Hyaluronan and the Interaction Between CD44 and Epidermal Growth Factor Receptor in Oncogenic Signaling and Chemotherapy Resistance in Head and Neck Cancer

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Objectives: To investigate whether hyaluronan (HA) and CD44 (hereinafter HA-CD44) promotes head and neck squamous cell carcinoma (HNSCC) chemotherapy resistance and whether HA-CD44 promotes epidermal growth factor receptor (EGFR)–mediated oncogenic signaling to alter chemotherapy sensitivity in HNSCC. Hyaluronan, a glycosaminoglycan component of the extracellular matrix, is a ligand for the transmembrane receptor CD44, which acts through multiple signaling pathways to influence cellular behavior. We recently determined that HA-CD44 promotes phospholipase C–mediated calcium signaling and cisplatin resistance in HNSCC.

Design: Cell line study.

Main Outcome Measures: Tumor cell growth with various chemotherapeutic drugs (methotrexate, doxorubicin hydrochloride, adriamycin, and cisplatin) was measured in the presence or absence of HA and other inhibitors of the EGFR-mediated signaling pathway. Immunoblotting was used to study EGFR signaling. Migration assays provided one measure of tumor progression.

Results: The addition of HA, but not HA plus anti-CD44 antibody, resulted in a 2-fold reduced ability of methotrexate and an 8-fold reduced ability of adriamycin to cause HNSCC cell death. Immunoblotting studies demonstrated that HA can promote an association between CD44 and EGFR as well as CD44-dependent activation of EGFR-mediated signaling. Migration assays demonstrated that HA-CD44 can promote tumor migration with EGFR signaling. The presence of AG1478, an EGFR inhibitor, and U0126, an extracellular signal–regulated kinase inhibitor, inhibited HA-mediated tumor growth, migration, and chemotherapy resistance.

Conclusions: Our results indicate that HA promotes CD44/EGFR interaction, EGFR-mediated oncogenic signaling, and chemotherapy resistance in HNSCC. Perturbation of HA-CD44–mediated signaling may be a promising and novel strategy to treat HNSCC.


HEAD AND NECK SQUAMOUS CELL CARCINOMA (HNSCC) is the sixth most common cancer worldwide. Patients with advanced-stage HNSCC continue to have poor 5-year survival rates (0%-40%), which have not significantly improved in the last 30 years. Understanding the mechanisms underlying HNSCC tumor progression and resistance to standard treatment are critical to improving outcomes for patients with this disease. Tumor progression (ie, tumor invasion, migration, and metastasis) is determined by both genetic mutations and the tumor's microenvironment, including the interaction between tumor cells and extracellular matrix molecules. Tumor drug resistance involves multiple mechanisms, including reduced drug accumulation, decreased apoptosis, alterations in drug target, and increased DNA damage repair. The interaction between tumor cells and their microenvironment can lead to the activation of oncogenic signaling pathways that promote both cancer progression and drug resistance.

Hyaluronan (HA) is a glycosaminoglycan component of the extracellular matrix and has well-known biophysical properties. More recently, HA has been studied with regard to its interaction with various cell signaling pathways. Hyaluronan is the primary ligand for the transmembrane receptor CD44, which is expressed in many different benign and malignant cell types. In cancer cells, HA interaction with CD44 promotes multiple signaling pathways that influence tumor cell progression behaviors, includ-
ing abnormal adhesion, migration, and invasion.\textsuperscript{4,6,9} The interaction of HA and CD44 (hereinafter HA-CD44) with these signaling pathways is incompletely understood, and little is known about the role of HA and CD44 in HNSCC.

Epidermal growth factor receptor (EGFR)–mediated signaling seems to be important in many types of epithelial cancers, including HNSCC.\textsuperscript{10-12} One of the well-known targets of EGFR signaling is mitogen-activated protein kinase (also known as extracellular signal–regulated kinase [ERK] 1 and ERK 2). Activation of EGFR signaling results in a variety of tumor progression behaviors, including migration, proliferation, and invasion. Research efforts focused on down-regulating EGFR signaling, through inhibition of the EGFR, have met with considerable but incomplete success.\textsuperscript{13-15} These observations suggested to us that there could be other molecular factors that interact with EGFR signaling and that thus contribute to tumor progression. We hypothesized that HA-CD44 interaction could promote EGFR-mediated oncogenic signaling.

