HYALURONAN AND ITS RECEPTORS IN MUCOEPIDERMOID CARCINOMA

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Accepted 25 May 2005
Published online 30 November 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/hed.20307

Abstract: Background. Hyaluronan (HA) is a prominent extracellular matrix component undergoing continuous production and degradation. Increased HA levels have been described in a variety of tumors. The objective of this study was to examine the staining patterns of HA and two of its associated receptors (CD44 and HARE) in relation to the metastatic potential of mucoepidermoid carcinoma (MC). Immunohistochemical staining of preserved surgical specimens was used.

Methods. Tissues from 12 patients with a histologic diagnosis of salivary MC (10 parotid, one submandibular gland, one minor salivary gland) were studied. Half (six of 12) of the patients had regional metastases. Tumor, normal salivary tissue, and regional lymph nodes were stained for HA, CD44, and HARE expression. Specimens were graded for staining intensity and a percent of the specimen stained.

Results. Normal salivary tissue did not demonstrate epithelial cell surface HA expression, whereas HA was expressed on tumor cells and in regional lymph nodes containing metastases. These differences were both significant using Student's t test ($p < .00002$, and $p < .0022$, respectively). Tumors with positive nodes tended to have greater cell surface HA. Decreased expression or downregulation of HARE was also noted in involved lymph nodes. No differences in CD44 expression were seen between primary specimens and lymph nodes. The observed staining patterns for CD44 and HARE were not reflective of the metastatic potential of the primary MC.

Conclusions. Increased HA expression was seen on mucoepidermoid carcinoma cells compared with adjacent normal salivary gland epithelium. This observation may assist in explaining the development of regional metastasis in these tumors. We did not identify specific HA, CD44, or HARE staining patterns in primary lesions that were predictive of regional metastases.

Keywords: HARE; CD44; hyaluronan; mucoepidermoid carcinoma

The ability of tumor cells to invade locally and metastasize to regional and distant sites is influenced by a multitude of factors. Hyaluronan...
Hyaluronan, a prominent extracellular matrix (ECM) component, has been investigated for its role in these events.\(^1,2\) Tumor-associated production of hyaluronan (HA) can significantly alter a tumor's local milieu. Its postulated roles in the local spread of tumor cells include the creation of a framework for receptor-mediated migration of tumor cells, osmotic expansion and distortion of the ECM, immunoprotection, and stimulation of angiogenesis.\(^2-5\) Receptors for HA, including CD44 and HA receptor for endocytosis (HARE), can also undergo tumor-mediated modulation, which can affect intercellular adhesion properties and the turnover of HA.\(^1,6\)

Previous investigations into the altered expression of HA (and its associated receptors) in salivary gland malignancies are limited.\(^7-10\) This study examined the staining patterns of HA and two HA receptors and whether these patterns were associated with the metastatic potential of mucoepidermoid carcinoma (MC).

**MATERIALS AND METHODS**

A review of the pathology archives at the University of Rochester Medical Center for the diagnosis of MC of salivary gland origin identified 12 cases that had been treated with surgical resection and neck dissection. Of the 12 patients, 10 had parotid primary tumors, one submandibular gland, and one minor salivary gland tumor. A chart review was performed to assess patient demographics, clinical presentation, treatment, and clinical outcomes. Archived tissues from the primary tumor site and neck dissection contents were stained for HA, CD44, and HARE.

