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Clinical and molecular biological aspect of the hyaluronidases: basis and clinical overview for oriental medical application

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SUMMARY

Components of extracellular matrix and the matrix-degrading enzymes are some of the key regulators of tumor metastasis and angiogenesis. Hyaluronic acid (HA), a matrix glycosaminoglycan, is known to promote tumor adhesion and migration, and its small fragments are angiogenic. Until now, we have compared levels of hyaluronidase, an enzyme that degrade HA, in normal adult prostate, benign prostate hyperplasia and prostate cancer tissues and in conditioned media from epithelial explant cultures, using a substrate (HA)-gel assay and ELISA-like assay (Kim *et al.*, unpublished results). The present review described an overall characterization of hyaluronidases and its application to human diseases. The hyaluronidases are a family of enzymes that have, until recently, deed thorough explication. The substrate for these enzymes, hyaluronan, is becoming increasingly important, recognized now as a major participant in basic processes such as cell motility, wound healing, embryogenesis, and implicated in cancer progression. And in those lower life forms that torment human beings, hyaluronidase is associated with mechanisms of entry and spread, e.g. as a virulence factor for bacteria, for tissue dissection in gas gangrene, as a means of treponema spread in syphilis, and for penetration of skin and gut by nematode parasites. Hyaluronidase also comprises a component of the venom of a wide variety of organisms, including bees, wasps, hornets, spiders, scorpions, sh, snakes and lizards. Of particular interest is the homology between some of these venom hyaluronidases and the enzyme found in the plasma membrane of mammalian spermatozoa, attesting to the ancient nature of the conserved sequence, a 36% identity in a 300 amino acid stretch of the enzyme protein. Clearly, hyaluronidase is of biological interest, being involved in the pathophysiology of so many important human disorders. Greater effort should be made in studying this family of enzymes that have, until recently, been overlooked. Also, oriental medical application of the hyaluronidase will be discussed with respect to inhibition and suppression of inflammation and malignacy.

Key Words: tancer; angiogenesis; disease; cancer; glycosidase glycosaminoglycan; hyaluronidase; hyarluronan; oriental medicine;

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INTRODUCTION

Hyaluronidases are a family of enzymes (Lokeshwar *et al.*, 1996) that degrade hyaluronic acid(HA). In vertebrates, hyaluronidase can be categorized into two classes, those active at neutral pH (pH optimum, 5.0) and those active at acidic pH (pH optimum, 3.5-4.0). For example, testicular hyaluronidase is of the neutral type, whereas the liver enzyme has an acidic pH optimum. Recently, the cloning and sequencing of porcine liver hyaluronidase has revealed that it is identical to hemopexin, a heme biding beta-glycoprotein that is abundantly present in the serum. However, the majority of the hyaluronidase activity present in serum appears to be distinct from hemopexin. The concerted actions of both hyaluronidases are know to play important roles during embryonic development, vasculogenesis, vascular remodeling, and immune surveillance. Although to date the presence of hyaluronidase in tumor tissues has not been established, it has been known that hyaluronidase levels are elevated in the uterine of Wilm's tumor(nephroblastoma) patients. Therefore, it is possible that hyaluronidase levels may be elevated in tumor metastasis, and the amount secreted may correlate with tumor progression, indicating possible suppression of the enzyme results in tumor progression.

Hyaluronidase have recently taken on greater significance, due to the increasing attention being given to their substrate, hyaluronan (hyaluronic acid, HA). However, these enzymes, with a few exceptions, have deed purification and denitive characterization, largely because of the enormous difficulties involved in their isolation. This situation now seems to be changing rapidly, and this review is undertaken with the knowledge that it will very soon be outdated, perhaps prior to publication. Excellent reviews of the hyaluronidases appear approximately once each decade (Meyer and Rapport, 1952; Meyer, 1971; Rod *et al.*, 1989; Lokeshwar *et al.*, 1996). The current review, clearly not in synchrony, should be taken rather as an harbinger of the imminent onslaught of new information.

The hyaluronidases are a class of endoglucosaminidase enzymes of considerable complexity and heterogeneity. It might be expected that these enzymes, found in the salivary gland of three disparate organisms, bee, man, and leech, might bear some superficial resemblance to each other. Instead, these three examples are paradigms of the three major classes of the eukaryotic enzyme, respectively neutral- and acid-active endo-, beta-N-glucosaminidases, and an endo-, beta-glucuronidase.

Hyaluronidases were first identified as a "spreading factor" in an extract from mammalian testes that facilitated diffusion of subcutaneously injected dyes and drugs (Duran-Reynals, 1928). It became apparent thereafter that the spreading factor was an enzyme, and that its substrate was HA (Chain and Duthie, 1939). However, prior to this, an enzyme that degraded HA had been identified in bacteria (Meyer *et al.*, 1937). Similar hyaluronidase-like enzymes were identified subsequently from a large number of tissues and organisms. Though these enzymes have the same or similar substrates, their mechanisms of action differ widely. The hyaluronidases have already been tabulated by category according to their disparate mechanisms (Lokeshwar *et al.*, 1996; Meyer, 1971). Various properties have been utilized to categorize the hyaluronidases, whether acid- or neutral-acting, according to substrate specificity, or by their reaction products. The problem with such attempts at categorization

are the exceptions to the rules. For example, the hyaluronidase from Streptomyces has a unique enzymatic mechanism, and the enzyme from Stonesh venom has a mechanism of action comparable to the testicular group of hyaluronidases, except absolute substrate specificity, like that of the bacterial hyaluronidases. Molecular genetics and sequence homologies will provide the ultimate strategy for making category assignments (Fig. 1). Such data are just beginning to become available.

