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REVIEW

Devising a pathway for hyaluronan catabolism: are we there yet?

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Hyaluronan is a negatively charged, high molecular weight glycosaminoglycan found predominantly in the extracellular matrix. Intracellular locations for hyaluronan have also been documented in cytoplasm, nucleus, and nucleolus. The polymer has an extraordinarily high rate of turnover in vertebrate tissues. The focus here is to formulate a metabolic pathway for hyaluronan degradation using all available data, including the recently acquired information on the hyaluronidase gene family. Such a catabolic scheme has defied explication up to now. In somatic tissues, stepwise processing occurs, from the extracellular high molecular weight space filling, antiangiogenic ~10^7-kDa polymer, to intermediate sized highly angiogenic, inflammatory, and immune-stimulating fragments, and ultimately to tetrasaccharides that are antiapoptotic and potent inducers of heat-shock proteins. It is proposed that the high molecular weight extracellular polymer is tethered to the cell surface by the combined efforts of hyaluronan receptors and hyaluronidase-2 (Hyal-2). The hyaluronan is cleaved to a 20-kDa intermediate-sized fragment, the limit product of Hyal-2 digestion. These fragments are delivered to endosomal- and ultimately lysosomal-like structures. Further catabolism occurs there by Hyal-1, coordinated with the activity of two lysosomal β-exoglycosidases, β-glucuronidase and β-N-acetyl-glucosaminidase. A membrane-associated mini-organelle is postulated, the hyaluronasome, in which coordinated synthetic and catabolic enzyme reactions occur. The hyaluronasome can respond to the physiological states of the cell by a series of membrane-bound and soluble hyaluronan-associated receptors, binding proteins, and cofactors that trigger enzymatic events and signal transduction pathways. These in turn can be modulated by the amounts and sizes of the hyaluronan polysaccharides generated in the catabolic cascade. Most of these highly dynamic interactions remain to be determined. It is also proposed that malignant cells can commandeer some of these interactions for facilitating tumor growth and spread.

Key words: β-endoglycosidases/β-exoglycosidases/CD44/hyaluronan/hyaluronidase

Introduction

Hyaluronan (HA, hyaluronic acid) is a straight-chain glycosaminoglycan (GAG). It is the only GAG not attached to a core protein, and of the major GAGs, it is the only one not sulfated. It is a β-chain polymer composed entirely of repeating disaccharides of β-D-glucuronyl-(1 → 3)-N-acetyl-D-glucosamine connecting through β(1 → 4) linkages (Figure 1A). There are many areas of HA metabolism that continue to confuse and intrigue. The synthesis of HA does not take place in the Golgi, as do all other GAGs, but occurs instead within a complex on the cytoplasmic surface of the plasma membrane (Prehm, 1984), products of a family of HA synthases (Weigel *et al.*, 1997; Itano and Kimata, 2002). HA is extruded from the cell through the plasma membrane into the extracellular space. HA chains found in the extracellular matrix (ECM) can reach enormous sizes, between 10^5 and 10^7 Da. Because of its strong

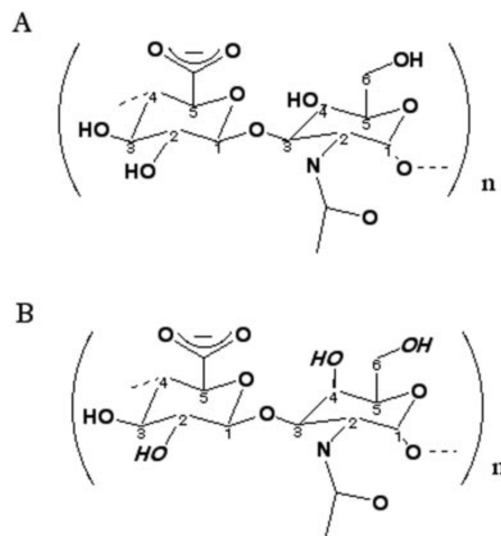

Fig. 1. (**A**) Structure of hyaluronan. The polymer is built of alternating units of glucuronic acid (GlcUA, left) and N-acetylglucosamine (GlcNac, right). Degradation by both bacterial and vertebrate hyaluronidases occurs between the two structures shown. The bacterial HA lyases are eliminases, generating unsaturated disaccharide units. The vertebrate hyaluronidases are hydrolases. All the glycosidic linkages are β type, 1-3 between GlcUA and GlcNac, and 1-4 between GlcNac and GlcUA. (**B**) Structure of chondroitin. The polymer is built of alternating units of GlcUA (left) and N-acetylgalactosamine GalNac (right). The glycosidic linkages are all of the β type. Epimerization of the hydroxyl group on carbon 4 is the only difference between the two polymers between GlcNac and GalNac. The vertebrate hyaluronidases and chondroitinases cleave between the two structures shown. The hyaluronidases can cleave HA as well as chondroitin and CSs, albeit at a slower rate. The vertebrate chondroitinase (Hyal-4) appears to have specificity for chondroitin and CSs and does not cleave HA. Hydroxy radicals in italics indicate potential sulfation sites.