The technique used for specimen preparation and immunohistochemical staining for CD44 and HARE was performed as previously described.\(^8\) Tissue was fixed in 10% neutral buffered formalin and paraffin embedded, at the time of original surgery, using routine methods. Sections were cut at 5 \(\mu\)m and dried overnight at 60°C. Slides were deparaffinized through a series of xylene and alcohol washes, and endogenous peroxidase was quenched with 3% hydrogen peroxide. Antigen retrieval was performed by placing the slides in prewarmed solution of 16 mg pepsin in 50 mL of 0.1 N HCl with a 15-minute incubation at 37°C. Slides were washed in phosphate-buffered saline (PBS: 0.137 \(M\) sodium chloride, 0.015 \(M\) potassium chloride, 0.0014 \(M\) sodium phosphate dibasic, and 0.00147 \(M\) potassium phosphate monobasic) and incubated for 60 minutes at room temperature in PBS containing the appropriate primary antibody or preimmune serum negative control. CD44 antibody from DAKO (clone DF1485, DAKO, Carpinteria, CA) was used at a 1:200 dilution, and anti-HARE antibody #154\(^4\) was used at a 1:500 dilution. Slides were rinsed in PBS followed by 30-minute incubation with secondary antibody (biotinylated horse anti-mouse, 1:200 DAKO) at room temperature in PBS. Slides were rinsed briefly in PBS followed by incubation with streptavidin peroxidase (1:1000 in PBS) followed by a PBS and water wash. Color development was performed for 5 minutes with 2.0% v/v aminoethylcarbazine and hydrogen peroxide according to manufacturer instructions (ScyTek, Logan, UT). Hematoxylin counterstain was used. Staining for HA was similar except that the primary antibody was replaced with a biotinylated...
HA-binding protein (Seigagaku Corp.). Nonspecific staining was assessed by pretreatment of the slides with *Streptomyces* hyaluronidase in digestion buffer (0.05 M sodium acetate, pH 5.5).

Results were graded by an experienced head and neck pathologist (CTM) using light microscopy and scored on a 0 to 3 scale for both the percentage of tissues stained and intensity of staining. Percent staining was graded as follows: 0, no staining; 1, 1% to 33% stained; 2, 34% to 66% stained; 3, >66% stained. The intensity of staining scores were judged 0, no staining; 1, mild staining; 2, moderate staining; and 3, intense staining.

The scores for staining and intensity for HARE, CD44, and HA expression in primary site and neck dissection specimens were compared by Student’s *t* test to assess for statistical correlations.

**RESULTS**

The study population was composed of four women and eight men with a mean age of 51 years (range, 18–81 years). Based on histologic features, 83% (10 of 12) of tumors were high-grade/poorly differentiated lesions. The other two cases were intermediate grade; no low-grade lesions were studied. Fifty percent (six of 12) of tumors had associated regional metastasis. All tumors (six of six) with regional metastases were poorly differentiated. No patient had a history of prior head and neck radiation or operative intervention other than biopsy of the primary tumor.

Normal salivary tissue expresses small amounts of interstitial stromal HA but did not express HA on epithelial cell surfaces (Figure 1). A significant increase in tumor cell surface and stromal HA expression was noted in portions of

**FIGURE 2.** (A) Lymph node with mucoepidermoid carcinoma (hyaluronan binding protein, original magnification, ×20). (B) Hyaluronidase-treated negative control (original magnification, ×20). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

**FIGURE 3.** (A) Normal lymph node (hyaluronan receptor for endocytosis stain, original magnification, ×20). (B) Normal mouse serum negative control (original magnification, ×20). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
salivary tissue invaded by MC compared with normal salivary tissue (intensity, $p = 1.6 \times 10^{-5}$; number, $p = 5.4 \times 10^{-7}$) (Figure 1). We also noted an increase in HA expression in lymph nodes with metastatic disease versus normal lymph nodes (intensity, $p = .002$; number, $p = .001$) (Figure 2).

HARE expression was not seen in normal salivary tissue or MC tumor. Interestingly, a difference in staining of HARE was observed in N+ lymph nodes versus uninvolved (N-) lymph nodes with respect to intensity of staining ($p = .0094$) and percentage of tissue staining HARE ($p = .054$) (Figures 3 and 4). No differences were observed in CD44 expression between primary specimens in normal glandular tissue or tumor or in lymph nodes. We did not observe any correlations between primary tumor HA, CD44, or HARE staining patterns and the presence of regional lymph node metastases. Tissue staining results are seen in Table 1. A summary of $t$ test results is given in Table 2.

**DISCUSSION**

HA is a ubiquitous hydrophilic polysaccharide that is found as an extracellular matrix component of all soft tissues. HA is continuously synthesized by fibroblasts and other cell types, whereas clearance is the responsibility of the liver and the lymphatic circulation. The lymphatics account for 85% of the turnover of HA, and the liver catabolizes the remaining 15%. Receptors for HA include CD44, the receptor for HA-mediated motility (RHAMM), and HARE. HARE, which was a focus of this study, mediates the endocytosis of HA in the medullary sinuses of lymph nodes, venous sinuses of the spleen, and the sinusoids of the liver.6,11,12