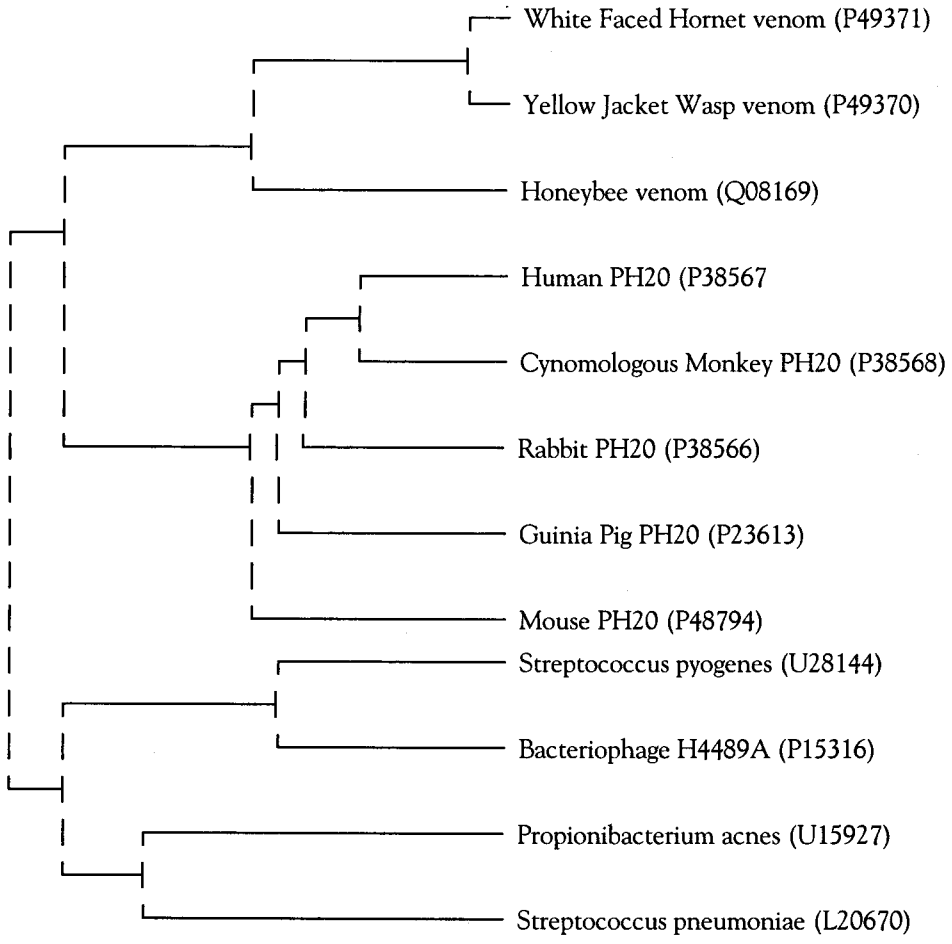

Fig. 1. Dendrogram of selected hyaluronidases. Alignments of selected hyaluronidases from various species were performed. Accession numbers are in parentheses. Amino acid sequences were analyzed through a pileup algorithm (Gap Creation Penalty = 3.0, Gap Extension Penalty = 0.10) using UPGMA, or unweighted pair-group method and arithmetic averages (138). Pairwise alignments were performed through the method of Needleman and Wunsch (137).

1. Biochemical assays of hyaluronidase activity

1) Classical and modernized assays of hyaluronidase activity

The original assays for hyaluronidase activity were not sufficiently rapid nor sensitive for enzyme purification. The ubiquitous occurrence of hyaluronidase inhibitors, particularly in the early stages of a purification procedure made the task particularly arduous. Early assays were based on "mucin clot" prevention upon acidification, or on turbidity or viscosity reduction. A more quantitative procedure was the estimation of the aldehyde groups released upon rupture of the bond releasing N-acetylglucosaminidic groups. The general colorimetric procedure was the most practical of this class of assay (Reissig *et al.*, 1955; Linker, 1974). Because this procedure measures both terminal reducing N-acetylglucosamine and free N-acetylglucosamine, the assay is sensitive not only to the hyaluronidases, but also to the combined activities of beta-D-glucuronidase and N-acetyl-beta-D-hexosaminidase. Each of these exoglycosidases are likely to be present in crude biological preparations.

Also, sensitive ELISA-based assays are available. One of the rst was based on a brain-derived HA-binding protein, hyaluronectin, using antibodies against that molecule (Delpech *et al.*, 1987). The intrinsic difficulties in preparing such antibodies were overcome with the development of an ELISA-based assay (Stern and Stern, 1992) using the high affinity biotinylated HA-binding peptide derived from tryptic digests of proteoglycan core protein from bovine nasal cartilage and the avidin-biotin reaction (Tengblad, 1979). The HA-coated plates are incubated with serial dilutions of the enzyme preparation, and the undegraded HA remaining on the plate is measured.