We have previously reported that HA can promote CD44-dependent cisplatin resistance in HNSCC.\textsuperscript{8} In this study, we sought to determine whether HA can promote CD44-dependent HNSCC drug resistance to 2 other chemotherapeutic agents, methotrexate and doxorubicin hydrochloride. We also sought to determine whether HA-CD44 interacts with EGFR-mediated signaling to promote tumor progression and chemoresistance.

## METHODS

### CELL CULTURE

The cell line, HSC-3 (Japan Cancer Research Resources Bank, Tokyo), was established in 1985 from a primary oral squamous cell carcinoma removed from the tongue of a 64-year-old male patient. 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.

### IMMUNOBLOTTING AND IMMUNOPRECIPITATION TECHNIQUES

After growing in serum-free media for 24 hours, HSC-3 cells underwent one of the following procedures: incubation with or without 30 µg/mL of HA for 5 minutes; pretreatment with anti-CD44 antibody (1:1000) or AG1478 (30nM) or U0126 (100nM), followed by the addition of HA (5 minutes); or EGF (30 ng/mL) for 30 minutes. Subsequently, cells were solubilized in 50mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 150mM sodium chloride, 2mM magnesium chloride, 10% NP-40, 0.2mM sodium orthovanadate, 0.2mM phenylmethylsulfonyl fluoride, 10 µg/mL of leupeptin, and 5 µg/mL of aprotenin. After brief centrifugation, the samples were either first subjected to immunoprecipitation using rat anti-CD44 antibody followed by incubation with agarose bead-conjugated anti-IgG or directly solubilized in sodium dodecyl sulfate buffer, electrophoresed on 4% to 12% Tris-glycine gels (Novex; Invitrogen, Carlsbad, Calif), and blotted onto nitrocellulose. After blocking nonspecific sites with 5% milk, the nitrocellulose filters were incubated with rabbit anti-EGFR antibody, mouse anti-phospho-EGFR antibody, rat anti-CD44 antibody, rabbit anti-ERK, or mouse anti-phospho-ERK antibody followed by incubation with horseradish peroxidase–labeled anti-rat IgG. The blots were then developed by the enhanced chemiluminescence system (GE Healthcare Biosciences, Piscataway, NJ).

### ANTIBODIES AND REAGENTS

Monoclonal rat anti-human CD44 antibody (clone, 020; isotype, IgG2b), which recognizes a common determinant of the CD44 class of glycoproteins, was obtained from CMB-TECH Inc (San Francisco, Calif). Polyclonal rabbit anti-EGFR antibody (1005: sc-03) was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). Polyclonal rabbit anti-p44/p42–mitogen-activated protein kinase (ERK 1 and ERK 2) antibody (No. 9102) was obtained from Cell Signaling Technology (Beverly, Mass). Monoclonal mouse anti–phospho-EGFR antibody (1045: No. 05-483) and monoclonal mouse anti–phospho-mitogen–activated protein kinase (ERK 1 and ERK 2) (No. 05-481) were obtained from Upstate Co (Lake Placid, NY). The EGFR inhibitor, AG1478, and the ERK inhibitor, U0126 (No. 662005), were obtained from Calbiochem (La Jolla, Calif). Healon hyaluronan polymers (roughly 500 000-Da polymers) obtained from Healon Pharma (Erlangen, Germany) were prepared by gel filtration chromatography using a SephacrylS1000 column (GE Healthcare Biosciences). The purity of the high-molecular-mass HA polymers used in our experiments was further verified by anion-exchange high-performance liquid chromatography.

