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Degradomics of matrix metalloproteinases in inflammatory diseases

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1. ABSTRACT

Organisms have evolved to react to stress, tissue damage and pathogen invasion to assure their survival. Leukocytes are the primary responders and they regulate repair, immune defense and inflammation with the aid of a wide variety of other cells (e.g. epithelial, fibroblasts). To assure proper responses, a plethora of proteins are involved including signaling molecules, chemokines and proteases to orchestrate a step-by-step reaction. Inflammation is an essential biological process, however, when it persists, it can lead to various diseases that are challenging to heal or cure. The technologies and techniques covered in this book chapter can be applied to study all proteases and their inhibitors although will be centered on the matrix metalloproteinases (MMPs). It will focus on the proteolysis performed by MMPs, their various beneficial and detrimental effects in inflammation and the novel methods to study their roles on human diseases.

2. MATRIX METALLOPROTEINASES: ZINC-DEPENDENT MOLECULAR SCISSORS

Every single protein encounters proteolysis, either by the cleavage of its signal peptide, pro-peptide activation, processing or degradation. Proteolysis is irreversible and is performed by five different classes of proteases in humans including aspartic, metallo, cysteine, serine and threonine (1-3). The metalloprotease superfamily contains 194 members and the matrix metalloproteinases (MMPs) family consists of 23 members in humans and 24 in mice due to the duplicity of the MMP1 gene (MMP1a and MMP1b) (1,4,5).

The basic structure of most MMPs consists of five typical domains (figure 1): 1) a signal peptide that directs MMPs to the secretory pathway; 2) a ~80-90 amino acids prodomain that confers latency to the enzyme; 3) a zinc-dependent catalytic domain; 4) a 15-65 highly flexible hinge region linking the catalytic domain to the hemopexin domain; and 5) a ~200 residues hemopexin-like domain that mediates substrates interactions and multimerization (dimers, trimers and tetramers) (5). Additional MMP domains include transmembrane domains (MMP14, -15, -16 and -24), cytoplasmic domains (MMP14, -15, -16 and -24), glycophosphatidyl inositol-anchoring (GPI) domains (MMP17 and -25), fibronectin type II modules (MMP2 and -9), Ig-like domain (MMP23) and C-terminal extension (CTE) domain (MMP27) (5,6). There is a zinc-binding motif in the active site of each MMP that consists of HEBXHXBGXHS (where H is histidine, E is glutamic acid, B is a bulky hydrophobic amino acid, G is glycine, X is a variable amino acid and S is serine) where the three zinc-binding histidines and a glutamate activates a zinc-bound water molecule acting as the nucleophile cleaving peptide bonds (7-9). Mutation of the glutamate residue in the active site ablates catalytic activity (7,10).