2) Zymography for hyaluronidase activity staining

Impregnation of acrylamide gels with HA prior to polymerization led to the development of substrate gel procedures. Incubation of the enzyme-containing gel after electrophoresis and digestion of the HA substrate is followed by staining of the gel. Undigested substrate is stained, and the hyaluronidase activity is visualized as a clear band, corresponding to the migration position of the enzyme. Such an assay was introduced by Fiszer-Szafarz (1984; 1990), but has the disadvantage of being light-sensitive, plus other intrinsic difficulties. A reproducible HA-substrate gel procedure has been developed utilizing double staining, Alcian blue, followed by Coomassie blue, that enhances sensitivity (Guntenhoener, 1992). The HA-substrate gel procedures have the advantage that hyaluronidase and its inhibitors are separated, making it possible to detect enzyme activity in crude biological preparations that would not otherwise be possible. A more recent modification is the incorporation of additional glycosaminoglycans (GAGs), such as chondroitin sulfate, into the procedure. These lower molecular weight GAGs are chemically modified so that they become immobilized in the gel, making it possible to examine substrate specificity of individual hyaluronidases(Miura, 1995). A survey of assorted assays for hyaluronidase activity has appeared recently (Hynes and Ferretti, 1994).

2. The substrates for the hyaluronidases

The predominant substrate for these enzymes is HA, a glycosaminoglycan (GAG), a repeating disaccharide straight chain polymer with the structure (N-acetyl-D-glucosamine -D-glucuronic acid). It is the only GAG not associated with a core protein, and the only naturally occurring GAG that is not sulfated.

That the glycosidic linkages of HA are all of the beta type is of more than passing interest. Changing the alpha linkage of poly-D-glucose to a beta linkage converts glycogen to cellulose, and poly-D-N-acetyl glucosamine containing beta linkages generates chitin. The beta linkage is therefore a critical structural feature, and not a curiosity promulgated by carbohydrate chemists. The hyaluronidases, once structure-function relationships are clarified, and their active sites defined, are likely to have more in common with chitinases and cellulases that act upon beta linkages, than with the glycosidases that have soluble carbohydrate polymer substrates and recognise alpha bonds, such as amylases and glycogen phosphorylase.

Hyaluronan has a key role in the structure and organization of the ECM (Fig. 2).

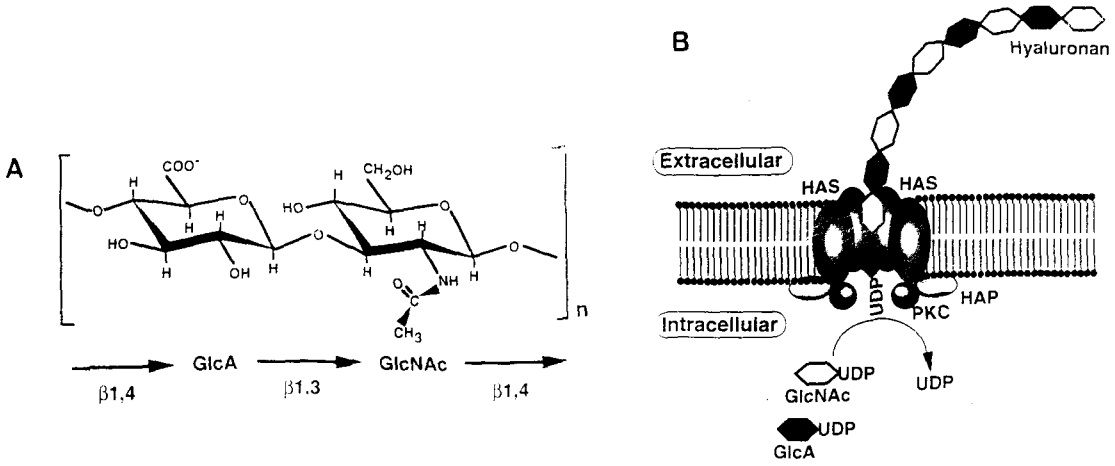

Fig. 2. Structure (A) and biosynthesis (B) of hyaluronan. The polymer is composed of the repeated disaccharide units of N-acetylglucosamine and glucuronic acid. The schematic illustrates the model of that the HAS moleculae involves in secretion/transport of growing hyaluronan chain through a channel- structures as well as polymerization on the inner surface of plasma membrane. PKC, protein kinase C; HAP, HAS associate protein.

It is a major component of the ECM during embryogenesis and whenever rapid tissue proliferation, regeneration, and repair occur. It is involved in cell motility (Turley and Torrance, 1984). A burst of HA synthesis occurs just prior to mitosis (Toole *et al.*, 1972; Tomida *et al.*, 1974; Mian, 1986; Brecht *et al.*, 1972) enabling cells to become dissociated from neighboring cells and their ECM in preparation for division. Hyaluronan inhibits cell differentiation (Kujawa, 1986; 1983) creating an environment that promotes cell proliferation. Hyaluronan is also concentrated in the stromal environment of malignant tumors (Pauli and Knudson, 1988). However, despite the obvious importance of HA in many basic biological processes, the catabolism and turnover of HA is not well understood. More than half of the HA in the mammlian body is in skin, predominantly in the dermis. The turnover rate there is rapid, with from 50-75% being turned over every 24 hours (Reed *et al.*, 1990). A portion of the HA goes in to the circulation, wherein the half life has been measured to be two to five minutes (Fraser *et al.*, 1981; 1985).

Hyaluronan exists in the vertebrate body in several forms. In the matrix, it may be firmly intercalated with binding proteins and proteoglycans, or it may be firmly associated

with cells, or bound to cell surfaces through specific binding proteins. However, much of the HA of the body exists in a freely soluble form that circulates in the lymphatic and cardiovascular system. Even in this relatively free form, HA has a complement of binding proteins, or hyaladherens (Toole, 1990; Knudson and Knudson, 1993) that decorate it in both covalent(Yoneda *et al.*, 1990; Zhao *et al.*, 1995) and electrostatic associations. An underlying question in a survey of HA degradation, then, is to correlate the possible states of the HA substrate and attendant catabolic reactions, and also to understand how the various states of the HA substrate affect enzyme kinetics. These questions are further confounded by the observation that some hyaluronidases are HA-binding proteins, even at a pH when an enzymatic reaction does not occur.

Hyaluronan has potent biological effects that are in part related to its molecular size. Low molecular mass HA is highly angiogenic (West *et al.*, 1985; 1989), while high molecular mass HA is inhibitory to angiogensis (Feinberg and Beebe, 1983). The transition from an antiangiogenic to a highly angiogenic state in wound healing, embryogenesis, or in malignant progression must be controlled by a hyaluronidase reaction. In embryogenesis, as shown in a number of developmental systems, there is an early HA-rich morphogenetic stage (Toole; Turley and Roth, 1980). Rapid expansion and motility of undifferentiated cells occur during this phase. The HA takes on a large volume of water of hydration that loosens the matrix, creating spaces through which cells can easily move. This is followed by removal of HA and the onset of programs of cytodifferentiation. Clearly the removal of HA by a hyaluronidase -like enzyme controls this transition between the two stages.

In the prokaryotes and invertebrates, hyaluronidase tends to be specific for HA, but from enzyme in vertebrates is also able to degrade chondroitin-4- and 6-sulfate, albeit at a much slower rate. There is specific binding that occurs between the carbohydrate chains of chondroitin sulfate and HA (Turley, 1980). It is possible that "vertebrate hyaluronidase" is actually an enzyme for the degradation of a naturally occurring complex formed between HA and chondroitin sulfate.

3. Classification of hyaluronidases

1) Bacteriophage-associated, Bacterial and fungal hyaluronidases

Bacteriophage isolated from a strain of Streptococcus pyogenes have a hyaluronidase unrelated to the bacterial enzyme (Hynes and Ferretti, 1989). Of interest is the presence of a collagenous domain, 10 repeats of the collagenous gly-x-y repeat in the enzyme, with ve proline residues in the x-y position (Stern and Stern, 1992). The 10 triplet collagen repeat is the minimal length suficient to provide stability for a triple helical structure. The single strand can also interact with intact triple helical collagen molecules. Thus, the phage enzyme may function as a virulence factor, helping the bacteria to attach to collagenous tissues of the host, while simultaneously enabling the bacteriophage to penetrate the HA capsule of the group A Streptococci. Analysis of a second bacteriophage hyaluronidase gene from Streptococcus pyogenes has a deletion of precisely this collagenous sequence (Hynes *et al.*, 1995). It is tempting to speculate that other viruses, particularly the viruses that attack mammalian cells, might produce a broad spectrum of interesting hyaluronidases. This is virtually an unexplored area of hyaluronidase biology.

On the othe hand, the bacterial hyaluronidases act as endo-N acetyl hexosaminidases by a beta elimination, yielding disaccharides, which differs from the mechanism of action of the vertebrate enzymes. Two hyaluronidase enzymes have been obtained from Streptococci,

products of two genes with 50% sequence identity (Berry *et al.*, 1994; Lin *et al.*, 1994). From the dendrogram of hyaluronidases (Fig. 1), it appears that the bacteriophage hyaluronidase of Streptococcus pyogenes has closer homology to the enyme of its host, than do the enzymes from the two strains of Streptococci to each other.

The hyaluronidases of Staphylococci also have multiple forms (Abramson, 1967). Of intrinsic clinical interest would be to establish whether Staphylococcus hyaluronidase perhaps is involved in the pathogenesis of toxic shock syndrome. The enzyme in Clostridium perfringens is a virulence factor, acting in conjunction with collagenases on connective tissues, and the dissection of fascial planes, in the pathogenesis of gas gangrene (Canard *et al.*, 1994). The hyaluronidase associated with the syphilis bacterium Treponema pallidum facilitates dissemination (Fitzgerald and Repesh, 1987). Antibodies to hyaluronidase restrict tissue spread of the treponemae, and in particular, prevent their attachment and penetration through capillaries.