### MTT GROWTH ASSAYS

Logarithmically growing cell lines were cultured, washed, counted, and plated at 3000 cells per well in triplicate wells of 96-well plates and incubated in serum-free media overnight. The following day, the cells were treated with various concentrations of cisplatin or methotrexate or adriamycin, with or without HA (30 µg/mL), anti-CD44 antibody, the EGFR inhibitor AG1478 (30nM) or the ERK inhibitor, U0126 (100nM). Two days later, we performed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazolium bromide (MTT) assays according to the manufacturer’s protocol (Roche Diagnostics, Mannheim, Germany). 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 control was plotted as a linear function of drug concentration. Each assay was repeated at least 3 times. The 50% inhibitory concentrations (IC\textsubscript{50}) were identified as the concentration of drug required to achieve a 50% growth inhibition relative to the untreated control.

### TUMOR CELL MIGRATION ASSAYS

Twenty-four Transwell units (Costar Corp, Cambridge, Mass) were used for monitoring in vitro cell migration as described previously.\textsuperscript{4} Specifically, 8µM porosity polycarbonate filters (Costar Corp, Cambridge) were used for the cell migration assays. HSC-3 cells in the presence of or absence of anti-CD44 antibody (30 µg/mL), AG1478 (50nM), or U0126 (100nM) were placed in the upper chamber of the Transwell unit. Medium lacking or containing HA (30 µg/mL) or EGF (50 ng/mL) was placed in the lower chamber of the Transwell unit. After an 18-hour incubation at 37°C in a humidified 95% air and 5% carbon dioxide atmosphere, cells on the upper side of the filter were removed by wiping with a cotton swab. Cell migration processes were determined by measuring the cells that migrated to the lower side of the polycarbonate filters by standard cell number counting methods. Cell counting was performed by 2 observers (S.J.W. and L.Y.B.), including 1 who was blinded to the sample being assessed. The CD44-specific cell
migration was determined by subtracting nonspecific cell migration (ie, cells that migrated to the lower chamber in the presence of anti-CD44 antibody treatment). Each assay was performed in triplicate and repeated at least 3 times. The number of tumor cells that migrated in untreated HSC-3 cells (control) is designated 100%.

RESULTS

HA AND CD44/EGFR ASSOCIATION IN HSC-3 CELLS

Investigations of several cell model systems have indicated that HA-CD44 can promote a variety of cell signaling pathways such as those involving EGFR, which is known to play an important role in HNSCC tumor progression.10,13 To address the question of whether there is a physical linkage between CD44 and EGFR in HNSCC, we first performed immunoprecipitation with anti-CD44 antibody, followed by anti-EGFR antibody immunoblotting (Figure 1A) or reblotting with anti-CD44 antibody (B) as a loading control. Lane 1, untreated cells; lane 2, cells treated with hyaluronan.

Figure 1. Analysis of the CD44 and epidermal growth factor receptor (EGFR) association in the head and neck squamous cell carcinoma cell line HSC-3. HSC-3 cells were solubilized in 1% NP-40 and immunoprecipitated (IP) with anti-CD44 antibody, followed by immunoblotting using anti-EGFR antibody (A) or reblotting with anti-CD44 antibody (B) as a loading control. Lane 1, untreated cells; lane 2, cells treated with hyaluronan.

is an obvious increase in their association following HA treatment of HSC-3 cells.

HA- AND CD44-DEPENDENT EGFR PHOSPHORYLATION IN HSC-3 CELLS

Epidermal growth factor receptor is known to be activated by phosphorylation of its tyrosine residues. We found that HA signaling also stimulates EGFR activity. Using anti-EGFR antibody-mediated immunoblot analysis, we found that EGFR is expressed in HSC-3 cells incubated under various conditions (eg, untreated [Figure 2B, lane 1]; treated with HA [lane 2]; pretreated with anti-CD44 antibody, followed by HA treatment [lane 3]; treated with EGF [lane 4]; or pretreated with AG1478, followed by HA treatment [lane 5]). We also observed that EGFR phosphorylation was significantly up-regulated by HA treatment as detected by anti–phospho-EGFR antibody-mediated immunoblotting (Figure 2A, lane 2). The level of EGFR phosphorylation was relatively low in untreated cells (Figure 2A, lane 1) or in cells pretreated with anti-CD44 antibody, followed by HA treatment (lane 3). The level of EGFR phosphorylation was also up-regulated in cells treated with EGF (lane 4) whereas the level of EGFR phosphorylation was down-regulated in cells pretreated with the EGFR inhibitor AG1478 for 30 minutes, followed by HA treatment (lane 5). These findings support the conclusion that, independent of EGF, HA-CD44 interaction promotes EGFR phosphorylation in HSC-3 cells.

