Biochimica et Biophysica Acta 1840 (2014) 2560–2570

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Cysteine cathepsins and extracellular matrix degradation ☆

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ARTICLE INFO

Article history:
Received 17 January 2014
Received in revised form 16 March 2014
Accepted 22 March 2014
Available online 27 March 2014

Keywords:
Cathepsin
Extracellular matrix
Lysosome
Collagen
Osteoporosis
Glycosaminoglycan

ABSTRACT

Background: Cysteine cathepsins are normally found in the lysosomes where they are involved in intracellular protein turnover. Their ability to degrade the components of the extracellular matrix *in vitro* was first reported more than 25 years ago. However, cathepsins were for a long time not considered to be among the major players in ECM degradation *in vivo*. During the last decade it has, however, become evident that abundant secretion of cysteine cathepsins into extracellular milieu is accompanying numerous physiological and disease conditions, enabling the cathepsins to degrade extracellular proteins.
Scope of view: In this review we will focus on cysteine cathepsins and their extracellular functions linked with ECM degradation, including regulation of their activity, which is often enhanced by acidification of the extracellular microenvironment, such as found in the bone resorption lacunae or tumor microenvironment. We will further discuss the ECM substrates of cathepsins with a focus on collagen and elastin, including the importance of that for pathologies. Finally, we will overview the current status of cathepsin inhibitors in clinical development for treatment of ECM-linked diseases, in particular osteoporosis.
Major conclusions: Cysteine cathepsins are among the major proteases involved in ECM remodeling, and their role is not limited to degradation only. Deregulation of their activity is linked with numerous ECM-linked diseases and they are now validated targets in a number of them. Cathepsins S and K are the most attractive targets, especially cathepsin K as a major therapeutic target for osteoporosis with drugs targeting it in advanced clinical trials.
General significance: Due to their major role in ECM remodeling cysteine cathepsins have emerged as an important group of therapeutic targets for a number of ECM-related diseases, including, osteoporosis, cancer and cardiovascular diseases. This article is part of a Special Issue entitled Matrix-mediated cell behaviour and properties.

1. Introduction

Extracellular matrix (ECM) is a complex macromolecular structure present in all organ and tissue types [1]. Besides providing basic physical scaffolding it also creates biochemical and biomechanical environment, which is vital for tissue homeostasis, morphogenesis and differentiation. ECM is a dynamic structure that is constantly remodeled through a large variety of post-translational modifications, which can be enzymatic or non-enzymatic. Among the enzymatic modifications, proteolytic processing and degradation are among the most important. Through proteolysis, proteases can modify the ECM structure and also release the bioactive factors, which influence growth, morphogenesis and pathological processes, thereby restructuring extracellular matrix in a rapid and irreversible manner. Due to their extracellular localization and enzymatic stability at neutral pH, metalloproteases and serine proteinases were traditionally considered to be the main agents of ECM degradation [2,3]. In contrast to that, lysosomal cysteine proteases or cysteine cathepsins, which require acidic pH for optimum activity, were mainly regarded as intracellular proteases involved in a general protein turnover. This view was shifted by the discovery that under specific physiological and pathological conditions, the cathepsins can also be secreted into the extracellular space where they can remain proteolyticaly active and degrade various components of the extracellular matrix [4,5]. Moreover, inhibition of their extracellular activity was also shown to be highly relevant in clinical applications [6–10]. In this review, we will present an overview of cysteine cathepsins and discuss how they can remodel the extracellular matrix including their importance in disease.

2. Cysteine cathepsins

Cathepsins are a diverse group of proteases which is composed of various catalytic types. Among them are serine cathepsins (cathepsins A and G), aspartic cathepsins (cathepsins D and E) and cysteine cathepsins which are the most abundant. Cysteine cathepsins belong to the C1 family (papain family) of CA clan of cysteine proteases [11]. Human

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http://dx.doi.org/10.1016/j.bbagen.2014.03.017
0304-4165/

genome sequencing data revealed the existence of 11 human cysteine cathepsins (cathepsins B, C, F, H, K, L, O, S, V, X and W) [12]. They are monomeric proteins with molecular mass in the range of 20–35 kDa, the only exception being cathepsin C, which is a tetrameric protein with molecular mass around 200 kDa. Regarding their substrate preference, cysteine cathepsins are mainly endopeptidases (cathepsins F, K, L, O, S, V and W), whereas the aminopeptidase cathepsin C and the carboxypeptidase cathepsin X are strict exopeptidases. Although primarily exopeptidases, cathepsins B and H can also act as endopeptidases (Table 1). The majority of cathepsins are ubiquitously expressed in human tissues, while cathepsins K, S, V and W show more tissue-specific distribution [4,9,13–19]. Such diverse tissue-specific expression of some cysteine cathepsins suggests that their action is not limited solely to the bulk protein degradation but is also involved in the regulation of specific cellular functions. This was confirmed by the finding that active cysteine cathepsins are not localized strictly in the lysosomes but can also be found in other cellular compartments, including the nucleus, where they perform very specific functions [20–22]. All these help the cathepsins to regulate numerous important physiological processes, among others intracellular protein turnover, immune response, bone remodeling and prohormone processing [5–7,14,23].

2.1. Structure and specificity

Cysteine cathepsins have a characteristic papain-like fold, which is composed of two domains: left (L-), which is formed by three α-helices, and right (R-), which has a β-barrel structure. The two domains form an active site cleft, where the two active-site residues (Cys25 and His159, papain numbering) are located (Fig. 1a). The two residues form a thiolate-imidazolium ion pair, which is critical for the proteolytic activity. Binding surfaces for the substrate residues are provided by both domains and substrate binds along the active-site cleft with its side chains alternately oriented towards the L- and R- domains [4,5,14]. According to the Schechter and Berger nomenclature, substrate amino acid residues (P) and substrate binding pockets (S) are numbered according to their position relative to the scissile peptide bond (Fig. 1b). The positions towards the N-terminus are denoted as the "non-prime" residues and sites (P1–Pn; S1–Sn), whereas those towards the C-terminus are denoted as the "prime" residues and sites (P1′–Pn′ and S1′–Sn′) [24]. Structural studies have shown that in cysteine cathepsins only the S2, S1 and S1′ binding sites are well defined and that the S2 and S1′ sites are the main determinants of the substrate specificity [25]. Contrary to some other cysteine proteases such as caspases or legumain, cysteine cathepsins exhibit much broader specificity [25]. This suggests that their specificity is determined by a cumulative effect of interaction with multiple binding sites and is not governed by a single site. Even the S2 subsite, which is structurally the most stringently defined (it is the only one which forms an actual pocket"), can be occupied by various hydrophobic or aromatic amino acid residues, such as leucine, isoleucine, valine, tyrosine and phenylalanine [25–28]. In addition, profiling substrate specificity of cysteine cathepsins using a combinatorial peptide library revealed a unique specificity for cathepsin K for Pro and Gly in the P2 and P3 positions, respectively, which matches well the cathepsin K specificity on ECM proteins such as collagen [27]. In addition, cysteine cathepsins that exhibit exopeptidase activity (cathepsins B, C, H and X), have additional structural features, which limit the substrate access to the active site and regulate exo- and endopeptidase activities of cathepsins B and H [5,29–35].

2.2. Localization and regulation of cathepsin activity

Cysteine cathepsins are expressed as inactive preproenzymes in the endoplasmic reticulum. The signal peptide is proteolyticaly removed during the transport through the lumen of the rough endoplasmic reticulum, followed by procathepsin glycosylation in the Golgi. In the trans-Golgi network, glycosylated procathepsins are tagged with the mannose-6-phosphate marker, which enables their sorting to the endolysosomal compartments [36]. However, lysosomal sorting of cathepsins can also be independent of the mannose-6-phosphate pathway [37]. Inside the lysosome, procathepsins are then activated by proteolytic removal of their prodomains. Their activation can be autocatalytic or catalyzed by other proteases [14,38]. However, even during autocatalytic activation, activation is always in trans, *i.e.* one cathepsin or procathepsin molecule activating another procathepsin molecule [39]. In contrast to endopeptidases, exopeptidases cathepsins C and X cannot be autocatalytically activated but require an endopeptidase such as cathepsin L or S for their activation [40,41]. A major role in facilitating autocatalytic activation of cysteine cathepsins also has various glycosaminoglycans (GAGs) and other negatively charged surfaces [42–47]. It seems that the molecular mechanism involves a conformational change in the cathepsin zymogen, induced by GAG binding, which loosens the interaction between the propeptide and the mature part of the enzyme, thereby enabling easier processing of another procathepsin molecule [44]. Moreover, GAG and other negatively charged polymers such as dextran sulfate also facilitated autocatalytic activation of the cathepsins at pH values close to neutral, which may have an important function in disease states [43,48]. In addition, GAG binding was also found to have a stabilizing role thereby protecting the cathepsins against pH-induced inactivation [49]. However, there is no conserved GAG-binding surface that would be common to all cathepsins.

For their optimal activity, cathepsins require reducing and slightly acidic environment such as found in the lysosome. This linked with their expression in the form of inactive zymogens and their endo/lysosomal compartmentalization led to the general idea that cathepsins are primarily endolysosomal enzymes involved in general protein turnover. Moreover, their localization inside the lysosomes, a general instability at neutral pH and a requirement for reducing conditions were long believed to protect the cell and limit unwanted proteolysis. If any of these would fail, activity of cysteine cathepsins that are released from the lysosomes or secreted is additionally regulated by the endogenous

Table 1
Localization and specificity of cysteine cathepsins.

Name	Gene symbol	Substrate cleavage mode	Extracellular localization	Tissue and cell specificity
Cathepsin B	CTSB	Carboxydipeptidase endopeptidase	Secreted membrane bound	Ubiquitous
Cathepsin C	CTSC	Aminodipeptidase	n.d.	Ubiquitous
Cathepsin F	CTSF	Endopeptidase	Secreted	Ubiquitous
Cathepsin H	CTSH	Aminomonopeptidase endopeptidase	Secreted	Ubiquitous
Cathepsin L	CTSL	Endopeptidase	Secreted	Ubiquitous
Cathepsin K	CTSK	Endopeptidase	Secreted	Predominant in osteoclasts and synovial fibroblasts
Cathepsin O	CTSO	n.d.	n.d.	ubiquitous
Cathepsin S	CTSS	Endopeptidase	Secreted	Predominant in antigen presenting cells
Cathepsin V	CTSL2	Endopeptidase	n.d.	Thymus, testis
Cathepsin W	CTSW	n.d.	n.d.	Cytotoxic lymphocytes
Cathepsin X	CTSZ	Carboxymonopeptidase	Secreted membrane bound	Ubiquitous

Several properties of cysteine cathepsins are shown, including their preference to cleave substrates as endo- or exopeptidases, their tissue and/or cell specific expression preference, as well as information about their potential extracellular localization (n.d., not determined). Original references can be found within [4,6,9,14,23,52,83].

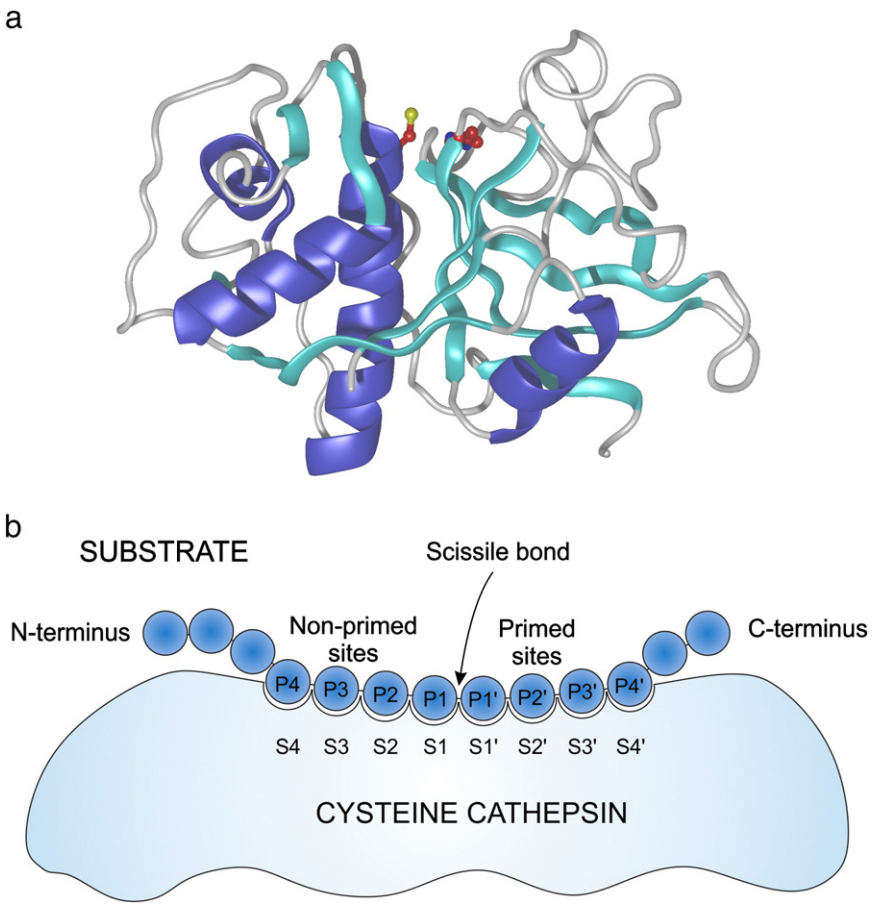

Fig. 1. (a) A fold of a typical cathepsin endopeptidase is shown using cathepsin V as an example ([216]; PDB ID: 3H6S). Secondary structure elements are colored in blue (alpha helices) and cyan (beta strands). The side chains of catalytic residues Cys25 and His164 are shown in ball-and-stick representation with S, N, and C atoms colored yellow, red, and blue, respectively. Figure was prepared with MAIN software [217]. (b) Schematic representation of a protein substrate binding to a cathepsin according to Schechter and Berger [24]. The surface of the cathepsin (light blue) able to accommodate a side chain of a single substrate residue (dark blue ball) is called the subsite. Subsites are numbered S1–Sn towards the N terminus of the substrate (non-primed sites), and S1′–Sn′ towards the C terminus (primed sites), starting from the scissile bond, which is marked. The substrate residues that bind into the subsites are numbered P1–Pn, and P1′–Pn′, respectively.

protein inhibitors such as stefins, cystatins and kininogens, serpins and alpha$_2$ macroglobulin, which are believed to provide another level of safety [14,50,51].

However, it became evident that under certain physiological and pathological conditions, cathepsins can be released into the cytosol, translocated to the cell surface or secreted into the extracellular space, which suggested that the cathepsins are active also in less favorable conditions [7,13,52]. One such example is apoptosis, where cathepsins were found to be released from the lysosomes into the cytosol, where they were found to process various Bcl-2 homologs and thus contribute to the progression of apoptosis [13,53,54]. Cathepsins were also found to be associated with the cellular membrane and such membrane-associated form of cathepsin B was found in various cancer cell types [52,55]. In colorectal carcinoma cells, cathepsin B was found to bind to the annexin II tetramer and localized in the caveolar membrane microdomain [56]. Its membrane localization pattern correlates well with the serine protease urokinase plasminogen activator (uPA) and urokinase plasminogen activator receptor [52,57]. Moreover, cathepsin B was shown to activate pro-uPA in ovarian carcinoma cells *in vitro*, which was suggested to increase cancer cell invasion, and a similar mechanism was proposed also for colorectal carcinoma [58]. Similarly, cathepsin L was also found to be located at the membrane of melanoma cells, where it was suggested to participate in metastasis spread [59]. Another protease found to be associated with membranes is cathepsin X [60]. However, the enzyme, which is predominantly expressed by the immune cells, is apparently not involved in ECM degradation, but rather in regulation of cell adhesion through activation of integrin receptors, mediated by the integrin-binding motifs RGD present in the proform and ECD present in the mature form of the enzyme [61–64]. While procathepsin X primarily binds to β3 integrin receptors, mature cathepsin X is responsible for the activation of β2 integrin receptors Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18) [65].

Contrary to the membrane localization, which was observed only in cancer cells, secretion of cysteine cathepsins into the extracellular millieu was also found during normal physiological processes such as bone remodeling, wound healing and prohormone processing [23]. During bone remodeling, cathepsin K is secreted by osteoclasts and influences bone resorption [66–68]. In a similar manner, cathepsins B, L and K are secreted from the thyroid epithelial cells, where they release thyroid hormones from the extracellular thyroglobulin [23,69,70], whereas secreted cathepsin B is also involved in the keratinocyte migration during wound healing [71]. However, more often excessive secretion of cysteine cathepsins is implicated in various pathological states and is often linked with inflammation [6,7,9,23,52].

In all these conditions cathepsins were found to be active. There are several explanations for such activity. First, the cathepsins, with the exception of cathepsin S that is a very stable enzyme, were shown to survive for at least a short time at neutral pH, which is sufficient for their efficient cleavage of the substrates [72–75]. Second, the cathepsins were also found to be relatively resistant towards oxidative stress [76,77], which further explains their ability to function outside lysosomes. In agreement with this, a significant cathepsin activity was detected in the synovial fluid of arthritic patients [78]. Third, the cathepsins are often secreted as inactive zymogens [52], which could have been at

least *in vitro* activated to their active forms in the presence of GAGs (see above). A constant efflux of cathepsins could thus have been provided, which could be sufficient for a sustained activity of even the short-lived cathepsins. Finally, in a number of such conditions, especially in pathology, the pH of the local microenvironment was often found to be acidic and not neutral. This is true not only for apoptosis [79,80] and cancer [52,81], but also for arthritis, especially at the later stages of the disease [82,83]. Under acidic conditions cathepsins are far more efficient proteases than at neutral pH, partially due to the instability of their substrates, and partially due to their higher stability [13], thereby suggesting that extracellular cathepsins may even have a leading role in the degradation of ECM [83]. A further confirmation of such cathepsin activity was obtained by *in vivo* imaging approaches using activity-based probes against different cathepsins [84–86].

3. Cathepsins as effectors of ECM remodeling

In general, extracellular matrix can be functionally divided into interstitial matrix and basement membrane. The basement membrane forms a thin layer, which underlies the epithelial cells, separating them from the connective tissues, while the interstitial matrix fills the intercellular space, separating cells and clusters of cells [1]. Biochemically, ECM is composed of two main classes of macromolecules, proteoglycans and fibrous proteins. Members of both molecular classes have already been reported as substrates of cysteine cathepsins (Table 2) [6,7,10,52,83]. However, it should be noted that ECM degradation by cathepsins is not limited to the extracellular space. Many cell types can internalize ECM components through endocytosis and degrade them inside the lysosomes [52]. However, one should be aware that for a number of these proteins the evidence has only been provided *in vitro*, whereas *in vivo* validation, which is a major issue in understanding the physiological role of proteases, is still missing [87]. Here we will focus on the ECM components, which have been shown to be processed or degraded by cysteine cathepsins with major focus on proteoglycans and fibrous proteins.

3.1. Proteoglycans

Proteoglycans are the major components of the interstitial matrix. They are glycosylated proteins, composed of a core protein with covalently attached glycosaminoglycan molecules (GAGs). GAGs have been shown to have a major role in cathepsins' role as ECM remodeling enzymes. Cathepsins are namely known to form complexes with some GAGs, such as chondroitin sulfate, keratan sulfate or heparan sulfate, which have, in addition to having a major effect on the cathepsin activation and stability (see above), a major role in regulating cathepsin activity. However, different GAGs have opposing effects on cathepsin activity. Chondroitin sulfate, for example, inhibits degradation of elastin by cathepsins V and K [88] and collagenolytic activity of cathepsin S [89]. Similarly, collagenolytic activity of cathepsin K was reduced in the presence of dermatan, heparan sulfate, and heparin [90]. In contrast, chondroitin and keratan sulfate strongly increase the collagenolytic activity of cathepsin K [90–92]. Besides forming complexes with their glycosaminoglycan components, cathepsins also cleave the proteoglycan protein core. Aggrecan is the most abundant proteoglycan component of the cartilage, where it forms aggregates with hyaluronan and gives tissue the ability to resist mechanical compression. It is composed of three globular domains (G1–G3), which are separated by an E1 domain and a more extended E2 domain. The E2 domain contains numerous O-linked glycosaminoglycans such as keratan and chondroitin sulfates. Both E1 and E2 domains are highly susceptible to proteolysis by various matrix metalloproteases and cysteine cathepsins [93]. Among the cysteine cathepsins capable of cleaving aggrecan are cathepsins B, K, L and S. Cathepsin B cleaves aggrecan as an endoprotease within the E1 region (G^{344}) in close proximity to the MMP cleavage site (N^{341}) [93,94]. Cathepsin K on the other hand, was reported to cleave the G1, E1 and E2 domains of aggrecan. Degradation of the E2 domain enables the formation of complexes between cathepsin K and released GAGs, which increases the collagenolytic activity of cathepsin K and further stimulates cartilage degradation [95].

Besides proteoglycans of the interstitial matrix, cathepsins were also reported to cleave proteoglycan components of the basement membrane. Perlecan is a heparan sulfate proteoglycan, which contains a multidomain core protein and three glycosaminoglycan chains at its N-terminus. It maintains the endothelial barrier function and plays an important role in cardiovascular, cartilaginous and neural development [96]. Cathepsin L was reported to process perlecan during apoptosis and release the LG3 peptide, also called endorepellin, from its C-terminus [97], whereas cathepsins B and L were shown to be involved in the generation of neuroprotective LG3 during cerebral ischemia [98]. This might have important consequences as endorepellin is an inhibitor of angiogenesis and blocks endothelial cell adhesion to fibronectin and type I collagen [99]. Another basement membrane proteoglycan found to be cleaved by cathepsins is nidogen. Both nidogen variants, nidogen-1 and nidogen-2, were shown to be efficiently degraded by cathepsin S. Several cleavage sites were identified within the G2 and G3 domains, which interact with other ECM components such as collagen VI, perlecan and laminin [100]. Since nidogen serves primarily as a structural crosslink between collagen and laminin, its degradation could significantly affect the viscoelastic properties of the basement membrane. Moreover, laminin was also found to be cleaved by cathepsins B and L [45,101], whereas accumulation of laminin and collagen was observed in the colon of cathepsin K-deficient mice

Table 2
ECM substrates of cysteine cathepsins.

Interstitial ECM proteins	Cathepsin	Pathophysiological function	Reference
Aggrecan	Cathepsins B, K, L, S	Osteoarthritis	[93–95]
Type I collagen, Type II collagen	Cathepsins B, K, L, S	Osteoporosis, rheumatoid arthritis, osteoarthritis, cardiovascular diseases, lung diseases, cancer	[106,107,110,113,123,148,218]
Elastin	Cathepsins K, L, S, V	Cardiovascular diseases, cancer	[68,88,118,119]
Fibronectin	Cathepsins B, L, S	Adipogenesis, cancer	[45,101,121,167]
Osteocalcin	Cathepsins B, H, L, S	Osteoporosis	[122,124]
Osteonectin/SPARC	Cathepsins B, K	Osteoporosis	[122,123,125]
Basement membrane ECM proteins	**Cathepsin**	**Pathophysiological function**	**Reference**
Perlecan	Cathepsins B, L	Neuroprotective activity in brain ischemia, vascular remodeling	[97,98]
Nidogen	Cathepsin S	n.d.	[100]
Type IV collagen	Cathepsins B, S	Cancer progression, intestine trauma	[218–221]
Tenascin C	Cathepsin B	Cancer progression	[103]
Laminin	Cathepsins B, L, S	Cancer progression, intestine trauma	[45,101,220,221]

Interstitial and basement membrane ECM proteins are listed together with the cathepsins reported to process or degrade them and some relevant references. The known pathologies, where cathepsin-mediated processing and/or degradation of these proteins have been found, are also shown (n.d., not determined).

[102], in agreement with an important role of cathepsins in regulation of the basement membrane function. A further support of that came through the findings that cathepsins cleave also other basement membrane components such as collagen IV and tenascin C [57,103].

3.2. Fibrous proteins

Collagens are the most abundant fibrous proteins and represent the main structural element within the interstitial ECM. They provide tensile strength, regulate cell adhesion, migration and direct tissue development [104]. Although there are 28 known types of collagen, the vast majority represents type I collagen, which forms ~90% of bone organic matrix of [83]. Type I and II collagens have a triple helical structure which is highly resistant to proteolysis. Human proteases known to degrade triple helical collagen are matrix metalloproteases (MMP-1, MMP-8, MMP-13, MT1-MMP, MT2-MMP), serine protease neutrophil elastase and cysteine protease cathepsin K [105]. Although collagen was the first ECM protein found to be degraded by various cathepsins *in vitro* [106], cathepsin K is nowadays the only physiologically relevant collagenase among the cathepsins [107]. The collagenolytic activity of cathepsin K strongly depends on its complex formation with GAGs, in particular chondroitin sulfate-4 [91]. The crystal structure of the cathepsin K chondroitin sulfate complex shows that the GAG binding site is located on the opposing side of the cathepsin active site and is formed by several positively charged lysines and arginines [108]. Mutational analysis revealed two crucial GAG interaction sites, composed of cluster 2 (K103, K106, R108, R111) and cluster 3 (K122, R127) [109]. GAG binding sites in cathepsin K are also highly conserved between all vertebrates and it was suggested that the GAG-cathepsin K complex formation enables unfolding of the collagen triple helix [109]. Type I collagen molecules consist of two α_1 chains and one α_2 chain. Both terminal regions of the α chains are not part of the triple helix and are cross-linked by pyridinoline–deoxypyridinoline to form the telopeptide region. Regular collagenases such as MMPs and neutrophil elastase cleave the triple helix at a single site between the amino acid residues 775 and 776. In contrast, cathepsin K can cleave collagen triple helix at multiple sites, which makes it unique among the known collagenases [110,111]. Besides the triple helix, cathepsin K can also cleave the telopeptide region of the collagen, which is a common feature of other cathepsins (cathepsins B, L and S) and produces collagen monomers [68]. Another important feature which distinguishes cathepsin K from other cysteine cathepsins is its ability to accept proline residues in the P1 and P2 positions [27,112]. This is essential for the degradation of collagen, since two highly repetitive motifs in the triple collagen helix are Gly-Pro-X and Gly-X-Hyp (X and Y represent various amino acid residues). However, the active site preference for proline is not sufficient for efficient cleavage of the triple helix collagen. This was confirmed by generating a cathepsin K-like variant of cathepsin L by mutagenesis of the S2 binding site, which exhibited cathepsin K-like preference for small substrates, but showed no triple helix collagenolytic activity [113]. This further supports the idea that a combined effect of GAG complex formation and active site specificity for Pro is critical for the efficient triple helix cleavage. In support of this idea, cathepsin V as the only other cathepsin known to form a complex with chondroitin sulfate, does not accept proline in the P2 position [27] and does not exhibit any collagenolytic activity [107].

However, cathepsins do not degrade only collagens. Cathepsin L was thus suggested to be, in addition to metalloproteases, an important enzyme in generation of endostatin, an angionesis inhibitor, by processing the C-terminus of collagen XVIII in both mouse and human systems [114,115]. In addition, cathepsin S was found to be another enzyme, which was capable of generating endostatin in human umbilical vein endothelial cell-based assay [114].

Elastin is another major ECM fibrillar protein, which is closely associated with collagen and enables tissue to recoil from stretching forces [116]. Cathepsins K, S and V are all known as potent elastases, while cathepsin L only has weak elastinolytic activity, despite its 78% homology with cathepsin V, which is the most potent elastase known so far [88,117]. This discrepancy was resolved only recently with the discovery of two hydrophobic exosites, which stabilize the complex between elastin and cathepsin V. Both exosites are distant from the active site and their replacement generates a non-elastinolytic cathepsin V variant similar to cathepsin L [118]. Binding of elastin to the exosites was suggested to enable the correct positioning of the cleavage susceptible region into the cathepsin active site. Such a mechanism was previously proposed also for the elastinolytic activities of cathepsins S and K [119]. It should be noted that in mouse models cathepsin L is often referred to as a very potent elastase. However, whereas in humans we find cathepsins L and V, which are very closely related enyzmes, mice express a single enzyme, cathepsin L that is functionally more similar to human cathepsin V [7].

Fibronectin is the third fibrous protein which is closely involved in the organization of the interstitial ECM through its interaction with integrins. Cellular fibronectin is secreted as a soluble dimer and is later assembled into insoluble fibrillar matrix. Fibronectin interacts with a large variety of ECM proteins and plays an important role in cell adhesion, migration, growth and differentiation [120]. Fibronectin was found to be cleaved by cathepsins S, L and B [45,101,121]. The functional role of these cleavages was not further investigated although the protein has major roles in cancer and adipogenesis, where cathepsin S-mediated fibronectin degradation was suggested to be important in the early steps of differentiation [121].

3.3. Other ECM-related cathepsin substrates

Cathepsins were also found to degrade two bone ECM proteins, osteocalcin and osteonectin [122–125]. Osteocalcin is a noncollagenous protein that is secreted by osteoblasts and is involved in bone formation and insulin metabolism [126], whereas osteonectin/SPARC is an acidic glycoprotein that plays a major role in interactions of matrix with cells and in collagen binding [127,128]. Osteocalcin is also often used as a serum biomarker of bone mineral density (BMD) during treatment with various osteoporosis drugs, but it has only very seldom been used as a biomarker for measurement of BMD during the evaluation of cathepsin K inhibitors [129,130].

Another protein that is tightly involved in the regulation of ECM homeostasis and is a substrate of cathepsins is heparanase. Heparanase is an endo-β-D-glucuronidase that degrades heparan sulfate in the extracellular matrix and on the cell surface and is, in contrast to most other proteins described here, not degraded by cathepsins but instead cathepsin L was found to be a critical enzyme involved in its activation [131].

4. Clinical aspects of cathepsin-based ECM degradation

Impaired ECM remodeling caused by upregulated extracellular cathepsin activity has been related to numerous diseases, including osteoporosis, osteoarthritis and rheumatoid arthritis, cancer, atherosclerosis and various lung diseases. The majority of information was obtained using clinical samples and mouse models of disease that provided unambiguous evidence of cathepsin involvement [6,7,9,132]. Most of these diseases are linked with inflammation so it is not surprising that the infiltrated immune cells including macrophages are the major source of such secreted cathepsins. In all these diseases cathepsins were found to be highly overexpressed and secreted into the extracellular milieu by various cell types. There are numerous mechanisms that lead to cathepsin overexpression, with a number of them linked with transcriptional regulation [52]. Among the important factors inducing cathepsin overexpression are various cytokines [117,133–138] and collagen-derived fragments [139], which may create various feedback loops, thereby providing a constant influx of cathepsins into the disease area.

4.1. Osteoporosis

Osteoporosis is clearly a disease of excessive ECM degradation, where cathepsins, in particular cathepsin K as the most efficient collagenase are really crucial for its development and progression. Cathepsin K secreted by osteoclasts is namely of crucial importance for the effective bone resorption, which has become evident when the enzyme was genetically ablated and cathepsin K-deficient mice developed osteopetrosis as a result of insufficient matrix degradation [66,140]. During bone resorption, cathepsin K is secreted to the acidified resorption lacunae as an active enzyme [67], and degrades there type I collagen, which is the major protein constituent of the bone organic matrix [68,95,111,141]. However, increased type I collagen degradation caused by the hyperactivity of osteoclasts decreases mineral bone density, which is the main symptom of osteoporosis [9]. Moreover, overexpression of cathepsin K in mice resulted in enhanced bone resorption, supporting its role as the major protease in bone resorption [142] and as a major drug target for osteoporosis (see below).

4.2. Osteoarthritis and rheumatoid arthritis

Osteoarthritis is the most prevalent age-related degenerative joint disease. A key feature of osteoarthritis is a gradual loss of articular cartilage and deformation of bone at the margin, which eventually results in the impairment of joint function. The primary cause of cartilage destruction is elevated levels of active proteases, largely produced by articular chondrocytes, although some may also come from the inflammatory cells, especially at the later stage of the disease. Among them the most important are matrix metalloproteases, ADAMTSs and cathepsins. In contrast, rheumatoid arthritis is characterized by chronic synovitis, which has a key role in the destruction of articular cartilage and bone. In rheumatoid arthritis, extracellular cathepsins largely originate from the cells attracted to the site of sustained inflammation, including activated macrophages and synovial fibroblasts that are recruited to the cartilage lining of a joint where they secrete large quantities of proteases [143–146]. In rheumatoid arthritis as well as in osteoarthritis, cathepsin K was shown to be the most important cysteine cathepsin involved in the degradation of type II collagen and aggrecan, which leads to the degeneration and progressive loss of articular cartilage [9]. Moreover, the leading role of cathepsin K in the two diseases is supported by the findings that its expression and/or secretion was found to be increased in patients with osteoarthritis and rheumatoid arthritis [82,147–151] as well as in mouse models of the diseases [152–154]. Furthermore, an important role of cathepsin K in osteoarthritis as compared to metalloproteases is supported by the finding that the pH of the cartilage decreased with the severity of the disease reaching values as low as 5.5 in grade 3 osteoarthritis [82]. Finally, pharmacological inhibition of cathepsin K activity was shown to reduce bone erosion, cartilage degradation and inflammation in collagen-induced arthritis in mice, thereby suggesting that it might also be a suitable target in the human disease [155]. In addition to cathepsin K, cathepsins B, L and S were also suggested to participate in both diseases and were identified in synovial fluids of the patients [78,149,156–160] and in mouse models of joint inflammation, where they could have been detected by imaging approaches [86,161]. In addition, an important role of cathepsin L in the pathogenesis of rheumatoid arthritis was supported in the antigen-induced mouse model of arthritis, where genetic ablation of cathepsin L reduced the severity of the disease. However, this was not found to be associated with the ECM degradation, but rather by influencing the selection of T helper cell populations in the thymus [162].

4.3. Heart diseases

Simultaneous with the findings that several cathepsins, in particular S, V and K, are very potent elastases (see above), the interest in cathepsins as disease-modifying factors in cardiovascular diseases has emerged and Sukhova et al. detected significantly elevated levels of cathepsins S and K in human atheroma [117]. A number of subsequent studies also detected significantly elevated levels of cathepsins B, L, S and K in the atherosclerotic plaques. The latter are invaded with macrophages and smooth muscle cells, which are the main source of extracellular cathepsins in the atheroma. Moreover, localization of cathepsins also coincides with atheroma regions with increased elastin degradation, which all point towards cathepsins as important mediators of atherosclerosis [133,163]. However, a major support for the role of different cathepsins as major effector proteases in atherosclerosis came from animal studies. Ablation of cathepsin S thus significantly reduced the signs of disease in the LDL receptor knock-out mice including degradation of ECM proteins [164]. Similar results were obtained by pharmacological inhibition of the enzyme in another animal atherosclerosis model, the ApoE knock-out mice [165]. Moreover, ablation of cathepsins L and K also reduced the symptoms of the disease, indicating that multiple cathepsins are involved [166,167]. However, the role of cathepsins in atherosclerosis is not limited to ECM degradation, but is also linked to the immune response. In addition to atherosclerosis, cysteine cathepsins were found to be important also for other cardiovascular diseases, such as abdominal aortic aneurisym (AAA), atrial fibrillation, hypertensive cardiac hypertrophy and cardiomyopathy [168,169]. There is increasing amount of information especially about the role of cathepsins S, L/V and K in AAA, which is likely associated with their elastolytic properties [168,170–172]. In addition, cathepsins were found to have a potential as plasma biomarkers in these diseases [173,174].

4.4. Cancer

Since the discovery that cathepsin B is associated with metastatic potential of tumor cells [175], cysteine cathepsins have always been tightly linked with cancer research. Cathepsin B remained the cathepsin most often associated with various processes of tumor progression followed by cathepsins L, S, H and more recently K and also X. Most of the early studies were linked with evaluation of the cathepsin role as ECM-degrading enzymes, largely based on *in vitro* cleavages of ECM proteins. In addition, due to their overexpression and secretion, cathepsins are considered potent prognostic and diagnostic markers for numerous cancer types. Usually, elevated levels of serum cathepsins were associated with poor prognosis for patients [52,132,176–183]. A major breakthrough was achieved when pharmacological inhibition of cathepsins by a broad spectrum epoxide type irreversible inhibitor significantly delayed various stages of tumor progression in the pancreatic islet tumors mouse model [184], a finding confirmed by a reversible broad spectrum cathepsin inhibitor VBY-285 [185]. Moreover, combination of cathepsin inhibition with chemotherapy significantly enhanced the efficacy of chemotherapy in this tumor model [186]. Similarly, cathepsin inhibition was found to delay tumor progression in the mammary gland mouse tumor model [187]. In addition, tumor progression was significantly delayed by genetic ablation of cathepsins B, L, H and S in the mammary gland and/or pancreatic islet model [188–191], whereas cathepsin L was found to have an antitumorigenic function in the squamous carcinogenesis mouse model [192], suggesting that the cathepsins may have different and even opposing roles in cancer progression, depending on the cancer type. Cathepsins in the tumor microenvironment originate from tumors as well as tumor-associated cells. Although tumor cells are probably the first that start secreting cathepsins, the major source of extracellular cathepsins are tumor-associated macrophages (TAMs) that are known to promote tumor invasion [193]. Moreover, they are also significantly contributing to sustained inflammation, one of the hallmarks of cancer progression, where immune cells such as macrophages are recruited to the invasive front of the tumor. Inhibiting such macrophage-derived cathepsins in combination with standard chemotherapy enhanced the therapeutic efficacy of

taxol in breast cancer model, supporting the idea of a major role of macrophages and cathepsins in reducing the efficacy of chemotherapy [194]. Another important factor contributing to extracellular cathepsin-mediated ECM degradation is the slightly acidic pH of the tumor microenvironment because of the hypoxia-induced glycolysis [195]. However, it is now clear that cathepsins have a far more complex role in cancer than just the degradation of ECM proteins [81,196], but we need to identify the signaling pathways that cathepsins trigger in the tumor microenvironment for a better understanding of their function.

4.5. Other diseases

There is considerable evidence that cathepsins contribute to ECM degradation in a number of other diseases, including those associated with the lungs such as lung fibrosis [197,198], lung emphysema, bronchial asthma [199] and even cystic fibrosis, although the latter seems to be associated with the disease by blunting the innate immune response through cathepsin S-mediated degradation of the surfactant protein A [200]. In addition, due to their critical role in MHC II-mediated antigen processing and presentation, cathepsins have been found to be linked to a number of inflammation-associated diseases, which has been discussed elsewhere.

5. Therapeutic targeting of cathepsins

Because of their important roles in various diseases, several cathepsins have been considered as valuable therapeutic targets. Among them the most important are cathepsins K and S. