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Mini review

Inhibitors of the hyaluronidases

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Abstract

The inhibitors of hyaluronidase present in mammalian sera, first described half a century ago, have remained uncharacterized. Because of increased interest in hyaluronidases and their hyaluronan substrate, a study of these inhibitors was undertaken recently. The predominant serum inhibitor is magnesium-dependent and is eliminated by protease or chondroitinase digestion, and by heat. Kinetics of inhibition are similar against hyaluronidases from testis, snake and bee venom. The inhibitor has no effect on Streptomyces hyaluronidase; indicating inhibition is not through protection of the hyaluronan substrate. Circulating inhibition levels are increased in mice following carbon tetrachloride or interleukin-1 injection, inducers of the acute-phase response. Reverse hyaluronan gel zymography reveals a predominant band of 120 kDa relative molecular size. Additional studies indicate that the inhibitor resembles a member of the Kunitz type inter-α-inhibitor family. Inhibition of hyaluronidase activity is observed using purified inter-α-inhibitor and is reversed by antibodies specific for inter-α-inhibitor. This molecule, found in the hyaluronan-rich cumulus mass surrounding mammalian ova and the pericellular coat of fibroblasts and mesothelial cells, may function to stabilize such matrices by protecting against hyaluronidase degradation. Turnover of circulating hyaluronan is extraordinarily rapid, with a half-life of two to five min. Prompt increases in levels of serum hyaluronan occur in patients with shock, septicemia or massive burns, increases that may be partly attributed to suppression by these acute phase reactants of the constant and rapid rates of hyaluronan degradation by hyaluronidase. A literature survey of other hyaluronidase inhibitors is also presented.

Keywords: Hyaluronidase; Hyaluronidase inhibitor; Hyaluronan; Inter-α-inhibitor; Acute phase reactants

1. Introduction

Enzyme inhibitors have a very old history. Remarkably, they are often ignored. However, inhibition of enzyme activity may be as significant as the activity itself in the regulation of biological processes, as exemplified by the metalloproteinases (MMPs) and their tissue inhibitors (TIMPS). Very often, it is energetically more efficient to inhibit a constantly active catabolic reaction, than to stimulate synthesis, particularly the high molecular weight polymeric substrates that constitute the extracellular matrix (ECM). This is particularly the case when a rapid response is required or when finely tuned temporal and spatial ECM activities must be regulated. This appears to be the situation that best describes the hyaluronidases in mammalian tissues. In cultured animal cells, hyaluronidase activities are rapidly secreted into the culture medium. This may reflect the situation in vivo. However, it is unlikely that extracellular hyaluronidases are retained in an active form where they would

Abbreviations: CS, chondroitin sulfate; ECM, extracellular matrix; GAGs, glycosaminoglycans; HA, hyaluronan, hyaluronic acid; IL-1, interleukin-1; IαI, inter-α-inhibitor; PαI, pre-α-inhibitor; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of MMPs; TSG-6, tumor necrosis factor-stimulated gene-6

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cause great havoc. If present in the ECM, they would most likely be in an inactive or suppressed form, bound to inhibitors.

Inhibitors of enzymes are frequently encountered in tissue extracts during isolation procedures. They are disposed of as quickly as possible, as they often interfere with the task at hand. The hyaluronidase enzymes themselves have been relatively neglected (Kreil, 1995), largely because they are present in such small amounts and are endowed with very high specific activities that are unstable in the absence of detergents and protease inhibitors. However, their inhibitors, that are also encountered frequently, have been even more neglected. A number of new procedures have been developed that facilitates their study (Stair-Nawy et al., 2001; Mio and Stern, 2001; Mio et al., 2001). An inhibitor of hyaluronidase, detected in human and mouse sera (Mio et al., 2000), is the first to be identified of this widely distributed class of molecules. The tissue inhibitors of hyaluronidases may be more complex than the hyaluronidase enzyme family itself.

