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Reduction of Hyaluronan-CD44-Mediated Growth, Migration, and Cisplatin Resistance in Head and Neck Cancer Due to Inhibition of Rho Kinase and PI-3 Kinase Signaling

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Abstract

Objectives—To investigate whether hyaluronan (HA), a ligand for the transmembrane receptor CD44, and CD44, which acts through multiple signaling pathways to influence cellular behavior, promote Rho kinase– and phosphatidylinositol 3 (PI-3) kinase–mediated oncogenic signaling to alter cisplatin sensitivity and stimulate tumor cell proliferation, migration, and matrix metalloproteinase secretion in head and neck squamous cell carcinoma (HNSCC).

Design—Laboratory investigation using the HNSCC cell line HSC-3.

Setting—University laboratory.

Main Outcome Measures—Rho kinase and PI-3 kinase activity, myosin phosphatase and AKT phosphorylation, tumor cell growth, migration, and matrix metalloproteinase secretion were measured in the presence or absence of HA, cisplatin, and inhibitors of Rho kinase and PI-3 kinase.

Results—The addition of HA, but not HA plus anti-CD44 antibody, resulted in increased Rho kinase and PI-3 kinase activity. Results of immunoblotting studies demonstrated that HA promotes Rho kinase-mediated myosin phosphatase phosphorylation and PI-3 kinase-mediated AKT

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phosphorylation. Hyaluronan was shown to promote migration and increased matrix metalloproteinase secretion through Rho kinase-mediated signaling. Hyaluronan treatment promoted increased tumor proliferation and resulted in a 12-fold reduced ability of cisplatin to cause HNSCC cell death. On the other hand, the presence of Y-27632, a Rho kinase inhibitor, and LY-294002, a PI-3 kinase inhibitor, blocked HA-mediated cisplatin resistance by HNSCC.

Conclusions—Our results suggest that HA and CD44 promote Rho kinase- and PI-3 kinase-mediated oncogenic signaling and cisplatin resistance. Perturbation of HA-CD44-mediated Rho kinase and PI-3 kinase signaling pathways may be a novel strategy to treat HNSCC.

Head and Neck Squamous cell carcinoma (HNSCC) is an aggressive malignant neoplasm associated with major morbidity and mortality. The 3-year survival rate for patients with advanced-stage HNSCC who are treated with standard therapy is only 30% to 50%.^{1,2} Resistance to standard therapy continues to be a limiting factor in the treatment of HNSCC. Nearly 40% to 60% of patients with HNSCC subsequently develop locoregional recurrences or distant metastases.^{1,2}

Hyaluronan (HA), an important glycosaminoglycan component of the extracellular matrix (ECM), and its major cell surface receptor, CD44, have been suggested to be important cellular mediators influencing tumor progression and treatment resistance.^{3–12} Our group previously reported that HA-CD44 interaction promotes intra-cellular Ca^{2+} mobilization and the activation of epidermal growth factor receptor (EGFR)-mediated oncogenic signaling in HNSCC.^{4,5} These 2 mechanisms trigger survival pathways that lead not only to HNSCC tumor progression (ie, abnormal growth, migration, and invasion) but also to resistance to several chemotherapeutic drugs such as cisplatin, methotrexate sodium, and etoposide.

In tumor cells, HA-CD44 interaction leads to the activation of multiple cell-signaling pathways that result in tumor progression. The Rho subclass of guanine triphosphatases has been implicated in the control of diverse processes, including cell proliferation and motility as well as transformation and metastasis.^{8–15} Several studies in breast, ovarian, and esophageal carcinoma have demonstrated that HA-CD44 interaction is capable of activating the Rho pathway effector Rho kinase, which regulates several cytoskeletal proteins (eg, myosin light chain phosphatase) that are highly involved in tumor migration and promote the secretion of matrix metalloproteinases (MMPs), which degrade the ECM during tumor invasion.^{9,11,12,14–16}

The interaction of HA and CD44 also has been shown to promote a malignant tumor phenotype through activation of the Ras pathway in breast cancer and HNSCC.^{9,10} One of the downstream molecular targets in the Ras pathway is phosphatidylinositol 3 (PI-3) kinase. Phosphatidylinositol 3 kinase is capable of catalyzing the conversion of phosphatidylinositol 3,4-bisphosphate (PIP₂) to phosphatidylinositol 3,4,5-triphosphate (PIP₃), which subsequently results in the activation of AKT.^{17–20} Activated AKT downregulates the cell's apoptotic potential to promote increased cell proliferation and tumor survival. The PI-3 kinase/AKT pathway is also thought to play a key role in the development of resistance of cancer cells to certain chemotherapeutic agents such as cisplatin, which causes tumor death through proapoptotic mechanisms.^{18–21} Recently, Rho kinase has been described as an effective communicator between the Rho and Ras pathways. In particular, Rho kinase appears capable of increasing the activity of PI-3 kinase, to enhance the survival mechanisms of the cell and promote chemoresistance.⁹

