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Emerging roles for hyaluronidase in cancer metastasis and therapy

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Abstract

Hyaluronidases are a family of five human enzymes that have been differentially implicated in the progression of many solid tumor types, both clinically and in functional studies. Advances in the past five years have clarified many apparent contradictions, (1) by demonstrating that specific hyaluronidases have alternative substrates to hyaluronan (HA) or do not exhibit any enzymatic activity, (2) that high molecular weight HA polymers elicit signaling effects that are opposite those of the hyaluronidase-digested HA oligomers, and (3) that it is actually the combined overexpression of HA synthesizing enzymes with hyaluronidases that confers tumorigenic potential. This review examines the literature supporting these conclusions and discusses novel mechanisms by which hyaluronidases impact invasive tumor cell processes. In addition, a detailed structural and functional comparison of the hyaluronidases is presented with insights into substrate selectivity and potential for therapeutic targeting. Finally, technological advances in targeting hyaluronidase for tumor imaging and cancer therapy are summarized.

Keywords

Hyaluronan; hyaluronidase; tumor biology; metastasis; tumor imaging; cancer therapy

1. Introduction

Hyaluronan (HA) is abundant as a polymer in joint and tissue matrices (Fraser et al., 1997; Laurent et al., 1996), where its roles in hydration, cushioning, and shock absorption have been well studied. Seemingly contrary to this architectural role, HA has also been well defined as a specific biological stimulus, critical for facilitating cellular proliferation and motility (Toole, 1997). A dramatic illustration of this role was elegantly demonstrated in embryonic heart development, in which HA is both a migration substrate and a signal for EMT, promoting the timed transformation and movement of pericardial cells to form the atrioventricular septum (Camenisch et al., 2000). HA is a simple linear polymer of alternating glucuronic acid and N-acetylglucosamine that can be repeated several hundred times. This relative chemical homogeneity may seem difficult to reconcile with the array of functional outcomes in which HA is implicated. However, advances in the past 20 years have revealed that the information content of the HA stimulus is highly context dependent

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and may contribute radically different phenotypic outcomes based on processing or degradation by hyaluronidase enzymes and reactive oxygen species, and differential engagement of cell surface receptors and intracellular signaling cascades.

Newly synthesized HA polymers are generated by HA synthases HAS1, HAS2, and HAS3, which are integral plasma membrane enzymes with an intracellular active site that catalyzes alternating monosaccharide addition to an average mass of 100–2,000 kDa (Itano et al., 1999), concurrent with extrusion of the polymer to the exterior of the cell. Normal HA synthesis is activated transiently for cell division or motility, after which HA is rapidly cleared from the site by endocytic uptake and/or hyaluronidase-catalyzed hydrolysis. There are five human protein-coding sequences assigned to the hyaluronidase family on the basis of overall homology and active site conservation (*hyal1-hyal4* and PH20)(Csoka et al., 2001). Three of these have measurable activity for endolytic HA hydrolysis, one acts primarily on chondroitin sulfate, and activity of the fifth has not yet been determined but it is inactive toward HA. We discuss these enzymes in detail below.

Cellular responses to HA polymers and HA oligosaccharides are executed through multiple mechanisms, involving both cytoskeletal reorganization upon direct binding of HA to surface receptors such as CD44 (Aruffo et al., 1990; Bourguignon et al., 2000; Legg et al., 2002), and receptor-mediated internalization of HA-bound complexes through endosomal pathways (Harada and Takahashi, 2007; Tammi et al., 2001). These complex receptor mediated events and their aberrant behavior in cancer are the subject of several reviews within this volume. Through these receptors, specific sizes and quantities of HA have opposing impact on cell growth and tissue remodeling. For example, HAS overexpression leads to HA polymer accumulation that can promote tumor growth and/or metastasis (Enegd et al., 2002; Itano et al., 2004; Jacobson et al., 2002; Kosaki et al., 1999). These effects of HA are dependent on its steady state levels, and excess HA polymer suppresses tumor growth (Bharadwaj et al., 2007; Itano et al., 2004). Depending on actual chain length, HA oligomers may promote proliferation and angiogenesis, or induce apoptosis (Zeng et al., 1998). In this review, we will discuss hyaluronidase-catalyzed processing of HA polymers to shorter fragments and oligomers and their effects on functional outcome.

2. Of mole rats and men: insights about hyaluronan and cancer

2.1 Hyaluronan and hyaluronidase accelerate human cancers

Respective functions of HA polymers and HA oligomers, resulting from altered gene expression of HAS or Hyal, respectively, have been carefully dissected in functional studies of cancer progression. The clinical significance of concurrent excess HA and Hyal overexpression in resected or biopsied human tissue specimens confirms the relevance of such mechanisms for human cancer. HA accumulation is a clinical feature of prostate cancers of Gleason sum >4, regardless of the patient's hormone status. However, HA detection is confined to the stromal compartment until later stages of cancer, when HA can be observed in association with abnormal glandular epithelial cells as well (Aaltomaa et al., 2002; Lokeshwar et al., 2001). Overexpression of Hyal1, combined with excess HA detection, clinically predicts prostate cancer biochemical recurrence and reduced five-year survival (Ekici et al., 2004; Gomez et al., 2009; Posey et al., 2003). Combination of Hyal1

and HAS2 expression is prognostic for bladder cancer recurrence and expression of Hyal1 is an independent marker for disease-specific mortality in this study (Kramer et al.). Levels of Hyal1 expression measured in clinically invasive resected prostate tumors are tumor promoting in mice (Lokeshwar et al., 2005). Similarly, the combined overexpression of HAS and either Hyal1 (Tan et al., 2011a) or Hyal2 (Udabage et al., 2005) is specifically observed at the invasive front in human breast cancer and associated functionally with breast cancer progression in mice.

Excess HA suppresses tumor growth in the absence of hyaluronidase. Stable HAS overexpression in prostate carcinoma cells that normally make negligible HA and HAS significantly reduces tumor take and tumor growth kinetics in either the subcutaneous (Bharadwaj et al., 2007; Simpson, 2006) or the orthotopic primary injection site (Bharadwaj et al., 2009). In orthotopically implanted animals that bore tumors, tumor vascularity was not appreciably different in HAS-overexpressing tumors and lymph node metastasis was not observed. Accumulation of HA in the tumors suggested poor clearance of HA produced by HAS-overexpressing tumor cells is antiproliferative. In fact, tumor cell proliferation was found to be inversely and temporally coincident with HA production, and these effects could be reversed either by coexpression with Hyal1 or exogenous hyaluronidase addition. Moreover, exogenously added HA did not affect cell growth. This implies that HA production within a tumor must be altered at the level of the tumor cell to impact growth, while the sources of Hyal1 could be numerous.

In contrast to HAS overexpression, HAS+Hyal1 co-overexpression potentiates both tumorigenesis and metastasis (Bharadwaj et al., 2009; Kovar et al., 2006). Prostate tumor cells that produce endogenous large quantities of HA polymer are more metastatic to lymph nodes when injected intraprostatically in mice, but only if they express Hyal1 (Patel et al., 2002). Low HA-producing prostate tumor cells are normally not metastatic, but when transfected with Hyal1 alone, cells disseminated rapidly to lymph nodes following orthotopic implantation (Kovar et al., 2006). When these cells co-overexpressed HAS and Hyal1, there was a 6-fold increase in tumor size and all tumors exhibited lymph node metastasis. Knockdown of HA synthesis abrogated ≈90% of spontaneous lymph node metastasis of highly metastatic prostate tumor cells (McCarthy et al., 2005). Thus, HA production by tumor cells in prostate cancer may enhance the aggressive potential of the tumor by providing substrates for Hyal1-dependent autocrine proliferation. However, stromal HA production, activated by cytokines, may also serve to recruit macrophages to tumor sites and thereby enhance angiogenesis indirectly. Macrophages can induce degradation of HA polymers to oligomers through a combination of hyaluronidase-mediated cleavage and reactive oxygen species induced HA chain scission (Ohnuma et al., 2009).