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negative charge, the polymer with a pseudo-random coil configuration in aqueous solvents occupies a large volume of solvent. It is thus a space-filling molecule, loosening tissues, creating spaces for cell motility, decreasing cell–cell contacts, and impeding intercellular communication (Laurent, 1998). It also has the ability to confer motility directly on cells through receptors that interact with the cytoskeleton (Entwistle *et al*., 1996; Bourguignon *et al*., 2001).

HA is present predominantly in the ECM, particularly in embryonic and malignant tissues. It occurs early in the ECM of the healing wound (Longaker *et al*., 1991) and whenever rapid repair and regeneration occur. It is also highly expressed in the glycocalyx, the pericellular coat that ensheathes most cells, and is particularly prominent on the apical surface of endothelial cells (Henry and Duling, 1999). HA can also be taken up by cells (Collis *et al*., 1998) through receptors such as CD44 (Culty *et al*., 1992; Hua *et al*., 1993; Kaya *et al*., 1997) and the receptor for HA-mediated motility (RHAMM) (Cheung *et al*., 1999). It is possible that not all the HA taken up by cells is immediately degraded. Intact HA chains have been detected within cells, in cytoplasm, in nucleus, and even within the nucleolus (Evanko and Wight, 1999; Evanko *et al*., 1999; Tammi *et al*., 2001). HA is primarily the product of stromal cells. However, it is now well established that epithelial cells also synthesize HA (Pasonen-Seppanen *et al*., 2003). Levels of such synthesis are often underestimated (Lin *et al*., 1997). A scenario can be envisioned in which stromal cells supply HA taken up secondarily by epithelial cells. It has not been established whether intracellular HA represents newly synthesized material or HA taken up secondarily by cells.

Despite the increasing importance of HA in biology (Lee and Spicer, 2000; Toole, 2000; Toole and Hascall, 2002) and the recognition of its extraordinarily rapid rate of turnover, surprisingly little is known about the degradation of HA. The purpose of this review is to formulate a catabolic scheme for HA based on the available, though limited data. A 70-kg individual has 15 g of HA, a third of which turns over daily. Between different tissues, rates of turnover vary widely. In the blood stream, the $t_{1/2}$ for HA is rapid, 2–5 min (Fraser *et al*., 1981). The HA of skin, making up half that of the body, turns over in 1–2 days in the epidermal compartment. The turnover rate in the dermis of skin has not been determined. In a tissue as relatively inert as cartilage, HA turnover occurs in 1–3 weeks.

The hyaluronidase class of enzymes is responsible for much of this catabolism. These enzymes have been relatively neglected (Kreil, 1995), largely due to the difficulty in assaying their activity and to their instability. The "spreading factor" in testicular extracts (Duran-Reynals, 1928) was identified as a hyaluronidase activity (Chain and Duthie, 1940) and became the first vertebrate hyaluronidase to be isolated. An acid-active hyaluronidase activity in serum had been recognized for many years (De Salegui and Pigman, 1967). However, isolation of this serum enzyme, the first hyaluronidase from somatic tissues, took another 30 years (Afify *et al*., 1993; Frost *et al*., 1997), facilitated by the development of a rapid assay (Frost and Stern, 1997) and the recognition that detergents and protease inhibitors were necessary throughout the purification procedure (Frost *et al*., 1997; Csoka *et al*., 1997b). The sequencing of the purified circulating enzyme, together with information from the Human Genome Project and its Expressed Sequence Tag databank, facilitated the recognition, finally, of a family of hyaluronidase-like proteins.

Classification of the hyaluronidases

The various kinds of hyaluronidases fall into three distinct classes, as first formulated by Karl Meyer (1971). This classification system was based on biochemical analyses of the enzymes and their reaction products. With the advent of molecular genetic data, we now know that Meyer's classification scheme was remarkably accurate.

1. Bacterial hyaluronidases (E.C. 4,2,99,1) are endo-β-acetyl-hexosaminidases that function as β-eliminases, yielding predominantly disaccharides as end products. Most of these enzymes have substrate specificity for HA.
2. Hyaluronidases that are endo-β-glucuronidases (E.C. 3.2.1.36) are found in leeches (Hovingh and Linker, 1999), some other parasites, and crustaceans. It is a major activity of krill (Karlstam *et al.*, 1991; Karlstam and Ljungloef, 1991). These enzymes generate tetra- and hexasaccharide end products.
3. The mammalian hyaluronidases (E.C. 3.2.1.35) are hydrolytic, generating tetra- and hexasaccharides as the predominant end products. They lack absolute substrate specificity, being able to also digest chondroitin and chondroitin sulfates (CSs), specifically C4-S and C6-S, albeit at a slower rate.

The mammalian-type hyaluronidases have additionally some transglycosylation activity, with the ability *in vitro* to generate cross-linked chains, not only inter-HA chains but also hybrid HA-CS molecules. Whether such a reaction occurs *in vivo* or whether a cross-linked HA-CS reaction product might have some biological activity is yet to be examined.

Mammalian hyaluronidases

Six hyaluronidase-like gene sequences are present in modern mammals. Until nonplacental mammals are examined, it cannot be assumed that all mammals have the same repertoire of hyaluronidases. A case can be made that the en masse block duplication of three to six hyaluronidase genes occurred when placental mammals separated from nonplacental mammals.