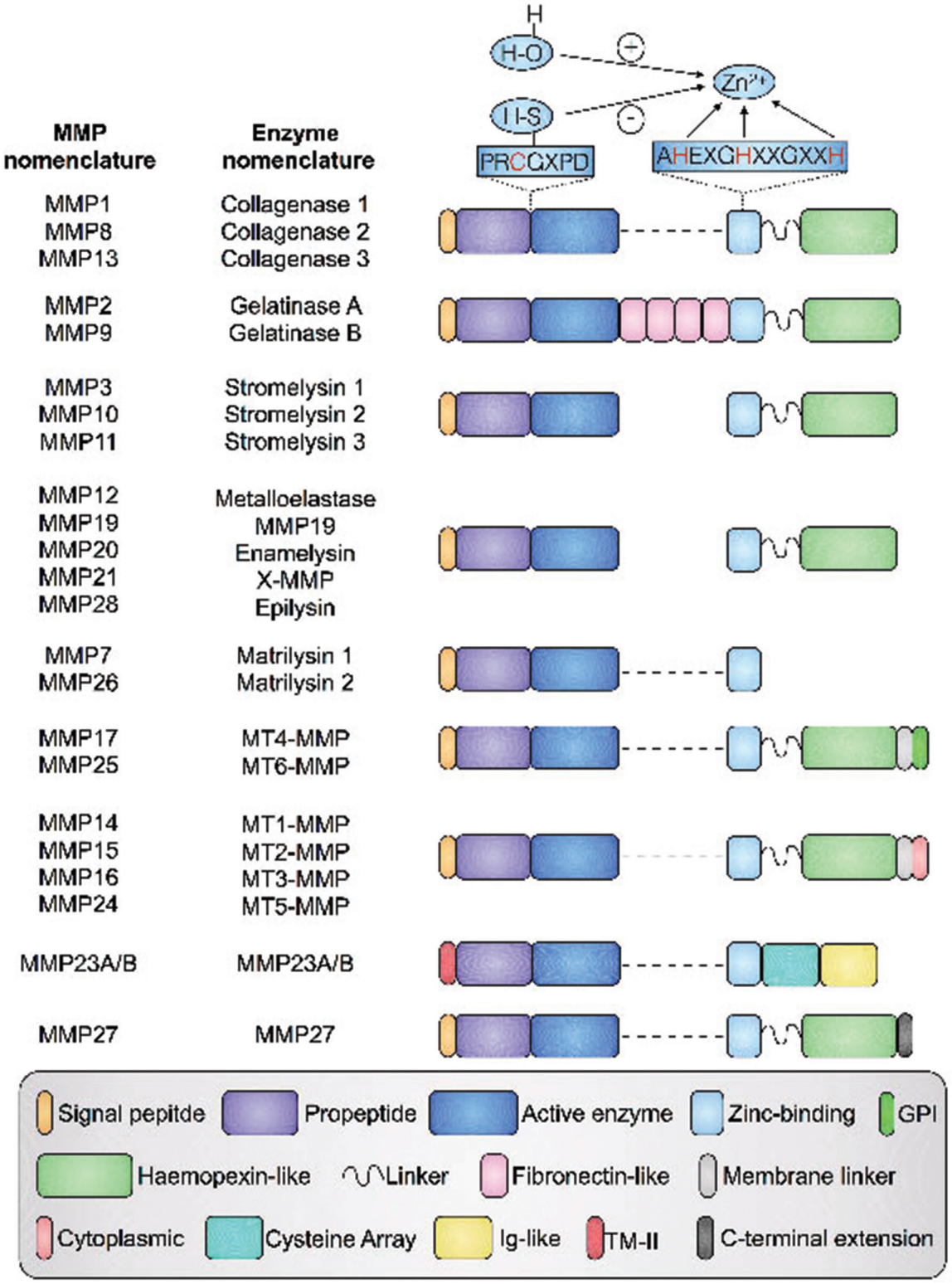

Figure 1. Schematic representation (modified from Hu et al. (20) and Overall and Lopez-Otin (5)) of the 23 human matrix metalloproteinases (MMPs). Various domains of MMPs include a signal peptide (necessary for secretion), a propeptide, catalytic domain (active enzyme and zinc-binding), a linker, a haemopexin-like domain, a fibronectin-like domain, a membrane linker, a GPI (glycosylphosphatidylinositol) domain, a type II transmembrane (TM-II) segment, a cysteine array (CA), immunoglobulin (Ig)-like domain and a C-terminal extension (CTE) domain. Reproduced with permission from (5, 20).

Once activated, MMPs can cleave numerous substrates in virtually all tissues including blood; therefore, they must be tightly regulated by various endogenous inhibitors such as the tissue inhibitors of metalloproteinases (TIMP1, -2, -3 and -4), β-amyloid precursor protein, alpha2-macroglobulin, tissue factor pathway inhibitor-2, endostatin, the reversion-inducing cysteine-rich protein with kazal motifs (RECK), the noncollagenous NC1 domain of type IV collagen, secreted leukocyte protease inhibitor, procollagen C-terminal proteinase enhancer and cystatins (11-13). A perfect balance between MMPs and their inhibitors must be maintained in tissues and blood to avoid misbalance and to limit the risk of diseases.

3. THE FLAME KEEPS BURNING: THE ROLE OF MATRIX METALLOPROTEAINASES IN INFLAMMATORY DISEASES

In normal tissue homeostasis, MMPs are tightly regulated by several endogenous inhibitors e.g. TIMPs. The TIMPs bind MMPs in a stoichiometric ratio of 1:1 (1,4,15). During inflammation, this balance between MMPs and TIMPs often shifts in favor of MMPs, leukocytes traffic to the site of injury/stress and respond by creating a chemotactic gradient, clearing pathogens or apoptotic cells and remodeling/renewing the extracellular matrix (ECM) (16-17). At first, MMPs are beneficial, coordinating appropriate immune responses, however, as inflammation persists, MMPs often become harmful (18-20). As shown in table 1, MMPs are correlated with both detrimental and beneficial roles in inflammatory diseases including cancer, asthma, endotoxin shock and arthritis (4, 20-22). It is therefore critical to understand their precise roles and investigate the substrates they cleave to properly make use of MMP inhibitors in inflammatory diseases.

3.1. Bacteramia, septic and endotoxin shocks

Systemic inflammatory response syndrome (SIRS) such as bacteramia, septic and endotoxin shocks cause multi-organ failures in patients which is the most frequent cause of death in intensive care units (4,5,23,24). Following a response to microorganisms, fast and excessive host-inflammatory responses can become impossible to overcome and several MMPs have been implicated in SIRS. Using the caecal ligation and puncture (CLP) model, a murine model of sepsis, MMP1a was found to be upregulated a few hours after initation (1,5,25). An MMP1 and MMP8 inhibitor, MMP-Inh-1, protected mice from CLP-induced lethality whereas a specific MMP1 antibody had a lesser inhibitory effect suggesting that both MMPs might contribute to sepsis (1,5,6,26). Inhibition of MMP1 activity was improved in WT and *Par2*$^{-/-}$ mice but was ineffective in *Par1*$^{-/-}$ animals suggesting a MMP1/PAR1 dependent effect (1, 27). In humans, levels of proMMP1 was increased by 18-fold and active MMP1 by 8.7.-fold during sepsis and was linked to a decrease in survival (1, 28).

MMP3 was demonstrated to facilitate neutrophil influx by the cleavage of claudin-5, occludin and laminin-alpha1, therefore disrupting the blood-brain barrier (BBB) in a model of intracerebral LPS injection (29, 30). The WT animals had highly disrupted BBB and a higher neutrophil counts in the brain as compared to *MMP3*$^{-/-}$ animals (30,31).