Mechanistic studies in vitro have provided insights to the correlate processes of tumor progression impacted by HAS and Hyal. Coexpression of Hyal1 with HAS2 or HAS3 diminished HA retention, but restored rapid proliferation in culture that was suppressed by HAS, which supports a combined role for excess HA synthesis and processing in maximizing unrestricted growth of prostate cancer cells. Stable HAS transfectants retain HA at the cell surface, grow significantly more slowly in culture, and exhibit 50–90% reduced adhesion and motility on extracellular matrix protein substrates. Adhesion is dependent on

differential engagement and cell surface presentation of β1 integrin receptors, which also alter motility. Integrin binding to ECM ligands is linked both to motility and to cell viability, and in fact, HAS overexpressing cells have higher levels of p21/p27 cyclin-dependent kinase inhibitors and commensurate increased time to S phase in cell cycle analysis (Bharadwaj et al., 2011). These effects were opposite in Hyal1 overexpressing tumor cells, which did not have altered adhesion receptor expression, were more motile and exhibited more rapid cell cycling. It is apparent that uncleared excess HA accumulation as a result of HAS overexpression can lead to alterations in cell surface receptor function.

2.2 Naked mole rats resist cancer

Validation of the respective functions of HA in tumor suppression and hyaluronidase in eliminating protection against tumorigenic insults was provided recently from an unexpected source: the naked mole rat, a mouse-sized hairless organism with a 20–30 year lifespan. Naked mole rats are resistant to cancers of any kind, whether exposed to chemical carcinogens, ultraviolet irradiation, injected tumor cells, or other type of cancer-inducing insult. One protective element in mole rats was found to be an unusually high level of stromal, interstitial, and subcutaneous HA of very high molecular mass, nearly double the average size of HA polymers found in humans and other rodents (Tian et al., 2013). The quantity of HA production was attributable to elevated expression of a single isozyme orthologue of the human HA synthase HAS2, which also produces HA of high molecular mass in humans and other mammals. Two substitutions at the HAS2 putative active site, both serine residues that are conserved glutamine residues among other mammalian HAS2 orthologues, may promote increased processivity in the naked mole rat HAS2, but this has not yet been experimentally demonstrated. Also contributing to the steady state levels of polymeric HA is that the hyaluronidase activity of all tissues tested in the mole rat is significantly lower than in human cells or other rodent tissues. Therefore, the mole rats do not degrade HA appreciably.

Importantly, naked mole rat skin fibroblasts can be transformed by overexpression of constitutively active ras and SV40 large T antigen, and will grow in culture, but not in soft agar or in mice. These cells became tumorigenic and grew anchorage independently when their high level of HA polymer production was reduced by knocking down HAS2, or antagonized by increasing HA turnover with overexpression of hyaluronidase (Tian et al., 2013). This is consistent with results discussed above in human breast and prostate cancers overexpressing HAS, which suppresses tumor growth, while concurrent HA synthesis and turnover with both HAS and hyaluronidase present, significantly accelerates tumor growth and metastasis. The authors further confirmed in vitro that the growth suppressive response to HA, manifest as early contact inhibition, was dependent upon signaling through the known axis of the CD44 HA receptor, Nrf2, and ultimately $p16^{INK4a}$. These results are important because the respective roles for HAS2 and Hyal2 in naked mole rats were identified through unbiased approaches that validate mechanisms defined in systems that are not truly cancer resistant. Thus, HA turnover as a cause of cancer progression, and the absolute cancer-protective role of intact HA polymers, are concepts that could be firmly established in the intact naked mole rat, which has not been possible in cancer-susceptible organisms.

3: Hyaluronidase Expression in Cancer

3.1 Hyal1

The expression and functional importance of hyaluronidases in cancer has been most widely characterized with respect to the Hyal1 enzyme. Hyal1 is normally expressed in many cell types and is found within cells, partially compartmentalized to vesicles that traffic in a manner distinct from clathrin or caveolin endosomal routes, and partially to lysosomes ((Puissant et al., 2014) and McAtee, et al, submitted). As such, its role in housekeeping levels of HA and glycosaminoglycan (GAG) turnover is well accepted. However, Hyal1 is also a secreted protein that can be found in tumor interstitial fluid (Lokeshwar et al., 2005; Tan et al., 2011a) and in conditioned media of tumor cells in culture (Lokeshwar et al., 2005; Simpson, 2006; Tan et al., 2011b), so its utility as a diagnostic and prognostic biomarker has been extensively validated and exploited.

Genitourinary cancer—Hyal1 is an accurate urinary diagnostic marker for bladder cancer, because it is significantly overexpressed in tumors and shed to the urine of cancer patients (Eissa et al., 2012a; Eissa et al., 2010; Eissa et al., 2012b). Hyal1 mRNA was increased 4- to 16-fold in bladder cancer specimens, and elevated Hyal1 expression predicted metastasis and mortality (Kramer et al., 2011). In addition, higher expression of Hyal1 correlated with bladder cancer progression to muscle invasion (Kramer et al., 2010). In prostate cancer, Hyal1 expression in biopsy samples predicted recurrence (Gomez et al., 2009).

Colorectal cancer—Hyal1 was detected in serum samples from colorectal carcinoma patients, where serum Hyal1 was reduced in cancer patients compared to levels in normal samples (Kolliopoulos et al., 2013). This phenomenon could be attributable to localization of the free Hyal1 at the primary tumor site rather than in the circulation. A separate study showed that multiple isoforms of Hyal (Hyal1, 2, 3, and PH-20) had increased activity in colorectal cancer patient samples and the expression of Hyal1 and Hyal2 was especially associated with more aggressive stages of cancer (Bouga et al., 2010).

Breast cancer—Clinically, Hyal1 expression can be used to predict invasive breast cancer progression in patients with benign ductal hyperplasias (Poola et al., 2008). Hyal1 overexpression in breast cancer enhances motility and anchorage independent growth in vitro, and angiogenesis in vivo (Tan et al., 2011b). Tumors in mice contained excess HA, resulting from increased HA fragment production by overexpression of Hyal1. Hyal1 levels are high in breast cancer cell lines MDA-MB-231 and MCF-7, and also in metastatic lymph nodes of breast cancer patients (Tan et al., 2011a). Knockdown of Hyal1 in MDA-MB-231 and MCF-7 lines decreased invasion, adhesion, proliferation, and xenograft tumorigenesis, which confirms its role as a tumor promoter in this cancer type.

Ovarian cancer—Hyaluronidase activity and Hyal1 transcript levels are elevated in mucinous and clear cell epithelial ovarian cancers relative to benign or normal ovarian tissue, concurrent with decreased expression of estrogen receptor ERα (Yoffou et al., 2011). When ERα was overexpressed, Hyal1 expression decreased by approximately half,

suggesting a loss of ERα function may be a mechanism by which Hyal1 expression becomes elevated in these tumors (Yoffou et al., 2011). In grade 3 serous ovarian cancer, levels of Hyal1 transcript are lower and HA staining consequently higher in comparison to normal ovary (Nykopp et al., 2009). Thus, the expression of Hyal1 is associated with a range of malignant phenotypes, even within the same tissue.

Pancreatic and lung cancers—A study of pancreatic ductal adenocarcinoma showed a correlation between modest Hyal1 expression and lower survival rates (Cheng et al., 2013). In the same study, accumulation of HA was found enhanced in cancerous tissues compared to normal tissues. Hyal1 and Hyal3 were also confirmed to have high expression in lung cancer cells when compared to normal control and stromal cells (de Sa et al., 2012). These Hyals, as well as the HAS proteins, are predicted to have a mechanistic role in the invasiveness of lung cancer (de Sa et al., 2013). Defining the correlation between cancer stage, HA production, and Hyal expression could contribute to the development of more individualized treatments for cancer patients.

Hyal1 splice variants—Hyal1 can undergo alternative splicing, and its splice variants have also been studied in cancer. Hyal1 splice variants are enzymatically inactive because they are all missing a 30 amino acid region that is required for wild type activity (Lokeshwar et al., 2002). The Hyal1-v1 splice variant has been studied in a bladder cancer model, where it is proposed that it forms a complex with wild type Hyal1 and thus lowers its activity, causing decreased growth and increased apoptosis (Lokeshwar et al., 2006). Differential expression of many of the Hyal splice variants is also associated with cancer outcomes. In a study of lung carcinomas, a better prognosis was associated with higher expression of the Hyal3v1 splice variant, whereas poor prognosis correlated with expression of Hyal1 wild type (de Sa et al., 2012). Hyal1-v3, Hyal3-v1, and Hyal3-v2 splice variants were also shown to be associated with low tumor recurrence and low Gleason score in prostate cancer (de Sa et al., 2009).

3.2 Hyal2

Hyal2 expression has been more recently correlated with progression of multiple cancers with the availability of isozyme-specific antibodies. In some cases, loss of expression has been reported. For example, in a small scale study of human lung cancer, loss of Hyal2 expression correlated with the presence of tumors (Li et al., 2007). Similarly, mRNA levels of Hyal2 were reduced in endometrial cancer relative to normal tissue (Nykopp et al., 2010). In contrast, expression of Hyal2 was significantly increased in pre-malignant and malignant melanomas (Siiskonen et al., 2013), and in breast cancer specimens (Tan et al., 2011a). Hyal2 was specifically expressed at the expanding margins of invasive breast cancer (Udabage et al., 2005).

Epigenetic modifications controlling Hyal2 expression have also been examined in cancer biomarker discovery. The methylation profile of numerous genes including Hyal2 can be used to identify normal versus head and neck squamous cell carcinoma (Langevin et al., 2012). Hyal2 splice variants also show varying expression in gastric cancer samples (Ohnuma et al., 2009). Presence of the splice variant Hyal2ex2-3 was associated with gastric

tumor cell lines (Ohnuma et al., 2009). This splice variant is found in the 5′UTR of the Hyal2 gene, which may contribute to its differential expression in cancerous and normal tissues (Ohnuma et al., 2009).

Hyal2 is GPI-anchored at the plasma membrane and associated with cholesterol-rich lipid rafts (Andre et al., 2011), where it acts in complex with CD44 and Hyal1 to promote uptake and endocytic internalization of HA (Harada and Takahashi, 2007). Overexpression of Hyal2 in fibroblasts has been shown to reduce pericellular retention of HA and proteoglycan-rich matrix, partially by increased turnover and partly through loss of CD44 from the cell surface (Duterme et al., 2009). In astrocytoma cells, the overexpression of Hyal2 significantly accelerated tumor growth through enhanced angiogenesis upon implantation in the HA-rich intracerebral microenvironment (Novak et al., 1999). As in breast cancer, Hyal2 was associated with increased invasive protrusions in the Hyal2-overexpressing astrocytoma tumors. Results of Hyal2 manipulation in these models are reproduced in the naked mole rat model, in which normally non-tumorigenic skin fibroblasts became highly tumorigenic upon Hyal2 overexpression when implanted in HA-rich tissue (Tian et al., 2013).

Hyal2 has been shown to associate with RON tyrosine kinase in epithelial cells, sequestering it at the plasma membrane and suppressing its activity (Danilkovitch-Miagkova et al., 2003). Upon ligation of Hyal2 by infection with a transforming sheep retrovirus, RON kinase was released and activated EMT through the Akt/ERK pathway. Since the region of Hyal2 required for viral recognition is proximal to the active site (Duh et al., 2005), it is probable that ligation of Hyal2 by HA or other GAGs triggers signaling through RON kinase. HA-ligated Hyal2 may also signal through the WOX1 (a.k.a.WWOX) apoptosis inducer and Smad4 (Chang et al., 2010; Hsu et al., 2009). In these studies, it was suggested that binding of TGF-β1 to Hyal2 at the plasma membrane caused WOX1 association and complex formation, leading to nuclear translocation and induction of apoptosis through WOX1 association with nuclear transcription factors including p53, ErbB2, and ErbB4 (Hsu et al., 2009). However, the interaction between WOX1 and Hyal2 was found to occur in the Hyal2 catalytic domain, which would not be expected to be available for binding to the intracellular WOX1 if it was engaged on the cell surface by TGF-β1, so the physiological significance of this mechanism needs further examination. It is still possible that the interaction of Hyal2 with WWOX protein at the plasma membrane could prevent it from functioning as a tumor suppressor (Hsu et al., 2009). Overexpression of Hyal2 in fibroblasts also reduces CD44-ERM interaction, which was actually found to result in lower ERM activation and decreased motility of these cells (Duterme et al., 2009). Further study of these complex putative mechanisms will be needed to better define the impact of Hyal2 on cancer progression.

3.3 Hyal3, Hyal4, PH-20

The other three human hyaluronidase family members, Hyal3, Hyal4, and PH-20, have been studied to a lesser extent in relation to cancer. Hyal3 does not have a GPI attachment site, but Hyal4 and PH-20 are GPI anchored at the plasma membrane. Of these three, only PH-20 has hyaluronidase activity but its expression is testis-specific and does not change in

multiple tumor cell lines (Patel et al., 2002). Nonetheless, there is evidence for their involvement in the progression of certain types of cancers.

Hyal3 lacks any detectable enzymatic activity to date, but its overexpression was shown to impact hyaluronidase activity in mice by increasing Hyal1 levels (Hemming et al., 2008). Expression of Hyal3 transcripts and splice variants has been examined for potential diagnostic or prognostic value. In lung cancer, expression of the Hyal3v1 splice variant correlated with lower risk of death from disease (de Sa et al., 2012). In a small study of lung squamous cell carcinoma patients, ≈10% of patients carried one of two heterozygous mutations in exon 1 or 2 of the *HYAL3* gene, found only within tumor tissue and not present in normal surrounding tissue (Zhang et al., 2013). Presence of these mutations was correlated with lymph node metastasis, but not with other clinical parameters such as tumor size, grade, or remote metastasis. A modest elevation in Hyal3 transcript was observed in conjunction with those for HAS3 and RHAMM, in nodular basal cell carcinomas (Tzellos et al., 2011). In a panel of breast tumor cell lines, mRNA expression of Hyal3 was associated with relatively low invasive potential (Udabage et al., 2005).

