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Commentary

Tumor-Associated Hyaluronan

Providing an Extracellular Matrix that Facilitates Invasion

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The report by Wang et al[1] in this issue of the American Journal of Pathology details the abundance and spatial distribution of the extracellular matrix macromolecule hyaluronan in normal and neoplastic epithelia of the esophagus, stomach, and colon. Their results demonstrate the enrichment of hyaluronan in the tumor-associated stroma of all the esophageal-gastrointestinal carcinomas and the variable staining intensities associated with tumor parenchyma, dependent on the epithelium of origin. Their demonstration of positive staining in esophageal squamous cell carcinoma *in situ* suggests that changes in extracellular matrix components such as hyaluronan may represent early events in tumor progression. Apart from the actual data and significance of the results, this paper highlights advances that have been made in visualizing hyaluronan within tissue sections. However, to put this topic in better perspective, it may be helpful to first address the question, "What is hyaluronan and why is it important to study?"

Hyaluronan (better known by its former name, hyaluronic acid) is a member of the family of complex carbohydrates known as glycosaminoglycans. It is a linear polysaccharide composed of a seemingly simple structure of repeating *N*-acetylglucosamine, glucuronic acid disaccharide units (Figure 1). Hyaluronan has many features that are unique as compared to the more well-known glycosaminoglycans such as heparin or chondroitin sulfate. For example, unlike most complex carbohydrates, hyaluronan is not synthesized within the golgi apparatus of cells and is not synthesized upon a protein core precursor. Instead, hyaluronan is synthesized *de novo* and extruded into the extracellular milieu via a yet-to-be-isolated synthase/pore complex embedded within the plasma membrane.[2] The hyaluronan synthase in bacteria has been identified as a single 42-kd protein.[3] Complete synthesis of hyaluronan results in a polyanionic macromolecule with molecular mass between $1-6 \times 10^6$ d, making hyaluronan one of the largest nonbranching polysaccharides found in animal tissues. However, aside from these properties, hyaluronan is of more significance because of its physiological role within tissues. High concentrations of hyaluronan deposited within the extracellular matrix cause tissue spaces to become highly hydrated and to expand because of increased osmotic pressure.[4] Many have suggested that this expanded and water-enriched environment deforms the normally compact, restrictive architecture of extracellular matrices and facilitates cell movement.[5,6] Interest in hyaluronan peaked when investigators discovered that the deposition of large amounts of hyaluronan was temporally regulated and coincided with particular morphogenic events in development, ie, the onset of migration and proliferation of cells. Large depositions of hyaluronan were observed coincident with the migration of presumptive fibroblasts into the primary corneal stroma,[7] mesenchymal cell proliferation in the limb bud,[8] cushion cell migration into the cardiac jelly of the embryonic heart,[9,10] and neural crest cell migrations along the dorsal trunk and cranial regions.[11,12] Reduction of hyaluronan levels within the matrix often signaled the cessation of cell movements and the onset of cytodifferentiation.[5] Enrichment of extracellular matrices with hyaluronan was also observed in adult tissues including forma-

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tion of the early blastema during amphibian limb regeneration,[13] dedifferentiation and cell movements in the healing rabbit tendon[14] and ear cartilage,[15] and the early stages of fracture callus formation.[16] In most of the above examples, the presence of hyaluronan accompanied the migration and proliferation of relatively undifferentiated cells. Thus, it was not too surprising that, as early as 1939, reports by Kabat[17] and others[18,19] studying virus-induced chicken sarcomas described an enrichment of the tumor tissue with a viscous mucinous compound that was identical to a polysaccharide recently isolated from umbilical cord and vitreous and synovial fluids, called hyaluronic acid. Since that time numerous tumors, both of epithelial and connective tissue origins, have been shown to be selectively enriched in hyaluronan as compared to their normal tissue counterparts.[20] Elevated levels of hyaluronan are most evident in human mesotheliomas and Wilm's tumor where hyaluronan can be detected not only locally within the tumor but also in the serum or lung lavage fluids of tumor-bearing patients.[21,22] Using animal models, Toole et al. further demonstrated that the accumulation of hyaluronan correlated with the relative invasiveness of the tumor, as the benign form of the rabbit V2 carcinoma was associated with considerably less hyaluronan than the highly invasive form.[23]

In the majority of the studies described above, hyaluronan was quantified biochemically following extraction of the tumor tissue. Quantification of hyaluronan typically relied on assays measuring the appearance of reducing equivalents following treatment with hyaluronidases, ultraviolet absorbance of unsaturated nonreducing termini following these treatments, content of bound basic dyes, or, more recently, the use of specific ELISA or RIA methodologies. Even as the assays have become more sensitive and specific, increases in hyaluronan continue to be observed in these tumors regardless of the assay employed. All of these results have led to the hypothesis that increased levels of hyaluronan may provide an "embryonic-like" environment that facilitates tumor cell migration (Figure 2).