Rho kinase and PI-3 kinase are key signaling regulators in tumor survival, migration, and metastasis. However, the interaction of HA and CD44 with Rho kinase and PI-3 kinase in HNSCC has not been fully described. In this study, we sought to determine whether HA and CD44 interact to promote Rho kinase and PI-3 kinase signaling in HNSCC progression. We

found that specific inhibitors of Rho kinase and PI-3 kinase signaling could downregulate HA-mediated mechanisms that are involved in the promotion of proliferation, migration, invasion, and cisplatin resistance of HNSCC.

METHODS

CELL CULTURE

The cell line HSC-3 (Japan Cancer Research Resources Bank, Tokyo, Japan), was established in 1985 from a primary oral squamous cell carcinoma removed from the tongue of a 64-year-old man. The HSC-3 cells were maintained in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum. Cells were routinely serum starved (and therefore deprived of serum HA) before adding HA.

ANTIBODIES AND REAGENTS

We used the following antibodies and reagents. Monoclonal rat anti-human CD44 antibody (clone, 020; isotype, IgG2b; CMB Technologies, Milford, Massachusetts) recognizes a common determinant of the CD44 class of glycoproteins. Polyclonal rabbit anti-phospho-MYPT1 (Thr696) antibody (Millipore Corp, Chicago, Illinois) recognizes the phosphorylated myosin phosphatase regulatory subunit. Polyclonal rabbit anti-phospho-AKT1/2/3 antibody (Santa Cruz Biotechnology, Santa Cruz, California) recognizes the phosphorylated AKT; in addition, we used polyclonal goat anti-actin (I-19) (Santa Cruz Biotechnology). The Rho kinase inhibitor Y-27632 (No. 688000) and the PI-3 kinase inhibitor LY-294002 (No. 440202) were also used (Calbiochem, La Jolla, California). Healon HA polymers (roughly 500 000-Da polymers) (Healon Pharmacia, Erlangen, Germany) were prepared by gel filtration chromatography using a superfine gel column (Sephacryl S1000; GE Healthcare Biosciences, Piscataway, New Jersey). The purity of the high-molecular-mass HA polymers used in our experiments was further verified by anion-exchange high-performance liquid chromatography.

RHO KINASE ACTIVITY ASSAY

The HSC-3 cells were plated in 10-cm dishes at 0.5×10^6 cells per dish and were serum starved overnight before various treatments, including no treatment, HA treatment, blocking with Y-27632 (5 μ M) followed by HA treatment (50 μ g/mL), or blocking with rat anti-CD44 antibody followed by HA treatment (50 μ g/mL). Ten minutes after HA treatment, cells were immediately prepared in NP-40 buffer (50mM Hepes [pH, 7.5], 150mM sodium chloride, 20mM magnesium chloride, 1% NP-40, 1mM sodium orthovanadate, 1mM sodium fluoride, complete protease inhibitor cocktail [Roche Applied Science, Indianapolis, Indiana], 1mM phenylmethylsulfonyl fluoride, and 1 $\times$ Halt phosphatase inhibitor cocktail [Pierce Protein Research Products, Rockford, Illinois]) at 4°C and centrifuged to obtain the lysates. Equal amounts (verified by immunoblotting) of total lysates approximately 10 μ g or immunoprecipitation-purified Rho kinase obtained by preincubating lysates (approximately 100 μ g) with a rabbit anti-Rho kinase antibody and agarose-conjugated antirabbit secondary antibody were assayed for Rho kinase activity using a kit (catalogue No. CY-1160; CycLex Co, Ltd, Nagano, Japan), following a protocol provided by the vendor. Briefly, samples were incubated with a kinase reaction buffer with 0.1mM adenosine triphosphate at 30°C for 45 minutes in plates precoated with a Rho kinase substrate corresponding to the C-terminal of the recombinant myosin-binding subunit of myosin phosphatase, which contains a threonine residue that can be phosphorylated, and the product was detected by a horseradish peroxidase—conjugated antibody AF20 recognizing Thr696 of the myosin-binding subunit. The horseradish peroxidase-mediated color reaction was then measured in a spectrophotometric plate reader at dual wavelengths of 450/540 nm. The absorbance data were analyzed. Control samples include a solvent control (no protein lysate) and an inhibitor control (10 μ M Y-27632 with protein lysate). Each assay was repeated at least 3 times.