Figure 2. Detection of epidermal growth factor receptor (EGFR) phosphorylation (p-EGFR) in the head and neck squamous cell carcinoma (HNSCC) cell line HSC-3. Detection of EGFR by immunoblotting with anti-p-EGFR antibody (A) or anti-EGFR antibody (B) in HNSCC tumor cell lysates obtained from untreated cells (lane 1) or from cells treated with hyaluronan (HA) (lane 2); cells pretreated with anti-CD44 antibody, followed by the addition of HA (lane 3); cells treated with EGF (lane 4); or cells pretreated with AG1478 (the EGFR inhibitor), followed by the addition of HA (lane 5).

HA- AND CD44-DEPENDENT ERK PHOSPHORYLATION IN HSC-3 CELLS

Both ERK 1 and ERK 2 are known downstream targets of activated EGFR signaling. Extracellular signal–regulated kinase is known to be activated by phosphorylation of threonine residues. We next investigated whether HA could
stimulate ERK activity. Using anti-ERK antibody-mediated immunoblot analysis, we found that both ERK 1 and ERK 2 were expressed in HSC-3 cells incubated under various conditions (eg, untreated [Figure 3B, lane 1]; treated with HA [lane 2]; treated with EGF [lane 3]; pretreated with anti-CD44 antibody, followed by HA treatment [lane 4]; pretreated with AG1478, followed by HA treatment [lane 5]; or pretreated with U0126, followed by HA treatment [lane 6]). We also observed that ERK 1 and ERK 2 phosphorylation was significantly up-regulated by HA treatment as detected by anti–phospho-ERK antibody-mediated immunoblotting (Figure 3A, lane 2). The level of ERK 1 and ERK 2 phosphorylation was comparatively low in untreated cells (Figure 3A, lane 1) or in cells pretreated with anti-CD44 antibody, followed by HA treatment (lane 4). The level of ERK 1 and ERK 2 phosphorylation was also up-regulated in cells treated with EGF (lane 3) whereas the level of ERK 1 and ERK 2 phosphorylation was down-regulated in cells pretreated with the EGFR inhibitor AG1478 for 30 minutes, followed by HA treatment (lane 5) or pretreated with the ERK inhibitor U0126 for 30 minutes, followed by HA treatment (lane 6). These findings support the conclusion that independent of EGF, HA-CD44 interaction promotes EGFR-dependent ERK phosphorylation in HSC-3 cells.

HA- AND CD44-DEPENDENT TUMOR GROWTH AND MIGRATION IN HSC-3 CELLS

We studied HSC-3 cells for HA-dependent tumor growth and migration activity (Figure 4). Using MTT growth assays, we observed that HSC-3 cells had greater proliferation with HA treatment relative to untreated cells (Figure 4A). This increased level of tumor growth was eliminated when cells were pretreated with anti-CD44 antibody and was significantly reduced when cells were pretreated with AG1478 or U0126, followed by the addition of HA. Similarly, using in vitro migration assays, we observed that HSC-3 cells actively migrated during HA treatment relative to untreated cells (Figure 4B). However, the level of tumor cell migration was similar to untreated cells when cells were pretreated with anti-CD44 antibody or AG1478 (or, to a lesser extent, U0126), followed by the addition of HA. HSC-3 cells treated with EGF alone also demonstrated active migration. Examples of HA-induced cell migration and inhibition of HA-induced cell migration by AG1478 are shown in Figure 4C and Figure 4D, respectively. Our observations support the conclusion that HA-CD44 interaction promotes tumor cell growth and migration, and EGFR signaling plays a role in these HA-mediated processes.

HA- AND CD44-DEPENDENT METHOTREXATE AND ADRIAMYCIN RESISTANCE IN HSC-3 CELLS

We have previously reported that HA promotes CD44-dependent cisplatin resistance in HNSC. For this study, tumor cell growth with methotrexate was measured with an MTT assay in the presence or absence of HA (50 µg/mL), or anti-CD44 antibody plus HA (Table). In the absence of HA, methotrexate inhibited tumor cell growth (IC₅₀ of 10µM). The addition of HA reduced the ability of methotrexate to cause HNSCC cell death (IC₅₀ of 20µM), indicating that HA can promote these tumor cells to become methotrexate resistant. Furthermore, pretreatment of HNSCC tumor cells with anti-CD44 antibody followed by the addition of HA eliminated the HA-mediated drug resistance, suggesting that HA acts through CD44 to promote cell survival in the presence of methotrexate (data not shown). Similarly, tumor cell growth with adriamycin was measured with an MTT assay in the presence or absence of HA (50 µg/mL), or anti-CD44 antibody plus HA (Table). In the absence of HA, adriamycin inhibited tumor cell growth (with IC₅₀ of 2µM). The addition of HA reduced the ability of adriamycin to cause HNSCC cell death (IC₅₀ of 16µM), indicating that HA can promote these tumor cells to become adriamycin resistant. Furthermore, pretreatment of HNSCC tumor cells with anti-CD44 antibody followed by the addition of HA eliminated the HA-mediated drug resistance, suggesting that HA acts through CD44 to promote cell survival in the presence of adriamycin (data not shown).

EGFR-MEDIATED SIGNALING PATHWAY INHIBITORS AND HA-MEDIATED CHEMOTHERAPY RESISTANCE

To investigate whether chemotherapy resistance in HNSC might be mediated through HA-CD44 interaction with EGFR signaling, we performed MTT assays with HSC-3 in the presence of increasing concentrations of a chemotherapeutic agent combined with inhibitors of EGFR signaling (Figure 5 and Table). Because cisplatin is the most commonly used drug in anti-head and neck cancer chemotherapy, tumor growth inhibition with this agent is shown in Figure 5. At
50nM concentration, the EGFR inhibitor AG1478 alone caused minimal inhibition of tumor cell proliferation (Figure 5A, inset). However, the same concentration of AG1478 when combined with cisplatin nearly eliminated the HA-mediated cisplatin resistance in HSC-3 cells (Figure 5A). Because ERK 1 and ERK 2 are known targets of EGFR signaling, we next used the ERK inhibitor, U0126. At 100nM concentration, U0126 alone caused minimal inhibition of tumor cell proliferation (Figure 5B, inset). However, when the same concentration of U0126 was combined with cisplatin, HA-mediated cisplatin resistance was significantly reduced (Figure 5B). Similar results were obtained when combining the 2 EGFR pathway inhibitors with methotrexate and adriamycin (Table). These results suggest that EGFR-signaling pathways play a role in HA-CD44-mediated chemotherapy resistance.

The major cell surface receptor for HA, CD44, has been implicated in tumor progression behaviors such as growth, migration, invasion, and metastasis in a variety of malignancies (breast, ovary, colon, lung, brain, and head and neck). In addition, HA-CD44 has also been linked to chemotherapy resistance in several tumor models. Hyaluronan oligosaccharides, which competitively inhibit HA-CD44 interaction, were shown to inhibit anchorage-independent growth in a lung and breast carcinoma cell line, and these oligosaccharides sensitized an adriamycin-resistant breast carcinoma cell line to a variety of chemotherapeutic agents. Another study found that enhanced expression of a variant CD44 isoform was associated with carmustine resistance in a colon carci-

**Figure 4.** Analysis of head and neck squamous cell carcinoma (HNSCC) cell line HSC-3 tumor cell growth and migration. A, HSC-3 cells were placed in triplicate in 96-well plates (3000 cells per well) and grown in serum-free culture overnight. The next day, the cells were incubated without or with hyaluronan (HA) (50 µg/mL) or were pretreated with anti-CD44 antibody or AG1478 (epidermal growth receptor inhibitors) (50nM) or U0126 (extracellular signal–regulated kinase inhibitor) (100nM), followed by the addition of HA. After 2 days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed. The cell growth in cells without any treatment is designated as 100%. The values represent an average of triplicate determinations of 3 experiments. Error bars represent calculated SDs. B, HSC-3 cells (incubated without or with HA, 50 µg/mL) were pretreated with anti-CD44 antibody or AG1478 (50nM) or U0126 (100nM), followed by the addition of HA or were incubated with epidermal growth factor (EGF) (50 ng/mL) and then placed in the upper chamber of a Transwell unit (Costar Corp, Cambridge, Mass). Cell migration processes were determined by measuring the cells that migrated to the lower side of the polycarbonate filters (with or without 50 µg/mL of HA) as described in the “Methods” section. The cell migration in cells without any treatment is designated as 100%. The values represent an average of triplicate determinations of 3 experiments. Error bars represent calculated SDs. C, Photomicrograph of HA-induced migration of HSC-3 cells (original magnification ×400). Note cellular projections. D, Photomicrograph of HSC-3 cells pretreated with AG1478 followed by the addition of HA (original magnification ×400).
Hyaluronan-CD44 interaction with various signaling pathways, such as the EGFR signaling pathway, is being increasingly examined as a possible mechanism for tumor progression. Several authors have reported that CD44 physically associates with EGFR in cancer cells and promotes EGFR-mediated signaling and tumor progression. CD44 was found to colocalize with EGFR in a large percentage of cervical carcinomas, and overexpression of CD44 in these cancers contributed to growth and metastasis through interactions with EGFR and other related type 1 receptors. Similarly, CD44 colocalized with EGFR and Erb-b2 predominantly in metastasizing breast carcinoma cells, suggesting a novel prognostic marker for aggressive mammary cancers. In addition, epidermal growth factor was found to up-regulate CD44 expression and CD44-mediated invasion in astrocytoma cells. Epidermal growth factor receptor was also found to augment cellular response to HA in glioblastoma cell lines. In the current investigation of the HNSCC cell line HSC-3, we demonstrate that HA can promote CD44 association with EGFR as well as EGFR phosphorylation and EGFR-dependent ERK 1 and ERK 2 phosphorylation. Inhibition of HA-mediated tumor growth, migration, and chemotherapy resistance by inhibitors to EGFR and ERK suggest that EGFR signaling also plays a role in these CD44-dependent tumor behaviors. To our knowledge, this is the first report of a link between HA-CD44 and EGFR signaling in HNSCC.

Several recent commercially released chemotherapeutic agents against human cancers are proposed to work through inhibition of EGFR or EGFR-mediated signaling. Efficacy as single agents or in combination with standard chemotherapeutic drugs or radiation therapy has been reported. However, constitutive resistance in many patients, as well as the development of acquired resistance in responders, has limited the effectiveness of the new EGFR inhibitors. Although EGFR inhibitor resistance remains a largely unexplored topic, speculation as to the mechanisms of resistance include the activation of alternate receptor signaling pathways, independent activation of intracellular effectors downstream of EGFR, and EGFR gene mutations. Although

### Table. Measurement of Hyaluronan (HA)-CD44-Dependent Chemotherapy Sensitivity in the Head and Neck Squamous Cell Carcinoma Cell Line HSC-3

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Cisplatin, IC50, µM</th>
<th>Methotrexate, IC50, µM</th>
<th>Doxorubicin Hydrochloride, IC50, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated (control)</td>
<td>4</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>HA alone</td>
<td>20</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>AG1478 alone</td>
<td>6</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>AG1478 + HA</td>
<td>6</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>U0126 alone</td>
<td>10</td>
<td>14</td>
<td>6</td>
</tr>
</tbody>
</table>

**Abbreviation:** IC50, 50% inhibitory concentrations.

*The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed with HSC-3 cells from the head and neck squamous cell carcinoma cell line in increasing concentrations of chemotherapy agent without or with HA plus various inhibitors AG1478 (epidermal growth factor receptor inhibitor); U0126 (extracellular signal–kinase inhibitor), as described in the “Methods” section.