Hyal4 catalyzes degradation of other GAGs than HA, specifically chondroitin sulfate, which could impact tumor cell surface proteoglycan turnover and contribute to cancer progression. Hyal4 is endogenously overexpressed in clear cell renal carcinoma and papillary tumors of the kidney compared to oncocytomas (Chi et al., 2012). Moreover, expression of Hyal4 independently distinguished between benign oncocytoma and renal cell carcinoma, suggesting a possible functional link to progression. An unbiased comparison of chromosome aberrations in low versus high grade glioma patients revealed a significant association between high grade glioma and the overrepresentation of specific portions of chromosome 7, including the *HYAL4* gene (Li et al., 2013).

PH-20 expression has been examined in the context of breast cancer (Madan et al., 1999b), where it was found in normal, carcinoma in situ, invasive, and metastastic breast tissue, but elevation was significantly associated with invasive and metastatic cancer in African American women (Beech et al., 2002). This was a small study but suggests potential for PH-20 in early detection and prognosis. Similar association between high PH-20 expression and prostate (Madan et al., 1999a) or laryngeal cancer (Godin et al., 2000) has also been reported. In breast tumor cells, expression of PH-20 was functionally associated with upregulation of p53 and WOX proteins, which allowed cells to be more susceptible to tumor necrosis factor (TNF) (Chang, 2002). Like Hyal2, PH-20 enhances the expression of WOX1, which is pro-apoptotic and therefore can function as a tumor suppressor (Chen et al., 2004).

Overall, the differential expression profile of the hyaluronidase family members and their splice variants could be one of the determining factors in cancer prognosis but more systematic and larger scale studies are needed to understand the significance of these profiles.

4: Hyaluronidase function and the metastatic process

4.1 Vesicle trafficking and cell motility

Many studies have investigated how the internalization of HA and its processing enzymes contribute to endocytic trafficking patterns and thus manipulate signaling pathway cascades controlling proliferation and migration, among other processes. HA is internalized from the keratinocyte plasma membrane through receptor mediated or bulk endocytosis by a route not involving clathrin or caveolin (Tammi et al., 2001). Using chemicals that disrupt lysosomal function, there was a shift to a higher molecular weight average fragment size of internalized HA, meaning that a portion of the internalized HA was targeted to lyosomes for degradation after uptake. Excessive or aberrant internalization and trafficking of HA has been demonstrated in tumor cells, which is proposed to result in incomplete HA breakdown products being recycled back to the cell surface (Fig 1), where they act as pro-angiogenic signaling molecules, or complete breakdown into precursor molecules to be fed into multiple pathways.

Murine macrophages secrete Hyal1 to the extracellular matrix, from whence its re-uptake involves the mannose receptor, but is mannose-6-phosphate independent (Puissant et al., 2014). A portion of the internalized Hyal1 undergoes a single cleavage event in endocytic vesicles that are targeted to lysosomes, where cleaved Hyal1 retains enzymatic activity and likely completes degradation of GAGs. In HT1080 human fibrosarcoma cells, treatment with basic fibroblast growth factor (bFGF) increased HA production and decreased Hyal2 expression, which caused build up of high molecular weight HA in the extracellular matrix (Berdiaki et al., 2009). Treatment of the cells with high molecular weight HA ($3–4 \times 10^6$ Da) impaired migration and treatment with low molecular weight HA (31 kDa) enhanced migration in a scratch wound healing assay (Berdiaki et al., 2009). Thus, the net effect of hyaluronidase-mediated HA turnover is increased motility in a variety of cell and tumor types.

HA turnover has been shown to affect levels of plasma membrane proteins, which translated to significant impact on cell adhesion and motility signaling pathways (Bharadwaj et al., 2011; Bharadwaj et al., 2009; Bharadwaj et al., 2007). In prostate tumor cells, overexpression of HAS3 resulted in lower expression of N-cadherin on the plasma membrane, reduced motility, and delayed cell cycle re-entry, while overexpression of Hyal1 produced opposite effects irrespective of HAS3 (Bharadwaj et al., 2009). Prostate tumor cells overexpressing Hyal1 have enhanced endocytic activity as measured by the rate of fluorescently labeled transferrin internalization (McAtee et al, submitted). It is probable that overall endocytic rate affects steady state levels of receptors at the plasma membrane. This observation would also explain many of the differences in receptor expression and internalization that have been reported to occur with differential expression of Hyals.

One way HA homeostasis could affect overall motility of the cell is through the formation and disruption of focal adhesions. In esophageal cancer cells, inhibiting HA synthesis by HAS3 knockdown or by diminishing the HA precursor pool with 4-methylumbelliferone disrupted filopodia and focal adhesions, which subsequently decreased proliferation and migration (Twarock et al., 2010). These outcomes occurred downstream of HA synthesis

inhibition following cleavage of focal adhesion kinase (FAK), which led to decreased activation of ERK. Since FAK plays a pivotal signaling role in multiple functions such as proliferation, motility, and invasion, a better understanding of the complex relationship between FAK, ERK, HA synthesis and HA turnover will provide insights to the role of HA in cancer progression.

4.2 Vesicle shedding

Increased vesicle shedding is an emerging hallmark of cancerous cells. Tumor cells secrete vesicles that contain proteins, microRNA, and other nucleic acids. Shedding vesicles or microvesicles are produced by budding from the plasma membrane, while the smaller (≤50 nm diameter) exosomes are generated by inward budding within large intracellular organelles called multivesicular bodies that fuse with the plasma membrane to release their exosomal contents. Vesicle shedding was originally proposed to be a mechanism for cellular waste disposal, but strong evidence shows that exosome production and secretion in particular is a tightly regulated process with specific functional relevance. Exosomes are released by tumor cells, have been correlated with cancer progression, and carry relevant biologically active epigenetic regulators capable of transforming target cells. Vesicle shedding suggests a mechanism for local stromal-epithelial crosstalk at the tumor primary site, and for communication with cells in remote tissues to promote metastatic susceptibility. HA and Hyals have been implicated in these processes.

Both levels of cellular HA production and hyaluronidase Hyal1 overexpression have been found to correlate with the rate of vesicle secretion from the cell. Cells with high endogenous HAS levels had a higher rate of shedding vesicle release than cells with low HAS expression, and inducing overexpression of HAS3 in a low HAS background increased release of vesicles (Rilla et al., 2013). The origin of these vesicles was a combination of the tips of microvilli, the pinching off of the plasma membrane, and secreted exosomes. The shedding vesicles contained HAS3 protein and retained a perivesicular HA coat. Hyaluronidases were not examined in this study, but independently it has been shown that Hyal1 was contained in both exosomal and microvesicle fractions isolated from conditioned media of prostate tumor cells overexpressing Hyal1 (McAtee et al, submitted). The ratio of exosome- to microvesicle- associated Hyal1 was higher in cells overexpressing wild-type Hyal1 versus a catalytically inactive mutant (E131Q). Thus, the enzymatic activity of Hyal1 is necessary for trafficking of Hyal1 into exosomal vesicles released by the cell.

