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Minireview

Are matrix metalloproteinases the missing link?

F Mannello[1], G Tonti[1], S Papa[2]

[1]*Istituto di Istologia e Analisi di Laboratorio, Università Studi Carlo Bo , Urbino, Italy*
[2]*Istituto di Scienze Morfologiche, Università Studi Carlo Bo , Urbino, Italy*

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Abstract
It is more and more evident that the matrix metalloproteinase (MMP) system is not a characteristic feature of vertebrate animals only, as it can also be found in many invertebrate organisms. This endopeptidase family has been widely studied since its first member was described 40 years ago during metamorphosis in tadpole tails. Many researches have been carried out in mammals in order to elucidate and analyze the several and important roles these endopeptidases play, both in physiological pathways and in pathological processes. The evolving researches of these multifaceted enzymes enter the very interesting and fascinating world of the invertebrates, where these enzymes seem to be in the front line during important biological events. MMP-like enzymes and their inhibitors have been found in insects, crustaceans, mussels, sea urchins and also in organisms as simple as hydra. In these species MMPs partake in several fundamental processes, such as extracellular matrix (ECM) remodelling, embryonic development, cell growth and differentiation and also in defense mechanisms thus highlightening their intriguing and unexpected functional importance in invertebrate life too.

Key words: development; embryogenesis; extracellular matrix; invertebrates; metalloproteinases; tissue inhibitors of metalloproteinases

Introduction

The discovery of the first member of the matrix metalloproteinase (MMP) family set the way to a new line of research dealing with this novel class of enzymes. An extraordinary number of new discoveries are contributing to put together the many pieces of this intruiging MMP puzzle world, adding more and more informations on how these enzymes work. The first enzyme with the capacity of degrading interstitial collagenase was found in the tail of a tadpole (Gross and Lapiere, 1962) and from then onwards the search continued and brought to the identification of more than 25 vertebrate MMPs (Brinckerhoff and Matrisian, 2002; Mott and Werb, 2004; Mannello *et al.*, 2005a).

Corresponding Author:
Ferdinando Mannello
Istituto di Istologia ed Analisi di Laboratorio, Via E. Zeppi, snc, Università Studi "Carlo Bo", 61029 Urbino (PU), Italy
E-mail: f.mannello@uniurb.it

At the beginning there were the vertebrate MMPs ...

MMPs are endopeptidases characterized by their zinc-dependece and by a highly conserved sequence that contains three histidines necessary for binding the zinc ion at the catalytic site, and a conserved methionine turn that lies beneath the active site zinc. (Stöcker *et al.*, 1995). MMPs are classified according to their substrate specificity and are subdivided into collagenases, gelatinases, elastases, stromelysins and membrane-type MMPs; moreover, they are also classified depending on their domain structure. The N-terminal portion of all MMPs (pre-domain) is a signal sequence due to be removed whose function is to guide the MMP synthesis to the endoplasmic reticulum and their following secretion in the extracellular environment. MMPs are secreted in a zymogenic form and the latency of these enzymes is maintained by a mechanism known as the "cysteine switch"; the unpaired cysteine in the pro-domain of latent MMPs forms a bridge with the catalytic zinc, thus maintaining the enzyme inactive as zymogen until this interaction is abolished by mechanical disruption or cleavage by

other proteinases yielding a fully active enzyme (Vu and Werb, 2000). The third fundamental domain of MMPs is the catalytic domain where the zinc ion is coordinated with three histidine residues and its fourth ligand is represented by a water molecule (Overall, 2004). Most MMPs are characterized by a hemopexin/vitronectin-like sequence that is linked to the catalytic domain by a hinge region that can vary in length (Baragi *et al.*, 1994). Two MMPs, known as gelatinases, are peculiar as they show the insertion of three fibronectin-like repeats within their catalytic domain which are necessery for binding and degrading specific substrates (Shipley, 1996). MMPs are not only secreted enzymes, as there are membrane-type MMPs which have a single-pass transmembrane domain and a short cytoplasmic C-terminal tail, or a short C-terminal hydrophobic region (Itoh *et al.*, 1999). MMP activity is specifically inhibited by endogenous tissue inhibitors of metalloproteinases (TIMP) which reversibly bind previously activated MMPs in a 1:1 stoichiometric ratio and differ in their expression patterns and MMP affinity (Gomez *et al.*, 1997; Mannello and Gazzanelli, 2001). Also nonspecific inhibitors (e.g., $á_2$-macroglobulin) can control MMP activity (Mannello *et al.*, 2005b). The level of expression of MMPs by unstimulated cells and in intact tissues is generally low. The MMP expression is inducible by cytokines, growth factors, physical stress, oncogenic transformation, by cell-cell and cell-matrix interactions (Stamenkovic, 2003), even if their expression is regulated primarily at the level of transcription and their proteolytic activity requires zymogen activation (Mannello *et al.*, 2005b). MMPs can degrade almost every component of the extracellular scaffold but their role is not limited only to the breakdown of structural components, as they have been found to be strongly involved in many physiological processes, such as cell migration, tissue morphogenesis, wound healing and in the modulation of the bioavailability of active molecules (Vu and Werb, 2000), and in pathological situations such as inflammation, cancer development and metastasis (Stamenkovic, 2003; Mannello *et al.*, 2005b).

...but something else emerges from the depth of the sea world...

One of the first invertebrates that has been analyzed in this context is the sea urchin in its different embryogenetic stages and it has been demonstrated that normal developmental processes (i.e. spiculogenesis and gastrulation) necessitate collagen deposition and ECM modifications (Spiegel *et al.*, 1989), thus evidencing the necessity of enzymes with proteolytic activity (Wessel *et al.*, 1984). During the blastula early stages the sea urchin regulates the transcription and secretion of the hatching enzyme (envelysin) which degrades the protective envelope. This collagenase-like enzyme is structurally very similar to the vertebrate counterpart as it is characterized by domains displaying the same functional roles found in mammalian collagenases; in addition, it posesses its own distinctive sequences. (Lepage and Gache, 1990; Roe and Lennarz, 1990; Nomura *et al.*, 1997). During the following embryonic developmental stages, the sea urchin expresses a 41 kDa protease which shows substrate specificity towards gelatin and extraembryonic collagen components (Mayne and Robinson, 1996; Mayne and Robinson, 1998). This gelatinase has been found in both the hyaline and basal lamina, which contain components similar to those found in vertebrate ECMs (Wessel *et al.*, 1984). This 41 kDa enzyme is secreted on the apical surface of the embryo and prior to its secretion it can be detected in the cortical and in the yolk granules (Mayne and Robinson, 1998). The structural organization of this collagenase is closely related to that of vertebrate MMPs as it has a signal and a pro-peptide, a Zn^{2+}-binding catalytic domain and a hemopexin-like C-terminal domain (Nomura *et al.*, 1991). In the gastrula and pluteus stages the sea urchin expresses an 87 kDa protease which can specifically cleave gelatin and can control shape-changes, cell-cell and cell-ECM interactions during the gastrula and pluteus stages through the regulation of ECM composition (Robinson, 1997). This enzyme is Ca^{2+} and Zn^{2+} dependent, but its activation mechanism may be different from that of the known "cysteine switch", as demonstrated by inhibitory and activating studies. The two proteinases (41 and 87 kDa) described up to now are both Zn^{2+}-dependent and also need low affinity Ca^{2+} binding for activity; moreover, Mg^{2+} seem to have an inhibitory effect on the enzyme (Robinson, 2000). This contrasting effect of Ca^{2+} and Mg^{2+} on the gelatinase activity could cause the fine regulation and modulation of the enzymatic activity on the cell surface, as small variations in the ion concentrations, obtained through the binding capacity of extraembryonic matrix, can regulate the enzyme activity (Robinson and Mayne, 1998). The hyaline layer of the sea urchin was analyzed for MMP activity during the transition from early to late stage embryos and enzymes (initially secreted as proenzymes and subsequently proteolytically activated) with molecular masses of 94/117, 90 and 45 kDa with gelatin specificity and Ca^{2+}-Zn^{2+} dependence were found and suggested to be matrix metalloproteinases (Flood *et al.*, 2000). The exact relationship between the 94/117 kDa and 90 kDa species and those found in the sea urchin embryo is still unclear. Also the process of skeleton formation and the process of spiculogenesis seems to be correlated to the action of metalloproteinases, as inhibitors of these enzymes block these morphological events (Ingersoll *et al.*, 2003).

In echinoderms, MMPs are not only involved during embryonic development, but play important roles also during tissue/organ regeneration due to the modifications that need to occur in the ECM. Evidence of this is the expression and activity of MMPs during early stages of intestinal regeneration in the sea cucumber *Holothuria glaberrima* (Quinones *et al.*, 2002).

A gelatinase has also been found in the digestive tissues of the crab *Scylla serrata*. All the identified crustacean collagenases belong to the serine protease family, while this novel enzyme results as a metalloproteinase with gelatin specificity. This enzyme has high proteolytic activity at low temperatures and acts on a wide range of substrates (this could be due to the fact that lower animal collagenases are necessary for the digestion of collagen containing tissues of the prey that these animals feed on) but its

preferential substrate is represented by denatured collagen (Sivakumar *et al.*, 1999).

The barnacle *Balanus amphitrite* is a thoracican cirriped crustacean that is due to undergo many complex and substantial morphological changes before it metamorphoses to the final stage of larval development: the lecithotrophic cypris larva. These developmental processes depend on the activity of specific extracellular matrix-degrading enzymes as *B. amphitrite* naupliar stages contain several proteinases specific towards different gelatin substrates evidencing their involvement in all phases of larval growth and development. Substrate and activity analyses collocate these proteolytic enzymes in the MMP family as they are specific towards gelatin substrates and dependend on Zn^{2+} and Ca^{2+} ions (Mannello *et al.*, 2003). These enzymes could also be dependent on Mg^{2+} suggesting that the enzyme activity could be regulated by these cations, as can be seen also in the sea urchin embryo (Robinson, 2000) and in the mussel *Mytilus galloprovincialis* (Mannello et al., 2001). Even though the activation mechanism of the barnacle MMPs is quite similar to the one observed for mammals it is probably not regulated by the same cysteine switch trigger but has its own unique setup (Robinson, 1997; Mannello *et al.*, 2001).

The serum and hemocytes of the eastern oyster *Crassostrea virginica* have been analyzed for ECM-degrading activity underlining the role of ECM ptoteolytic enzymes in both normal and diseased molluscs (Ziegler *et al.*, 2002). A MMP-like enzyme has been fonud in the hemocytes, but not in the serum of *C. virginica*. This enzyme has been proven to be an MMP due to its gelatin and collagen degrading activity and to its inhibition profile. This MMP is not involved in the degradation of interiorized phagocytosed materials, but it is probably active in the external environment. These findings support the hypothesis that hemocyte derived MMP-like activity may control the remodelling and development of ECM and may be also involved in the response towards pathogen invasion (Ziegler *et al.*, 2002). The new exciting role MMP may have during deseased or damaged states has been further investigated in the Pacific oyster *Crassostrea gigas* and a MMP inhibitor named Cg-TIMP with functional characteristics extremely similar to vertebrate was identified (Montagnani *et al.*, 2001). This inhibitor binds and blocks MMPs but posesses other functions probably regulated by an additional pair of cysteine residues in the carboxy-terminal domain (this could be a characteristic of invertebrate TIMPs as it is present also in *Drosophila* TIMP). *Cg*-TIMP could be strongly involved in pathogen protection or in wound healing processes as it was only expressed in hemocytes and showed increased activity after shell damage or bacterial infection, suggesting new insights for the anti-microbial defense of marine invertebrates (Bachere *et al.*, 2004).

Gelatinolytic activity similar to that found in *C. virginica* was discovered in the hemocyte and serum homogenates of the mussel *M. galloprovincialis* (Mannello *et al.*, 2001). This proteolytic enzyme was similar to known MMPs due to its gelatinase and collagenase activity and to its ionic requirements, but exhibited different activating and inhibiting processes suggesting that in molluscs the activation mechanism differs from the vertebrate “cysteine switch”. In healthy mussels this gelatinase may regulate normal physiological functions such as cell migration and tissue infiltration, but MMP activity goes beyond these roles being of great importance during cell-mediated and humoral immune responses (Chen and Bayne, 1995) and moreover in wound repair, inflammation, internal defense and also in pathological conditions as hematopoietic neoplasia (Riginos and Cunningham, 2005).

It is clearly emerging that these MMP-like enzymes touch several aspects of oyster and mussel biology as they take part to tissue homeostasis and are also strongly involved in defence mechanisms, or because they get produced directly by the pathogenic agent (Norqvist *et al.*, 1990; Lepore *et al.*, 1996) or because they are stimulated to be secreted by the pathogen itself (Okamoto *et al.*, 1997).

MMP-like enzymes have been found in a member of the Cnidaria family as hydra; the entire body wall of this organism is structurally reduced to an epithelium bilayer (ectoderm and endoderm) with an intervening extracellular matrix that contains basement membrane components such as laminin and interstitial matrix components such as a unique type I fibrillar collagen. The simple structure of this metazoan and the relation between developmental processes and cell-ECM interaction led to the search for MMP-like enzymes (Zhang and Sarras, 1994). A single hydra matrix metalloproteinase, HMMP, with a strong sequence similarity to human MMPs was identified, (even though it contains some unique amino acid stretches) (Leontovich *et al.*, 2000). HMMP is not only structurally similar to vertebrate MMPs, but it also shares functional characteristics as inhibition by specific MMP-inhibitors and substrate specificity towards hydra ECM molecules and gelatin. Activation studies on HMMP demonstrate that it can be activated intracellularly by a furin-like enzyme and that there can be an intermediate step before reaching the fully active enzymatic form. HMMP has been studied during foot and head regeneration processes (Leontovich *et al.*, 2000). These results clearly evidence a direct implication of this enzyme during morphogenetic events as HMMP is involved in cell transdifferentiation (Werb and Chin, 1998) and regenerative processes being implicated in biogenesis (Shimizu et al., 2002). HMMP is secreted by the cells belonging to the endoderm evidencing that although hydra ECM has a symmetrical structure, its components are synthesized in a non-symmetrical manner (Shimizu *et al.*, 2002). Besides having a important role during regenerative events and also in the maintainance of the differentiated state of certain cells (Leontovich *et al.*, 2000), HMMP may participate in the regulation of the bioavailability of signalling molecules which are sequestered by the ECM, and can be released by a HMMP dependent proteolytic cleavage (Muller, 1996). HMMP demonstrates to be a fundamental element in several processes of the hydra underlining the importance of ECM-related mechanisms. Molecules similar to MMP inhibitors have been found in low metazoans such as sponges; *Callyspongia truncata* produces callysponginol sulphate A (Fujita *et al.*, 2003a) and *Agelas nakamurai* expresses a novel MMP inhibitor, ageladine A which also possesses antiangiogenic activity (Fujita *et al.*, 2003b).

... and spreads into the sky...

Drosophila melanogaster expresses two matrix metalloproteinases: *Dm1*-MMP and *Dm*-2-MMP. *Dm1*-MMP, as vertebrate MMPs, contains a signal sequence necessary for secretion, a pro-peptide with a Cys residue that maintains the enzyme in an inactive zymogenic form, a catalytic domain with the zinc-binding site and finally a hinge region and a N-terminal hemppexin domain. One more aspect of this newly discovered proteinase is that its activation could also be regulated by furin-like proteases, due to the presence of a furin-like cleavage site located at the end of the pro-peptide (Roebroek *et al.*, 1993; Llano *et al.*, 2000). Studies on substrate specificity of recombinant *Dm1*-MMP evidenced proteolytic activity towards extracellular matrix and basement membrane proteins, such as fibronectin and type IV collagen which are present in *Drosophila* (Fessler and Fessler, 1989). On the bases of several reports it can be to hypothesized that this MMP could be directly involved in the guidance and extention of axons during the nervous system development, as it was found to be secreted by the glial cells of larval tissues (Menne *et al.*, 1997; Llano *et al.*, 2000); this could depent or on the brakdown of extracellular barriers or on the release of hidden, not yet available signal molecules. *Dm2*-MMP, is similar to *Dm1*-MMP by posessing the MMP distinctive structural domains, but it differs from *Dm1*-MMP because of the presence of a 200 amino acid-long insertion in the hinge region (Llano *et al.*, 2002) and also because this enzyme is expressed in all of the developmental stages of the fly, while *Dm1*-MMP is present in the developing embryo at stages 12 and 13 (Llano *et al.*, 2000). One more difference is that *Dm1*-MMP is a secreted enzyme, while *Dm2*-MMP is a membrane bound proteinase. Due to the differential pattern of expression of the two MMPs it could be possible that they have different functional roles. It has been hypotesized that *Dm2*-MMP may be involved in photoreceptor growth cone guidance and cell rearrangement in the retina and nervous system (Llano *et al.*, 2002). *Dm1*-MMP seems to be determinant for larval tracheal growth and pupal head reversion, while *Dm2*-MMP participates to larval tissue histolysis and epithelial fusion during metamorphosis and both enzymes seem to be required for tissue remodeling (Page-McCaw *et al.*, 2003). For a regulated MMP activity the presence of MMP inhibitors is fundamental, and infact one inhibitor of matrix metalloproteinases has been found in the fly (Pohar *et al.*, 1999) and it is structurely closely related to mammalian TIMPs (Wei *et al.*, 2003). It is clear that *Drosophila* in its relative semplicity unravels a highly regulated functional proteolytic system involved in several biological and physiological processes which could account for a common origin with the fully evolved vertebrate MMP system (Wei *et al.*, 2003). The larvae of the greater wax moth, *Galleria mellonella*, is peculiar in that it containes the first insect inhibitor of metalloproteinases (IMPI) with no similarity at all with other known vertebrate or invertebrate counterparts. It is possible that this IMPI is implicated during the response of *G. mellonella* to invading pathogens as it is released during the humoral immune response (probably stimulated by particular peptidic fragments) and is able to protect the insect from exogenous metalloproteinases of pathogen origin such as bacterial thermolysin (Wedde *et al.*, 1998; Vilcinskas and Wedde, 2002).

... and into every nook and craggy ...

Three gene products (MMP-C31, H19 and Y19) encoding matrix metalloproteinases have been found in the nematode *Caenorhabditis elegans*. These enzymes have a domain organization very similar to human MMPs, in particular the catalytic sites of these newly discovered MMPs show high sequence homology with human interstitial collagenase. Interestingly, two of these nematode MMPs were inhibited by specific human MMP inhibitors evidencing a similar MMP system between mammalian and *C. elegans* MMPs (Wada *et al.*, 1998).

A glycoprotein similar to *C. elegans* MMPs was analyzed in the larvae of *Gnathostoma spinigerum* and this protein showed to possess an N-terminal signal peptide necessary for its secretion and the catalytic domain which are two elements distinctive of other known MMPs (Uparanukraw *et al.*, 2001).

Considerations

It is evident that in all organisms the role of the ECM goes beyond that of a mere scaffold having only structural functions, but controls many more complex and important processes such as cell shape, growth, migration and differentiation as a consequence of its capacity of sequestering signal molecules. The involvement of the ECM components in such biological pathways led to a great amount of rersearch in order to better understand the mechanisms that regulate the organization of extracellular environment. Key elements in the regulation of ECM components are the proteinases belonging to the MMP family; these enzymes seem to hide many surprises as their functional roles touch many aspects of physiological and pathological processes and have been widely studied not only in vertebrate models but also in invertebrates (Massova *et al.*, 1998). MMP-like enzymes with their relative inhibitors have been discovered in animals belonging to distantly related taxa, evidencing the existence and importance of such a proteolytic system also in relatively simple organisms. Even though MMPs and relative inhibitors are present in such a varied range of organisms, they share many features characteristic of the vertebrate MMP family, suggesting a possible common ancient origin. These vertebrate and invertebrate enzymes may share structural similarities, but it is amazing to note that invertebrate MMPs take part in many processes such as embryogenesis, differentiation, cell migration, wound healing and immune defense, evidencing that also in these organisms MMPs are far more than simple structure destroyers. It is evident that in all these organisms MMPs and their inhibitors are closely linked to ECM remodelling displaying multiple roles that touch many aspects of animal physiology and demonstrating that in such evolutionary distant organisms several biological pathways follow similar laws.

Finally, this overview add force to the emerging concept that MMPs and TIMPs are universal and ubiquitous in animals, and that invertebrates may provide further novel information for the understanding of the multifaceted physiological roles of this ancient proteolytic system (Mannello *et al.*, 2005a); numerous evidences suggest that both MMP and TIMP expression and their remodelling functions appear well conserved in invertebrates, laying the hypothetical basis on their possible biological link between proterostomata and deuterostomata.

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