620 cells by transfection with antisense HAS2 and/or antisense HAS3 constructs diminished pericellular HA retention and secretion and decreased Matrigel invasion.

Differential expression of HAS isozymes has been shown to impact tumor growth in other malignancies such as fibrosarcoma (15) and murine mammary carcinoma (16). HAS3 in particular appears to be crucial in cancer progression. For example, overexpression of HAS3 in prostate cancer cells increases tumor cell growth *in vitro* and in animal models (17, 18). Our previous work has demonstrated that HAS3 expression is increased in metastatic colon carcinoma cells and that HAS3 inhibition decreases anchorage-independent growth (19). The studies presented herein demonstrate that inhibition of either isozyme is capable of inhibiting invasion, although inhibition of HAS3 has a greater effect. Because either isozyme is capable of synthesizing the pericellular HA coat, the difference in effect on growth and invasion may result from the unique enzyme kinetics and regulation of each isozyme (14, 53–57). For example, although enzy-

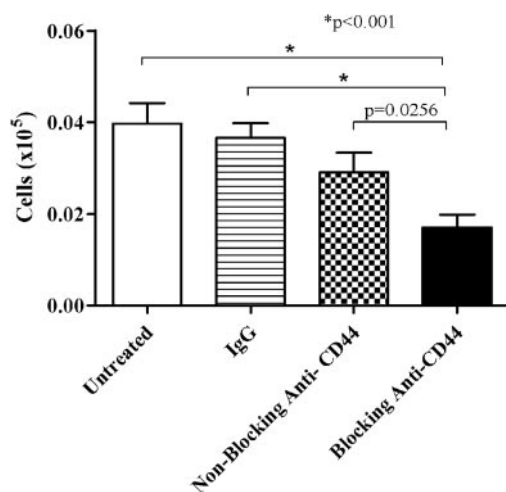


Fig. 5. Incubation with anti-CD44 antibody inhibited adhesion to hyaluronan (HA). To confirm that the anti-CD44 antibody was capable of blocking HA-CD44 interaction, adhesion of SW620 cells to HA-coated wells was tested after incubation with anti-CD44 antibody (10 μ g/ml). We coated 96-well plates with HA and incubated them overnight at 37°C. Cells were incubated with a blocking anti-CD44 antibody, a nonblocking anti-CD44 antibody, IgG, or no antibody (growth medium) for 30 min at 4°C. Cells were added to the HA-coated plates and incubated for 1 h at 37°C. The plates were then washed and 20 μ l of CellTiter 96 Aqueous One Solution added. After 3 h, the absorbance was read at 490 nm and number of cells determined by comparison to a standard curve (cell number versus absorbance). Data are presented as the mean of three experiments; bars, \pm SE.

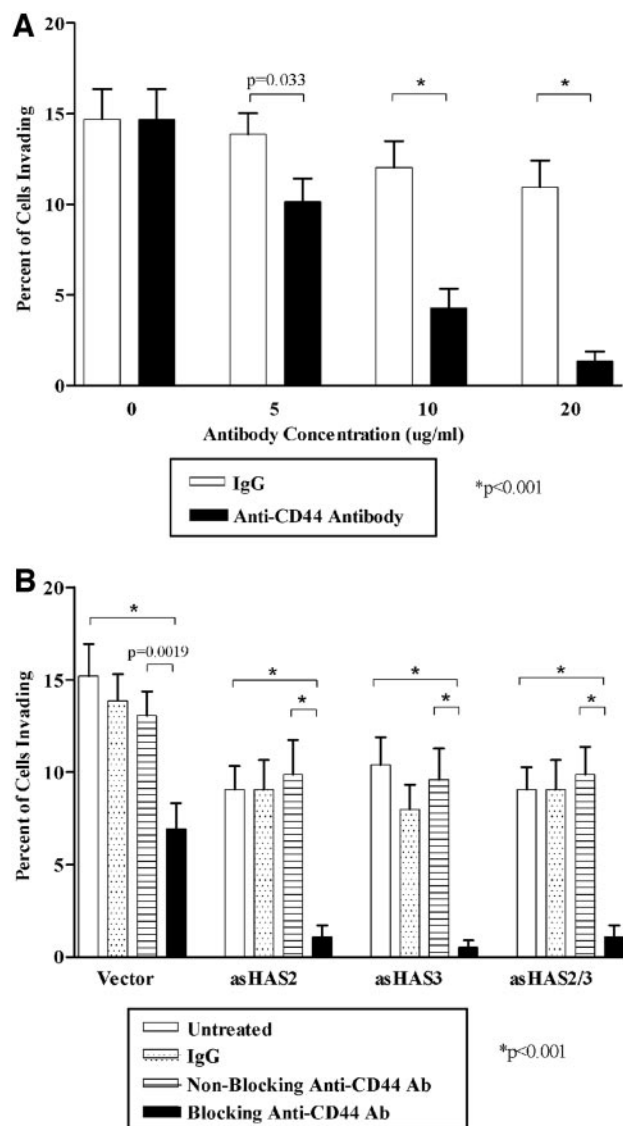


Fig. 6. Matrigel invasion is inhibited in the presence of an anti-CD44 antibody that blocks hyaluronan (HA)-CD44 binding. To determine whether HA-CD44 interaction is necessary for Matrigel invasion, invasion was assessed after incubation with anti-CD44 antibody. A, the SW620 cells were incubated with anti-CD44 antibody or IgG at concentrations of 0, 5, 10, and 20 μ g/ml. Invasion assays were then repeated as described in "Materials and Methods." Anti-CD44 antibody inhibited invasion in a dose-dependent manner. B, experiments were then repeated using Matrigel supplemented with HA (1 mg/ml). Anti-CD44 antibody (20 μ g/ml) inhibited invasion of stable antisense transfectants. IgG and a nonblocking anti-CD44 antibody had no effect on invasion. Data are presented as the mean of three experiments; bars, \pm SE.

matic activity (K_m) is similar for HAS2 and HAS3, HAS2 has a faster elongation rate than HAS3. HAS2 also synthesizes HA polymers that are roughly twice as large as those synthesized by HAS3 (2×10^5 to 2×10^6 daltons versus 1×10^5 to 1×10^6 daltons; Ref. 14). Although it is not known whether these *in vitro* differences in HA synthesis are important *in vivo*, it is possible that differences in the synthetic capabilities of each isozyme contribute to the differential effect on cellular behaviors such as growth and invasion. Alternatively, interaction between HAS2 and HAS3 may be required to affect invasion. At present, the relative contribution of HAS2 versus HAS3 to the invasive capability of colon carcinoma cells remains an open question. The impact of HAS2 and/or HAS3 inhibition in other colon carcinoma cells, such as SW480 primary tumor cells, has not been assessed. Studies are in progress in our laboratory to determine the effect of inhibiting HAS2 and HAS3 in these and other colon carcinoma cell

lines to better delineate the mechanisms by which these biosynthetic enzymes affect cancer progression.

The size of the HA polymer also may impact the biological function of this molecule. For example, increased production of HA increases growth of several tumor cell lines (15, 58, 59), whereas small HA oligosaccharides (8–10 disaccharide units, $M_r \sim 2.5$ molecular weight) inhibit cellular growth (60). Our previous study demonstrated addition of 54 kD HA markedly increased anchorage-independent growth of colon carcinoma cells transfected with an antisense HAS3 construct, but the addition of 220 kDa HA did not (19). In contrast, both 54kD and 220kD HA were capable of increasing Matrigel invasion by antisense HAS transfectants, suggesting that size alone may have less impact on cancer cell invasion than on other properties such as tumor growth.

The addition of exogenous HA in these studies increased the invasive capability of the antisense transfectants, additionally supporting the contention that pericellular HA facilitates basement membrane penetration. However, whereas exogenous HA increased invasion of these cells, it did not restore invasion to the level seen in either the parental SW620 cells or vector control cells. The amount of HA required to enhance invasion under these experimental conditions also greatly exceeded the amount of HA normally secreted by SW620 or vector control cells (~ 100 ng/ml per 10^4 cells). This difference may reflect decreased availability of HA imbedded in Matrigel when compared with HA synthesized at the cell surface. Although HA supplementation of the Matrigel makes HA available to the cells, it is likely that the pericellular concentration of HA is less than the concentration when HA is synthesized by membrane-associated HAS. Alternatively, pericellular matrix assembly and retention may be impaired by the absence of HAS2 and/or HAS3, thus decreasing the efficiency by which exogenous HA can reconstitute the pericellular matrix and facilitate invasion. However, the observation that HA supplementation dramatically increased invasion by the antisense transfectants suggests that the availability of HA to the cell is crucial for this function.

The ability of HA to enhance cancer cell invasion may result from the biomechanical properties of the HA matrix, from specific HA-receptor interactions, or from HA activation of matrix degrading enzymes. Pericellular HA creates a hydrated matrix and, thus, may “loosen” the stroma surrounding cancer cells, promote detachment, or diminish contact inhibition (61–64). Large HA polymers increase the hydration state and decrease the viscosity of the extracellular matrix to a greater extent than small HA polymers (14); therefore, if mechanical properties alone were responsible for enhancing invasion, one would expect 220 kD HA to increase invasion to a greater degree than 54 kD HA, an effect that was not seen in our experiments. Moreover, if mechanical disruption of the matrix was solely responsible for increasing invasion, exogenous HA would be expected to facilitate invasion by the SW620, SW480, and vector control cells. In our experiments, exogenous HA had limited effect on invasion by these cell lines. Finally, chondroitin sulfate A, which possesses polyanionic properties that are similar to HA, had no effect on invasion. These observations suggest that the biomechanical effects of HA are inadequate to fully explain the facilitation of colon cancer cell invasion, and additionally suggest that specific interactions between the pericellular HA matrix and the cancer cell surface are responsible for this process.