MMP7 is a key regulator in mucosal innate immunity through the cleavage of alpha-defensins (cryptdins) to promote innate host defense; indeed, *MMP7*$^{-/-}$ mice lacked mature cryptdins and have decreased antimicrobial activity (7, 11, 32). In acute inflammation, MMP7 has a pro-inflammatory role; *MMP7*$^{-/-}$ mice, in contrast to WT counterparts, were protected from LPS-induced lethality associated with reduced levels of systemic cytokines (7, 33).

MMP8 has been shown to have both pro- and anti-inflammatory roles. Using an airpouch model, *MMP8*$^{-/-}$ mice had a much lower polymorphonuclear neutrophils (PMNs) influx after LPS stimulation as compared to WT counterparts; in mice, MMP8 cleaved LPS-induced CXC chemokine LIX at two positions: between serine^{4}-valine^{5} and lysine^{79}-arginine^{80} (7,34). LIX is not present in humans but its orthologues CXCL5/ENA-78 and CXCL8/IL-8 were also processed by MMP8 in a *cis* feed-forward activation mechanism for PMNs migration (7, 16, 19, 34). Using an acute lung injury model by injecting LPS through the intratracheal route, *MMP8*$^{-/-}$ mice had a ~2-fold greater accumulation of PMNs in their alveolar space after 24 hours (7, 35). *MMP8*$^{-/-}$ mice also had a ~3-fold elevated myeloperoxidase (MPO) activity as compared to WT mice (18, 35, 36). Inhibition of MMP8, MMP9 and ADAM17 by regasepin1 prolonged the survival of mice against lethal endotoxinemia through both intraperitoneal and intravenous injections of LPS (21, 23, 25-28, 37). A different report demonstrated that a general MMP inhibitor, BB-94, completely protected the mice against LPS-induced hypothermia and death (7, 9, 38). *MMP8*$^{-/-}$ mice were also protected in the same model and these effects were associated with the detrimental MMP8 activity on the CNS barrier integrity, the cytokines levels and the brain inflammation (38).

In neutrophils, MMP9 is released within ~20 minutes into the blood after contact with proinflammatory mediators such as LPS, CXCL-8/IL-8, tumor necrosis factor-alpha (TNF-alpha) and granulocyte colony-stimulating factor (G-CSF) (39). In monocytes, ~7 hours is needed for MMP9 to be detectable due to the fact that no preformed proMMP9 is present thus relying on *de novo* protein synthesis (39, 40). In a murine model of abdominal sepsis, *MMP9*$^{-/-}$ mice had reduced leukocyte recruitment to the site of *Eschericia coli* infection and a more severe distant organ damage (41). However, in a different model of endotoxin shock, *MMP9*$^{-/-}$ mice were

Table 1. Biological roles of MMPs in various diseases

Diseases	MMPs	Biological Roles	References
Sepsis and endotoxemia	MMP1	1 - Disrupts endothelial barrier, intravascular coagulation, lung vascular permeability and cytokine storms by activation of PAR1 in the CLP murine model 2 - High levels of proMMP1 and MMP1 correlated with decrease survival in humans	(1)
	MMP3	1 - Expressed in endotoxemia 2 - MMP3 contributes to the disruption of blood-brain barrier after intracerebral LPS injection by cleavage of claudin-5, occludin and laminin-alpha1	(30, 86)
	MMP7	1 - *MMP7*$^{-/-}$ mice are protected from LPS-induced lethality associated with reduced levels of systemic cytokines	(33)
	MMP8	1 - Inhibition of MMP8 by regasepin1 protects against endotoxin shock (mouse model) 2 - *MMP8*$^{-/-}$ mice display lower polymorphonuclear neutrophils (PMNs) influx after LPS stimulation 3 - *MMP8*$^{-/-}$ mice are protected against hypothermia and death caused by LPS	(34, 37, 38)
	MMP9	1 - High levels of proMMP9 and MMP9 correlated with decrease survival in humans 2 - Expressed in endotoxemia 3 - Inhibition of MMP9 by regasepin1 protects against endotoxin shock (mouse model) 4 - In one model, *MMP9*$^{-/-}$ mice has an impaired host defense against *Escherichia coli* abdominal sepsis and in another model, *MMP9*$^{-/-}$ mice are protected against endotoxin shock	(37, 39, 41, 42, 86-88)
	MMP10	1 - Expressed in endotoxemia	(86)
	MMP12	1 - Expressed in endotoxemia	(86)
	MMP13	1 - Expressed in endotoxemia 2 - *MMP13*$^{-/-}$ mice display a strong protection in both LPS - and caecal ligation and puncture-induced sepsis	(44, 86)
	MMP14/ MT1-MMP	1 - Expressed in endotoxemia	(86)
Arthritis	MMP1	1 - Higher levels in the synovial fluids of rheumatoid arthritis patients as compared to controls 2 - Mediates irreversible joint destruction	(60, 64)
	MMP2	1 - *MMP2*$^{-/-}$ mice display more severe clinical and histopathological arthritis than WT animals	(61)
	MMP3	1 - Higher levels in the serum and synovial fluids of rheumatoid arthritis patients as compared to controls 2 - *MMP3*$^{-/-}$ mice have a decreased cartilage damage as compared to WT	(60, 89, 90)
	MMP8	1 - Higher levels in the serum and synovial fluids of rheumatoid arthritis patients as compared to controls 2 - *MMP8*$^{-/-}$ mice has earlier and more severe joint inflammation than WT	(60, 62, 63)
	MMP9	1 - Higher levels in the serum and synovial fluids of rheumatoid arthritis patients as compared to controls	(60)
	MMP10	1 - Activates procollagenase and contributes to tissue destruction	(91)
	MMP13	1 - *MMP13*$^{-/-}$ mice have reduced ankle swelling and less local inflammatory responses in a murine model of antibody-induced arthritis 2 - Mediates irreversible joint destruction	(64, 92)
Asthma	MMP2	1 - Plays a protective role in a murine model	(45, 46)
	MMP8	1 - Plays an anti-inflammatory role by the regulation of inflammatory cell apoptosis in a murine model	(49)
	MMP9	1 - Cleaves CCL7, CCL11 and CCL17 to induce the transepithelial chemokine gradients in a murine model 2 - Mediates the transmigration of several leukocytes in an allergen-induced airway inflammation model	(46-48)
Cancer	MMP1	1 - Increases migration, intravasation and metastasis of cancer cells 2 - *MMP1a*$^{-/-}$ mice exhibit decreased growth and angiogenesis of lung tumors 3 - Increases skin carcinogenesis and papilloma formation	(7, 54, 93-98)