2. Historical background

Hyaluronidase inhibitors are ubiquitous and potent, as is evident to those who have attempted to purify hyaluronidases from crude tissue sources. The first studies documenting the existence of a circulating inhibitor of hyaluronidase were published half a century ago (Haas, 1946; Dorfman et al., 1948). These studies were followed by clinical reports that increased inhibitor levels occur in the serum of patients with cancer (Hyman et al., 1955; Kolarova, 1975), liver disease (Snively and Glick, 1950) and dermatological disorders (Grais and Glick, 1948). These inhibitors are high molecular weight thermolabile glycoproteins (Newman et al., 1955) requiring magnesium ions for full activity (Freeman et al., 1949; Mathews et al., 1952). The inhibitor in cancer patients is qualitatively different and does not require magnesium for activity (Fiszer-Szafarz, 1968). Despite the fact that evidence for such inhibitors has been available for over half a century, that they have clinical importance and even though a review appeared that summarized these findings (Mathews and Dorfman, 1955), no further characterization was made.

3. The hyaluronidase inhibitors in mammalian serum

Using newer techniques, a study of the hyaluronidase inhibitor present in the mammalian circulation was undertaken (Mio et al., 2000). Levels of inhibition activity were examined in mouse serum at pH 7.5 using bovine testicular hyaluronidase as the test enzyme, a neutral-acting hyaluronidase. Testicular hyaluronidase activity is suppressed by mouse serum in a dose-dependent manner, with 50% inhibition obtained at 0.35% vol. of the standard reaction mixture. The thermolabile nature of the inhibitor was confirmed (Newman et al., 1955), as well as proteinase sensitivity. No loss of activity occurs with collagenase or heparinase digestion.

The pH for optimal inhibitory activity occurs between pH 6 and 8, in sharp contrast to the wide pH range of activity of the test enzyme. The serum inhibitory activity was examined using other neutral-acting hyaluronidases. Several microorganisms, as well as the venom from insects and many species of snakes, contain hyaluronidases active at neutral pH. Snake venom from *Crotalus horridus horridus*, the Timber rattlesnake; bee venom from the honeybee, *Apis mellifera*; and *Streptomyces* hyaluronidase from *Streptomyces hyaluronlyticus* were examined. The serum inhibitor with potent inhibitory activity against bovine testicular hyaluronidase inhibited the snake and bee venom enzymes, but not *Streptomyces* hyaluronidase

A curious observation made in the course of these studies was that plasma contains far less inhibitory activity than does serum. To address this question the effect of various cations was examined. Cations are removed by the chelating agents used as anti-coagulants in the preparation of plasma. Of a broad range of divalent cations, only Mg^{2+} was effective in restoring activity, confirming earlier observations (Freeman et al., 1949).

4. A cancer-specific hyaluronidase inhibitor

Levels of hyaluronidase inhibitor activity are elevated, more than twice normal, in the circulation of cancer patients, an observation also made more than a half century ago (Hakanson and Glick, 1948; Fiszer-Szafarz, 1968).

Hyaluronan promotes tumorigenesis, the level of HA on the surface of cancer cells correlating with aggressiveness and poor outcome (Zhang et al., 1995). Hyaluronan endows cells with motility, as well as creating spaces through which cells can move. Increased HA deposition enhances tumor growth (Kosaki et al., 1999). Inhibitors of hyaluronidase activity can facilitate accumulation of HA, thus participating in cancer progression, cancer cell motility, and metastatic spread.

5. The acute phase response

In many systems in mammalian biology, an increase

in critical biologicals results not from enhanced rates of synthesis, but by inhibition of degradation reactions. This provides a rapid response mechanism, far more rapid than could be provided by enhanced synthesis. Such a scenario has recently been identified for rapid increases in levels of circulating HA with the discovery of a family of hyaluronidase inhibitors that are also acute phase substances (Mio et al., 2000). The classic experimental model for the acute phase response is the intraperitoneal administration of CCl_4 to the adult mouse (Kinoshita et al., 1989), drawing blood samples at 24 and 48 h. Using this model, a three-fold increase in hyaluronidase inhibitor activity was obtained. Administration of interleukin-1 (IL-1), also an inducer of the acute phase response, generated similar results (Mio et al., 2000).