PI-3 KINASE ACTIVITY ASSAY

The HSC-3 cells were plated in 10-cm dishes at 0.5×10^6 cells per dish and were serum starved overnight before various treatments, including no treatment, HA treatment, blocking with LY294002 (5 μ M) followed by HA treatment (50 μ g/mL), or blocking with a rat anti-CD44 antibody followed by HA treatment (50 μ g/mL). Ten minutes after HA treatment, cells were rinsed with ice-cold buffer A (20mM TRIS hydrochloride [pH, 7.4], 137mM sodium chloride, 1mM calcium chloride₂, 1mM magnesium chloride₂, and 0.1mM sodium orthovanadate) and immediately lysed in NP-40 buffer at 4°C to obtain the lysates. The PI-3 kinase activity was assayed using a competitive enzyme-linked immunosorbent assay kit (catalogue No. K-1000; Echelon Biosciences, Salt Lake City, Utah) because of its ability to produce PIP₃ from PIP₂. In brief, approximately 200 μ g of lysates from each sample were incubated with an anti-PI-3 kinase and p85 antibody (catalogue No. 06-195; Millipore Corp), followed by an agarose-conjugated goat antirabbit antibody. After extensive washing with buffer A plus 1% NP-40 (3 times); with 0.1M TRIS hydrochloride (pH, 7.4), 5mM lithium chloride, and 0.1mM sodium orthovanadate (3 times); and with 10mM TRIS hydrochloride [pH, 7.4], 150mM sodium chloride, and 5mM EDTA containing 0.1mM sodium orthovanadate (2 times), the beads were incubated with PI-3 kinase reaction buffer (5mM Hepes [pH, 7], 2.5mM magnesium chloride₂, and 25mM adenosine triphosphate) and 240pmol PIP₂ for 2 hours at room temperature. The reaction was stopped by adding PIP₃ detector protein, and the supernatant was transferred to the PIP₃-coated detection plate and incubated for 1 hour at room temperature with agitation. The PIP₃ detector protein binding to the plate was detected by a peroxidase-linked secondary detector, and the colorimetric readout, which was inversely proportional to the amount of PIP₃ produced by PI-3 kinase, was determined at 450 nm in a plate reader. The PIP₃ standards were used as references for the experimental samples, and control samples containing no enzyme and no lipid were also included. Each assay was repeated at least 3 times.

IMMUNOBLOTTING TECHNIQUE

After growing in serum-free media for 24 hours, HSC-3 cells underwent one of the following procedures: incubation with or without HA, 50 μ g/mL, for 10 minutes, or pretreatment with anti-CD44 antibody (1:1000), Y-27632 (5 μ M), or LY-294002 (5 μ M), followed by the addition of HA for 10 minutes. Subsequently, cells were solubilized in 50mM Hepes (pH, 7.5), 150mM sodium chloride, 20mM magnesium chloride, 1.0% NP-40, 0.2mM sodium orthovanadate, 0.2mM phenylmethylsulfonyl fluoride, 10- μ g/mL leupeptin, and 5- μ g/mL aprotinin. After brief centrifugation, the samples were directly solubilized in sodium dodecyl sulfate buffer, electrophoresed on 4% to 12% TRIS-glycine gels (Novex; Invitrogen, Carlsbad, California), and blotted onto nitrocellulose. After blocking nonspecific sites with 5% milk, the nitrocellulose filters were incubated with rabbit anti-phospho-AKT antibody or anti-phosphomyosin phosphatase antibody followed by incubation with horseradish peroxidase-conjugated goat antirabbit IgG. The blots were then developed by the enhanced chemiluminescence system (GE Healthcare Biosciences).

GROWTH ASSAYS

Logarithmically growing HSC-3 cells were cultured, washed, counted, and plated at 3000 cells per well in triplicate on 96-well plates and incubated in serum-free media overnight. The following day, the cells were treated with various concentrations of cisplatin without or with HA (50 μ g/mL), anti-CD44 antibody, the Rho kinase inhibitor Y-27632 (5 μ M), or the PI-3 kinase inhibitor LY-294002 (5 μ M). Two days later, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays according to the manufacturer's protocol (Promega, Madison, Wisconsin). The MTT assay measures cell survival based on mitochondrial conversion of MTT from a soluble tetrazolium salt into an insoluble colored formazan precipitate, which is dissolved in dimethyl sulfoxide and quantitated by

spectrophotometry. The percentage of absorbance relative to controls was plotted as a linear function of drug concentration. Each assay was repeated at least 3 times. The 50% inhibitory concentrations (IC₅₀) were identified as a concentration of drug required to achieve a 50% growth inhibition relative to the untreated control.

TUMOR CELL MIGRATION ASSAYS

A scratch wound was made by scraping the cell monolayer across the cover glass with a sterile pipette. We changed the culture medium immediately after scraping to prevent the medium from being conditioned with cell debris and factors released from detached cells. Scratched cultures were pretreated with anti-CD44 antibody, the Rho kinase inhibitor Y-27632 (5 μ M), or the PI-3 kinase inhibitor LY-294002 (5 μ M) for 1 hour before adding HA (50 μ g/mL). The progress of cell migration was recorded with an inverted phase-contrast microscope at 0 hours and 24 hours from the time HA was added.

GELATIN ZYMOGRAPHY

Gelatin zymography for detecting picograms of MMP-2 and MMP-9 and nanograms of other MMPs and proteases was performed. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed in 7.5% or 10% polyacrylamide containing a 0.33-mg/mL concentration of gelatin. The gels were then rinsed twice in 0.25% surfactant (Triton X-100; Dow Chemical Company, Midland, Michigan) and incubated in the assay buffer (0.05M TRIS hydrochloride [pH, 7.5], 0.2M sodium chloride, 0.01M calcium chloride₂, 1mM zinc chloride, 3mM phenylmethylsulfonyl fluoride, 0.02% sodium azide, and 0.005% polyoxy-ethylene lauryl ether [Brij 35; Caledon Laboratories Ltd, Georgetown, Ontario, Canada]) at 37°C for 18 hours. Gels were then stained with Coomassie blue 1995 R-250. Latent and active forms of gelatinases or other MMPs produce clear areas in the gel.

STATISTICAL ANALYSIS

From the experimental results of the in vitro assays, a mean value and standard error of the mean were calculated for each experimental group. We used a *t* test with the resultant *P* value representing a 2-sided test of statistical significance. Significance was set at 95% (*P* = .05).

RESULTS

HA- AND CD44-DEPENDENT RHO KINASE-MEDIATED FUNCTION IN HNSCC

Rho kinase mediates cell shape, motility, and invasiveness of cancer cells by reconfiguring the structure of the cytoskeleton and the attachments that the cell makes with its physical surroundings.^{14,15} The function of Rho kinase has previously been linked to HA-CD44-mediated signaling,^{8–13} and HSC-3 cells express CD44.^{4–6} Using an in vitro assay, we measured Rho kinase activity in HSC-3 cells in the presence of HA or the Rho kinase inhibitor Y-27632 to determine whether Rho kinase activation was sensitive to HA-CD44-mediated signaling in HNSCC (Figure 1). Compared with untreated cells and cells pretreated with anti-CD44 antibody followed by HA addition, we observed increased Rho kinase activity after HA treatment. However, treatment with the Rho kinase inhibitor Y-27632 or pretreatment with Y-27632 followed by HA addition significantly reduced Rho kinase activity (*P* < .001). These results suggest that Rho kinase activity is sensitive to HA-CD44-mediated signaling in HSC-3 cells.

Myosin phosphatase is an important regulator of cell motility that is phosphorylated by Rho kinase to promote tumor cell migration. In this investigation, we sought to determine whether HA promotes the phosphorylation of myosin phosphatase, to increase HSC-3 cell migration (Figure 2). Using activated myosin phosphatase antibody-mediated immunoblot analysis, we

found that myosin phosphatase phosphorylation was upregulated by HA treatment (Figure 2A, lane 2). The level of myosin phosphatase phosphorylation was comparatively low in cells that were untreated (Figure 2A, lane 1). The level of myosin phosphatase phosphorylation was significantly decreased in cells pretreated with the Rho kinase inhibitor Y-27632 for 1 hour followed by HA treatment (Figure 2A, lane 3). These findings support the conclusion that HA promotes Rho kinase–dependent myosin phosphatase phosphorylation in HSC-3 cells.

Because Rho kinase–mediated myosin phosphatase phosphorylation has previously been shown to promote cell motility, we next studied HSC-3 cells for HA-dependent tumor migration activity (Figure 3 A). Using an in vitro scratch wounding assay, we observed that HSC-3 cells actively migrated during HA treatment (Figure 3A, lane 2). However, the level of tumor cell migration was significantly reduced in cells without HA treatment or when cells were pretreated with Rho kinase inhibitor Y-27632 followed by the addition of HA (Figure 3 A, lanes 1 and 3, respectively). Our results support the conclusion that HA promotes Rho kinase–dependent tumor cell migration in HSC-3 cells.