With respect to lymphatic metastases, it is interesting to note that the subcapsular location of the medullary sinus within a lymph node is also the site where initial tumor implantation frequently occurs. On the basis of these observations, models for regional metastasis that use cell surface HA as a bridging ligand can be postulated. For example, if a tumor cell migrating through the lymphatics, coated with CD44 receptors or hyaluronan synthases occupied by HA, entered a lymph node, the free end of the tumor-bound HA could be recognized by medullary sinus HARE allowing tumor implantation to occur. A similar hypothesis could also explain liver and spleen metastases, given the presence of HARE populations at these sites. Furthermore, the possibility of a mutated CD44 isoform that does not recognize HA offers a potential mechanism whereby a tumor could evade lymphatic capture by HARE. This could help explain those patients who are initially seen with distant metastases without regional disease.

Elevated tumor-associated HA levels have previously been reported with salivary gland tumors, including MC, pleomorphic adenoma, adenoid cystic carcinoma, adenocarcinoma, and squamous cell carcinoma.7–10,13,14 In addition,
metastatic prostate carcinoma cells lines with increased hyaluronan synthase activity and increased pericellular HA coats show increased adhesion to bone marrow endothelium.\textsuperscript{15,16} However, the bone marrow endothelial cell molecule mediating this HA-dependent adhesion has not been identified. Before obtaining our results, we hypothesized that increased HA levels associated with the tumor growth would parallel an increase in tumor-bound CD44 levels. We anticipated that there would also be associated upregulation of HARE within the draining regional lymph nodes. These factors could theoretically enable the metastatic implantation of MC within a lymph node. Although we found a significant increase in HA expression with MC both at the primary site and within a lymph node, CD44 expression was not significantly different between normal tissue and tumor. This observation suggests that some of the HA associated with MC may be interstitial HA produced during tumor growth. The effects that HA can have on the local tissue environment are significant and alone can promote local spread of tumor. Other explanations for the lack of a concomitant increase in CD44 staining include the presence of another as yet unidentified HA receptor, aCD44 splice variant not detected by our staining technique, or an altered glycosylation of CD44 inhibiting antibody recognition.\textsuperscript{17}

The results from our study suggest that HARE is capable of downregulation in lymph nodes harboring regional MC metastases. The HARE concentration within the normal lymph node is prominent. However, in our samples, when metastatic deposits appeared within a lymph node, a peritumoral zone of clearance of HARE was noted. A possible explanation for this downregulation of HARE is a tumor-induced manipulation of its surrounding microenvironment. With a lower concentration of HARE surrounding a lymphatic metastasis, distant spread or extracapsular extension of the tumor might be more likely to occur. Although we did not witness this correlation, the number of patients with regional metastases within our study was limited, and additional investigation is required to validate this hypothesis.

Prior studies examining HA relationship to various malignancies have yielded several important findings. Patients with metastatic disease have elevated serum levels of HA.\textsuperscript{13} In breast carcinoma, increased local concentrations of HA have been found in invasive peripheral areas compared with central tumor. This peripheral upregulation of HA has been considered to be tumor-directed modulation of stromal elements. A highly concentrated zone of HA may play a role in protection from immune attack, hydrodynamic expansion of the local architecture, decrease in cell-to-cell adhesiveness, receptor-mediated signaling of increased cell motility, and promotion of angiogenesis.\textsuperscript{2} Alone or combined these factors favor metastatic spread of tumor.

Other reports in head and neck squamous cell carcinoma have demonstrated altered tumor-associated glycosaminoglycans similar to our results. Laryngeal squamous cell cancer had a 3.5-fold increase in tumor HA compared with normal tissue.\textsuperscript{18} However in oral squamous cell carcinomas, reduced HA staining in the tumor correlated with decreased overall and disease-specific survival, suggesting tumors with less HA are more aggressive.\textsuperscript{19} However, our results in MC indicate increased tumor HA in patients with involved lymph nodes dissimilar to the oral squa-

### Table 2. \textit{p} value results of \textit{t} tests.

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<td>Stromal staining 1.0 .42</td>
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Abbreviations: HA, hyaluronan; HARE, hyaluronan receptor for endocytosis; LN+, lymph node positive; LN−, lymph node negative.
Hyaluronan and Receptors in Mucoepidermoid Carcinoma

HEAD & NECK February 2006

ACKNOWLEDGMENTS

We thank Judith Cornenko for technical assistance.

REFERENCES