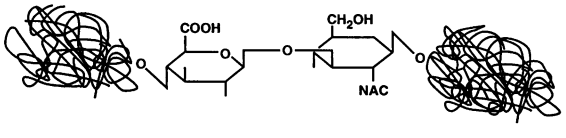

Figure 1. *The structure of hyaluronan. The linear polymer has a repeating disaccharide unit consisting of β1,4-glucuronic acid linked β1,3 to* N*-acetylglucosamine. The native molecule is believed to assume the structure of an expanded random coil in solution. Reproduced from Knudson and Knudson, in Tumor Matrix Biology, Roza Adany, editor, CRC Press, Boca Raton, FL, Chapter 3, Overproduction of hyaluronan in the tumor stroma, pp 55–79. With permission.*[20]

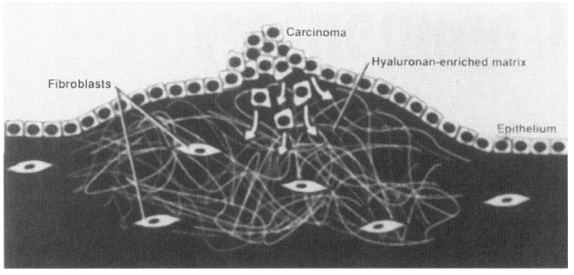

Figure 2. *Increased deposition of hyaluronan within adjacent stromal connective tissue. The increased synthesis and deposition of hyaluronan in tumor tissues results in an expansion and hydration of the matrix, severely distorting the normally compact tissue architecture. The hyaluronan-enriched extracellular matrix associated with invasive carcinomas may provide a highly hydrated milieu conducive to cellular invasion and migration. Reproduced from Knudson and Knudson, in Tumor Matrix Biology, Roza Adany, editor, CRC Press, Boca Raton, FL, Chapter 3, Overproduction of hyaluronan in the tumor stroma, pp. 55–79. With permission.*[20]

Determination of the spatial distribution of hyaluronan within tumor tissues presented investigators with a more difficult challenge. Hyaluronan, being a polysaccharide and relatively ubiquitous to all animals (as well as some bacteria), is essentially nonantigenic. As discussed above, no precursor core protein exists for detection purposes and the hyaluronan synthase is yet to be characterized. In a few studies, hyaluronan was quantified chemically after careful dissection of tumors. For example, when working with the rabbit V2 carcinoma, Toole found that the capsular connective tissue immediately surrounding the invasive tumor contained three times as much hyaluronan as the central tumor parenchyma.[23] Using radiolabeled organ culture slices, Bouziges et al showed that the peritumor region of dissected human colorectal adenocarcinomas displayed a higher level of incorporation of ^{3}H-glucosamine into hyaluronan than slices of the tumor parenchyma itself, which in turn had a significantly higher level of incorporation than normal tissue.[24] Studies such as these provided hints that hyaluronan distribution within tumor tissues was not homogeneous.

Aside from chemical quantification, several studies have attempted to determine the spatial pattern of tumor hyaluronan deposition via conventional histochemical techniques. These localization methods have relied principally on alcian blue or other basic dyes at particular electrolyte concentrations and pH, with or without pretreatment with *Streptomyces* hyaluronidase. In general, these methods are satisfactory for the detection of sulfated glycosaminoglycans such as chondroitin sulfates or heparin, but are significantly less sensitive for the detection of hyaluro-

nan. A second problem is that hyaluronan, being a highly soluble polyanion, readily leaches from tissue sections. This problem may be compounded in tumor tissues where the structures that normally retain matrix components are increasingly destroyed during progressive invasion. To counter this problem, investigators have traditionally included quaternary ammonium salts such as cetylpyridinium chloride to precipitate and fix all polyanionic macromolecules. However, the ammonium salts occupy the same sites on the hyaluronan as the basic dyes, reducing the staining sensitivity. More recent studies (including the paper by Wang et al[1] in this issue) have utilized proteins that bind specifically and with high affinity to hyaluronan as morphological probes. Proteins such as the cartilage-derived proteoglycan termed aggrecan, link protein or hyaluronectin naturally bind to hyaluronan with high affinity. These hyaluronan-binding proteins have been modified (eg, biotinylated, iodinated) or coupled with antibinding protein antibodies for use in ELISAs, RIAs, and immunohistochemistry.[25] This type of indirect immunohistochemical methodology was first utilized for analysis of glioblastomas and astrocytomas, human brain tumors that exhibit elevated hyaluronan levels.[26,27] In one of the studies, the tumor parenchyma was found to stain relatively weakly whereas the adjacent infiltrated stroma showed intense staining for hyaluronan.[27] In areas where cells were highly intermingled within the stroma, it was impossible to distinguish tumor from stroma and the hyaluronan appeared distributed throughout the entire extracellular matrix.[26] In both studies, hyaluronan was observed associated with neighboring blood vasculature. Our laboratory used a chondroitinase-digested aggrecan (cartilage proteoglycan) to visualize hyaluronan within sections of human lung carcinoma. The chondroitinase digestion created "neoepitopes" on the proteoglycan that are recognized by a commercially available monoclonal antibody.[28] As with the studies on brain tumors, indirect immunofluorescence revealed little hyaluronan staining associated with the nests or clusters of infiltrating tumor cells, but did reveal positive hyaluronan staining confined to the remnants of the associated connective tissue stroma.[20] The more comprehensive study by Wang et al in this issue, describes similar results concerning the involvement of stroma and vasculature. This study suggests that the carcinoma, if relatively well differentiated, exhibits patterns of hyaluronan expression similar to their epithelium of origin.[1] In their study, the quaternary ammonium salt, cetylpyridinium chloride, was used to preserve hyaluronan within the tissue sections, as there is less interference of the salt with interactions of binding-protein probes and hyaluronan. Other recent reports have demonstrated that microwave fixation greatly improves the retention of hyaluronan in tissues and may be superior to the use of cetylpyridinium chloride.[29]

One outcome of the studies on hyaluronan localization within tumor tissues is insight as to the cell type responsible for hyaluronan synthesis. Although many tumors have been found to be enriched in hyaluronan *in vivo*, studies on tumor cell lines *in vitro* have been more ambiguous.[20] Many tumor cell lines (eg, fibrosarcomas) have the capacity to produce copious levels of hyaluronan, enough to turn their culture medium into a "gel-like" consistency. Others, particularly carcinoma cell lines, synthesize relatively little hyaluronan in culture.[20] In our early studies we found that the murine A-10 carcinoma had little capacity to synthesize hyaluronan *in vitro*, but subcutaneous or intramuscular tumors generated by these same cells *in vivo* exhibited a 7- to 18-fold increase in hyaluronan as compared with normal mouse subcutaneous or muscle tissues (approximately 3.5 mg hyaluronan per gram dry weight of tumor tissue).[30] These results suggested that other cells within the tumor, such as adjacent normal connective tissue cells, may be participating in the synthesis of hyaluronan. This suggestion is corroborated by many of the histological localization studies of hyaluronan in tumor tissues.

Several mechanisms have been proposed for the accumulation of hyaluronan within tumor-associated stromal connective tissue. One suggestion is that invasive tumor cells, or products secreted by these cells, induce a stimulation of hyaluronan synthesis by stromal cells such as fibroblasts or smooth muscle cells.[20] Such interactions have been shown to regulate the synthesis of vertebrate collagenase as well as tumor-associated cathepsin B activity.[20] We have shown that four human carcinoma cell lines, each possessing a low capacity to synthesize hyaluronan *in vitro*, induce a significant stimulation of hyaluronan synthesis when co-cultured with normal human fibroblasts, because of an increase in fibroblast hyaluronan synthase activity.[31] Similar results were demonstrated by other investigators following the interaction of human fibroblasts with cells derived from mammary carcinoma, colon carcinoma, glioma, and mesothelioma tumors.[20] It is interesting to note that epithelial-mesenchymal interactions that occur during embryonic limb morphogenesis also appear to promote a stimulation of hyaluronan synthesis.[32] Induction of hyaluronan synthesis via

these heterologous cell interactions has been attributed to the release of soluble factors such as basic FGF, the BB isoform of PDGF or TGF-β (ie, paracrine stimulation) as well as interactions requiring direct cell-cell contact (juxtacrine stimulation).[20] In addition, however, other inflammatory mediators such as IL-1 and TNF-α, soluble cytokines secreted by peripheral blood mononuclear cells or macrophages, also have the potential to up-regulate hyaluronan production by fibroblasts. Thus, the interplay of the host immune system during invasion may also play a role in accumulation of tumor-associated hyaluronan.