Metastatic tumor cells are capable of degrading the ECM barrier to migrate out of the primary tumor location and establish new sites of metastasis. The breakdown of the ECM can be traced to the action of 1 or more MMPs.^{22,23} The MMPs are secreted as proenzymes and become activated outside the cell. The secretion and activation of several MMPs, including the 72-kD gelatinase MMP-2 and the 92-kD gelatinase MMP-9, correlate with the invasiveness of solid tumors. Secretion of MMP-2 and MMP-9 have previously been linked to HA-CD44–mediated invasion in breast carcinoma and HNSCC.²² To investigate a putative role for HA-CD44–mediated Rho kinase–dependent HNSCC invasion and metastasis, serum-free conditioned media from HSC-3 cells treated with HA or pretreated with the Rho kinase inhibitor Y-27632 followed by HA treatment were obtained and gelatin zymography was performed (Figure 3B). The upper bands represent MMP-9 and the lower bands represent MMP-2. Activation of MMP-2 is shown by the presence of a second lower band in lane 2, indicating the cleavage of the proenzyme into its active enzymatic form. Compared with the untreated control media, we found that treatment with HA promotes increased secretion of MMP-9 as well as increased secretion and activation of MMP-2 (Figure 3B, lanes 1 and 2, respectively). However, pretreatment with Y-27632 clearly reduced the secretion of MMP-9 and MMP-2 and eliminated the activation of MMP-2 (Figure 3B, lane 3). These observations establish the fact that HA promotes Rho kinase–dependent MMP secretion and activation in HSC-3 cells, with consequent greater capability for tumor cell invasion.

HA- AND CD44-DEPENDENT PI-3 KINASE-MEDIATED FUNCTION IN HNSCC

Phosphatidylinositol 3 kinase is known to mediate various cellular activities, such as the suppression of apoptosis, the stimulation of proliferation, and the promotion of tumor cell survival.^{17–20} Activity of PI-3 kinase has been linked to HA-CD44 signaling in other cell model systems.^{9,21} In this study, we used an in vitro kinase activity assay to determine whether PI-3 kinase activity was sensitive to HA-CD44 signaling in HSC-3 cells (Figure 4). Compared with untreated cells and cells pretreated with anti-CD44 antibody followed by HA addition, we observed increased PI-3 kinase activity after HA treatment. However, treatment with the PI-3 kinase inhibitor LY-294002 or pretreatment with LY-294002 followed by HA addition significantly reduced PI-3 kinase activity ($P < .001$). These results suggest that PI-3 kinase activity is sensitive to HA-CD44–mediated signaling in HSC-3 cells.

One of the well-established effector molecules for PI-3 kinase is AKT, which is known to be an important mediator of cell survival and growth signaling pathways. AKT facilitates cell survival by suppressing the cell's own built-in suicide program of apoptosis.^{19,20} Using phospho-AKT antibody–mediated immunoblot analysis, we found that the level of AKT phosphorylation was comparatively low in cells that were not treated with HA (Figure 5A, lane

1). On the other hand, AKT phosphorylation was significantly increased after HA treatment (Figure 5A, lane 2). Pretreatment with the PI-3 kinase inhibitor LY-294002 for 1 hour followed by HA treatment resulted in reduced AKT phosphorylation (Figure 5A, lane 3). Our results support the notion that HA promotes AKT phosphorylation, and PI 3-kinase is an important regulator of this process.

Activation of AKT promotes cell proliferation and survival.^{17–20} We previously reported that HA is able to promote HNSCC growth in a CD44-dependent manner.^{4–6} In this study, we investigated the role of PI-3 kinase to mediate HA-CD44-dependent proliferation in HSC-3 cells, using MTT assays. Compared with untreated control cells, we observed increased growth when HSC-3 cells were treated with HA (Table 1). On the other hand, when cells were pretreated with the PI-3 kinase inhibitor LY-294002 before the addition of HA, there was significantly ($P = .004$) less proliferation compared with the HA-treated cells. When the HSC-3 cells were pretreated with the Rho kinase inhibitor, Y-27632, there was reduced proliferation compared with the HA-treated cells, although the reduction was less than what was observed with PI-3 kinase inhibition. These results suggest that PI-3 kinase and Rho kinase are involved in HA-CD44-mediated promotion of tumor cell growth.

HA- AND CD44-DEPENDENT CISPLATIN RESISTANCE IN HNSCC

Cisplatin is one of the most commonly used anticancer drugs in HNSCC. We next investigated the role of HA to promote cisplatin resistance in HSC-3 cells. Compared with the untreated control cells (cisplatin IC_{50} of 4 μ M), we found that the addition of HA reduced the ability of cisplatin to cause cell death (IC_{50} of 12 μ M), indicating that HA can promote cisplatin resistance in these tumor cells (Figure 6A and Table 2). Furthermore, pretreatment of HSC-3 cells with anti-CD44 antibody followed by the addition of HA sensitized these cells to treatment with cisplatin (IC_{50} of 1 μ M), suggesting that HA interacts with CD44 to promote tumor survival.