The appearance of HAS3 and Hyal1 in vesicles is a novel mechanism by which HA homeostasis in tumor cells may influence cancer progression. Tumor cells that overexpress Hyal1 combined with HAS are more tumorigenic and metastatic, through autocrine-enhanced proliferation and motility. However, the well-accepted angiogenic potential of HA oligomers may be partially or largely mediated by vesicle-associated HA delivery. The presence of HA and Hyal1 in exosomes indicates that it could be carried from the primary tumor site through the circulation and arrive at target cells in distant tissues. In this way, a tumor overexpressing HAS or Hyal1 could initiate events to prepare distant tissues for metastasis (Fig 1). HA or Hyal1 at the vesicle surface may facilitate docking and uptake of the vesicle and its contents by other cells. HA and Hyal1 could also produce active HA

fragments upon arrest in a remote site, irrespective of cellular uptake, and thereby initiate proliferation or migration. These mechanisms remain to be examined.

Exosomes produced by tumor cells are known to bear unique contents that can affect the proliferation and motility of non-tumorigenic cells. In a study of rat pancreatic adenocarcinoma cell lines, wild type cells and CD44 knockdown cells secreted exosomes with altered expression of ECM proteases, including hyaluronidase (Mu et al., 2013). Tumor-derived exosomes enhanced the migration of rat endothelial, fibroblast, and stromal cell lines. Exosomes are also involved in epithelial to mesenchymal transition in cancer progression. Application of exosomes with higher expression of TGF-β to primary fibroblasts can upregulate expression of α-smooth muscle actin and induce formation of a thick HA coat, both hallmarks for myofibroblast differentiation and cancer-associated stromal activation (Webber et al., 2010; Webber et al., 2014). Thus, exosome associated signals can also affect HA homeostasis of target cells and drive the epithelial to mesenchymal transtition.

4.3 Products of hyaluronidase: fragments versus oligos

The complex information content of HA as a molecular signal in cancer is largely attributable to polymer length and HA quantity, both of which are influenced by hyaluronidase processing. Only Hyal1 and Hyal2 are thought to contribute to processing of HA in most tumors. Both isozymes are capable of generating a range of fragment sizes, and tetrasaccharides are the complete digestion product. Since Hyal2 is GPI anchored, its activity in vivo is thought to be limited to short polymer generation. HA oligomers, specifically of 4–25 disaccharides, have been shown to stimulate angiogenesis (West et al., 1985), despite the antiproliferative effect of larger HA polymers on endothelial cells that suppresses angiogenesis (Rooney et al., 1995; West and Kumar, 1989). The antiangiogenic effect of HA polymers on endothelial cells is irreversible once engaged except in the sustained presence of antagonistic HA oligomers (Deed et al., 1997). This normal function of HA may allow tumor cells to directly signal their own vascular development and has been exploited in therapeutic targeting. HA decasaccharides reduced proliferation, motility, and invasion of breast tumor cells and prevented osteolytic lesions in mice (Urakawa et al., 2012).

Several reports have implicated Hyal1 processing of HA in prostate tumor angiogenesis. For example, inhibition of HA polymer synthesis suppressed growth (McCarthy et al., 2005; Simpson et al., 2002) and reduced vascular density of prostate tumors by ≈80%. Seemingly contrary to this finding, excess deposition of HA can suppress angiogenesis of prostate tumors (Bharadwaj et al., 2007). This supports a requirement for HA in angiogenesis, but clearly shows further metabolism of the polymeric form is critical for the angiogenic response. HA fragments (20–30-mers) have been detected in high-grade prostate cancer tissues (Lokeshwar et al., 2001) and knockdown of Hyal1 also impairs angiogenesis (Lokeshwar et al., 2005). HA production in relatively low quantities can promote angiogenesis in prostate tumors (Simpson, 2006), consistent with motility experiments in which low HA concentrations stimulate, while high levels inhibit, migration. This effect

suggests there is a threshold HA polymer level that saturates or antagonizes hyaluronidase activity.

HA fragments stimulate cellular chemokinesis. For example, the cervical cancer cell line Hela-S3 exhibited spontaneous chemokinesis that was reduced by knocking down HAS2, Hyal2, or CD44 (Saito et al., 2011). Addition of exogenous high molecular weight HA (230 and 920 kDa) did not affect this motile phenotype, whereas short HA polymers (23 kDa) were able to restore the chemokinetic process in the Hyal2 knockdown background. Low molecular weight HA also enhances the migration and proliferation of human papillary thyroid carcinoma cells, acting through the toll-like receptor 4 (Dang et al., 2013). Low molecular weight HA (3–5 kDa) was shown to associate with CD44 and toll-like receptors to induce an inflammatory response in breast cancer cells (Bourguignon et al., 2011). HA size impacts CD44 clustering, which is stimulated by HA polymers and dispersed in the presence of oligomeric HA, leading to altered cell adhesion (Yang et al., 2012). HMW-HA treatment of embryonic fibroblasts stimulates Snail2 expression, and epithelial to mesenchymal transition (Craig et al., 2009).

4.4 Products of hyaluronidase: beyond hyaluronan

Chondroitin sulfate is a GAG with similar structure to HA that is covalently attached to proteoglycans at the cell surface and abundantly accumulated in the extracellular matrix of malignant tissue. Sulfated polysaccharides can have differential effects on HA homeostasis. Dextran sulfate has been shown to inhibit degradation of HA by PH-20 in vitro, but had complex net effects in cultured cells because the treatment also resulted in lower expression of CD44 and HAS, concurrently with enhanced expression of hyaluronidases (Udabage et al., 2004). Hyal1, Hyal2, Hyal4 and PH-20 have all been shown to have significant activity toward specific chondroitin sulfates. For example, fragments of chondroitin sulfate E have the ability to activate CD44 signaling, thus promoting tumor cell motility through cytoskeletal rearrangement and increased formation of filopodia (Sugahara et al., 2008).

Recently, quantitative measurement of degraded GAG products by 2-aminobenzidine derivatization and fluorescence monitoring of HPLC anion exchange fractionation has facilitated the direct comparative assessment of substrates for Hyal1, Hyal4, and PH20 (Honda et al., 2012; Kaneiwa et al., 2010). Because this assay method eliminates some of the pitfalls of other previously used methods, new insights about substrate specificity and enzyme activity parameters have been possible. In particular, there were modest but potentially significant differences in substrate preference depending upon the pH at which activity was assayed. Comparison of enzyme efficiency (Vmax/Km) revealed approximately 3-fold more efficient degradation of chondroitin 4-sulfate (CS-A) relative to HA at the frequently reported optimal pH of 4, and also at 4.5, but catalytic efficiency was comparable for both substrates at pH 3.5 (Honda et al., 2012). Hyal1 was also able to degrade chondroitin 6-sulfate (CS-C) and chondroitin, but this activity was lower by approximately an order of magnitude. At pH 4–4.5, recombinant, bead-immobilized PH20 was significantly less efficient than Hyal1 in catalyzing depolymerization of any of these substrates, but showed the greatest activity using HA and CS-A, followed by chondroitin. Neither enzyme

showed particularly strong size dependence for HA polymer cleavage by this method, which notably quantifies cleavage events stoichiometrically, in contrast to most other methods.