HA binds to several cell-surface receptors, and the specificity of these interactions may mediate the biological effects of the molecule. One of the best-characterized HA receptors is CD44, and this molecule has been implicated in the growth and progression of a number of malignancies including colon cancer (42–44, 65, 66). CD44 and its splice variants have been implicated in cancer progression; however,

the mechanism by which this molecule impacts tumor growth and metastasis is not well understood, and existing data are contradictory. For example, Yamada *et al.* (44) have detected overexpression of CD44s and CD44 variant 6 (CD44v6) in advanced colon cancer. Conversely, Asao *et al.* (42) have demonstrated that expression of CD44s is decreased in lymph node metastases from human colon cancer specimens. Similarly, the introduction of CD44s into HT-29 colon carcinoma cells decreases tumor formation in an animal model of liver metastasis (43). In contrast, Ghatak *et al.* (60) have shown that the effect of HA on anchorage-independent growth of HCT-116 colon carcinoma cells is mediated via HA-CD44 interaction and that inhibition of HA-CD44 interaction with small HA oligomers decreases tumor cell growth. Because little is known about the impact of manipulating HAS expression and HA synthesis in these or other colon carcinoma cells, it is possible that the differential effect of CD44 results from variability in pericellular HA production. We have determined that HT-29 cells do not secrete or retain pericellular HA and express very little HAS, whereas HCT-116 cells secrete and retain large HA matrices and express HAS3.⁵ Thus, the contradictory effects of CD44 seen in these two cell lines may result from differences in the presence or absence of pericellular HA. The studies presented herein additionally suggest that in the presence of elevated pericellular HA, such as is found in SW620 cells, CD44 plays a crucial role in facilitating invasion, thus implicating this cell surface receptor in the process of basement membrane invasion and colon cancer progression.

To invade, cancer cells must adhere to the surrounding matrix, migrate, and degrade matrix macromolecules; HA and CD44 may impact each of these processes. For example, HA enhances prostate cancer cell adhesion to bone marrow endothelium (17), and HA-CD44 interaction mediates adhesion of prostate carcinoma cells and COS cells via interaction with the cytoskeletal protein ankyrin (67, 68). Similarly, HA binding to CD44 facilitates adhesion of TA3/St murine mammary carcinoma cells to the peritoneum in an animal model of metastasis (69). Because adhesion to the extracellular matrix is a prerequisite for invasion, it is possible that the pericellular HA matrix facilitates invasion by colon carcinoma cells via enhanced adhesion. Alternatively, HA has been shown to increase migration of both malignant and nonmalignant cells (45, 64, 70–73). Thus, the ability of pericellular HA and HA-CD44 interaction to facilitate Matrigel invasion may reflect improved cellular motility through the matrix. The presence of HA also has been implicated in proteinase activation. HA production induces gelatinase (matrix metalloproteinases 2 and 9) activity in benign epithelial cells (74), glioblastoma cells (75), lung carcinoma cells (76), and breast carcinoma cells (77). Although SW620 cells do not appear to synthesize gelatinases, these cells are known to express the metalloproteinase matrilysin, and matrilysin activity is required for Matrigel invasion (78). It is possible, therefore, that the pericellular HA matrix facilitates matrilysin activity and thereby enhances Matrigel invasion. Alternatively, the matrix may trap and/or activate other proteolytic enzymes that could contribute to matrix degradation. Characterization of proteinase expression and activity in both SW620 cells and stable transfectants will be necessary to determine whether inhibition of HA retention and secretion will alter production and activity of these enzymes. Finally, the presence of pericellular HA may help to stabilize the subcellular distribution of CD44, thus enhancing CD44 function, or may increase CD44 expression and synthesis or decrease CD44 degradation. The mechanism by which HA affects CD44 remains poorly understood, and ongoing

⁵ Unpublished observations.

studies in our laboratory are focused on defining these effects in colon carcinoma cells.

In summary, we have shown that metastatic colon carcinoma cells that are known to secrete and retain large amounts of pericellular HA invade artificial basement membrane (Matrigel) more readily than cells from a primary tumor that produces less HA. Inhibition of the biosynthetic enzymes HAS2 and/or HAS3 decreases the pericellular HA matrix and significantly inhibits invasion, whereas addition of HA restores invasive capability. Inhibition of HA binding to CD44 inhibits invasion to a similar degree, suggesting that HA-CD44 interaction is responsible in part for Matrigel invasion. These data suggest that sustained HA overproduction by colon carcinoma cells may play a role in metastasis by promoting invasion of the basement membrane and that this process is mediated by interaction with CD44.

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