Previous reports suggest that apoptosis is responsible for cisplatin-induced cell death.²⁴ Because AKT suppresses apoptosis, its activation by PI-3 kinase has been suggested to play a key role in cisplatin resistance.^{19,20} Likewise, Rho kinase has also been reported to promote drug resistance, possibly through cross talk with the PI-3 kinase/AKT signaling pathway.^{9,10} To investigate whether cisplatin resistance in HNSCC might be mediated through HA-CD44 activation of Rho kinase and PI-3 kinase, we performed additional MTT assays with HSC-3 cells in the presence of increasing concentrations of cisplatin combined with inhibitors of Rho kinase and PI-3 kinase (Figure 6B and Table 2). Tumor cell growth with cisplatin was measured by MTT assay in the presence of HA treatment alone (50 μ g/mL), LY-294002 plus HA, Y-27632 plus HA, or LY-294002 and Y-27632 plus HA. Pretreatment with LY-294002 or Y-27632 alone reduced HA-mediated cisplatin resistance (IC_{50} of 6 and 7 μ M, respectively). Combined pretreatment with LY-294002 and Y-27632 followed by HA treatment eliminated HA-mediated cisplatin resistance in HSC-3 cells (IC_{50} of 4 μ M). These findings support the conclusion that simultaneous inhibition of Rho kinase and PI-3 kinase effectively blocks HA-mediated cisplatin resistance in HSC-3 cells.

COMMENT

Hyaluronan and its major cell surface receptor, CD44, have been implicated in tumor progression behaviors such as growth, migration, invasion, and metastasis in a variety of malignant neoplasms. In addition, HA and CD44 have been linked to chemotherapy resistance in several tumor models. We previously reported expression of CD44 in a panel of HNSCC cell lines and demonstrated that HA-CD44 signaling promotes tumor progression behaviors in several HNSCC cell lines, including HSC-3, HOC313, SCC-4, and MDA1483.^{4–7,10} In the present investigation, we studied HA-CD44 interaction to upregulate Rho kinase- and PI-3

kinase-mediated signaling pathways, thereby contributing to tumor progression. The HSC-3 cell line was selected to study the role of Rho kinase and PI-3 kinase in HA-CD44 signaling because previous studies indicated that it expressed CD44 and was sensitive to HA-dependent growth, migration, and cisplatin resistance.^{4–710} Our study results with HSC-3 cells will need to be confirmed in other HNSCC cell lines or, preferably, other in vivo models to establish their broader applicability. Figure 7 represents our proposed model of HA-CD44-mediated activation of PI-3 kinase and Rho kinase to promote HNSCC progression and cisplatin resistance.

Members of the Rho subclass of guanosine triphosphatases (eg, RhoA, Rac1, and Cdc42) are known to transduce a variety of signals regulating many different cellular processes, including invasion and motility, which are obvious prerequisites for metastasis.¹⁴¹⁵ Rho kinase is a key effector of Rho guanosine triphosphatase-mediated signaling. Activated Rho kinase phosphorylates a number of cytoskeletal proteins such as myosin phosphatase and adducin. Effective phosphorylation of myosin phosphatase by Rho kinase results in its inactivation, which is necessary for successful myosin light chain phosphorylation to generate actomyosin-mediated membrane motility and cell migration. In the present investigation, we showed that Rho kinase activation is necessary for HA-CD44-mediated myosin phosphatase phosphorylation. We demonstrated that inhibition of Rho kinase decreased myosin phosphatase phosphorylation and reduced the capacity of HA to promote migration of HSC-3 cells.

Rho kinase also promotes tumor invasion by modulating the motility of cancer cells and their ability to degrade the ECM.^{16,23} Two important enzymes secreted by cancer cells and surrounding stromal cells, MMP-2 and MMP-9, are thought to play a role in the degradation of the basement membrane because of their ability to cleave type IV collagen. It has recently been established that MMP-2 is activated by membrane-type 1 MMP on the cell surface of cells, whereas MMP-9 is secreted as an inactive zymogen that is activated extracellularly by serine proteinases.¹⁶ In osteosarcoma cells, the Rho/Rho-kinase pathway was shown to participate in the degradation of the ECM by increasing the expression of membrane-type 1 MMP, which subsequently activates MMP-2.²³ Our results are in agreement with previous reports suggesting Rho kinase involvement in HA-CD44-mediated MMP secretion and activation. By suppressing the activity of Rho kinase, we observed diminished secretion of MMP-9 and diminished secretion and activation of MMP-2.