Thus, the existing view in the field that the hyaluronidase activities of this family of enzymes are the most physiologically relevant bears strong consideration. In the acidic context of a tumor, in which excess HA deposition, elevated proteoglycan expression, and aberrant GAG modification of cell surface proteins have all been well documented, the role of the hyaluronidase enzymes in remodeling the extracellular matrix may be equally or more significant because of their ability to cleave other GAGs. This method was also used to demonstrate that Hyal4 has chondroitinase activity, exhibiting a preference for chondroitin 2,6-bissulfate (CS-D) followed by CS-C and CS-A, with no detectable activity toward chondroitin or HA (Kaneiwa et al., 2010). To date, Hyal4 activity has not been associated with cancer, but has not been widely examined.

5. Hyaluronidase targeting in cancer therapy and imaging

5.1 Structural and functional features of human Hyals

Structure determination and enzymological characterizations of Hyal1 have helped define the key features of human hyaluronidases. In 2007, the crystal structure of human Hyal1 was described, providing the molecular details of the enzyme active site and the orientation of the catalytic domain relative to the C-terminal EGF-like domain (Chao et al., 2007). Given the close pairwise sequence identity between Hyal1 and the other four human Hyals (41–43%), we were able to generate highly credible homology models of each of the enzymes using Phyre 2 (Kelley and Sternberg, 2009). A comparison of the five human Hyals indicates that these enzymes have similar overall folds and conserved active site features. A pronounced substrate-binding cleft bisects the core of the protein, with several highly conserved residues located at the site of HA cleavage. Using site-directed mutagenesis and steady state kinetic analysis of recombinant purified protein, the contributions of several residues of Hyal1 to enzymatic activity were previously demonstrated (Zhang et al., 2009). In the proposed substrate-assisted mechanism, Tyr247 and Asp129 polarize the N-acetyl moiety of the N-acetylglucosamine residue to be cleaved, such that it forms an oxyanionic nucleophile to attack and hydrolyze its own glycoside bond. Glu131 protonates the hydroxyl leaving group and activates an incoming water to release the intramolecular HA intermediate. Arg265 also contributes to Hyal1 activity, as mutation to a leucine severely compromises its ability to cleave HA. However, its precise role in catalysis may be indirect through structural perturbations. Additional mutants at Tyr202 and Ser245 were shown to have full catalytic activity but impaired HA binding. Inspection of the models predicts that Trp321 and Tyr75 will also contribute to HA binding and not catalysis as both residues are remote from the site of cleavage. Recently, the thirteen amino acids in the loop between Cys207 and Cys221 in Hyal1 were replaced with four residues of alternating glycine and serine residues in an effort to extend the substrate binding cleft (Reitinger et al., 2009). Although comprehensive kinetic analysis was not reported, the engineered mutant had greater enzymatic activity at higher pH values relative to wild-type Hyal1. This observation and the high degree of sequence conservation at the enzyme active site suggest that the observed pH profiles of enzymatic activity for each of the hyaluronidases are unlikely to

reflect only the protonation state of catalytic residues, but rather the pH dependence of substrate association with the enzyme.

Comprehensive structural and mechanistic characterizations of the other four human Hyals are lacking. In comparison to Hyal1, human Hyal2 had limited hyaluronidase activity (Lepperdinger et al., 1998; Liu et al., 2003) and no detectable chondroitinase or heparanase activity (Lepperdinger et al., 1998) under the assay conditions employed. Hyal2 was reported to generate larger fragments of HA with an approximate molecular mass of 20kDa. From a structural standpoint, it is difficult to rationalize why a 20kDa fragment would not be degraded by the enzyme, given the similarities of the active site clefts of Hyal1 and Hyal2. This limited digestion may reflect structural domains of the HA substrate (Lepperdinger et al., 1998) rather than limitations of the enzyme active site. Alternatively, a soluble form of Hyal2 has been shown to degrade HA to considerably smaller fragments with extended incubation, and to a similar extent as with PH-20 (Vigdorovich et al., 2005). Hyal2 has also been shown to serve as a virus entry receptor (Vigdorovich et al., 2005). Residues that mediate this function are not highly conserved among the Hyals and are located adjacent to but distinct from the HA binding cleft (Duh et al., 2005). To date, hyaluronidase activity has not been demonstrated for mammalian Hyal3 (Atmuri et al., 2008; Hemming et al., 2008), despite conservation of key catalytic residues. A cursory examination of the substrate binding cleft suggests minor amino acid substitutions may preclude HA binding and/or alter substrate specificity. Interestingly, Hyal3 may still promote HA turnover by promoting Hyal1 activity (Hemming et al., 2008). Additional work in this area is needed.

Human Hyal4 was recently shown to be a chondroitin sulfate hydrolase, with limited to no hyaluronidase activity over the length of the assay (Kaneiwa et al., 2010). Subsequent studies confirmed the importance of Glu147 and Tyr218, which are equivalent to Glu131 and Tyr202 in human Hyal1. There are two significant active site differences between Hyal1 and Hyal4 (Kaneiwa et al., 2012). Both Tyr247 and Gln288 are proximal to the site of HA cleavage in Hyal1 (Fig 2). Human Hyal4 in contrast has a glycine (Gly263) and an arginine (Arg305) at these positions. Unpublished results indicate that mutation of Gly263 to a tyrosine did not alter the substrate specificity of human Hyal4 (Kaneiwa et al., 2012), but details of the characterizations are not yet available. In mouse Hyal4, a similar substitution, a mutation of Ser263 to a tyrosine, resulted in an enzyme with both hyaluronidase and chondroitinase activity. Interestingly, mouse Hyal4 has a glutamine residue at position 305 that is structurally equivalent to Gln288 of human Hyal1. Gln 288 is adjacent to the C6 of the N-acetylglucosamine and near the C4 as well. Perhaps residues at this position contribute to substrate specificity.

The biological functions of PH-20 have been studied extensively, particularly with respect to fertilization. However, relatively limited mechanistic studies have been reported. Asp 146 and Glu 148, equivalent to Asp 129 and Glu 131 in Hyal1, have been shown to significantly contribute to catalysis. Arg 211, Glu284, and Arg 287 of human PH-20 were also shown to be critical for optimal activity (Vigdorovich et al., 2005). Examination of the homology model of PH-20 indicates these residues are unlikely to be involved directly in substrate binding or catalysis, but are instead key structural residues involved in extensive hydrogen bond networks.

The C-terminal EGF-like domain of Hyal1 packs tightly against the catalytic core of the protein. This domain is proposed to mediate protein-protein interactions that may influence the localization and efficacy of this family of enzymes. An examination of the sequence conservation in this region reveals limited selective pressure and may indicate that different isozymes have different interacting partners or cellular localizations. In Hyal1, deletion of the EGF-like domain eliminated its hyaluronidase activity (Zhang et al., 2009). Of the reported Hyal1 variants, several would largely eliminate the EGF-like domain (Lokeshwar et al., 2006; Lokeshwar et al., 2002). As previously discussed (Chao et al., 2007), it is difficult to ascribe functional significance to these variants because the structural data indicate that it is unlikely a folded protein would be produced. This is also the case for the described Hyal3 variants. To date, recombinant or purified versions of Hyal1 or Hyal3 splice variants have not been purified and characterized.

5.2 Targeting of hyaluronidase for cancer therapy

The role of hyaluronidases Hyal1 and Hyal2 in liberating or “activating” pro-tumorigenic and pro-angiogenic HA fragments is well supported by the functional studies described in the above sections, making it an obvious choice for pharmacological targeting in chemotherapy. Its extracellular or cell surface localization increases its appeal as an accessible target, and general cytotoxicity or off-target effects may be reduced by limiting inhibition to the extracellular space.