All six genes are transcriptionally active and have similar genomic structures. The expression of each has a unique tissue distribution. These six genes define a novel paralogy group in the human genome. In the human, three genes (*HYAL1*, *HYAL2*, and *HYAL3*) are found tightly clustered on chromosome 3p21.3, coding for hyaluronidase-1 (Hyal-1), Hyal-2, and Hyal-3. Another three genes *HYAL4*, *PHYAL1* (a pseudogene), and *SPAM1* (Sperm Adhesion Molecule 1) are clustered in a similar fashion on chromosome 7q31.3. They code, respectively, for Hyal-4, transcribed but not translated in the human, and PH-20. This

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chromosomal pattern suggests two ancient gene duplications, followed by en masse block duplication, events that probably occurred before the emergence of modern mammals (Csoka *et al*., 1999, 2001).

Of the cluster on chromosome 3p, Hyal-1 and -2 constitute the major hyaluronidases of somatic tissues. Hyal-2 may be the more important enzyme (Lepperdinger *et al*., 1998, 2001). The Hyal-2 null mutation in the mouse is an embryonic lethal (Lepperdinger, personal communication), whereas the Hyal-1 mutation is not. A human genetic disorder with absent Hyal-1 activity has now been identified, termed Mucopolysaccharidosis IX (Natowicz *et al*., 1996; Triggs-Raine *et al*., 1999). This disorder is associated with circulating levels of HA that are 40 times normal, as is also observed in the Hyal-1 null mutation mouse (unpublished data).

Hyal-2 is anchored to the plasma membrane by a glycosylphosphatidylinositol (GPI) link, though a portion of Hyal-2 also occurs in a soluble form. Hyal-2 cleaves high molecular weight HA to a limit product of approximately 20 kDa, or about 50 disaccharide units. Hyal-1 appears to be a lysosomal enzyme that can cleave HA to small disaccharides, with the tetrasaccharide being the major product. Hyal-1 is also the circulating human plasma hyaluronidase (Afify *et al*., 1993; Frost *et al*., 1997). It is not clear why a presumably lysosomal enzyme should also occur in the circulation. It is also not understood why enzymes, such as Hyal-1, have pH optima of 3.8, well below the pH found in lysosomes, pH 4.5. Another example of an enzyme with a low pH optimum is cathepsin D, with a pH optimum of 3.4–3.6 (Bazel and Alhadeff, 1999). These may all be the results of artifacts of *in vitro* enzyme assays. Hyal-1 is also the only hyaluronidase present in human urine (Csoka *et al*., 1997b).

Hyal-1 is a candidate tumor suppressor gene (TSG) product, deleted in many tobacco-related lung tumors (Lerman and Minna, 2000; Csoka *et al*., 1998; Frost *et al*., 2000). On the other hand, Hyal-2 seems to be able to function as either an oncogene or a TSG. Overexpression of Hyal-2 accelerates tumor formation of murine astrocytoma cells (Novak *et al*., 1999). Hyal-2 is a cell surface receptor for some retroviruses, the envelope protein of which mediates oncogenic transformation (Rai *et al*., 2001; Maeda *et al*., 2001). However, evidence for TSG function of Hyal-2 is also available. Hyal-2 can accelerate apoptosis (Chang, 2002). Furthermore, an adenovirus–Hyal-2 vector suppresses growth of tumor xenografts in mice (Ji *et al*., 2002). Finally, Hyal-2 overexpressing clones of src-transformed fibroblasts have a reduced rate of proliferation (Flamion, personal communication).

Hyal-3 is a mystery. It is widely expressed, but no activity can be identified using the available hyaluronidase assays (unpublished data). Expression occurs in chondrocytes (Flannery *et al*., 1998) and increases when fibroblasts undergo chondrocyte differentiation (Nicoll *et al*., 2002). There may be coordinate expression of Hyal-2 and -3. They are both up-regulated by inflammatory cytokines, such as interleukin-1 and tumor necrosis factor α, whereas Hyal-1 is not (Flannery *et al*., 1998).

PH-20 is relatively specific for testes, the enzyme facilitating penetration of sperm through the cumulus mass that surrounds the ovum. The enzyme is also necessary for fertilization (Cherr *et al*., 2001). PH-20 is a multifunctional protein with a separate domain that binds to the zona pelucida (Myles and Primakoff, 1997).

By more sensitive techniques, including polymerase chain reaction (PCR) analysis, PH-20 can also be detected in the epididymis (Deng *et al*., 2000), the female genital tract (Zhang and Martin-DeLeon, 2003), breast (Beech *et al*., 2002), and placenta and fetal tissues (Csoka *et al*., 1999). Expression of PH-20 can also occur in certain malignancies (Madan *et al*., 1999a,b; Godin *et al*., 2000; Beech *et al*., 2002). The pseudogene *PHYAL1* contains an aberrant stop codon and, though not translated into active enzyme in the human, does appear to be translated in other species. This may explain why the mouse with the null mutation in the PH-20 gene is fertile (Baba *et al*., 2002).

The other genes coding for hyaluronidaselike sequences on chromosome 7p are *HYAL4* and *PHYAL1*. Based on preliminary evidence, Hyal-4 appears to be a chondroitinase, the first to be identified in vertebrate tissues. The anomaly is that Hyal-4 enzyme has absolute specificity for chondroitin and CS, with no ability to degrade HA. This is in marked contrast with the Hyal-1 and PH-20 hyaluronidases, which in addition to HA can also cleave CS, albeit at a slower rate.