Contd...

Table 1. Contd...

Diseases	MMPs	Biological Roles	References
	MMP2	1 - Increases migration, invasion, angiogenesis and metastasis 2 - *MMP2*$^{-/-}$ mice had reduced angiogenesis and tumor progression	(7, 54, 95, 99, 100)
	MMP3	1 - *MMP3*$^{-/-}$ mice have fewer and less-vascularized papillomas in a 7,12-Dimethybenz (a) anthracene (DMBA)-induced tumor model 2 - Promotes mammary carcinogenesis	(101, 102)
	MMP7	1 - Increases migration, proliferation, tumor growth rte and resistance cancer cell resistance to apoptosis 2 - Initiates and maintains metaplastic events and acinar cell transdifferentiation and apoptosis	(7, 103-108)
	MMP8	1 - *MMP8*$^{-/-}$ mice have increased incidence of skin tumors and altered inflammatory responses as compared to WT counterparts 2 - Dampens metastasis	(109, 110)
	MMP9	1 - Increases migration, invasion, cancer cell survival angiogenesis, tumor growth and metastasis 2 - Both anti-tumorigenic effects depending on the tumor stage and animal models	(7, 16, 19 , 22, 100, 111-114)
	MMP11	1 - Can be both a tumor enhancer and suppressor in a MMTV-ras tumor model 2 - Promotes homing and survival of malignant cells	(115-117)
	MMP12	1 - Better prognosis in colorectal carcinoma patients with high MMP12 expression 2 - Plays a protective role in lung tumor growth and metastasis	(118-120)
	MMP13	1 - Enhances metastases growth	(84)
	MMP14/ MT1-MMP	1 - Increases migration, invasion, tumor growth, angiogenesis and metastasis	(27, 114, 121, 122)
	MMP19	1 - *MMP19*$^{-/-}$ mice have decreased susceptibility to skin tumors in a methylcholanthrene (MCA) chemical carcinogenesis model 2 - *MMP19*$^{-/-}$ mice have increased tumor angiogenesis, cell invasion as compared to their WT counterparts	(123, 124)

protected suggesting a model dependent effect (42). Furthermore, inhibition of endotoxin shock by regasepin1, which inhibits MMP8, MMP9 and ADAM17, suggest that more proteases other than MMP9 alone are directly involved in this model (37). Lorente *et al*. demonstrated that patients with sepsis have low levels of MMP9/ TIMP1 complexes but higher levels of MMP10, TIMP1 and MMP10/TIMP-1 complexes as compared to healthy controls therefore implicating the ratios of not only MMPs but also their natural inhibitors (43).

MMP13 plays a role in LPS-induced systemic inflammation and lethality; *MMP13*$^{-/-}$ mice were significantly protected against LPS-induced hypothermia and death (44). In the CLP model, *MMP13*$^{-/-}$ mice were also protected and these effects were associated to the cleavage of pro-TNF into bioactive TNF (44).

Overall, several MMPs have been demonstrated to be potential drug targets for sepsis; however, some observations were model dependent, as in the case of MMP9 (table 1). Therefore, the role of each individual MMP must be teased apart and well characterized. The use of specific inhibitors might be needed in the treatment of endotoxin shocks in humans and will need further investigation.

3.1.1. Asthma

Protective roles of MMPs have been demonstrated in animal models of asthma. In the ovalbumin (OVA) model of asthma using *Aspergillus fumigatus* as an allergen, *MMP2*$^{-/-}$ mice suffered from a more severe asthma phenotype and a worse susceptibility to asphyxiation as compared to WT counterparts (45). Even if *MMP2*$^{-/-}$ mice had a reduced influx of the total number of cells into the bronchoalveolar lavage (BAL), they had an increased of inflammatory cells due to a reduced chemotactic activity (45). However, the role of MMP2 appears to be overridden by MMP9. In a later report, it was shown that MMP9 was broadly involved in the resolution of allergic inflammation, whereas MMP2 played a more limited role (46). The role of MMP2 was limited to the cleavage of eotaxin (CCL11) to induce a transepithelial chemokine gradient, whereas MMP9 cleaved eotaxin (CCL11), MARC (CCL7) and TARC (CCL17) (46). MMP9 mediated the transmigration of several leukocytes including dendritic cells, eosinophils and lymphocytes in an allergen-induced airway inflammation model (47, 48). It is still debatable if the role of MMP9 is detrimental, beneficial or both, as different reports have conflicting results depending on the mice strains and the types of protocols used to induce the allergic reaction (46-48).

In OVA-sensitized model of asthma, *MMP8*$^{-/-}$ mice had an augmented neutrophilic inflammation in their bronchoalveolar lavage fluids (BALFs), an increase in the infiltration of neutrophils and eosinophils in the airway walls and an increase in IL-4, anti-OVA igE and IgG1 levels (49). Thus, it was shown that MMP8 plays an anti-inflammatory roles by the regulation of inflammatory cell apoptosis (49).

It is important to mention that the roles of only a few MMPs have been investigated in asthma but several beneficial roles have been noted. These observations need to be taken in account for the use of MMP inhibitors in patients with a clinical history of asthma.

3.1.1.1. Cancer

Inflammation is interwoven with cancer and constitutes an important factor to promote tumor progression (50, 51). A clinical evidence of the importance of the link between inflammation and cancer is apparent in the long-term users of nonsteroidal anti-inflammatory drugs (NSAIDS) which exhibit lower risk of various cancers including colon, oesophagus and stomach (52,53). Importantly, MMPs are not only produced by cancer cells by also by inflammatory and stromal cells. However, for over 30 years, MMPs were studied almost exclusively in the context of cancer cells. A plethora of MMP inhibitors have been designed and have been taken into phase III clinical trials before being removed for lack of efficacy and muscoloskeletal side effects in some patients (4, 54, 55). When MMP inhibitors entered clinical trials, only 3 (MMP1, -2 and -3) out of the 23 human MMPs were known. The initial concept was that MMPs degrade and remodel the ECM to allow cancer to invade out of the tumor and later on, metastasize. We now know that MMPs contribute to cancer progression in several other ways: they 1) increase the migration and invasion of tumor cells; 2) affect angiogenesis; 3) affect leukocyte recruitment through activation or inactivation of chemokines; 4) control several inflammatory processes; 5) prepare the metastatic niche and 6) affect cell survival/growth (4, 56). The roles of MMPs in cancer can be both beneficial and detrimental. It depends on the tissue location, the type and stage of cancer and the substrates being processed (4,54). Given these various roles shown in table 1, it is not surprising that broad-spectrum inhibitors had mixed outcomes during the cancer clinical trials. Several options have been proposed to better the efficacy of MMP inhibitors: 1) more selective inhibitors, 2) exosite (e.g. linker or hemopexin domains) inhibitors or 3) targeting the substrate of MMPs and has been discussed in details in several reviews (4,54).

3.1.1.1.1. Rheumatoid Arthritis

Rheumatoid arthritis is an autoimmune disease characterized by systemic inflammation of multiple tissues but mostly the synovial joints. It is accompanied by the degradation of the joint cartilage. The collagenases (MMP1, MMP8 and MMP13) have been linked to arthritis because of their ability to degrade type II collagen, an important component of cartilages (57,58). Following cleavage by collagenases, MMP9 can further degrade type II collagen resulting in the release of immunodominant epitopes affecting T-cell reactivity (58,59). Importantly, the levels of several MMPs (MMP1, -3, -8 and -9) was higher in the synovial fluids of rheumatoid arthritis patients in comparison to either control or osteoarthritis patients (60).

For example, in a model of antibody-induced arthritis, *MMP2*$^{-/-}$ mice had more severe clinical and histopathological outcomes than wild-type mice, whereas *MMP9*$^{-/-}$ mice displayed milder arthritis (61). For MMP8, even if its levels are higher in rheumatoid arthritis patients as compared to controls, it is difficult to know if the precise role of MMP8 is pro- or anti-inflammatory. In mice, using either a spontaneous model (MRL/*lpr*) or a K/BxN serum transfer model of arthritis, *MMP8*$^{-/-}$ mice had earlier and more severe joint inflammation than their WT mice counterparts. [62,63] In the spontaneous model, there was a massive accumulation of neutrophils within the synovium of *MMP8*$^{-/-}$ mice and a decrease in neutrophil apoptosis (62). As MMP8 is anti-inflammatory in animal models, extreme cautions should be applied for MMP inhibitors that would also inhibit MMP8 in human arthritis patients. Several MMPs contribute to the pathogenesis of arthritis through the destruction/remodelling of bones, tissues and extracellular matrix, however, MMP-2, -8 and -12 were shown to have protective roles therefore complicating the development of MMP inhibitors (20,61,62,64,65).

4. CHALLENGES FOR THE DEVELOPMENT OF MMP INHIBITORS

Several MMPs have been implicated in the pathology of cancer, rheumatoid arthritis and other diseases (table 1) leading to the testing of MMP inhibitors in clinical trials (reviewed in 4, 20, 54). Yet, one must be cautious. Several MMP inhibitors were tested in cancer clinical trials with limited success and were removed in phase III. Will the lessons learnt help for the treatment of rheumatoid arthritis or other pathologies (54,55) At least 10 MMPs have been validated as anti-targets in various diseases, therefore, the aim must now be to deliberately avoid such MMPs (4). Will more selective inhibitors have better outcome than the broad-spectrum drugs tested in advanced cancer patients? Will pharmaceutical companies contemplate developing MMP inhibitors when so many anti-target activities have been reported? For example, arthritis patients must take drugs daily and for long periods of time, therefore, inhibitors must be proven to not trigger or increase the susceptibility to other diseases where MMPs are implicated such as cardiovascular diseases, chronic obstructive pulmonary disease (COPD) or cancer. Nonetheless, the anti-target roles of some MMPs make these proteases even more fascinating to study and by

unraveling their roles, new potential avenues for the treatment of inflammatory diseases will be found.

Due to the complexity of immunological and inflammatory processes, new technologies are now available to investigate, on a global scale and in unbiased manners, the roles of proteases (i.e. MMPs) in various stages of cancer, autoimmune and inflammatory diseases. MMPs not only remodel the extracellular matrix but also affect the phosphorylation of signaling molecules, cleave cell surface receptors and process chemokines thus controlling immune processes and immunological responses (4). However, these events remain largely unknown and are challenging to detect without specific enrichment methods. The remainder of this book chapter will present an overview of these novel enrichment proteomics technologies for the study of proteolysis.

5. POST-TRANSLATION MODIFICATIONS: THE KEY THAT OPENS THE DOOR OF COMPLEXITY FROM GENOMICS TO PROTEOMICS

After the completion of the human genome, a decade ago, an important question has emerged: Why are there so few genes in humans, counting only 20,135 (66)? For example, flies have ~15,000 genes, mice carry ~25,000, grape plants count ~30,000 and cabbages ~40,000. On several levels, all these organisms are considered much less “biologically complex” than humans but yet contain a superior number of genes. Has nature played a game of gene redundancy or there is another level of complexity that governs biology? During translation, the genes still dictate what becomes a protein but later, additional complexity arises, in the post-translation modification (PTMs) of proteins. More than 300 PTMs can occur in human proteins including acetylation, phosphorylation, methylation, glycosylation, nitration, proteolysis, etc. For example, an average car contains ~30,000 different parts and yet, is built to perform a limited set of functions. Only a limited number of the car pieces can be modified before the car becomes unable to perform its task. Indeed, the *parts* of humans (or proteins and their PTMs) are more malleable than car parts. As observed in a plethora of genetic diseases, the lack or the mutation of a gene has critical outcomes, however, it makes it difficult to reconcile why this low number of genes is needed to reach such biological complexity seen in humans. Will PTMs unravel the secrets that lie within this unexpected complexity? PTMs can be rare events or present in small amounts even though they dictate critical biological responses. Using proteomics approaches, one need to enrich for specific PTMS in order to detect these differences.

6. DEGRADOMICS: A GLOBAL TALE

The function of a protease is defined by the substrates it interacts with and cleaves. Unraveling the substrate repertoire of a protease leads to the understanding of its mechanism and biological importance. In diseases, uneven ratios occur between the proteases and their endogenous inhibitors. Therefore, knowing the specific substrates being cleaved by a protease in disease will reveal critical information for rational drug design. Degradomics is defined as the system-wide genomics, proteomics and systems biology techniques to investigate the biological roles of proteases and their inhibitors (2). Genomics techniques have tremendously evolved in the past decade and a large amount of information is now available in various database incorporating analyses of several diseases (67). Even if genomics studies provide crucial information about the biology of diseases, by definition, it does not provide information about the state of the proteins. Knowing that the mRNA levels of a chemokine are elevated in a disease, one stills does not know whether the chemokine is latent or activate. For example, MMPs cleave chemokines and affect their outcomes by either converting them into agonists or antagonists (68). It is therefore critical to complement the genomics information with proteomics knowledge. The proteomics technologies have tremendously evolved in recent years thereby creating novel biochemical tools to study protein functions.

7. QUANTITATIVE SHOTGUN PROTEOMICS

Proteomics allow for large-scale analysis and determination of protein functions. Quantitative proteomics can be achieved in two ways: using label-free methods or by incorporating stable isotopic labeling (69). In label free quantification methods, the mass spectrometry signal of the same peptides from different samples are compared to one another (70). This approach is now far more robust than before and can be utilized in experiments where large ratio differences between samples are expected (more than fourfold) (69). Isotope-based methods incorporate light and heavy versions of chemical groups labeled to proteins or peptides of the samples; for example, the amide groups of peptides can be dimethylated by a normal isotope from formaldehyde or heavy versions using ^{15}N, ^{13}C or ^{2}H isotopes (71, 72). Other options include isobaric tags for relative and absolute quantitation (iTRAQ), tandem mass tags (TMT) and stable isotope labeling by amino acids in cell culture (SILAC) (69, 73, 74). Quantitative shotgun proteomics provide crucial information about the protein amounts in different samples. However, as mentioned before, several states of a single protein exist in nature and dictate important biological roles. It is therefore challenging to detect PTMs without specific enrichment methods and proteolysis is no exception.

8. PROTEOMICS OF POST-TRANSLATIONAL MODIFICATIONS: N-TERMINOMICS

Proteases generate new peptides after cleaving substrates thus enrichment for neo-N-termini is the

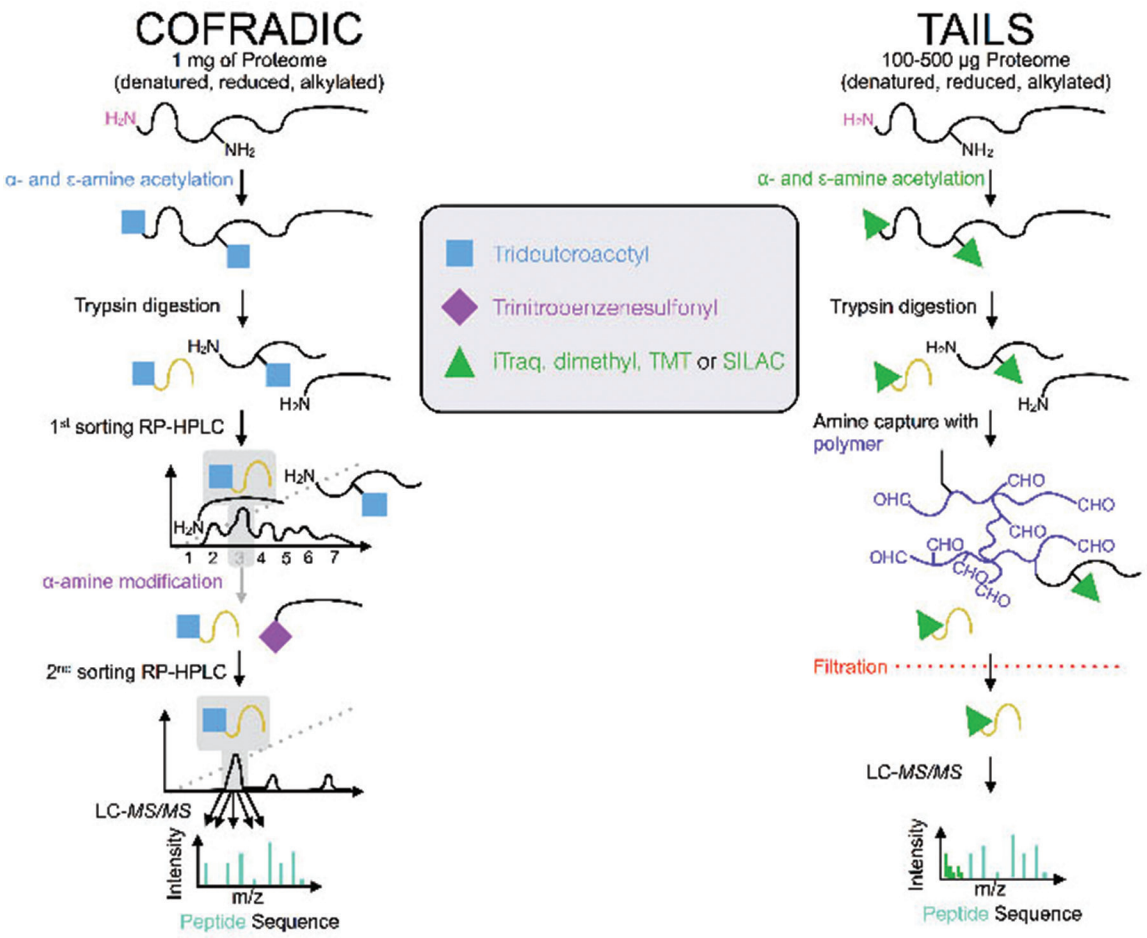

Figure 2. Schematic representation (adapted from Huesgen and Overall (125)) of N-terminal peptide enrichment strategies based on the removal of non-target peptides. COFRADIC and TAILS are methods designed to enrich for N-terminal by selective removal of internal peptides followed by N-terminal protection. These negative selection approaches also enrich for endogenous alpha-amine modified N-terminal peptides. See main text for method and biological application details. Reproduced with permission from (125).

method of choice to study post-translational proteolysis. N-terminomics allow for the identification of N-termini of both original N-termini and neo-N-termini generated by a protease. Positive selection methods can positively enrich for the neo-N-termini. For example, a subtiligase can be used to biotinylate the alpha-amines of the N-termini of peptides followed by the cleavage using the specific tobacco etch virus (TEV) protease before LC/MS/MS analysis (75). Negative selection methods are described in figure 2 and include combined fractional diagonal chromatography (COFRADIC) and terminal amine isotopic labeling of substrates (TAILS) (76,77).

8.1. Terminal amine isotopic labeling of MMP substrates

In the TAILS procedure, both alpha- and epsilon-amines are blocked by dimethylation (or other labels) followed by protein trypsinization. The water-soluble hyperbranched polyglycerol aldehyde polymer is then added to bind the neo-tryptic generated alpha-amine peptides. Due to the polymer's large size (more than10 kDa), by using ultracentrifigation, the tryptic bound peptides are then removed from the sample leaving behind the peptides cleaved by the protease of interest (figure 2) (77). The unbound N-terminal peptides are then send to the mass spectrometer followed by bioinformatics analysis. TAILS reduces sample complexity due to its depletion of tryptic peptides. Such enrichment methods enhances the identification of low abundance peptides in both *in vitro* and *in vivo* systems and offer a different coverage of peptides as compared to traditional proteomics techniques (77,78).

Terminomics approaches also present challenges. In a typical shotgun proteomics experiment, trypsin is often the protease of choice to digest proteins into peptides, cutting after arginine and lysine residues. In TAILS, trypsin is also used, however, due to the dimethylation of the free amines of lysine residues, trypsin is unable to cut after dimethylated lysines and only

cuts after arginine residues, resulting in peptides with an ArgC specificity i.e. semi-tryptic peptides. These N- and C-terminal peptides are not always amenable for LC-MS/MS analysis: they are often longer in length and risk to have different physicochemical properties for ionization and/or fragmentation (79). ArgC generated peptides also risk to have different charge states, therefore, acquisition methods utilizing different collision or electron transfer energy are needed. To solve this limitation, some groups have used GluC and chymotrypsin in parallel to trypsin to increase the number of MS-amenable peptides (77, 80).

Enriching for the N-terminome can lead to the detection of low abundance proteins or never before identified proteins. For example, Lange *et al*. (81) have identified 1369 natural and neo-N-termini corresponding to 1234 proteins in human erythrocytes using TAILS. Of these identified proteins, 281 were novel identified erythrocyte proteins and 6 were detected for the first time in the human proteome.

Several MMPs have already been investigated using TAILS. 146 high-confidence substrates were discovered using *MMP2-/-* fibroblast secretomes treated with either recombinant MMP2 or buffer (77). In a separate experiment, murine fibroblasts were treated with MMP2 or MMP9 and identified 3,152 unique N-terminal peptides corresponding to 1,054 proteins (82). Of these identified N-terminal peptides, 201 MMP2 cleavages and 19 MMP9 cleavages were detected (82). Novel substrates were biochemically validated including galectin-1, insulin-like growth factor binding protein-4 (IGFBP4), complement C1r component A, dickkopf-related protein-3 (Dkk-3) and thrombospondin-2 (TSP2) (82).

TAILS can also be used to study *in vivo* samples. Using a 12-*O*-tetradecanoylphorbol 13-acetate (TPA) model of skin inflammation, the skin of wild-type and *MMP2*$^{-/-}$ mice were analyzed (83). In wild-type animals, MMP2 cut serpin G1 at position ^{470}R↓S^{471}, leading to its inactivation (83). In the *Mmp2*$^{-/-}$ mice the high levels of intact functional serpin G1 blocked complement activation *in vivo*. In response to phorbol ester-induced inflammation, MMP2 inactivates the complement 1 (C1) inhibitor therefore increasing complement activation and bradykinin generation, thus, leading to an increase in vessel permeability (83).

Co-cultures of the human breast cancer cells MDA-MB-231 with the osteoblast MC3T3-E1 cells were analyzed by both the CLIP-CHIP® microarray and TAILS. Breast cancer cells induced MMP13 on both the mRNA and protein levels which lead to the analysis of the osteoblast secretomes with the addition of exogenous recombinant MMP13 or buffer (84). A total of 1280 proteins were identified and 48 proteins were significantly increased in the culture supernatants of MMP13 treated samples including novel substrates of MMP13 (4, 8). These cleavages of MMP13 affect biology in several ways: two chemokines (CCL2 and CCL7) are inactivated therefore representing a feedback mechanism in a monocyte recruitment pathway and platelet-derived growth factor-C (PDGF-C) is activated leading to an increase in the phosphorylation of ERK1/2. Other substrates including SAA3, osteoprotegerin, cutA and antithrombin III were also biochemically validated to be cleaved by MMP13 (4,8).

Several novel substrates of the neutrophil-specific protease membrane-type 6 matrix metalloproteinase (MT6-MMP/MMP25) were also discovered using TAILS. The secretomes from human fetal lungs (HFL-1) cells were incubated with either recombinant MT6-MMP or with the catalytically inactive form (MT6-MMP^{E234A}) and 58 high confidence novel substrates were identified. Five of these proteins were validated biochemically including cystatin C, insulin-like growth factor-binding protein-7 (IGFBP7), galectin-1, vimentin and secreted protein acidic and rich in cysteine (SPARC) (85). Furthermore, the cleavage of vimentin by MT6-MMP results in a loss of chemotaxis of the human monocytic cells THP-1 but an increase in phagocytosis (85). Therefore, MT6-MMP is implicated in macrophage chemoattraction and phagocytosis of apoptotic neutrophils by the cleavage of vimentin.

Using unbiased techniques like degradomics, the number of known MMP substrates has greatly expanded. Interestingly, many of these are proteins implicated in inflammatory diseases and pathogen-induced processes. These novel discoveries in the field of MMPs will modify our understanding on the biological roles of these proteases and how they affect and control immunity.

9. PERSPECTIVES: A SHORT-CUT CAN TELL TAILS

The function of a protease is defined by the substrate it cuts. Proteases can induce signaling pathways, activate or inactivate chemokines, increase or decrease cell migration, cell invasion, cell proliferation and angiogenesis (2,4,56). Importantly, still more than half of the proteases have no annotated substrates in MEROPS, the protease database (http://merops.sanger.ac.uk). Over the last decade, various degradomics tools have been developed leading to a significant increase of the discovery of protease substrates. MMPs play major roles in inflammation and remain of great interest in biology. As more novel and unexpected MMP substrates are being discovered using unbiased approaches like TAILS, new opportunities arise for developing MMP inhibitors. MMPs do not act alone. They are embedded in an interconnected protease web that is dynamic and altered in disease states. By understanding the role of MMPs within these complex networks, other therapeutics avenue will be discovered to treat inflammatory diseases.

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