Numerous naturally occurring and synthetic compounds have been characterized as inhibitors of PH-20 or Hyal1 in vitro. Since PH-20 exhibits relatively high hyaluronidase activity over a broad pH range, inhibition of its activity is frequently screened by loss of absorption by the cationic carbocyanine dye, Stains-All, or by its use to detect product size shifting in gel electrophoresis. Stains-All is sensitive to pH shifts so its signal is significantly diminished in the low pH conditions that are optimal for activity of Hyal1. Consequently, Hyal1 and other acid-active hyaluronidases are typically assayed by chemical derivatization of acetamido groups and measurement of the resulting colorimetric product, known as the Morgan-Elson reaction. This technique has the added advantage of stoichiometrically reporting cleavage events. A third method that has been used directly to compare inhibitors of PH20 and Hyal1 is a competitive binding assay, or ELISA-like assay, which is a plate-based assay that reports activity by loss of microplate-adsorbed HA polymers detected by a labeled HA binding protein. The limitation of this and the Stains-All assay is that the HA must be fully degraded before the positive signal is lost, so it is not able to quantify actual cleavage events catalyzed by the enzyme.

Using purified bee venom, bovine testicular, or recombinant human PH-20 hyaluronidases, the most potent inhibition was achieved with Vitamin C palmitate, also known as L-ascorbyl 6-hexadecanoate (Botzki et al., 2004; Hofinger et al., 2007), and inhibition was greatest when the length of the alkyl chain was 12–16 carbons. Glycyrrhizic acid was somewhat effective against Hyal1 that was expressed and purified from Drosophila cell culture (Hofinger et al., 2007). PH-20 was also expressed by “autodisplay” on E.coli cells and compared directly to bovine testicular hyaluronidase (BTH) for efficacy of known inhibitors (Kaessler et al., 2011). BTH is frequently used to screen for new hyaluronidase inhibitors,

and this comparison revealed that while BTH was effectively inhibited by vitamin C palmitate and by two indole acetamide/carboxamide derivatives, only vitamin C palmitate significantly inhibited PH-20. Such comparative studies are important in identifying lead compounds for isozyme-selective inhibitors, but it is important to consider that the authors use the Stains-All method, which is non-stoichiometric and loses sensitivity at the low pH optimal for Hyal1 or Hyal2 activity. A series of indole derivatives was characterized with the Morgan-Elson assay, and though the authors only tested BTH (Olgen et al., 2010; Olgen et al., 2007), increasing the lipophilicity of the compounds was found to enhance affinity, which is consistent with results of vitamin C ester comparisons above.

Polystyrene sulfonates and sulfated HA showed greater potency than glycyrrhizic acid and comparable inhibition to vitamin C palmitate, using partially purified Hyal1 or PH-20 from cell conditioned media assayed in the competitive plate assay (Isoyama et al., 2006). Sulfated HA partially (Isoyama et al., 2006) or fully (Toida et al., 1999) inhibits Hyal1 and PH-20, but it is not clear from examination of the Hyal1 structure where a sulfate group would be tolerated at the active site, which is relatively locked down and sterically constrained in the model with superimposed HA tetrasaccharide.

Partially sulfated HA polymers (≈300–600 kDa average molecular mass) are the only hyaluronidase inhibitor that has been tested for anti-tumor efficacy in vivo. When given twice weekly (intraperitoneally) beginning at the time of prostate tumor cell injection, this treatment delayed or inhibited subcutaneous tumor growth in mice (Benitez et al., 2011). The authors tested effects of sulfated HA in vitro to determine if phenotypic effects that are promoted by enhanced HA turnover were reversed. This was in part the case, since treatment with sulfated HA induced apoptosis and decreased proliferation, motility, and invasiveness of prostate tumor cell lines. Sulfated HA functioned mainly through inhibiting Akt signaling, and overexpression of Akt or application of non-sulfated HA oligos reversed the sulfated HA effects. However, the study used a subline derived from LNCaP cells, which reportedly lack measurable hyaluronidase activity in conditioned media. Hyal1 expression and activity in the subline were not shown, so it is possible that the response mechanism is not exclusively dependent on hyaluronidase activity, particularly in light of the findings discussed above with respect to other substrates. The authors also reported an effect of the sulfated HA treatment on signaling downstream of HA receptors CD44 and RHAMM, both of which also have the potential to be engaged by the sulfated HA directly. Regardless of targeting specificity, it does appear that sulfated HA has promise in preventing tumor growth, but it will be important for ultimate clinical translation to determine whether sulfated HA can regress established tumors.

Interestingly, PEGPH20 has recently been used to sensitize pancreatic cancer to gemcitabine (currently a first line chemotherapy for this disease), with significantly faster response in mice, though growth of tumors is only delayed by accelerating delivery, and they still reach lethal size. Since HA is highly hydrophilic, its accumulation in tumor stroma increases interstitial fluid pressure so significantly that molecular transfer from the tumor vasculature into the tumor is virtually undetectable. PEGPH20 delivery intravenously prior to gemcitabine treatment was shown to reduce stromal HA and interstitial pressure, thereby allowing the drug to reach the tumor cells and induce cell death (Provenzano et al., 2012).

The prospect for use of hyaluronidases in this manner is exciting, but is not without precedent for concern about effects of residual HA degradation products on surviving tumor cell proliferation and motility, as well as tumor angiogenesis.

5.3 Hyaluronidase-targeting agents for tumor imaging

As a cancer biomarker and potential therapeutic target, use of hyaluronidase substrates for noninvasive tumor imaging has received increasing attention. Many investigators have used HA to target nanoparticles, isotopic labels, fluorophores, and other imaging agents to HA receptors that are elevated on the tumor cell surface in many solid epithelial tumors. In addition, the clearance route of HA through lymphatic vessels and accumulation in lymph nodes facilitates its use for imaging tumor associated lymphatic flux (Proulx and Detmar, 2013; Sharma et al., 2007).

Another novel innovation is the use of HA as a probe for the activity of hyaluronidase. For example, subcutaneous tumors resulting from ovarian tumor cell line injection were effectively imaged by MRI using an intravenously delivered HA-DTPA conjugated to agarose beads, which chelated and partially shielded the contrast agent, gadolinium, subsequently releasing it specifically in the tumor, yielding a change in the imaging parameters (R1 and R2 relaxation rates). Hyal1 and Hyal2 activity in the ovarian tumor cells were both found to contribute in vitro to the degradation of the HA carrier, liberating the environmentally sensitive contrast agent (Shiftan et al., 2005). The HA-GdDTPA-bead targeting agent was further found to be sufficiently sensitive to report apparent kinetics of hyaluronidase activity in the tumors, and found the initial activity was localized primarily to the peripheral tumor, possibly concentrated in peritumoral lymphatics (Shiftan and Neeman, 2006). Since hyaluronidases are elevated in the stromal space and/or at the tumor cell surface, and may be involved in the cellular uptake of HA, imaging probes that give no signal until activated by hyaluronidase cleavage offer higher levels of sensitivity.

Besides MRI contrast agents, fluorescent probes have been developed that illustrate the clinical potential for this approach, which may be translatable to an intraoperative setting. The FDA-approved near infrared fluorescent dye, indocyanine green, is used successfully in the clinic for identifying tumor-involved lymph nodes and metastatic cancer. By encapsulating the dye in a HA nanogel, the dye is more effectively solubilized and stabilized in vivo. A proof of concept trial determined that fluorescence intensity could be significantly increased in mice upon hyaluronidase exposure to release the dye, but the agent needs to be validated using tumor xenografts with differential hyaluronidase expression (Kim et al., 2014; Mok et al., 2012). An additional intriguing use of HA was to provide the outermost envelope of multilayer liposomes. The liposomes contained the cytotoxic agent paclitaxel and were first coated with arginine/histidine-rich cell permeating peptides before coating with HA (Jiang et al., 2012). The HA screened the peptides from circulating proteases and targeted the particles to tumor sites enriched in hyaluronidase, whereupon the degradation of HA exposed the peptides, stimulating cellular uptake into vesicles that were dispersed in a pH-dependent manner to release paclitaxel to the cytosol. Specificity of HA targeting in mice was demonstrated by competition of the near infrared fluorescence signal in the tumor with free HA pre-injection, and by significantly reduced tumor growth and extended

survival time of animals treated with HA-targeted peptide-paclitaxel liposomes. Finally, fluorescent HA-based assays have been tested to improve the sensitivity of hyaluronidase detection in urinary samples, where it is effective in diagnosis of bladder cancer. Fluorescence correlation spectroscopy of autoquenched fluorescein-HA (Rich et al., 2012) and Forster Resonance Energy Transfer (FRET, (Chib et al., 2013)) upon cleavage of fluorescein-rhodamine double conjugated HA both showed promise for increasing the dynamic range of hyaluronidase detection and improving quantitative cancer staging with urinary samples.

6. Conclusions and future perspective

Hyaluronidase family enzymes have been broadly implicated in a variety of cancers, and have demonstrated potential for clinical utility both as biomarkers and therapeutic targets. However, a number of key questions remain to be answered before they can be fully exploited in cancer therapy. For example, systematic studies that examine structure-function relationships among the Hyals will be essential to understand the basis for substrate specificity, binding properties, and high affinity inhibition. Development of specific strategies to target individual Hyal proteins would be advanced by additional structural data in complexes of each Hyal with a putative substrate or inhibitor, so active site differences can be fully appreciated. Moreover, a more thorough comparison of tissue and tumor-specific effects of each Hyal is needed to clarify when hyaluronidase activity is beneficial (e.g.; by removing HA to improve drug delivery) and when this activity is pro-tumorigenic and pro-metastatic (e.g.; Hyal1 and Hyal2). Rigorous comparisons of each Hyal in the context of HA accumulation, as has been done with Hyal1, are needed to determine respective tumorigenic and metastatic proclivity conferred by each Hyal. Finally, more large-scale systematic studies that examine all five Hyals in the same sets of staged cancers would better inform us of the clinical relevance of each Hyal in cancer initiation, progression, and metastasis, and give insights about true prognostic power of Hyal expression.

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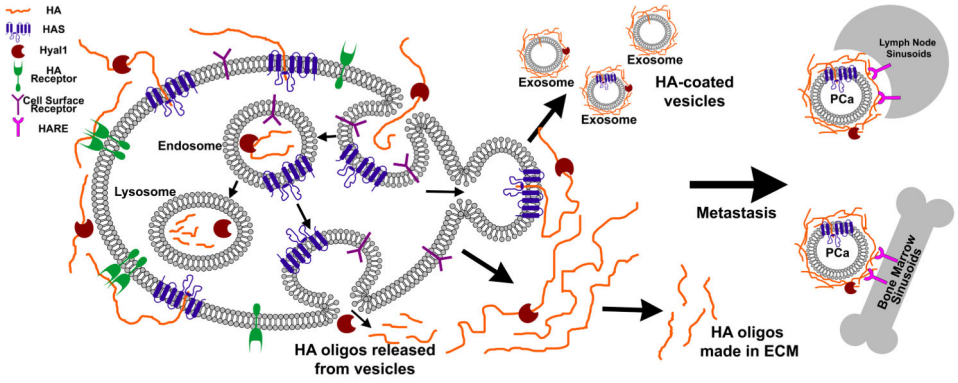

Figure 1. Model for HA impact on tumor progression

HA polymers are synthesized at the cell surface by membrane embedded HAS. Reuptake and/or degradation of HA may require secreted hyaluronidases to generate low MW oligomers of HA. HA is retained by ligation to specific cell surface receptors or residual association with HAS, and can act on both tumor cells and associated stromal cells. Tumor cells may signal in HA and/or Hyal1-dependent fashion to endothelial cells of lymphatic vessels, lymph node or bone marrow sinusoids via other HA receptors. These signals may be released at the primary site to prepare metastatic target tissues and render them hospitable for tumor invasion, or tumor cells bearing HA may generate signals locally upon arrest in metastatic target tissues. The context of the HA signal is assumed to be free extracellular HA polymer or HA oligomer, but HA and/or Hyal1 delivery via exosomes or microvesicles is an emerging possibility. HA internalized by tumor epithelial cells may contribute to cellular transformation, proliferation, motility, and ultimately may be required for sustained tumor growth and metastasis.

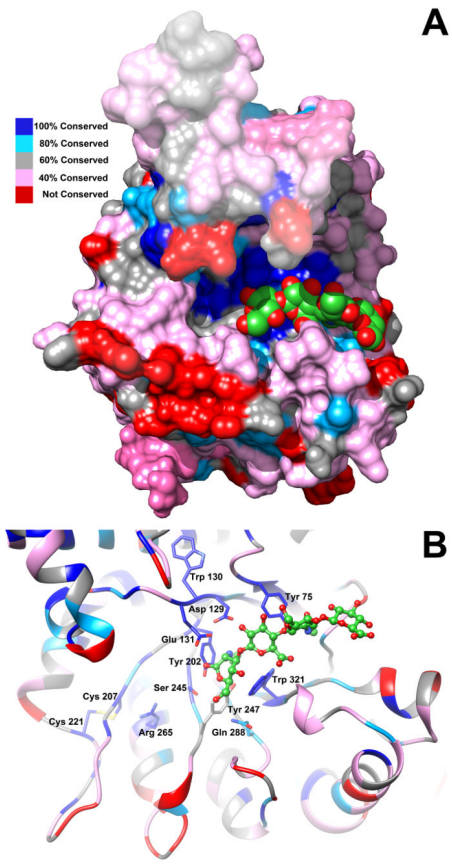

Figure 2. The structure of human Hyal1

(A) Surface representation of human Hyal1. A HA tetrasaccharide (solid spheres with carbons colored in green, oxygen in red, and nitrogen in blue) was docked in the enzyme active site in an orientation comparable to that observed in the bee venom hyaluronidase structure (pdb codes: Hyal1 2PE4; bee venom 1FCV). The surface of the protein is colored based on the sequence conservation among the five human hyaluronidases, with perfectly conserved residues colored in dark blue and residues with no sequence conservation colored in red. Residues found in 4 out of 5 hyaluronidases are colored in light blue, 3 out of 5 in grey, and 2 out of 5 in pink. The most highly conserved residues are generally located within the enzyme active site or key structural elements. **(B**) **Conserved active site features of human hyaluronidases.** A ribbon representation of Hyal1 is color-coded as in Panel A, with the HA tetrasaccharide shown in ball and stick representation. Key active site residues are shown in stick representation. Several perfectly conserved residues are clustered at the site of the substrate-assisted cleavage.