Pseudogenes may play a more active role in genomic dynamics than previously assumed. An expressed pseudogene can regulate the mRNA stability of its homologous coding gene (Hirotsune *et al*., 2003). It would be of intrinsic interest to establish such a possible role for *PYAL1* and the mRNAs of any of the hyaluronidase genes.

The genome of *Caenorhabditis elegans* has only one sequence with homology to the mammalian parology of hyaluronidases. However, the only GAG found in the nematode is chondroitin (Yamada *et al*., 1999), which is predominantly unsulfated (Beeber and Kieras, 2002), suggesting that the one hyaluronidase-like sequence is a chondroitinase. It is possible that the gene for Hyal-4 is the ancestral sequence. This would explain why Hyal-4 has absolute specificity for chondroitin and CS. The only difference between these two β-chain polymers, HA and chondroitin, is the replacement of the N-acetyl glucosamine with N-acetylgalactosamine, an epimer change of only one hydroxyl group (Figure 1B). Gene duplication and genetic drift of the second sequence could have permitted recognition of a new substrate, HA, while retaining residual recognition of the substrate for the original ancestral enzyme.

Hyaluronan seems to have appeared quite late in evolution. There is no indication in the genome of either *C. elegans* (Stein, 1999) or the sea squirt *Ciona intestinalis* (Dehal *et al*., 2002) for HA biosynthesis. The latter, with its vertebratelike notocord also has, based on a BLAST search, only one hyaluronidase-like sequence, which is probably a chondroitinase. However, the cephalochordate *Amphioxus* does have HA (Spicer, personal communication). This may be the organism in which gene duplication has occurred, with the second hyaluronidase-like sequence perhaps coding for an enzyme associated with true hyaluronidase activity.

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The magnificent seven or the seventh veil?

A seventh hyaluronidase is proposed (Heckel *et al.*, 1998), but there has been no further enzyme characterization since the initial description. This enzyme is an antigen that immunologically resembles a hyaluronidase that occurs in meningiomas. The hyaluronidase activity associated with this sequence has not been well established. Of note is the observation that there are no sequences in the *Drosophila* genome with homology to the family of six hyaluronidase-like sequences. However, the fruit fly genome does contain the one meningiomalike hyaluronidase sequence, as documented by BLASTP analysis. There is also homology to a sequence in *C. elegans*. Further analysis is required to clarify this situation and to establish how this putative enzyme will fit (if at all) into an overall scheme of HA catabolism. A splice variant of this gene produces a protein located in the nucleus with a β-N-acetylglusaminidase activity (Comtesse *et al.*, 2001).

Significance of enzyme isoforms is a mystery

The vertebrate hyaluronidases occur in a variety of states, all products of posttranslational processing. It is not yet possible to assign specific roles to these enzyme isoforms in catabolic schemes, nor has the substrate specificity of each isozyme been established. These facts alone indicate how tenuous any hypothetical scheme of HA catabolism must be at present.

The mature hyaluronidase enzymes undergo two different kinds of additional processing, either (1) two endoproteolytic cleavage reactions or (2) a single proteolytic step releasing GPI-anchored enzyme from the cell membrane. Hyal-1, an example of the first class of processing, exists in two sizes. A 54-kDa form of Hyal-1 is the only isoform present in plasma, but both a 54- and a 49-kDa isoform occur in urine. The 49-kDa size results from two endoproteolytic steps that remove 99 amino acids from near the carboxy terminus. Disulfide bonds presumably hold the resulting two polypeptides together. Whether the same protease is responsible for the two cleavage reactions is not known.

Chitin, another β-linked sugar, is a homopolymer of β1,4 N-acetylglucosamine. A chitinase present in monocytes and macrophages also occurs in two forms, processed and unprocessed, that parallel the two isoforms of Hyal-1 (Renkema *et al.*, 1997). The isoforms of chitinase and hyaluronidase do not reflect a zymogen and mature enzyme relationship, because both forms of each enzyme have comparable activity. Why two isoforms exist for these β-endoglycosidase enzymes is not known.

Three members of the hyaluronidaselike family are GPI-linked to plasma membranes, Hyal-2, Hyal-4, and PH-20. A proteolytically processed soluble form has been well documented for PH-20 (Cherr *et al.*, 1996; Meyer *et al.*, 1997). PH-20 decreases in molecular mass from 65 kDa to 53 kDa, though the precise scission point is undetermined. It would be of intrinsic interest to identify the proteases that process all of these enzymes and establish their control mechanisms.

All three enzymes may be proteolytically processed and released from a GPI anchor. It follows that all three enzymes may occur in both soluble and membrane-associated isoforms. However, this has not been established unequivocally for Hyal-2 and -4 under physiological conditions. A soluble form of expressed PH- 20 is detected following transfection of cells with cDNA (Lepperdinger *et al.*, 2001). Phospholipase C treatment can be used generally to release all GPI-linked enzyme from plasma membranes. Activities of the naturally processed soluble forms of these enzymes may be quite different from activities that are artificially solubilized from cell surfaces by phospholipase C. There may be differences in pH optima and subtle differences in substrate specificity or kinetics. It has been established, however, that the physiologically processed form of PH-20 has a pH optimum at 4.0, whereas the unprocessed form has an optimum at neutral pH (Oettl *et al.*, 2003). Differentiating between the roles of soluble and cell-associated forms and processed and nonprocessed isoforms of all the hyaluronidases in any catabolic scheme may be difficult to establish currently.