Patients with burns, septicemia or shock, particularly the shock associated with gram negative sepsis, have rapid increases in circulating HA levels (Engstrom-Laurent and Hellstrom, 1990; Onarheim et al., 1991a,b; Ferrara et al., 1991). The HA and its enormous volume of water of hydration, occupying 10 000 the volume of the original polymer, may function as a naturally occurring intravascular volume expander, to prevent circulatory collapse. The production of hyaluronidase inhibitors, as part of the acute phase response, may result in a suspension of rapid HA degradation and prolongation the rate of HA turnover, thus providing a rapid response for increasing circulating HA levels. It would be of intrinsic interest to measure the $t_{1/2}$ of circulating HA under such circumstances.

6. The inter-α-inhibitor complex

Biochemical and immunological studies suggest that the circulating hyaluronidase inhibitor has the characteristics of an inter-α-inhibitor (IαI)-like molecule. The IαI molecules are plasma proteins, a group of Kunitz-type protease inhibitors with a high molecular mass, ranging from 130 to 240 kDa (Heimburger, 1974), present in the circulation at approximately 0.2–0.7 mg/ml. The original form reported for IαI consist of two heavy chains and one light chain, the latter referred to as bikunin. The heavy chains are comprised of any combination of four separate gene products, H1, H2, H3 and H4. The four genes have homology, but have evolved independently (Nakatani et al., 1997; Daveau et al., 1998). Virtually nothing is known about the functional differences between the four chains. Recent studies have demonstrated the existence of even further divergence in IαI family members. In addition to the genes coding for heavy chains, divergence in the number and combination of peptides make IαI a complex family of molecules.

The IαI polypeptides are connected by covalent linkages to a CS chain (Sjoberg and Fries, 1990) and are by definition proteoglycans. The CS chain is covalently bound to bikunin through the normal -gal-gal-xyl-serine linkage. The unusual feature is the heavy chain attachment. The CS is bound by an ester bond between a sugar hydroxyl group and a heavy chain aspartate residue. Neither the enzyme that catalyzes this reaction nor the precise linkage moieties between CS and the heavy chain have been identified. Digestion with endogenous serum chondroitinase eliminated hyaluronidase inhibitory activity in mouse serum (Mio et al., 2000). The structure resulting from linkage to CS appears to be necessary for hyaluronidase inhibitory activity. The heavy chains of IαI also interact in some unknown way, but most probably covalently, with tumor necrosis factor-stimulated gene 6 (TSG-6) and HA (Hascall, personal communication).

The interaction with HA results in pericellular matrix stabilization, as has been demonstrated for fibroblasts and mesothelial cells (Blom et al., 1995), and for the cumulus mass that surrounds the mammalian ovum (Chen et al., 1994). This may be a general situation for the HA-rich pericellular matrix of all cells, but is more obvious in cells that have a particularly prominent pericellular matrix, which may be the case Fobroblasts and mesothedial cells (Hedman et al., 1979). We have observed significant immunohistochemical staining for IαI wherever there is abundant deposition of HA, such is in the dermis of normal human skin and in the stromal or desmoplastic reaction to human tumors (Mio et al., manuscript in preparation).

Transcriptions of all five gene products, the four various heavy chains, H1- H4 and the short chain, bikunin, occur in the liver, and are stimulated under conditions of acute inflammation. Such regulation indicates that IαI family members participate in the acute phase response (Daveau et al., 1998). Stimulation of the acute phase response by carbon tetrachloride or IL-1 administration to the mouse, as aforementioned, results in a marked increase in circulating hyaluronidase inhibitory activity (Mio et al., 2000).

It is unknown what chain arrangements are involved in hyaluronidase inhibition or whether chain specificity exists in such a function. Nor is the relationship between the structural HA component of IαI and the HA substrate being protected from hyaluronidase digestion known. However, protection of HA substrate is not the entire mechanism of action of the hyaluronidase inhibition, since there is no inhibition against bacterial hyaluronidase. Absolute

identification of the elusive circulating hyaluronidase inhibitor, however, has not been achieved. The 120 kDa band of hyaluronidase inhibitory activity observed by reverse HA-substrate gel zymography makes pre-α-inhibitor (PαI), with a reported size of 120–130 kDa, a promising candidate. The PαI member of the IαI family consists of one heavy chain and one light chain. It is so named because it precedes α1 globulin in serum electrophoresis and it is not a precursor to IαI.