Hyaluronan and CD44 are known to promote malignant tumor phenotypes through multiple signaling regulator proteins. One such regulator protein is EGFR, a membrane tyrosine kinase receptor that is often overexpressed in HNSCC. We previously reported that HA-CD44 interaction can influence EGFR-mediated mitogen-activated protein kinase signaling in HNSCC, leading to tumor migration, proliferation, and multidrug resistance.^{5,10} Several authors have reported that EGFR is capable of interacting with PI-3 kinase, which subsequently activates the AKT pathway to facilitate tumor cell survival.³¹⁰ Because HA-CD44 interaction influences EGFR-mediated signaling in HNSCC, we examined whether HA can promote the activity of PI-3 kinase. We observed HA-mediated CD44-dependent increased PI-3 kinase activity and enhanced AKT phosphorylation. On the other hand, inhibition of PI-3 kinase led to decreased AKT phosphorylation and subsequent diminished HA-mediated tumor cell growth and survival.

Resistance to standard treatment modalities is common in many aggressive cancers, including HNSCC. Cisplatin is the most commonly used chemotherapeutic drug in HNSCC, but its efficacy is limited by a low single-agent response rate (28%) and high toxicity.^{2,24} The currently accepted mechanism of action for cisplatin is that it binds cellular DNA through the formation of inter-strand cross-links.⁴ If the DNA adducts are not efficiently processed by the

cell machinery, the eventual result is activation of apoptosis and cell death. Interaction of HA and CD44 with various signaling pathways is being increasingly examined as a possible mechanism mediating chemoresistance. A previous report from our group indicated that HA-CD44 interaction with EGFR signaling and phospholipase C-mediated Ca^{2+} signaling promoted resistance to cisplatin, methotrexate, and adriamycin in HNSCC.^{4,5,7} There are likely multiple downstream signaling pathways through which HA-CD44-mediated chemoresistance occurs. In colorectal cancer, CD44 was reported to mediate chemoresistance through the activation of the PI-3 kinase/AKT pathway.²¹ Increased AKT phosphorylation is known to suppress the activation of proapoptotic signals generated as a result of the DNA damage caused by treatment with cisplatin and other chemotherapeutic drugs.^{18–21} Because activation of the PI-3 kinase/AKT signaling pathway is thought to promote cisplatin resistance, we studied whether PI-3 kinase might also mediate HA-CD44 promotion of cisplatin resistance in HNSCC. Because Rho kinase has been reported to interact with the PI-3 kinase/AKT pathway, we also investigated the role of this signaling protein in HA-mediated cisplatin resistance. We observed that simultaneous inhibition of Rho kinase and PI-3 kinase reduced cisplatin resistance in HNSCC to a greater degree than was observed with inhibition of either enzyme alone. These findings suggest that both Rho kinase and PI-3 kinase are involved in HA-CD44-mediated cisplatin resistance in HNSCC.

This study suggests the importance of Rho kinase and PI-3 kinase signaling in HA-CD44-mediated cisplatin resistance in HNSCC. We also illustrate how inhibition of these enzymes diminishes the capacity of HA and CD44 to promote malignant tumor phenotypes such as abnormal proliferation, migration, and invasion in a single HNSCC cell line. We believe that this study suggests that CD44 and its associated signaling molecules (ie, Rho kinase and PI-3 kinase) may be important targets for the future development of novel therapies for the treatment of head and neck cancer.

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Figure 1.

Rho kinase activity assays. In vitro Rho kinase activity in HSC-3 cells was measured as described in the “Methods” section. Compared with untreated cells and cells pretreated with anti-CD44 antibody followed by hyaluronan (HA) addition, there was increased Rho kinase activity after HA treatment. However, treatment with the Rho kinase inhibitor Y-27632 (D) and pretreatment with Y-27632 followed by HA addition significantly reduced Rho kinase activity. Each assay was performed in triplicate and repeated at least 3 times. Error bars represent calculated standard error of the means.

Figure 2.

Activated myosin phosphatase antibody-mediated immunoblot analysis. A, Detection of phosphorylated myosin phosphatase was performed with anti-phosphomyosin phosphatase antibody in tumor cell lysates obtained from HSC-3 cells without hyaluronan (-HA) treatment (lane 1), cells treated with HA (+HA) (lane 2), and cells pretreated with Rho kinase inhibitor Y-27632 followed by the addition of HA (lane 3). B, Anti-actin antibody detection of actin was used as a loading control. C, The ratio of phosphomyosin phosphatase to total actin (the loading control) was determined by densitometry, and the levels were normalized to untreated (no HA treatment) cell value (lane 1); the values expressed represent an average of triplicate determinations of 3 experiments with an SD of less than 5%. *Significantly different ($P < .001$) (compared with untreated control samples or HA-treated samples).

Figure 3.