In fungi, infections caused by Candida are the most common fungal infections affecting humans, their incidence having risen with the increasing use of wide-spectrum antibiotics, antitumor drugs, immunosuppressive agents, and most recently, with the AIDS epidemic. The various species of Candida produce hyaluronidases (Shimizu *et al.*, 1995). The enzymes have not been characterized, nor has it been established whether these enzymes are virulence factors or whether they play a role in pathogenesis. The enzyme is also present in Streptomyces, and is reported to have a unique mechanism of action (Ohya and Kaneko, 1977), but this claim for a novel reaction requires further elucidation.

2) Parasite-associated hyaluronidases

During skin penetration, infective hookworm (Anclystoma) larvae secrete a hyaluronidase (Hotez *et al.*, 1994), which is a major virulence factor for the organism. The enzyme has a 49 kDa and a second 87 kDa form. They have broad pH optima, between 6.0 and 8.0, and no activity against chondroitin sulfates. Two other parasitic nematodes, Ancyclostoma caninum and Anisakis simplex release hyaluronidases during the gastrointestinal invasive stage, and are believed to have a role in tissue histolysis and mucosal invasion, as well as in the pathogenesis of the associated enteritis (Hotez *et al.*, 1994).

3) Leech enzyme, Crustacean hyaluronidase and insect venom hyaluronidases

The hyaluronidase from the leech (Hirudo medicinalis) is specific for HA, and has no reactivity with chondroitin sulfate. It is unusual, being among the few endoglucuronidases among hyaluronidases, rather than an endo-N acetyl glucosaminidase. The potential role of a hyaluronidase as a spreading factor in the case of the leech is evident. It has a molecular weight of 28.5 kDa and a pH optimum of 5.3 (Yuki and Fishman, 1963).

A hyaluronidase from Antarctic krill (Euphausia superba) has been purified (Karlstam and Ljungloef, 1991). Krill describes a number of small marine crustaceans, and constitutes the major food source of baleen whales. These krill, which feed on phytoplankton, have a high content of a variety of carbohydrate polymers, and are characterized by rapid post morten autolysis caused by their high level of potent degradative enzymes. In fact the pancreatic function of these whales is barely extant, depending entirely on the digestive enzymes supplied by their food source. The puried enzyme has a pH optimum of 5.3, a molecular weight of 80 kDa. Though these creatures live in the Antarctic sea, the temperature optimum of the enzyme is 37℃. Preliminary

results suggest that the krill enzyme is an endoglucuronidase, similar to the leech enzyme.

The venom of bees, wasps, and hornets contain a hyaluronidase that is used as a "spreading factor" facilitating the diffusion of other venom components. The sequence structure of the enzyme from bee venom has been determined by molecular cloning of the cDNA (Gmachl and Kreil, 1993), as has that of the hornet (Lu *et al.*, 1995). They are secreted proteins, with a short pro segment, a signal peptide, and a mature enzyme of 349 and 331 amino acids, respectively. Expression of the cloned cDNA of bee venom hyaluronidase has been accomplished in an E. coli system yielding a product with enzymatic activity (Gmachl *et al.*, 1993).

3) The vertebrate venom hyaluronidases: The stonesh, snakes and lizards

The only marine hyaluronidase was isolated from the venom of the stonfiesh, Synanceja horrida (Sugahara *et al.*, 1992; Poh *et al.*, 1992) The purified enzyme is a 62 kDa glycoprotein with a pI of 9.2, and a pH optimum of 6.0. Intravenous injection of the enzyme does not result in the lethal effects of stonefish venom. As in the case of the leech, hyaluronidase may assisit in the rapid spread of venom through tissues. The enzyme is an endo-, beta-N-acetylglucosaminidase that does not act on chondroitin sulfate or dermatan sulphate, and is specic for HA. Product analysis indicates that the mechanism of action is comparable to testicular hyaluronidase, though it has the substrate specificity usually attributed to microbial hyaluronidases. It appears that this enzyme from a marine source is unique among vertebrate hyaluronidases. The specific activity of the purified enzyme is the highest to date of any hyaluronidase, 1.6×10^6 NFU/ mg protein.

Hyaluronidases are present in virtually all snakes venoms (Tu, 1977; 1982) but only a few have been characterized (Jaques, 1956; Pukrittayakamee *et al.*, 1988; Cevallos *et al.*, 1992) and isolation has been rarely reported. In all of the cases reported, the venom hyaluronidases act as spreading factors and have no intrinsic toxic activity. The hyaluronidase from the venom of a lizard, the Gila monster, Heloderma horridum, has been isolated and characterized. It is a 63 kDa glycoprotein with a pH optimum at 5.0, and here also acts as a sreading factor with no intrinsic toxic activity (Tu and hendon, 1983).

4) Vertebrate hyaluronidases

In vertebrates, the enzymatic degradation of HA by non-venom hyaluronidases takes place by the concerted activity of three separate enzymatic reactions, the endoglucosaminic reaction catalyzed by hyaluronidase, and by two exoglycosidases that remove terminal sugars, a beta-glucuronidase and a beta-N-acetyl glucosaminidase. The initial cleavage reaction by the hyaluronidases generate an ever increasing number of substrate molecules for the exoglycosidases. The hyaluronidase enzymes from mammals and other vertebrates have been difficult to investigate, largely because of the inability to isolate them in purified form. Despite inherent difficulties, a considerable amount of information has been accumulated (Lokeshwar *et al.*, 1996; Rod *et al.*, 1995; Fraser and Laurent, 1989). In rapid tissue repair and turnover, in embryological systems as well as in wound healing, the presence and removal of HA play key roles. None of the hyaluronidases from such sources have been characterized, with the single exception of a hyaluronidase from the resorbing tadpole tail (Silbert *et al.*, 1965).