It should also be considered that the increased hyaluronan accumulation in tumors *in vivo* may not necessarily result from a stimulation of hyaluronan synthesis. For example, the elevated levels of hyaluronan may result from a mere increase in cellularity of the tumor, with each cell producing basal levels of hyaluronan. Alternatively, lymphatic drainage, the physiological route of clearance of tissue hyaluronan, is often blocked in advanced malignancies. In other words, hyaluronan accumulation may also represent failure of normal turnover within the extracellular matrix. The mechanism for accumulation of tumor-associated hyaluronan should be resolved once the gene for the eukaryotic hyaluronan synthase is cloned and sequenced and probes become available for *in situ* hybridization studies.

One question that remains unresolved is whether accumulation of hyaluronan is a "cause" or "effect" of tumor invasion. Is hyaluronan deposition part of the desmoplastic response to "wall off" an invading tumor? It is interesting that the migration of some cell types, such as neutrophils, is completely inhibited in collagen gels enriched in hyaluronan whereas for other cells, such as fibroblasts, the migration rates are enhanced.[6] Collagen sponges enriched in hyaluronan are also being developed in hopes of facilitating cell migration and proliferation during wound healing. Similarities between tumor-associated hyaluronan accumulation during morphogenic events would, in addition, suggest that the presence of hyaluronan promotes or facilitates migration. For example, when stage-8 chick embryos received sub-blastodisc injections of *Streptomyces* hyaluronidase, the tissue spaces between somites and somitomeres, and between the overlying ectoderm, neural tube, and mesoderm, collapsed entirely.[33] The initial pattern of migration of emerging neural crest cells was maintained, but the continued "invasion" of these cells through the mesodermal connective tissue was totally blocked.

In addition to the hydrodynamic effect of hyaluronan on the extracellular matrix it has become clear that high concentrations of hyaluronan also play a direct, interactive role in cell migration—a role mediated via cell surface, hyaluronan-binding sites or "hyaluronan receptors." Several hyaluronan receptors have been identified in recent years.[20,34] The lymphocyte homing receptor CD44 represents one class of hyaluronan receptors expressed by many neoplastic cells. It has been suggested that expression of CD44 hyaluronan receptors provides a mechanism for tumor cells to adhere directly to hyaluronan and be translocated through hyaluronan-enriched extracellular matrices. Expression of alternatively spliced variants of CD44, particularly CD44 v6, is of particular current interest because of its close correlation with metastatic potential. However, this expression may have more to due with avoidance of immune surveillance during metastasis.[20] Whether the interaction between CD44 variants and hyaluronan is required for the promotion of metastatic potential of circulating tumor cells remains unknown. Structurally, CD44 is related to the hyaluronan-binding proteins described above, ie, aggrecan proteoglycan, link protein, and hyaluronectin. In fact, recombinant chimeric CD44/immunoglobulin constructs have also been used as morphological probes for hyaluronan.[35] Another hyaluronan receptor, unrelated to CD44, has also been found to be expressed by neoplastic cells. It has been termed RHAMM for "receptor for hyaluronan-mediated motility" because it appears to function primarily in stimulating a locomotory, chemokinetic response of tumor cells to soluble hyaluronan.[20] For example, in *c*-H-*ras*-transformed fibroblasts, a membrane-bound intracellular tyrosine protein kinase becomes activated upon the interaction of RHAMM with hyaluronan,[36] resulting in a chemokinetic response by the cells. RHAMM does not appear to be directly related to CD44 or hyaluronan-binding proteoglycans (ie, does not contain a B-loop structure) but may share common structural motifs within the actual hyaluronan-binding domain.

In summary, tumor-associated hyaluronan may provide an expanded, hydrated environment conducive to cell invasion. The presence of hyaluronan may also function as a support or substratum for receptor-mediated locomotion or even as an inducer of chemokinesis by tumor cells. Many of these mechanisms are being investigated in a number of *in vitro* and animal tumor model systems. However, the expression of hyaluronan in normal and neoplastic human tissues remains critical to our overall understanding. The article by Wang et al[1] in this issue

provides an exciting glimpse of work proceeding toward this goal.[1]

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