Recent studies indicate that two members of the IαI family, IαI and PαI, are critical in organizing and stabilizing the expanding ECM of cumulus–oocyte complexes in culture in vitro (Chen et al., 1994). The IαI molecules also form covalent interactions with HA within the ovulated cumulus ECM. Such IαI may participate in regulation of hyaluronidase activity of the testicular hyaluronidase, PH 20, during the penetration of the sperm through the cumulus matrix (see the review by Cherr et al., in this mini-series).

There may exist a dual matrix system for cells, an intimate pericellular matrix that is HA-rich, cross-linked by IαI and PαI, and bound to cell surfaces, in part, by HA receptors such as CD44. Surrounding this is the general ECM with loosely associated HA, as well as HA polymers tightly intercalated within proteoglycan structures. The HA of the ECM may have a turnover rate and a biology entirely different from that of the pericellular matrix. It may be formulated that all cells have a pericellular matrix, but that it is more prominent in cells such as fibroblasts, mesothelial cells and stem cells. It appears that the IαI family of molecules are consistently major components of the pericellular matrix, while their participation in ECM structures varies from tissue to tissue. The major proteoglycan of the pericellular matrix-cell surface membrane complex is heparan sulfate-rich and also possesses hyaluronidase inhibitory activity (vide infra).

7. Hyaluronidase inhibitor activity in urine and saliva

Hyaluronidase is present in human urine at 100 times the specific activity present in serum (Csoka et al., 1997). A hyaluronidase inhibitor activity also occurs in urine (Knudsen and Koefoed, 1961), but is uncharacterized. The kallikrein contained in urine is an inhibitor of hyaluronidase activity (Warren et al., 1962). Urine contains high levels of bikunin, the short chain of IαI complex of molecules. Whether bikunin alone possesses hyaluronidase inhibitory activity or whether bikunin constitutes part of the kallikrein family, is unknown.

Hyaluronidase inhibitory activity has also been described in saliva (Pogrel et al., 1999). It inhibits bacterial hyaluronidases as well, which is different from the serum inhibitory activity.

8. Heparin and heparan sulfate

Heparin, the most sulfated and most acidic of GAGs, is a well-characterized inhibitor of hyaluronidase (Wolf et al., 1984). It also promotes wound healing and has been recommended for use in burn patients, as a topical application (Saliba, Jr, 2001). Whether the beneficial effect of heparin on wound healing or on burn patients is through the ability to inhibit hyaluronidase has not been examined.

Inhibition of hyaluronidase activity is obtained at concentrations of heparin far greater than physiological levels. The concentration of normal plasma heparin cannot be detected by conventional means. However, heparin, released by mast cells in the process of degranulation, may inhibit hyaluronidase activity by formation of a local enzyme complex. Inhibition by heparin is non-competitive, as documented by a Lineweaver–Burke plot (unpublished observations), suggesting that heparin does not bind to the catalytic site of the enzyme. Heparan sulfate also inhibits hyaluronidase activity (unpublished). The heparan sulfate-rich proteolgycans, such as the syndecans, that are components of the basal lamina, on the surface of cell membranes and in the pericellular matrix, may constitute portions of the strategy for regulating extracellular hyaluronidase activity

Sucrose octa-sulfate (S.O.S., sucralfate, Carafate,® Hoechst Marion Roussel) was also examined. This compound, used in ulcer treatment, is reported to promote wound healing in the gastric mucosa (Maclaurin et al., 1985) and is also a highly sulfated sugar. It was postulated that enhanced hyaluronan deposition on the gastric surface by inhibition of hyaluronidase might be the mechanism of action. However, no inhibitory activity was observed, perhaps because sucrose is an α- rather than a β-linked disaccharide.