4. Human hyaluronidase

1) Liver hyaluronidase

The liver is the major organ for the degradation of circulating HA (Rod *et al.*, 1989). The non-parenchymal sinusoidal cells

are the major sites for this degradation. These cells have specific receptors for HA, and the HA is taken up for intracellular degradation (Raja *et al.*, 1965; Yannariello-Brown and Weigel, 1992; Gustafson, 1996). The liver enzyme has an acid pH optimum, and has been partially purified and characterized (Aronson and Davidson, 1967; 1967; Gold, 1982, Joy *et al.*). Clinically, patients with cirrhosis and other forms of severe liver disease have elevated levels of circulating HA, presumably because of an impaired degradative pathway (Nyberg *et al.*, 1988).

Recently the structure of a pig liver hyaluronidase has been deduced from a N-terminal sequence and cloning from a pig liver cDNA library (Zhu *et al.*, 1994). But the claim of identity with hemopexin was based on an artifact, on the ability of the cloned hemopexin to bind to HA and generate an artifact due to dye exclusion, plus the high levels of contaminating hyaluronidase activity in the hemopexin preparation (Hrkal *et al.*). The structure of liver hyaluronidase, and whether it is identical to serum hyaluronidase (Afify *et al.*, 1993), remains to be determined. Recent work from this laboratory indicates that mammalian hyaluronidases have a specific activity an order of magnitude greater than previously estimated. Small levels of exceedingly active enzyme protein account for the hyaluronidase activity associated with most biological extracts.

2) Kidney hyaluronidase

A portion of the circulating HA is excreted into the urine. It is apparently the smaller fragments of HA that can traverse basement membranes that are found in urine. Kidney and liver are the two major organs for HA degradation. Occlusion of either hepatic or renal vessels leads to a rapid rise in circulating HA, indicating that both liver and kidney are important in removal of HA from the blood (Engstroem-Laurent and Hellstroem, 1990). Patients with either hepatic or renal failure have increased levels of circulating HA because of the failure, of one of these two degradative mechanisms. Patients with acute renal failure however, have a lower molecular weight profile of serum HA than patients with hepatic failure (Kumar *et al.*, 1989). This suggests that the hyaluronidases of these two organs have a difference in preferential size for their HA substrate. A pH optimum for the kidney enzyme has been reported at 3.5, the most acidic to be documented (Afify *et al.*, 1993). The foregoing argue for the existence of a separate kidney enzyme different from that of most tissues, and perhaps a different gene product. Purification of a kidney enzyme has not been achieved.

Renal hyaluronidase has been invoked as the mechanism of action of antidiuretic hormone (Ginetzinsky, 1958). High levels of hyaluronidase appear in mammalian urine, particularly during dehydration. This suggests that reabsorption of water from the collecting duct lumen may be occurring secondary to increased permeability of the outer duct wall, following digestion of the HA by a renal hyaluronidase.

3) Hyaluronidase of the lymphatic system

One of the most enigmatic areas in HA metabolism is the lymphatic system. The major portion of circulating HA is in the lymphatics. Regional lymph nodes extract and degrade 90% of the HA before it reaches the bloodstream (Fraser *et al.*, 1988; Fraser and Laurent, 1989). What is the physiological basis of this enormous turnover of HA in the lymphatics? What is the identity of the cells that synthesize the HA and what are cells within lymph nodes involved in the catabolism, that extract and degrade HA, and that provide the hyaluronidase activity? These questions remains to be answered although hyaluronidase activity has been observed associated with macrophages (Goggins *et al.*, 1968; Fiszer-Szarfarz *et al.*, 1988). It is uncertain whether such cells can

account for HA catabolism in the lymphatic system.

Hyaluronan has profound immunosuppressive effects. Receptors on the surface of lymphocytes coated with HA are not available to their ligands, and therefore to cytokine or lymphokine signalling. Clearly, an additional mechanism of immune control would be a hyaluronidase regulating HA levels in lymph nodes, lymphatic channels, and on the surface of lymphocytes.

4) The skin hyaluronidase

Skin contains more than half the HA of the vertebrate body (Reed *et al.*, 1988). The turnover rate of HA in skin is also extraordinarily high, a significant portion being removed by the lymphatics in a 24 h period, catabolized by degradation in local lymph nodes and liver. (Laurent *et al.*, 1991). A hyaluronidase derived from skin has been identified (Cashman *et al.*, 1995; 1969) with a pH optimum at 3.7. A number of isozymes were observed that are interconverted by neuraminidase. Once appropriate antibodies are available for immunlocalization, it will be possible to establish whether a true "skin" hyaluronidase exists, or whether an activity from skin lymphatics was being isolated in these studies.