**Figure 5.** Analysis of epidermal growth factor receptor (EGFR) signaling inhibitors and cisplatin sensitivity. A, HSC-3 cells from the head and neck squamous cell carcinoma cell line grown in serum-free media were treated with cisplatin in the presence or absence of hyaluron (HA) (50 µg/mL) plus AG1478 (EGFR inhibitor) (50nM). B, AG1478 (50nM) + Cisplatin. Error bars represent calculated SDs. Inset, AG1478 alone had little effect on growth proliferation at 50nM. B, HSC-3 cells grown in serum-free media were treated with cisplatin in the presence or absence of HA (50 µg/mL) plus U0126 (extracellular signal–regulated kinase) (100nM). MTT assay was performed. Error bars represent calculated SDs. Inset, U0126 alone had little effect on growth proliferation at 100nM.
this study and others have focused on the role of EGFR signaling as a downstream effector of CD44-mediated tumor behaviors, given the association and interaction between CD44 and EGFR, it is interesting to speculate whether CD44 promotes EGFR-independent activation of downstream intracellular molecular targets of EGFR signaling and thus represents another mechanism for EGFR inhibitor resistance in HNSCC. Hyaluronan was recently shown to promote ERK signaling and ovarian cancer progression through an EGFR-independent interaction between CD44 and the IQ (isoleucine and glutamine) motif containing guanine triphosphatase-activating protein.22

Cisplatin, methotrexate, and adriamycin were 3 chemotherapeutic agents that we studied with regard to HA, CD44, and EGFR signaling. Cisplatin is the most common anti-head and neck cancer chemotherapy used today. Methotrexate is sometimes used as a single agent for palliation of recurrent, unresectable head and neck cancer. Although adriamycin is not typically used as an agent against HNSCC, previous studies of breast cancer demonstrated that this drug’s efficacy could be altered through HA signaling.18 Resistance to all 3 drugs is a frequently encountered problem. Various mechanisms of cisplatin resistance in head and neck cancer have been proposed with potential molecular effectors of resistance, including p53, glutathione S-transferase, and c-erb-b2.23–25

Mechanisms of methotrexate resistance that have been described include mutations in the target enzyme dihydrofolate reductase, mutated reduced folate carrier, mutations in enzymes involved in methotrexate polyglutamylation, or enhanced methotrexate efflux by adenosine triphosphate–dependent transporter.26 Our data suggest that HA, CD44, and EGFR signaling represent another mechanism for modulating sensitivity to cisplatin and methotrexate in HNSCC. Adriamycin is thought to bind DNA and inhibit nucleic acid synthesis and is most commonly used in the treatment of leukemia, sarcoma, breast carcinoma, and ovarian carcinoma. A previous study18 demonstrated that HA-CD44 signaling can alter adriamycin sensitivity in breast carcinoma, and in this study we found similar results in HNSCC.

**Figure 6** shows our current proposed model of HA-CD44, and EGFR-mediated promotion of head and neck cancer progression. In this model, the association of HA-CD44 with EGFR leads to EGFR and ERK 1 and ERK 2 phosphorylation, with subsequent tumor cell growth, migration, and chemotherapy resistance. It is probable that other signaling pathways also play a role in HA-mediated cancer progression. CD44 is known to be linked to various transmembrane and intracellular signaling pathways in addition to EGFR, including those involving PLC-gamma-1, PI3 kinase, and others.4,6,8,9,12,16,10,22 The incomplete inhibition of HA-mediated tumor progression and chemotherapy resistance by the ERK inhibitor U0126 also suggests that HA-CD44 interaction with EGFR triggers multiple EGFR-mediated signaling pathways.

In summary, we found that HA promoted resistance to multiple chemotherapeutic agents in an HNSCC cell line. We also found that, independent of epidermal growth factor, HA promoted CD44-EGFR interaction and EGFR and ERK 1 and ERK 2 phosphorylation. Furthermore, EGFR and ERK inhibitors significantly reduced HA-mediated EGFR and ERK phosphorylation, tumor growth, tumor migration, and chemotherapy resistance. The results of our study suggest that targeting HA-CD44-mediated pathways, such as EGFR-mediated ERK signaling, may be a potential therapeutic strategy for HNSCC patients resistant to standard chemotherapy.

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