Hyaluronidase and HA in embryology

HA is associated with the undifferentiated state and early embryological development. HA promotes cell proliferation and motility, and removal of HA by hyaluronidase is required for cells to commit to programs of differentiation (Toole, 1991, 2001). Neural crest cells bud from the neuroectoderm and travel in an HA-rich environment through the embryo. The HA is degraded when they reach their destination at the various sites (Pratt *et al.*, 1975), thus presumably depriving such cells of continued motility.

An experimental model for the importance of HA in embryology is the culture of trypsinized chick embryo cardiac cells. These grow, become confluent, fuse, synthesize cardiac actin and myosin, and ultimately begin to undergo waves of contraction. These same cells, however, cultured on HA, will grow but remain myoblasts, fail to undergo differentiation, and never undergo contractility (Kujawa *et al.*, 1986). Hyaluronidases are obviously critically important in embryology for the removal of the HA in the conversion from the morphogenetic and proliferative stages to the differentiating stages during development. The identities of the hyaluronidases in embryology are unknown. However, it has been established that Hyal-2 is expressed in early development, and Hyal-1 is not (Lepperdinger *et al.*, 1998; Csoka *et al.*, 1999). However, the role of HA and hyaluronidases in the multiple stages of development may be far more complex than formulated here. For example, HA synthesis is induced, rather than inhibited, during the differentiation of F9 teratocarcinoma cells to endodermal cells (Prehm, 1980).

CS is a GAG deposited at a late stage in wound healing and in fully differentiated tissues, such as bone and cartilage. A fillip for the glycobiology community to consider is the following. The appearance of HA occurs relatively late in the evolution of metazoan organisms. Why then does CS occur in evolution before HA? One possible explanation among many is that the appearance of HA in evolution may coincide with the need to partition off pluripotential stem cells that remain undifferentiated throughout the life

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of an organism. This provides a reservoir of undifferentiated cells for later retrieval and expansion, to fill in defects, for wound healing, and as a general adaptive repair technique. Organisms without HA may not have need for such compensatory mechanisms, possibly because all their cells maintain multipotentiality, whereas organisms higher on the evolutionary scale comprise predominantly cells that are terminally differentiated. Foci of pluripotential cells can be maintained in a fetal or stem cell–like state by an environment rich in HA, sequestering such cells from the remainder of the organism. Alternatively, the HA may facilitate migration of fetal cell over a considerable distance, a requirement not necessary for more primitive organisms.

The HA polysaccharides of varying length have different biological activities

The high molecular weight HA polysaccharides are generally space-filling molecules that hydrate tissues and are also antiangiogenic (Feinberg and Beebe, 1983). In addition, they are anti-inflammatory and immunosuppressive (McBride and Bard, 1979; Delmage *et al.*, 1986). The 20-kDa-limit fragments of Hyal-2 digestion stimulate synthesis of inflammatory cytokines (Noble, 2002). Smaller HA oligomers, in the 6–20-kDa size range, are potent activators of dendritic cells, the antigen presenting cells of the immune system (Termeer *et al.*, 2000, 2003). Thus lower molecular size HA fragments tend to be angiogenic, inflammatory, and immunostimulatory.

Very small HA oligosaccharides also have unique biological activities. Oligosaccharides of the 3–10-disaccharide size inhibit anchorage-dependent growth of tumor cells (Ghatak *et al.*, 2002). Tetrasaccharides are among the predominant products of Hyal-1 digestion. When added to cultured cells, these induce expression of heat-shock proteins. The tetrasaccharides are also antiapoptotic, suppressing cell death in cultures undergoing hyperthermia or when cells are serum-starved (Xu *et al.*, 2002). It is not understood precisely how intracellular products of HA degradation might accumulate and then function in a fashion that parallels tetrasaccharides added exogenously to cell cultures.

Increased generation of lower molecular size HA fragments tend to occur under conditions of inflammation, tumorigenesis, and tissue injury, perhaps the result of differential hyaluronidase activities. However, HA fragmentation can also occur in the presence of free radicals under oxidative conditions (Myint *et al.*, 1987; Uchiyama *et al.*, 1990). Free radicals and hyaluronidases may have coordinated HA chain scission activities under certain pathologic conditions.

Tertiary structures

Exciting developments in the field of hyaluronidase is occurring in the solution of their tertiary structures using crystallographic anaysis (Markovic-Housley *et al.*, 2000). The structure of the mammalian enzymes can to a large extent be modeled onto the bee venom enzyme, the bee venom hyaluronidase sharing 30% sequence identity with human PH-20. The crystal structure determined at 1.6 Å resolution resembles a classical eightfold β/α-triose phosphate isomerase (TIM) barrel, a structure common to many glycosyl hydrolases (Rigden *et al.*, 2003b), except that the barrel is composed of only seven strands. A long substrate-binding groove extends perpendicularly across the C-terminal of the barrel axis. Though the vertebrate hydrolytic hyaluronidases appear to be made up of a combination of β-pleated sheets and α-helices in the TIM barrel motif, the bacterial lyases or eliminase-type hyaluronidases are composed strictly of α/α barrels. Yet they retain a similar carbohydrate-binding groove (Rigden *et al.*, 2003a).