5) Testicular Hyaluronidase, the PH20 class of enzymes

Before successful gamete fusion can occur, mammalian sperm must pass through two structures surrounding the egg: the cumulus complex and the zona pellucida. The major extrascellular component of the cumulus complex which also permeates the zona pellucida and the perivitelline space is hyaluronic acid, a large polymer of alternating N-acetylglucosamine and glucuronic acid residues. It has been proposed that to successful negotiate the viscous intercellular milieu presented by hyaluronic acid in the cumulus matrix a sperm-associated hyaluronidase would hydrolyze the hyaluronic acid en route to the zona pellucida and the egg plasma membrane.

Paradoxically, many mammalian sperms, including those from mouse, undergo the acrosome reaction at the zona pellucida subsequent to penetration of the cumulus complex, suggesting that acrosomal hyaluronidase may serve some role other than penetration of the cumulus matrix. In addition to the soluble enzyme, there are reports indicating the presence of sperm surface associated hyaluronidase that may be released into the surroundings prior to fertilization or may be due to leakage from the acrosomal vesicle.

It has long been appreciated that extracts of mammalian testes contain high concentrations of hyaluronidase activity. These have been commercially available as medical therapeutics. The sperm must first penetrate a layer of cumulus cells that surrounds the egg before reaching the zona pellucida. The cumulus cells are embedded in a matrix rich in HA which is formed prior to ovulation, and the sperm hyaluronidase assists in this penetration process. There is also a suggestion that sperm hyaluronidase functions in sperm-egg adhesion during the process of fertilization. The sperm hyaluronidase, located in the plasma membrane and in the matrix of the acrosome, was first identified as a sperm antigen by a monoclonal antibody, and termed PH20 (Primakoff and Myles, 1983). Sequence analysis revealed homology with the known hyaluronidase contained in bee venom, and the identity of PH20 as a hyaluronidase was then established (Gmachl *et al.*, 1995). Structural studies of a number of venom proteins suggest that the venom apparatus may have evolved from the male reproductive system. In addition to the relationship between venom hyaluronidases and sperm PH20 a number of other venom proteins has strong homologies with sperm proteins

(Hoffman, 1996; Wofsbera *et al.*, 1995).

PH20 is a 64 kDA GPI-linked (glycosyl-phosphatidyl-inositol-linked)enzyme protein on the sperm membrane with a neutral pH optimum. However, a soluble 53 kDa form of the enzyme exists with an acid pH optimum, and whether this represents a cleaved or secreted form of PH20 or an entirely different gene product remains controversial (Laurent *et al.*, 1991; Cashman *et al.*, 1969). Northern analysis of poly(A+) mRNA has demonstrated that expression of the gene is strictly limited to the testis. Human PH20 has been mapped to chromosome 7q31.1 (Jones *et al.*, 1995).

6) Fibroblast hyaluronidase

One of the anomalies of HA metabolism has been the apparent absence of detectable hyaluronidase activity in cultured mammalian fibroblasts. There have been doubts that such an activity exists (Arbogast *et al.*, 1975), though indirect evidence for a fibroblast hyaluronidase enzyme has been documented (Nakamura *et al.*, 1990). In the pioneering work of Orkin and Toole (Orkin and Toole, 1980; 1980) hyaluronidase was described in chick embryo tendon fibroblasts. A hyaluronidase activity has also been found in granulation tissue fibroblasts of rabbit skin wounds by Bertolami (Bertolami and Donoff, 1978; 1982). A developmentally regulated neutral hyaluronidase activity from embryonic mesenchyme was described by Berneld et al. (Berneld *et al.*, 1984), possibly an endo- and beta-glucuronidase. However, further description has not been forthcoming.

In normal cultured mammalian fibroblasts, utilizing conventional extraction procedures, enzyme activity is not detectable. However, hyaluronidase activity can be detected in chicken fibroblasts when extracted in the presence of high salt (Lien *et al.*, 1990). The high salt may be disaggregating an enzyme-inhibitor complex. We have extended this observation to fibroblasts from a wide variety of species.

7) Hyaluronidases from other human tissues

Hyaluronidase from human placenta has been characterized (Yamada *et al.*, 1977), as well as from vitreous (Jones *et al.*, 1995), lung (Thet *et al.*, 1983), brain (Margolis *et al.*, 1972; Polansky *et al.*, 1974), gingiva (Goggins *et al.*, 1968; Tynelius-Bratthall, 1972), and salivary glands (Tan and Browness, 1968; 1968). An intriguing neutral-active hyaluronidase has been detected in a uterine malignancy, specic for HA. No activity was observed for chonondroitin sulfate degradation, unlike most other mammalian enzymes (Miura *et al.*, 1995). A hyaluronidase in malignant prostate tissue has been observed, with a pH optimum of 4.6, and with levels of activity that correlate with cancer progression (Lokeshwar *et al.*, 1996). In the urine of Wilms tumor patients, a hyaluronidase activity is detected that decreases following removal of the tumor and is therefore, probably of tumor origin (Stern *et al.*, 1991). Molecular genetic studies will determine ultimately how many separate mammalian hyaluronidases exist, and their relationship at the genomic level.

