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COMMENTARY

COLLAGENOLYTIC METALLOENZYMES OF THE HUMAN NEUTROPHIL

CHARACTERISTICS, REGULATION AND POTENTIAL FUNCTION *IN VIVO*

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The primary role of the human neutrophil is to destroy pathogenic microbes that have invaded the host [1, 2]†. Neutrophils accomplish this task by using their plasma membrane to engulf and sequester the microorganisms into the formed intracellular compartment which is termed the phagocytic vacuole. Simultaneous with the initiation of vacuole formation, a portion of the lysosomal granules of the neutrophil fuse with the encircling plasma membrane and discharge their complement of microbicidal proteins and proteases. In addition, a plasma membrane-associated NADPH oxidase is activated which consumes dissolved O_2 in order to generate a complex array of highly reactive oxidants. Together, the lysosomal contents and the oxygen metabolites efficiently participate in the destruction and degradation of the target.

Although the neutrophil is heavily girded for microbial battle, it is also known that the cell contains intracellular organelles whose contents are destined for extracellular discharge [3]. In this commentary we will discuss the potential functions of two oft-neglected metalloproteinases that are released from triggered neutrophils, collagenase and gelatinase. These enzymes are able to specifically degrade the collagens, a family of closely related, but distinct macromolecules that comprise the major structural proteins of all connective tissues [4, 5]. Given the apparent substrate specificity of the metalloenzymes, it seems clear that they do not exert a direct microbicidal effect, but their precise function in neutrophil physiology remains unclear. Indeed, despite the fact that the role of similar collagenolytic enzymes in physiologic and pathologic tissue degradation [6, 7], angiogenesis [8, 9] and tumor metastasis [10] is under intense scrutiny, few of these insights have been applied to our understanding of neutrophil function in host defense and inflammation. What are the characteristics of the neutrophil metalloenzymes? How are they regulated by the intact cell? What are their physiologic functions *in vivo*? By examining these questions we hope to focus attention on enzymes that potentially play critical roles in the regulation of cell movement and tissue degradation in health and disease.

What are the characteristics of neutrophil collagenase?

The triple helix of the interstitial collagens (i.e. types I, II, and III)‡ confers these macromolecules with a high degree of resistance against general proteases [4–7]. However, a number of mammalian cells including skin and synovial fibroblasts, uterine cells, bone cells, chondrocytes, macrophages and endothelial cells can synthesize and secrete "classic" vertebrate collagenases, i.e. they cleave native interstitial collagen at neutral pH and at only one specific locus within the helix 3/4 from the amino terminus [4–7]. In terms of its general properties, the neutrophil collagenase shares many characteristics with the more extensively studied tissue collagenases. All of these collagenases are calcium- and zinc-requiring enzymes, have neutral pH optima and express a strong preference for native, rather than denatured, collagens [6, 7].§ Despite these similarities, the neutrophil collagenase distinguishes itself from other collagenases by the fact that (1) the neutrophil collagenase is stored in a secretory granule and is not actively synthesized by the mature cell [6], (2) the neutrophil collagenase is structurally distinct and can be identified with monoclonal antibodies that do not identify other tissue collagenases [12], and (3) the neutrophil proteinase has a different substrate specificity for the individual collagen types [6, 13].

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† Whenever possible, readers will be referred to review articles or to recent publications where additional references can be found.

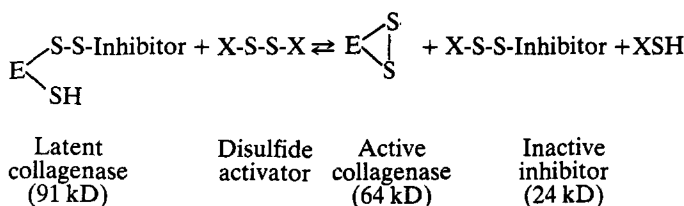
‡ Type I collagen is the most ubiquitous type of collagen in adult connective tissues and is found primarily in skin, bone, tendon, gingiva, large blood vessels and the uterine wall. Type III collagen has a tissue distribution similar to type I except that it is found in only small amounts in bone, tendon, and cornea. Type II collagen is primarily confined to the nucleus pulposus, hyaline cartilage and vitreous. For more extensive reviews on collagen distribution and chemistry, see Refs. 4 and 5.

§ A recent exception is the collagenase isolated from the rat uterus [11].

What is the molecular basis for the latency of neutrophil collagenase?