Venom hyaluronidases

Hyaluronidase is a component of venoms in a wide variety of organisms, including bees, wasps, hornets, spiders, scorpions, fish, snakes, and lizards (Frost *et al.*, 1996; Csoka *et al.*, 1997a). The activity functions in part as a spreading factor for other venom components. Many of these venom hyaluronidases have stretches of sequence with 36% identity with the mammalian spermatazoan PH-20. There are a number of proteins in the male ejaculate in addition to hyaluronidase that have homology with venom proteins. These include the disintegrin and metalloprotease family of proteins (Becherer and Blobel, 2003). This suggests that the venoms, including their hyaluronidases, may have derived originally from the male sexual apparatus. The radical feminists may be correct after all.

Genomic promiscuity

Why bee venom should contain a hyaluronidase activity with sequence homology to the sperm enzyme is not known. This may be an example of lateral gene transfer, because the HA substrate does not appear in evolution until amphioxus. There are abundant examples of other lateral gene transfers. A collagenous sequence is present in a bacteriophage hyaluronidase, a sequence obtained by lateral gene transfer from a vertebrate host. This functions presumably as a virulence factor for the bacterium, providing adhesion to collagenous tissue structures of the host (Stern and Stern, 1992).

Another example of lateral gene transfer can be observed in chlorella. Viral infections of chlorella frequently provide an HA synthase activity (DeAngelis *et al.*, 1997; Graves *et al.*, 1999), the only example of HA in the plant kingdom. Whether a hyaluronidase sequence also resides in this virus or its green algae host has not been determined.

Formulating a catabolic pathway for HA degradation

It is apparent that HA can be degraded in somatic tissues in a stepwise fashion and that the quantum decreases in polymer size are the specific products of different hyaluronidase activities. The discrete sizes of HA fragments have widely different biological activities, indicating that a highly controlled cascade of HA fragmentation occurs, suggestive of a catabolic pathway. From these observations, a putative metabolic scheme for HA catabolism can be formulated. High molecular HA is tethered to the plasma cell surface by HA receptors, combined possibly with an interaction with Hyal-2, the GPI-linked hyaluronidase anchored to

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the plasma membrane. Hyaluronan undergoes cleavage to 20-kDa intermediate-sized fragments by Hyal-2, which is about 50 disaccharide units.

The biological properties of HA in aqueous solution appear to be controlled by reversible tertiary structures, as defined by nuclear magnetic resonance (NMR) spectroscopy. The NMR evidence suggests a β-pleated sheetlike array stabilized by H- and hydrophobic bonds.

> Easy transitions between secondary and teritiary structures ... offer convenient and economic mechanisms for switching between functions dependent on these structures. ... The tertiary structure may present possibilities not present in the single stranded structure. ... 50 disaccharide units is around the size at which stable tertiary structures would be expected to form. (Scott and Heatley, 2002)

Such structures may provide the difference in substrate specificity between Hyal-2 and the other hyaluronidases, the 50-disaccharide stable tertiary structure being the limit product of Hyal-2 catalysis.

However, alternative explanations are available. The HA-binding proteins, or hyaladherens, may provide the various polymer organizations and solution properties that generate specific substrate specificities for the panoply of hyaluronidases (Day and Sheehan, 2001), independent of the self-association of HA chains.

The Hyal-2-generated HA fragments are internalized by receptor-mediated endocytosis. It cannot be surmised whether cleavage occurs externally or following partial internalization into specialized cell surface compartments. These fragments are delivered to endosomes and finally to lysosomes, where Hyal-1 activity degrades the 20-kDa fragments to small oligosaccharides. Two lysosomal β-exoglycosidases, β-glucuronidase and β-N-acetyl-glucosaminidase, participate in this degradation.

Evidence for the latter comes from the human disorder I-cell disease. Fibroblasts from patients with I-cell disease, lacking the mannose receptor pathway for lysosomal enzyme uptake, have an apparent HA storage disorder. These fibroblasts stain intensely for HA (Stern and Steinmann, unpublished data). The tetra- and hexasaccharide products of HA degradation are too small to detected by the HA-binding peptide staining reaction. This suggests that the β-exoglycosidases participate actively in the degradation of higher molecular size HA fragments all along the catabolic cascade, and not only at the terminal steps, cutting tetra- and hexasaccharides to the individual saccharides.

The specific defect in I-cell disease is the enzyme N-acetylglucosamine-1-phosphotransferase, an enzyme essential for the synthesis of the mannose-6-phosphate recognition marker that targets enzymes to lysosomes. Failure of this enzyme causes misrouting of most newly synthesized lysosomal enzymes. Plasma from patients with I-cell disease has normal levels of Hyal-1 but elevated levels of the two β-exoglycosidases (Natowicz and Wang, 1996), presumably because of defective uptake into cells. This suggests that Hyal-1 may be transported to the lysosome by a pathway other than the mannose-6-phosphate route. In the overall catabolic scheme for HA catabolism, the two β-exoglycosidases play an important role, participating in the intermediate as well as in the terminal steps of degradation. Without the action of the β-exoglycosidases, larger HA oligosaccharides may accumulate in lysosomes. What may be missing is the trimming of these HA fragments to a size sufficiently small to diffuse out of lysosomes into the cytoplasmic compartment either passively or by receptor-mediated exit.

Invoking a mini-organelle for HA metabolism, the hyaluronasome

Glycogen is a branched polymer of α-linked glucose. A glycogen mini-organelle occurs in both liver and muscle (Banhegyi and Mandl, 2001). It is proposed here that a similar mini-organelle may occur for the β-linked HA polymer.

> Readily visualized by the electron microscope, glycogen granules appear as bead-like structures localized to specific subcellular locales. Each glycogen granule is a functional unit, not only containing carbohydrate, but also enzymes and other proteins needed for its metabolism. These proteins are not static, but rather associate and dissociate depending on the carbohydrate balance in the muscle. Regulation takes place not only by allosteric regulation of enzymes, but also due to other factors, such as sub-cellular location, granule size, and association with various related proteins. (Shearer and Graham, 2002)

The very same observations might be applicable to a putative HA-containing granule, that can be termed a hyaluronasome. A multiprotein membrane-associated complex that contains HA synthetic activity is described by Mian (1986a,b). It is proposed that this HA synthase complex isolated from plasma membranes may constitute a component of such a mini-organelle, containing not only synthetic but catabolic activities. This hyaluronasome, located possibly on the cytoplasmic surface of plasma membranes, would be a functional unit providing an exquisite response mechanism to the metabolic state of the cell.

Preliminary data come from a variety of sources. Hyaluronidase treatment of cultured cells at very low concentrations stimulates HA synthesis (Philipson *et al.*, 1985; Larnier *et al.*, 1989). Treatment of isolated membrane preparations with low concentrations of hyaluronidase also stimulates HA synthesis (Philipson and Schwartz, 1984). This suggests that a feedback mechanism exists that enables cells to sense the levels of HA that have been synthesized. The exogenously added hyaluronidase cleaves newly synthesized HA chains as they are being extruded through the plasma membrane out of the cell into the extracellular space (Prehm, 1984), relaying to the cell the message that inadequate quantities of HA have been synthesized. Higher levels of hyaluronidase modulates the profile of expression of the HA receptor, CD44 (Tanabe *et al.*, 1993; Stern *et al.*, 2001). Levels of HA that cells synthesize or deposit respond to various physiological states, including growth phase (Tomida *et al.*, 1974), confluence, inversely related to cell density in both fibroblasts (Huey *et al.*, 1990) and keratinocytes (Tammi *et al.*, 2001), mitosis and cell detachment from the substratum (Brecht *et al.*, 1986), calcium concentrations (Frost and Stern, 1997), anoxia and lactate (Stern *et al.*, 2002), viral transformation (Ishimoto *et al.*, 1966), and serum stimulation (Tomida *et al.*, 1976; Decker *et al.*, 1989). Preliminary immunolocalization data indicate that

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some of the HA synthases and hyaluronidases colocalize (Spicer, personal communication). All of this evidence points to the existence of a mini-organelle, a hyaluronasome, that has synthetic and degradative activities and possesses sensitive sensor mechanisms that can respond to various metabolic states.

The glycogen granule contains, in addition to glycogen, glycogen synthase, glycogen branching and debranching enzymes, phosphorylases a and b, phosphorylase kinase, phosphorylase kinase kinase, phosphorylase phophatases, as well as binding sites for UDP-glucose, cyclic AMP, ATP, AMP, and glucose-1-P. The glycogen granule can respond to starvation, epinephrine, and glucogon. In skeletal muscle, glycogen granules respond to epinephrine, muscle contraction, and calcium ion concentrations. I suggest that the hyaluronasome may be a membrane-bound structure even more complex than glycogen granules. The hyaluronasome responding dynamically to extracellular and intracellular events may contain HA receptors, such as RHAMM (Cheung *et al.*, 1999) and CD44; the HA synthase enzymes-1, -2, and -3 (Weigel *et al.*, 1997; Itano *et al.*, 1999); the hyaluronidases, hyaluronidase inhibitors (Mio *et al.*, 2000; Mio and Stern, 2002) the β-exoglycosidases; and HA-binding proteins, such as HABP1 (Majumdar *et al.*, 2002). This mini-organelle may be able to regulate levels of HA deposition with exquisite precision by allosteric regulation of HA synthetic and degradative enzymes, using hyaladherins (Toole, 1990; Knudson and Knudson, 1993) and related proteins. Defining this structure and the attendant signal transduction pathways induced by size-specific HA oligosaccharides can provide many new opportunities for the glycobiology community.

The cancer conundrum

The hyaluronidase gene locations are presumptive TSG sites at both chromosomal locations (Zenklusen and Conti, 1996; Lerman and Minna, 2000). Tumor suppression at these sites results from toxic exposure, tobacco-related malignancies at 3.21.3, and exposure to other environmental toxins, particularly benzene, at 7q31.3 (Edelson *et al.*, 1997; Mateo *et al.*, 1999). The lesions associated with the latter include leukemias and lymphomas resistant to treatment, known clinically as the 7q syndrome (Hernandez *et al.*, 1997). Apparently, eradication of hyaluronidase activity is an important step in the multistep development of some cancers.