The serum inhibitor has no inhibitory effect against *Streptomyces* hyaluronidase and only 15% inhibition against human plasma hyaluronidase, Hyal-1. Heparin, however, exerts a very different profile of inhibition against these enzymes. Heparin inhibits snake venom hyaluronidase more efficiently than it does bovine testicular hyaluronidase and at lower concentrations. Heparin has no inhibitory effect against *Streptomyces* hyaluronidase, similar to the serum inhibitor, but has a major inhibitory effect against Hyal-1, suggesting that the mechanisms of action of heparin and serum hyaluronidase inhibitor are entirely

different in their ability to block hyaluronidase activity.

9. Hyaluronidase inhibition activity in anti-inflammatory drugs

Small fragments of HA are highly angiogenic (West and Kumar, 1989) as well as potent inducers of inflammatory cytokines (Horton et al., 1998; see the review by Noble in this mini-series), while high molecular weight HA polymers prevent angiogenesis (Feinberg and Beebe, 1983). Certain anti-inflammatory drugs possess hyaluronidase inhibitor activity, including salicylates (Guerra, 1946) and indomethacin (Szary et al., 1975). These drugs may exert a portion of their inflammatory activity by preventing generation of small HA fragments. Other drugs used to suppress allergic reactions, such as disodium cromoglycate and tranilast, that may function as hyaluronidase inhibitors (Kakegawa et al., 1985c) are used extensively in Japan.

10. Plant-derived hyaluronidase inhibitors

There is a rich tradition in ethno-pharmacology of compounds that function by blocking hyaluronidase activities. These have traditionally been used as contraceptives, to treat snakebites or to promote wound-healing, compounds that were subsequently demonstrated to possess anti-hyaluronidase activity. These include flavenoids (Kuppusamy and Das, 1991), tannins (Kakegawa et al., 1985a), hydrangenols from hydrangea (Kakegawa et al., 1988), curcumins from the spice, cumin (Tonnesen, 1989), glycyrrhizin from licorice (Furuya et al., 1997) and tranilast, a chemically modified cinnamic acid (Kakegawa et al., 1985b). Some of these compounds (Li et al., 1997) and their synthetic derivatives (Perreault et al., 1980) continue to be used as contraceptives, presumably through their ability to inhibit sperm hyaluronidase activity. Some of the same plant extracts are also used in folk medicines as anti-inflammatory drugs (Kushwah et al., 1978).

11. Concluding remarks and future directions

It is apparent that the description of hyaluronidase inhibitors in mammalian tissues has barely begun. Much of the literature cited in this review is nearly half a century old. After an initial flurry of activity, much of the work in the field ceased, largely due to a lack of sufficiently rapid and sensitive methods. However, techniques are now available and much progress is anticipated.

Many questions regarding the IαI or PαI molecules remain unanswered. What is the nature of the protein–protein interaction here, with the hyaluronidases whose activity they inhibit? What is the mechanism of inhibition? What is the significance of the HA and CS chains attached to these molecules and how do they participate in the inhibition? Indirect evidence indicates that CS is necessary for inhibition. Technical reasons preclude removal of the HA chain to test whether that is also required for inhibition. It has been our experience, in the process of purifying hyaluronidases, that virtually every mammalian tissue contains potent hyaluronidase inhibitory activity. Are all such activities related to the IαI family of proteins or are there a wide variety of molecules that possess similar inhibitory activity?

All of the vertebrate hyaluronidase also have the ability to digest chondroitin (CS), albeit at a slower rate. It would be of interest to establish whether the hyaluronidase inhibitor activity reported here has the ability to inhibit CS digestion as well. As reported in a prior article in this mini-review series (Csoka et al., 2001), one of the hyaluronidase-like sequences, Hyal-4,has chondroitinase activity. Can the circulating hyaluronidase inhibitor activity also suppress chondroitinase activity? This has major implications for the metabolism of bone and cartilage and for the pathologies of these tissues.

The modulation of hyaluronidases throughout the body is critical for normal homeostasis and aberrations are probably involved in a great number of disease processes. The challenge at the moment is to catalogue the inhibitor activities in various tissues, and to establish their molecular structure and mechanism of action. This review, finally, serves to draw attention to this neglected but important area of matrix biology.

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