One of the most intriguing aspects of the physicochemical properties of neutrophil collagenase is the fact that the enzyme can be isolated from the cell in a latent, inactive form [6, 7, 13]. This latency is not a general property of other lysosomal enzymes found in the neutrophil, but rather a characteristic of most, if not all, mammalian collagenases [6, 7]. All of the latent collagenases can be activated *in vitro* by an array of seemingly unrelated and many times physiologically irrelevant proteases (e.g. trypsin), thiol-reactive organomercurials (e.g. 4-aminophenylmercuric acetate) and chaotropic agents [6, 7]. In an attempt to explain these characteristics, the latent enzyme has been proposed to be either a proenzyme or an enzyme–inhibitor complex [6, 7, 14]. The proenzyme model was based on the observation that proteases activate latent collagenase while simultaneously decreasing its apparent molecular weight [6, 7, 14]. However, organomercurials and chaotropic agents also activate and decrease the molecular weight of the latent enzyme [6, 7, 14]. Because these compounds do not possess an intrinsic ability to mediate peptide bond cleavage, the data seemed to indicate that the enzyme was activated following the dissociation of an inhibitor. This controversy has been resolved recently in at least two cases. Studies with highly purified skin and synovial fibroblast collagenases have revealed that these metalloproteinases are proenzymes [6]. Organomercurials and chaotropic agents indirectly decrease the M_r of the skin collagenase by perturbing the conformation of the zymogen and unmasking an intrinsic, autocatalytic potential [15]. This allows the activated collagenase to then catalyze an intramolecular cleavage that is not a prerequisite for activation [15]. In contrast, synovial collagenase is activated indirectly after a proteinase or an organomercurial activates a latent proactivator which then acts on the procollagenase [6]. Under certain conditions, both the skin and synovial procollagenases can be shown to undergo activation without any detectable change in their molecular weights [6, 15].*

If highly purified, latent procollagenases can be activated by exogenous proteinases, organomercurials and chaotropic conditions, one might assume that the ability of these agents to also activate neutrophil collagenase would indicate that this latent enzyme is likewise a zymogen. However, a series of reports by Macartney and Tschesche [17–19] have marshalled support for the classification of neutrophil collagenase as an enzyme–inhibitor complex. Highly purified neutrophil collagenase was isolated as a 91 kilodalton (kD) latent enzyme that could be activated by either organomercurials or disulfides to yield active collagenase with an M_r of 64 kD and, in addition, a 24 kD inhibitor [19]. The inhibitor contained a single essential thiol, inactivated collagenase with an apparent 1:1 stoichiometry, and lost activity if incubated with a disulfide [17–19]. Based on these findings, the following model was proposed:



Thus, the latent enzyme was postulated to contain a disulfide-linked inhibitor and a free thiol. Following the addition of a disulfide, a series of thiol–disulfide exchanges occurred leading to the generation of the proposed disulfide-bridged active enzyme and an inactive disulfide-linked inhibitor. In support of this model, incubation of the activated collagenase with inhibitor regenerated latent collagenase with an apparent molecular weight of ~ 90 kD when estimated by gel filtration [19]. These data provide compelling evidence to support the contention that neutrophil collagenase is an enzyme–inhibitor complex and have led to the proposal that physiological changes in the thiol–disulfide redox couple might regulate neutrophil collagenase *in vivo*. However, the fact that all other well-characterized collagenases are proenzymes suggests that a cautious appraisal of these data is warranted. First, the presence of a thiol in the dissociated inhibitor has been demonstrated, but the model also predicts that the latent collagenase should contain a free thiol. The ability of the thiol-reactive organomercurials or disulfides to activate collagenase suggests that the latent enzyme might have a reactive thiol moiety, but this cannot be assumed. Indeed, although skin collagenase can be activated by organomercurials or disulfides, it is not an enzyme–inhibitor complex and does not contain any detectable thiols [15, 20]. Second, much of the early support for the concept that tissue collagenases were enzyme–inhibitor complexes was based on the fact that an excess of an inhibitor could also be detected in these cell culture media [14]. In a manner similar to the neutrophil system, the inhibitor formed a 1:1 complex with active collagenase to generate a species with characteristics that appeared almost identical to the original latent enzyme [14]. However, subsequent studies have demonstrated that the inhibitor is a distinct gene product, that it is not structurally related to the proenzyme, and that although it can be demonstrated to bind the active enzyme when assessed by gel filtration, it does not form a sodium dodecylsulfate (SDS)-stable complex

* As indicated by these investigators, small decreases in M_r (< 2000) may not be detected. For example, trypsinogen is a proenzyme that is activated following the removal of only the N-terminal hexapeptide [16].