Hyaluronidases have long been added to anticancer regimens. Tumors previously resistant to chemotherapy become sensitive when hyaluronidase is added (Baumgartner and Neumann, 1987; Maier and Baumgartner, 1989; Klocker *et al.*, 1998). It has been assumed that hyaluronidase functions in these regimens by enhancing the penetration of anticancer drugs and by decreasing the turgor of malignant tissues. However, hyaluronidase enzymes may themselves have intrinsic anticancer activities.

Evidence for the anticancer effects of hyaluronidases come from experimental tumor studies (Beckenlehner *et al.*, 1992). Human malignancies grown from tumor cell lines in SCID mice show dramatic regression following Hyal-1 treatment (Shuster *et al.*, 2002). Hyal-1 overexpression suppresses tumorigenicity in an experimental model for colon carcinoma (Jacobson *et al.*, 2002). Hyaluronidase treatment delays the appearance of carcinogen-induced tumors (Pawlowski *et al.*, 1973) and prevents lymph node spread in murine lymphomas (Zahalka *et al.*, 1995). The growth rate of murine malignancies correlates inversely with levels of circulating hyaluronidase (Maeyer and De Maeyer-Guignard, 1992).

It would be assumed that loss of hyaluronidase would be an excellent tumor marker. But clinical data have been inconsistent. Increases in both levels of hyaluronidase (Madan *et al.*, 1999a,b; Lokeshwar *et al.*, 2001) as well as of HA expression (Toole and Hascall, 2002; Toole, 2002; Hiltunen *et al.*, 2002; Wernicke *et al.*, 2003) correlate with tumor progression. This may reflect an overall increase of HA turnover, with increased rates of both synthesis and degradation in malignancies.

Loss of hyaluronidase expression occurs with Hyal-1 at the genomic DNA level, as in the classic TSG mode, with homozygous deletion or loss of heterozygosity. However, loss of Hyal-1 activity can also occur downstream, at the level of RNA. Alternative splicing, as triggered by epigenetic phenomena, can involve retention of introns that prevent or slow translation. This has been shown for *HYAL1* mRNA in tobacco-related carcinomas of the head and neck (Frost *et al.*, 2000). Ultimately, suppressor activity may also be found at the level of protein inactivation. Apparently the resilience and resourcefulness of malignant cells is such that any mechanism can be commandeered to eliminate activities that impede cancer growth and spread, at the level of either DNA, RNA, or protein.

Additional considerations for the anticancer effects of hyaluronidase include the phenomenon of the angiogenic switch (Folkman, 2002). Early in the course of malignancy, high molecular weight HA is necessary for opening tissue spaces to provide an avenue of flow for the nutrients at the primary site. When simple diffusion no longer suffices, the action of Hyal-2 provides the intermediate HA fragments that induce the requisite angiogenisis (West *et al.*, 1985). The resolution of endothelial cells and peritumor stromal cells from the specific cancer cells themselves and analysis of their individual HA catabolic pathways may help make conflicting data intelligible.

An exciting and powerful recent observation is the identification of a stem cell population within malignancies (Al-Hajj *et al.*, 2003). Only a minority of human cancer cells have the ability to form tumors in immunocompromised mice. As few as 100 of such cancer-derived stem cells can form tumors. These cancer stem cells are identified by surface marker flow cytometry as being CD44-positive, whereas tens of thousands of cells from the same tumor with alternate phenotypes fail to form tumors. Characterization of specifically these tumor stem cells regarding HA synthesis and catabolism may also help clarify the conundrum.

Summation

There are clearly many unanswered questions in a putative scheme for HA turnover and degradation, particularly in the control mechanisms involved. Hyaluronidase inhibitors

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would provide rapid response elements and may reflect why turnover rates are so very rapid. Immediate requirements for elevated HA levels could be accommodated by invoking hyaluronidase inhibitors. Such inhibitors have been described (Mio *et al*., 2000) and are present in most tissues. This is supported by the observation that apparent levels of total hyaluronidae activity are greatly elevated following a few initial steps in the purification process, as inhibitors become separated out. It may be that such inhibitors are more diverse and more ubiquitous than the hyaluronidases themselves.

The apparent substrate size specificity and site-specific location of the various hyaluronidases could be supplied by the hyaladherens. These would provide the necessary information for an otherwise monotonous polymer substrate (Day and Sheehan, 2001). The hyaladherens also promise to be a family of proteins of great diversity.

The problem then becomes enormously increased. What hyaladherens and what inhibitors are associated with each of the various hyaluronidases during the catabolic pathway for HA? Apparently, we have a ways to go.

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Abbreviations

CS, chondroitin sulfate; ECM, extracellular matrix; GAG, glycosaminoglycan; GPI, glycosylphosphatidyl-inositol; HA, hyaluronan, hyaluronic acid; Hyal, hyaluronidase; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; RHAMM, receptor for HA-mediated motility; TIM, triose phosphate isomerase; TSG, tumor suppressor gene.

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