Migration assays and zymography. A, In vitro scratch wounding analysis of cellular migration was performed in HSC-3 cells. Cellular migration during 24 hours was measured in cells without hyaluronan (–HA) treatment (lane 1), cells treated with HA (+HA) (lane 2), and cells pretreated with Rho kinase inhibitor Y-27632 followed by the addition of HA (lane 3). The data for migration are shown as mean percentage (SEM). *Significantly different ($P < .01$) compared with untreated control samples or HA-treated samples. B, Matrix metalloproteinases (MMPs) were detected using gelatin zymography. Secretion of MMP-9 and MMP-2 and activation were measured in cells without HA treatment (lane 1), cells treated with HA (lane 2), and cells pretreated with Rho kinase inhibitor Y-27632 followed by the addition of HA (lane 3). The upper bands represent MMP-9 and the lower bands represent MMP-2. Activation of MMP-2 is shown by the presence of a second lower band in lane 2, indicating the cleavage of the proenzyme into its active enzymatic form. *Activated MMP-2. MW indicates molecular weight.

Figure 4.

Phosphatidylinositol 3 (PI-3) kinase activity assays. In vitro PI-3 kinase activity in HSC-3 cells was measured as described in the “Methods” section. Compared with untreated cells and cells pretreated with anti-CD44 antibody followed by hyaluronan (HA) addition, there was increased PI-3 kinase activity after HA treatment. However, treatment with the PI-3 kinase inhibitor LY-294002 and pretreatment with LY-294002 followed by HA addition significantly reduced PI-3 kinase activity. Each assay was performed in triplicate and repeated at least 3 times. Error bars represent calculated standard error of the means.

Figure 5.

Anti-AKT antibody-mediated immunoblot analysis. A, Detection of AKT phosphorylation in HSC-3 cells was performed with anti-phospho-AKT antibody-mediated immunoblot analysis of tumor cell lysates obtained from HSC-3 cells without hyaluronan (HA) treatment (lane 1), cells treated with HA (lane 2), and cells pretreated with phosphatidylinositol 3 kinase inhibitor LY-294002 followed by the addition of HA (lane 3). B, Anti-actin antibody detection of actin was used as a loading control. The ratio of phospho-AKT to total actin (the loading control) was determined by densitometry, and the levels were normalized to the untreated (no HA treatment) cell value (lane 1); the values expressed represent an average of triplicate determinations of 3 experiments with an SD of less than 5%. *Significantly different ($P < .001$) compared with untreated (no HA treatment) control samples or HA-treated samples.

Figure 6.

Cisplatin sensitivity in HSC-3 cells. A, HSC-3 cells were grown in serum-free media in increasing concentrations of cisplatin in the presence or absence of hyaluronan (+HA or -HA, respectively) (50 μ g/mL) or anti-CD44 antibody plus HA. B, Analysis of the effect of Rho kinase and phosphatidylinositol 3 (PI-3) kinase inhibition on cisplatin sensitivity was performed in HSC-3 cells grown in serum-free media that were treated with cisplatin in the presence of HA, HA plus Rho kinase inhibitor Y-27632, HA plus PI-3 kinase inhibitor LY-294002, and HA plus Y-27632 and LY-294002. Fifty percent inhibitory concentrations (IC₅₀) are shown for each group by the dotted lines. The error bars represent calculated standard error of the means.

Figure 7.

Hyaluronan (HA)-CD44 mediated Rho kinase and phosphatidylinositol 3 (PI-3) kinase signaling in head and neck squamous cell carcinoma (HNSCC). This is our proposed model of HA-CD44 interaction to promote Rho kinase and PI-3 kinase signaling, resulting in HNSCC proliferation, migration, invasion, and cisplatin resistance. P indicates phosphorylation; PIP_2 , phosphatidylinositol 3,4-bisphosphate; and PIP_3 , phosphatidylinositol 3,4,5-trisphosphate.

Table 1Measurement of Growth in HSC-3 Cells ^a

Treatment	Cell Growth, Mean (SE), Arbitrary Units
No HA (Control)	1.00 (0.04)
HA alone	2.31 (0.06)
Anti-CD44 antibody + HA	0.98 (0.11)
LY-294002 + HA	1.22(0.07) ^b
Y-27632 + HA	1.86(0.12)

Abbreviation: HA, hyaluronan.

^aThe data for cell growth are shown as an average of triplicate determination of 3 experiments (calculated SEM)^bSignificantly different ($P=0.4$) compared with HA treatment alone samples.

Table 2

Measurement of HA-CD44-Dependent Cisplatin Sensitivity in HSC-3 Cells

Treatment	Cisplatin IC ₅₀ , μM
No HA (control)	4
HA alone	12
Anti-CD44 antibody + HA	1
LY-294002 + HA	6
Y-27632 + HA	7
LY-264002 + Y-294002 + HA	4

Abbreviations: HA, hyaluronan; IC₅₀, 50% inhibitory concentration.