[21–23].* Finally, it is difficult to rule out the possibility that neutrophil collagenase, like the tissue collagenases, is actually a proenzyme, but that it has undergone activation during its extraction from disrupted neutrophils. Following activation, the enzyme might then interact with a normally sequestered, 24 kD endogenous inhibitor that results in the isolation of the "latent" collagenase as an enzyme–inhibitor complex. Indeed, this proposed scenario has a precedent. Much of the thiol–disulfide model of neutrophil collagenase activation is based on an earlier series of reports by Steven and Podrazky [24, 25] on the regulation of a tumor cell protease. In a strikingly similar system, Ehrlich ascites tumor cells were shown to contain a trypsin-sensitive, latent neutral protease in their granular fractions and a thiol-containing protease inhibitor in the cytosolic post-granule supernatant fraction [24, 25]. If a similar compartmentalization occurs in the intact neutrophil, it will be important to analyze latent collagenase that has been rigorously prevented from interacting with cytosolic or other granule-associated factors. Indeed, Hasty *et al.* [26] may have circumvented this problem by isolating the collagenase that was released directly from intact triggered neutrophils. Using a combination of immunoaffinity chromatography and immunoblotting, neutrophil collagenase could be identified in latent forms with molecular weights of 75K and 57K [26]. Not only are these molecular weights substantially lower than the 91 kD species described by Macartney and Tschesche, but these two forms of collagenase could be activated by a chaotropic agent (NaSCN) without undergoing a detectable decrease in M_r [26]. Additional analyses of the stored latent collagenase and the compartmentalization of its inhibitor will be required before these differences can be resolved.

Can neutrophils activate latent collagenase and, if so, how?

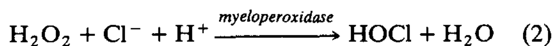
Regardless of the molecular basis underlying the latency of neutrophil collagenase, it is clear that the enzyme must be activated in an intact cell system before it can catalyze collagen degradation. Although a variety of processes have been postulated to play potential roles in the activation of neutrophil collagenase [6, 7], only a handful of studies have actually examined the regulation of the enzyme in an intact cell system [12, 27, 28]. Until recently, the accumulated data suggested that neutrophils could release latent collagenase extracellularly, but that the cells have almost no intrinsic ability to activate the enzyme [12, 27, 28]. (Indeed, it seems that the apparent inability of the neutrophil to activate its collagenase stifled further interest in the enzyme.) Perplexed by these observations, our group re-examined the ability of human neutrophils to release and activate endogenous collagenase [29]. In contrast to other studies, we demonstrated that neutrophils incubated with phorbol myristate acetate (a non-physiologic triggering agent that stimulates neutrophils to discharge granules and generate oxygen metabolites in a manner that mimics physiologic stimuli) or opsonized zymosan particles (an ingestable particle coated with plasma proteins that are recognized by receptors on the neutrophil membrane) activated ~50% of the released collagenase† [29]. These results clearly demonstrated that neutrophils could directly activate latent collagenase, but how was this accomplished?

Endogenously-derived serine proteinases, metalloproteinases, as well as non-proteolytic activators have been shown to play important roles in the activation of latent collagenases released by mouse bone cultures, alveolar macrophages, rheumatoid synovial cells, uterine and skin cells and even tumor cells [6, 7, 30]. In the neutrophil, early attention focused on the ability of the cell's two major serine proteinases, elastase and cathepsin G, to activate collagenase [13, 31]. However, studies with the isolated enzymes demonstrated that these enzymes actually destroy neutrophil collagenase [13]. Rather than focus our attention on potential protease-dependent mechanisms of activation, we became intrigued with the possibility that the ability of organomercurials to activate collagenase might provide a clue to the identity of the endogenous activator(s) generated by the neutrophil. Unlike almost all other cell types, the neutrophil not only releases lysosomal enzymes but also generates a family of highly reactive oxygen metabolites [1, 32]. In a series of catalyzed and spontaneous reactions, the triggered neutrophil generates superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl) and possibly the hydroxyl radical [1, 32]. Was it possible that microbicidal oxidants might function as "endogenous organomercurials" and activate collagenase by reacting with a critical thiol [17–19] or by perturbing its normal conformation [15]? Strong support for the participation of oxidants in collagenase activation was quickly provided from analyses of neutrophils isolated from individuals with chronic granulomatous disease [29]. This genetic disorder is characterized by the inability of the neutrophil to generate oxygen metabolites [1]. In contrast to our findings with normal neutrophils, the chronic granulomatous disease cells were only able to release latent collagenase and none of the enzyme could be detected in its active state [29]. These results clearly defined a critical requirement for oxygen metabolites in the expression of collagenolytic activity. Further studies with normal neutrophils led to the identification of HOCl as the key oxidant in the activation pathway [29]. This highly reactive oxidant (the active ingredient in bleach) is generated by a reaction dependent on H_2O_2, the lysosomal enzyme myeloperoxidase (a green hemoprotein that gives pus its distinctive color) and chloride (equation

* It should be noted that activation fragments released from zymogens can exert inhibitory effects on the active enzyme. For example, porcine pepsinogen is converted to enzymatically active pepsin by the loss of its 44 amino-terminal residues. This peptide can then be degraded by pepsin to generate potent inhibitors [16]. Thus, in such a system, enzyme activation could take on the appearance of the dissociation of an enzyme–inhibitor complex.

† These results contrast with those of Hibbs and coworkers [12] where PMA-stimulated neutrophils were not reported to release active collagenase. It is unclear why these investigators failed to detect the active collagenase.

2; Refs. 1 and 32):



$$H_2O_2 + Cl^- + H^+ \xrightarrow{myeloperoxidase} HOCl + H_2O \quad (2)$$

If neutrophils were triggered in the presence of catalase (consumes H_2O_2), azide (inhibits myeloperoxidase) or methionine (a competitive scavenger of HOCl), active collagenase could not be detected [29]. However, if these cell-free supernatant fractions were incubated with the organomercurial, 4-aminophenylmercuric acetate, or with reagent HOCl, the latent collagenase was activated [29]. Thus, neutrophils could activate their endogenous collagenase, and the activation process was linked to the generation of HOCl.

Is HOCl the final mediator in the activation sequence?

Regardless of the sequence of events that link HOCl generation to collagenase activation (see below), it is difficult to identify HOCl as the "final" mediator. Although HOCl is the final oxidant generated by the myeloperoxidase system, in a complex cell system the generated HOCl also participates in secondary reactions [33–35]. For example, HOCl can react with endogenous amines that are simultaneously released from the neutrophil to generate *N*-chloroamines (equation 3).

$$RNH_2 + HOCl \longrightarrow RNHCl + H_2O$$

These chlorinated products are two electron oxidants whose reactivity is partially dictated by the R group [33–35]. *N*-Chloroamines may be hydrophobic or hydrophilic, stable or unstable [33–35]. These derivative oxidants might mediate collagenase activation, but preliminary studies with *N*-chlorotaurine, the major hydrophilic *N*-chloroamine generated by the neutrophil [34, 35], indicate that this species is not as effective an activator as HOCl [29]. However, the fact that an oxidant with limited reactivity relative to HOCl (e.g. *N*-chlorotaurine preferentially reacts with thiols and thioethers at neutral pH) can partially activate collagenase may provide additional insights into the chemical basis of activation.

In considering the role that HOCl plays in collagenase activation, our attention should not necessarily be limited to the direct effects exerted by chlorinated oxidants. For example, HOCl or RNCl can oxidize thiols to disulfides and these products have been proposed to act as collagenase activators [17–19]. In a model system designed to mimic the intact cell, Tschesche and Macartney demonstrated that collagenase could be reversibly shifted from a latent to an active form depending on the ratio of glutathione (GSH) to oxidized glutathione (GSSG; Ref. 18). Oxidation of GSH to GSSG can be mediated by a variety of peroxidases including myeloperoxidase, glutathione peroxidase, horseradish peroxidase and lactoperoxidase [18]. Subsequently, the generated GSSG activates the latent collagenase by dissociating the putative enzyme–inhibitor complex (see equation 1). (This study is often misinterpreted by other investigators as indicating that cell-derived oxidants directly regulated collagenase activity. Actually, the H_2O_2-myeloperoxidase–Cl-system was incapable of activating collagenase unless exogenous GSH was oxidized to GSSG. Thus, peroxidases that do not release oxidants directly, e.g. glutathione peroxidase, were also capable of activating latent collagenase as long as they generated GSSG.) They concluded that alterations in the GSH/GSSG ratio that occur in triggered neutrophils would then regulate collagenase activity, but we consider this possibility unlikely. Decreases in GSH associated with phagocytosis are quite small (normal levels fall by ~30%) and only a portion of the lost GSH can be accounted for as GSSG [36]. In addition, the oxidation of GSH occurs in the cytosol [36], and it is not clear how GSSG might gain access to collagenase that has been released into the phagocytic vacuole or extracellularly. GSSG generated in the cytosol could be transported extracellularly [36], but we are unaware of any studies that have demonstrated GSSG efflux in the neutrophil. Finally, other investigators have been unable to demonstrate that GSSG can activate latent collagenase that had been released from neutrophils [29, 37]. At this time, a role for intracellular disulfides in the activation process remains unclear, but neutrophils can oxidize extracellular thiols present in plasma [38]. Thus, the possibility that these disulfides might regulate endogenous as well as exogenous collagenase activity at inflamed sites deserves careful attention.

How might HOCl regulate latent collagenase?

The mechanism(s) by which HOCl or HOCl-derived products activate collagenase is unknown. Ultimately, an elucidation of the reaction scheme underlying activation will depend on the construct of the latent collagenase as a proenzyme, an enzyme–inhibitor complex, or a combination of proenzyme and inhibitor. First, in the proenzyme model, HOCl could mimic the action of the organomercurials and directly activate latent procollagenase [6] or a latent activator of procollagenase [6, 39]. That is, HOCl-mediated oxidations or chlorinations could cause conformational changes in the targeted molecule which result in the activation of neutrophil collagenase. Second, if neutrophil collagenase is an enzyme–inhibitor complex, then HOCl could act by simply oxidizing the critical thiol proposed to control the dissociation of the inhibitor [17–19]. However, this model is complicated by the fact that Hasty *et al.* [26] could not detect the predicted 91 kD enzyme–inhibitor collagenase complex in supernatant fractions recovered from triggered neutrophils. Finally, if the neutrophil releases procollagenase and an independently functioning inhibitor of activated collagenase, a different scenario could be envisioned in which HOCl does not directly participate in collagenase activation. In this model, the proenzyme would be activated by an uncharacterized oxygen-independent process, but collagenolytic activity would not be detected unless the simultaneously released collagenase inhibitor was oxidatively destroyed. This model is not without precedent. Neutrophils contain large amounts of the lysosomal serine proteinase, elastase. Unlike collagenase, elastase is stored and released in an active form [40]. However, under physiological conditions, elastase activity is carefully regulated by the plasma antiproteinase, alpha-1-proteinase inhibitor (α-1-PI; Ref. 41). In turn, α-1-PI activity can be inhibited by

cell-derived chlorinated oxidants [40]. Thus, in a manner analogous to the proposed collagenase system, the elastase activity released by triggered neutrophils cannot be detected in the presence of α-1-PI unless the activity of the antiproteinase has been oxidatively down-regulated. In contrast to the elastase-α-1-PI system, the latent collagenase would first be required to undergo activation and the oxidizable inhibitor would necessarily be of endogenous origin. Interestingly, collagenase inhibitors have been described in the cytosol of human neutrophils, but their characteristics and potential extracellular release have not been examined [31, 42].

Could other factors play a role in collagenase activation?

The inability of chronic granulomatous disease neutrophils to activate significant amounts of collagenase indicated that alternate, oxygen-independent mechanisms of activation were not operative in our system [29]. However, it should be noted that cells were triggered for only short periods of time and in the absence of the collagen substrate (i.e. only the cell-free supernatant fractions were assayed for collagenase activity) or exogenous plasma factors [29]. Under these conditions, potential roles for collagenase autoactivation, substrate-dependent activation, or membrane-associated collagenase activity would not have been assessed, but do deserve consideration [6, 7]. In addition, neutrophils are generally considered to be devoid of any biosynthetic activity, but they are able to synthesize a number of proteins including plasminogen activator [43–45]. This serine proteinase can cleave the plasma protein plasminogen to form plasmin. In turn, plasmin has been shown to activate a number of tissue collagenases [6, 7]. In a preliminary study, we reported that neutrophils triggered in the presence of exogenous plasminogen did not activate collagenase by a plasmin-dependent process [29], but the conditions chosen may have been sub-optimal [45]. Furthermore, a search for additional activators should not necessarily be limited to endogenous factors. For example, early studies demonstrated that rheumatoid synovial fluids [46, 47] and dental plaque [48] contain factors that activated crude preparations of neutrophil collagenase. Indeed, the many similarities between the latent collagenases suggest that the ability of activators identified in other cell systems (e.g. kallikrein, cathepsin, and angiogenesis factors), to regulate neutrophil collagenase should also be considered [6, 7, 14]. Similarly, it will be interesting to determine if neutrophil-derived oxidants can activate other tissue collagenases or if exogenously introduced oxidants (e.g. cigarette smoke [49]; reagent H_2O_2 [50]) could also participate in the regulation of collagenase activity.

Characteristics of neutrophil gelatinase

Neutrophils contain a second metalloproteinase, gelatinase, [42, 51, 52], with characteristics similar to the gelatinase synthesized by a variety of connective tissue cells [53]. Like collagenase, neutrophil gelatinase is a latent enzyme, requires calcium and zinc for maximal activity, and possesses a neutral pH optimum [52]. However, unlike collagenase, neutrophil gelatinase is stored in an independently controlled secretory compartment and has a unique substrate specificity [13, 52]. Gelatinase does not attack native interstitial collagens, but can degrade denatured collagen (i.e. gelatin) as well as native type IV or type V collagen [13, 52].

Neutrophil gelatinase was first isolated in 1974 [54], and since that time a range of molecular weights and characteristics have been reported [52]. In the most recent study, Hibbs *et al.* [52] purified the latent enzyme released from triggered neutrophils by a combination of anion exchange and gelatin-affinity chromatography. Three bands with gelatinase activity could be identified on non-reduced gels with molecular weights of 92K, 130K and 225K. Upon reduction, all forms migrated as a single 92K band. These authors concluded that the multiple molecular weight forms were derived from a single proteinase, but they could not rule out the possibility that the enzyme had undergone cellular processing during secretion [52].

The molecular basis underlying the latency of gelatinase has not been studied as extensively as that for collagenase, but latent gelatinase can also be activated by proteases, organomercurials and chaotropic agents [13, 51, 52, 54]. At present, there seems to be more evidence supporting the concept that gelatinase is a proenzyme that undergoes a marked conformational change upon activation [13].

Can neutrophils activate latent gelatinase?

Little is known about the activation of latent gelatinase by intact neutrophils. Gelatinase has recently been localized to a highly responsive, but uncharacterized secretory compartment in the neutrophil [42, 51]. Thus, it is not surprising that the limited number of studies on neutrophil gelatinase have focused on the characterization of enzyme release rather than its activation. However, Hibbs *et al.* [42] recently reported that neutrophils triggered with high concentrations of the calcium ionophore A23187 release large amounts of active gelatinase [42]. Based on the fact that neutrophils appeared to have an endogenous activating system for gelatinase and that the latent enzyme has characteristics similar to those of latent collagenase, we initiated studies to examine the ability of oxidants to act as endogenous activators [55]. Indeed, triggered neutrophils were able to activate a portion of their released gelatinase by an HOCl-dependent process. As in the interstitial collagenase system, it might be argued that HOCl indirectly "activated" gelatinase by destroying an endogenous inhibitor. However, we also demonstrated that highly purified latent gelatinase could be activated by reagent HOCl [55]. These results suggest that neutrophils can use chlorinated oxidants to directly control at least one (and perhaps both) of their collagenolytic metalloenzymes. Structural analyses of the latent and HOCl-activated gelatinase may provide insights into the activation process at the molecular level.

HOCl played a major role in gelatinase activation, but antioxidants were not able to inhibit gelatinolytic activity by more than 75% [55]. The presence of an apparent HOCl-independent pathway of activation was confirmed in studies with chronic granulomatous

disease neutrophils [55]. Cells isolated from these individuals were able to activate gelatinase in amounts comparable to those detected with normal neutrophils that were triggered in the presence of antioxidants. Thus, neutrophils can activate gelatinase by an O_2-independent pathway, but potential roles for endogenous or exogenous proteinases (e.g. cathepsin G, plasmin) or an autoactivation process require further study [6, 13, 31].

How are collagenases regulated in the extracellular space?

Once activated, neutrophil collagenase or gelatinase will continue to degrade their respective substrates unless the enzymes are inhibited or destroyed [6, 14]. Inhibitors of collagenase activity have been detected in plasma, interstitial fluids, tissue extracts, platelets and neutrophils [6, 14, 56, 57]. With regard to the tissue collagenases, most of the emphasis has been placed on α-2-macroglobulin, β-1-anticollagenase and the tissue metalloproteinase inhibitors (TIMP). Each of these anticollagenases can effectively inhibit a variety of tissue metalloproteinases, but it is interesting to note that their less effective control of the neutrophil collagenases has not been stressed. Alpha-2-macroglobulin, a general plasma antiproteinase, has been reported to inhibit all mammalian collagenases, but the rate of inhibition of neutrophil collagenases appears quite slow [13, 58]. This could prove to be an important consideration *in vivo* if neutrophil collagenase, like other mammalian collagenases binds to fibrillar collagen and moves from cleaved molecules to new substrates without re-equilibrating in the fluid phase [6].

The large size of α-2-macroglobulin and its relative exclusion from tissue sites encouraged Woolley *et al.* [59] to search for a smaller antiproteinase that could regulate collagenase activity in the interstitium. Indeed, these investigators isolated a ~ 40 kD serum protein that inhibits gastric mucosal, rheumatoid synovial and skin collagenase activity by 40–80%, but decreased neutrophil collagenase activity by only 20% [59]. Recent studies suggest that β-1-anticollagenase cross-reacts immunogically with TIMP, a specific metalloproteinase inhibitor synthesized by connective tissue cells and by megakaryocytes that can be detected in plasma and the platelet α-granule [22, 56, 60]. The inhibitor (or family of inhibitors) has been characterized as a 28.5 kD glycoprotein that binds and inhibits tissue collagenases by forming a 1:1 complex.* However, Murphy *et al.* [13] reported that the total inhibition of purified neutrophil collagenase activity by TIMP (isolated from bone culture media) is difficult to obtain while the inhibition of gelatinase occurs slowly. An analysis of the inhibitory activity of plasma or platelet-derived TIMP would be of interest since TIMP isolated from different tissue sources appears to block neutrophil collagenase and gelatinase with varying degrees of effectiveness [62]. Alternatively, it is possible that the slow inhibition of collagenases by TIMP is not a reflection of physiologic irrelevance, but rather a control mechanism that allows released collagenases to function extracellularly for a limited period of time.