5. The CD44 pathway of HA degradation

Hermes antigen or CD44, one of the homing receptors on the surface of lymphocytes is now reconized as the predominant receptor for HA (Haynes *et al.*, 1989; Stamenkovic *et al.*, 1989; Arrufo, *et al.*, 1990; Underhill, 1992). It is an abundantly expressed glycoprotein on the surface of most cells (Stamenkovic *et al.*, 1989). CD44 is expressed in a great variety of isoforms, all products of a single gene, generated by alternative exon splicing (Lesley *et al.*, 1993). Much of CD44 function remains to be described, but the uptake and degradation of HA by CD44 receptor-mediated endocytosis has been documented (Culty *et al.*, 1992; Hua *et al.*,

1993; Pavesant *et al.*, 1994). The relationship between hyaluronidase activity and the CD44-mediated degradation of HA is unknown, nor is the identity known of the specific CD44 splice variants involved in this process of HA catabolism.

6. The caveolae connection

Caveolae are highly acidic endocytic pit-like compartments on the surface of cells. They appear to move materials into cells by a process called potocytosis, particularly materials involved in signal transduction (Anderson, 1993). It is tempting to argue that hyaluronidases are not lysosomal enzymes, as assumed heretofore, but rather function in caveolae. Most vertebrate hyaluronidases are glycosyl-phosphatidylinositol (GPI)-anchored, and most GPI-anchored proteins do not cluster into clathrin-coated pits, but into caveolae. The CD44 class of HA receptors have detergent characteristics suggestive of caveolae-associated proteins (Ko and Thompson, 1995; Neame *et al.*, 1995). CD44 has also been associated with the uptake and degradation of HA. The interaction of CD44 and hyaluronidases has not been investigated, but there is something clearly going on at the caveolae essential to the binding, uptake, and degradation of HA. Association of HA with caveolae in vascular endothelial and smooth muscle cells has been confirmed in ultrastructural studies (Eggli and grabber, 1995). This entire catabolic circuit awaits clarification.

7. Oriental medical and clinical applications of hyaluronidase

1) Hyaluronidase as an anticancer agent

Hyaluronidase has been utilized for some time as an additive to chemotherapeutic regimens, particularly in Europe, for augmentation of the anticancer effect. (Baumgartner and Moritz, 1988;Baumgartner *et al.*, 1988; Beckenlehner *et al.*, 1992; Spruss *et al.*, 1995). There is evidence, however, that hyaluronidase may have intrinsic anticancer effects and can suppress tumor development. Hyaluronidase-treated mice exposed to the carcinogen DMBA(dimethylbenzanthracene) had fewer and smaller tumors and a longer latent period prior to tumor development, compared to untreated controls (Pawlowski *et al.*, 1979). The growth rate of transplantable tumors in mice is regulated by serum hyaluronidase levels, the higher levels enhancing resistance to tumor development (DeMaeyer and Demaeyer-Guignard, 1992). And finally, hyaluronidase treatment has also been shown to block lymph node invasion by tumor cells in an animal model for T cell lymphoma (Zahalka *et al.*, 1995).

2) Hyaluronidase as a spreading agent

Prolonged use of intravenous flids with administration of medications often leads to extravasation. This can cause severe tissue damage and ischemic necrosis when anticancer drugs are being administered, such as the Vinca alkaloids. Tissue damage can become critical in neonates and infants when ordinary medications extravasate. Prompt subcutaneous injection of hyaluronidase prevents tissue destruction (Few, 1987; Bertelli *et al.*, 1994). In anesthesia, administration of hyaluronidase improves infiltration of the anesthetic agents, In all of these cases, it was believed that it is the "spreading factor" aspect of hyaluronidase that functions, to enhance permeation through tissues, through recent studies suggest that alteration of cell-cycle kinetics may also be involved (Roberts, 1988).

3) Other clinical application

Hyaluronidase was used at one time, with little obvious success, in patients with early myocardial infarction (Croix *et al.*, 1996). Most patients are quickly heparinized following a myocardial infarction. Heparin, however, is a

potent inhibitor of hyaluronidase activity. It would be valuable to substitute non-heparin anticoagulants in such patients, and to revisit the question of the efficacy of hyaluronidase administration, particularly since hyaluronidase dramatically reduces the extent of myocardial necrosis in an animal model for myocardial infarction (Baumgartner *et al.*, 1988). The mechanism of reduction of tissue injury may well be the removal of the HA-rich edema uid with a decrease in the extent of pressure necrosis. The edema that often follows organ transplantation may also be controllable by hyaluronidase administration (Haellgren *et al.*, 1990).

Hyaluronidase has also been used in primary closure of neglected or infected wounds. It is applied before final preparation of the wound, to soften the margins and to eliminate resistance to closure (Baurmash *et al.*, 1976), and is particularly useful in small or enclosed spaces, such as those in the hand or in the orofacial region.

4) Future prospects

The complete sequence of several mammalian hyaluronidases is imminent, in addition to the sequences already available for the PH20 superfamily. Structure-function relationships for the various classes of hyaluronidases will become apparent. Proof of structure of the active site and the substrate binding site for these enzymes will come from site-directed mutagenesis experiments. A tentative evolutionary map of the enzymes with homology to PH20 is given in Fig. 1, a multiple sequence analysis program (Feng and Doolittle, 1987; Needleman and Wunsch; Sneath and Sokal, 1973). Chromosomal assignment, and possible association with known tumor suppressor regions, considering the intrinsic anticancer effects of the enzyme, will constitute the next generation of information regarding this neglected, overlooked, underestimated but important family of enzymes.

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