TIMP can be found in human plasma or serum, but Macartney and Tschesche [63–65] isolated a different inhibitor with characteristics very similar to those described for the neutrophil collagenase inhibitor. The circulating inhibitor has an M_r of 30.5 kD, contains 1–2 free thiols and inhibits a range of active collagenases by a process that could be regulated by a thiol–disulfide interchange [64, 65]. Although they stated that the isolated inhibitor was β-1-anticollagenase, there are major differences between the thiol-dependent inhibitor and the collagenase inhibitor isolated from serum or fibroblasts [60, 64, 65]. TIMP does not contain free thiols and its inhibitory activity is not regulated by disulfides [60]. Thus, at the very least, there are two serum inhibitors, one with characteristics similar to TIMP and a second with characteristics similar to the inhibitor found complexed with neutrophil collagenase.

Finally, neutrophils also contain a cytosolic inhibitor of activated neutrophil collagenase [31, 42]. The inhibitor does not react with anti-TIMP antibodies [56] and does not regulate collagenase activity by a disulfide-sensitive process [42]. If the collagenase inhibitors described thus far do not effectively control the neutrophil's metalloenzymes, it may be that inhibitors released from dying neutrophils ultimately control collagen degradation.

A role for collagenolytic metalloenzymes in vivo*?*

Although many features of neutrophil collagenase, neutrophil gelatinase and their endogenous inhibitors remain to be characterized, it seems clear that the intact cell can release and activate collagenase and gelatinase, two metalloenzymes that arm the cell with the ability to degrade type I, II, III, IV and V collagens. Given the fact that the primary task of the neutrophil is the destruction of microbes, what roles might be postulated for these proteinases in host defense?

In response to the generation of an inflammatory stimulus in the extravascular tissues (e.g. an infection), the circulating neutrophil must leave the blood vessel, crawl to the affected site, and ingest the microbe [66]. In order to reach the invaders, the neutrophil must first sequentially traverse a series of collagenous barriers including the type IV and V collagens associated with the endothelial cells that line the vascular lumen [67–69]. After leaving the vascular bed, the neutrophil must then penetrate a dense network of interstitial collagens before finally reaching the infected site. Clearly, the order of presentation of collagenous barriers would necessitate the release of the appropriate collagenolytic enzyme. Indeed, current evidence suggests that the release of metalloenzymes from the neutrophil is specifically programmed for this type of response. Gelatinase and collagenase appear to be stored in at least two different intracellular compartments whose discharge can be separately controlled [42, 51]. The effect of increasing concentrations of chemotaxins

* TIMP has recently been sequenced and cloned in *Escherichia coli* [23]. Interestingly, TIMP is identical to a protein recently reported to have erythroid-potentiating activity [23]. In addition, regions of similarity have been detected between TIMP and the gag core proteins encoded by a number of retroviruses [61].

(compounds that can cause unidirectional locomotion of the neutrophil along a concentration gradient) on the release of collagenolytic metalloenzymes from neutrophils has been directly determined *in vitro* [42, 51]. At the lowest concentrations, neutrophils respond by first releasing gelatinase alone, but as the concentration of the chemotactic agent is increased (in a manner analogous to neutrophils moving up a concentration gradient as they migrate towards an infected site), both gelatinase and collagenase are released [42, 51]. Thus, this pattern of metalloenzyme release mirrors the expected order of presentation of their potential substrates. Despite the fact that so many features of this model are attractive (at least to us), it is surprising that so little data actually exist to support this scenario. What information is missing? What kinds of question might be asked?

(1) *Do neutrophils really need collagenolytic enzymes to penetrate the vessel wall?* The basement membrane does not consist of collagen alone, but also contains a complex mixture of specific glycoproteins (laminin, entactin, etc.) and heparan sulfate proteoglycans [10, 68]. If the type IV and type V collagens associated with the endothelial cell and its underlying basement membrane represent only a portion of the connective tissue barrier that must be penetrated, then other matrix degrading systems may also be required. The C-particle compartment not only contains gelatinase but also includes acid hydrolases and cathepsins [51]. The presence of these and other enzymes in this highly sensitive secretory compartment hints at their potential role in cell migration. Indeed, penetration of the basement membrane may require the concerted action of multiple enzymes, including gelatinase, plasminogen activator, heparanase and membrane-bound serine proteinases, as well as the mechanical disruption of the matrix by the migrating cell [10, 70–72]. Nonetheless, the fact that at least three different cell populations capable of traversing the vascular wall, neutrophils, endothelial cells [73] and tumor cells [10], all possess type IV and type V collagen-degrading activity cannot be easily dismissed. Biochemical, immunologic, and functional analyses of the regulation of gelatinase and collagenase activities during neutrophil migration through connective tissue barriers *in vitro* or *in vivo* [67, 74–76] should provide important insights into the role of these enzymes in the controlled dissolution of the basement membrane.

(2) *How are the metalloenzymes regulated during chemotaxis?* Neutrophils are able to activate gelatinase or collagenase by a process dependent on HOCl, an oxidant potentially generated by cells exposed to low doses of a chemoattractant [77]. However, oxygen-dependent activation of the collagenases must not be an absolute requirement for chemotaxis because chronic granulomatous disease cells do not express any gross abnormalities in cell migration *in vivo* [78]. A small portion of the released gelatinase can be activated by an oxygen-independent process *in vitro*, and the efficiency of this system under more physiologic conditions requires careful assessment. Other proenzymes are known to be activated by forming stoichiometric complexes with non-protease proteins [16, 30]. The effects of similar interactions between the collagenases, the endothelium or basement membrane are unknown. Similarly, plasma factors and proteins could potentially activate and enhance collagenolytic activity [79]. Finally, the endothelium should not be envisioned as a passive cellular barrier that only covers the basement membrane. The endothelium may play a direct participatory role in chemotaxis [80], and its ability to cooperatively regulate neutrophil collagenases remains an intriguing possibility.

In order for the collagenases to mediate orderly basement membrane degradation during chemotaxis, the enzymes must be activated and then inhibited. For example, collagenases may require shielding from plasma inhibitors (TIMP, α-2-macroglobulin, β-1-anticollagenase) in order to digest the basement membrane, but after the cell has migrated onward, the enzymes that remain tightly associated with the collagenous substrates must be inhibited. Membrane-bound collagenolytic enzymes would allow the neutrophil to sequester activity in the plasma-free environment at an interface created between the plasma membrane and its substrate [70]. In addition, cell-bound enzyme would not be left deposited on passed substrates. However, the presence of collagenolytic enzymes in the neutrophil plasma membrane has not been demonstrated nor would this model explain the apparent "decision" of the neutrophil to discharge gelatinase and collagenase into the extracellular milieu. Although other phagocytes may bind released proteinases to specific receptors on their plasma membrane, the ability of the neutrophil to localize enzymes in a similar manner is unknown [81]. Finally, the requirements for the sequential utilization and inhibition of the collagenases could be circumvented if plasma inhibitors are designed to slowly inhibit the activated enzymes. The activation and inactivation of the collagenases released from neutrophils migrating across an endothelial-coated matrix in the presence of plasma should prove amenable to *in vitro* analyses.

(3) *Do metalloproteinases participate in connective tissue degradation at the inflamed site?* Once neutrophils have reached the infected site, they can internalize the microbes and discharge additional C-particles and specific granules, as well as a third group of enzymes stored in the primary granules [3]. Thus, phagocytosable particles can stimulate the neutrophil to release large amounts of oxidants, collagenolytic metalloenzymes and serine proteinases, e.g. elastase [40, 82]. This enzyme can attack cross-links in non-helical portions of the interstitial collagens, helical portions of type III and type IV collagens, and a variety of connective tissue glycoproteins and proteoglycans [14, 83, 84]. It is within this milieu that oxidants, activated metalloproteinases and serine proteinases can form the complex "soup" that ultimately results in the formation of an abscess. The interplay between the ability of oxidants to directly attack collagen [85], to activate metalloproteinases, and to inactivate antiproteinases [40, 82] versus the ability of elastase to attack collagens while destroying free collagenase or gelatinase [13] may ultimately dictate the difference between physiologic and pathologic tissue degradation. The study of neu-

trophil-mediated collagen degradation *in vitro* coupled with biochemical and immunological analyses of fluids recovered from inflammatory foci *in vivo* [86] should allow investigators to elucidate the relative roles of these factors in tissue destruction.

In closing, arming the neutrophil with potentially destructive collagenolytic mechanisms may be the price we pay for a mobile host defense system that must be able to invade any normal tissue that harbors pathogenic microbes. The "tricks" used by the neutrophil to cross connective tissue barriers may be similar to those employed by migrating endothelial cells, fibroblasts, macrophages and even tumor cells. The ability of these cells to mediate tissue degradation on a small, controlled scale during migration may be part of the continuum of collagenase-mediated effects that can result in massive, uncontrolled collagen destruction in inflammatory disease states. Studies designed to elucidate the processes by which neutrophils regulate their own collagenolytic enzymes may not only serve as a prototype for the study of other cell populations, but also foster the development of therapeutic interventions that prove effective in the control of inflammation, angiogenesis and tumor invasion.

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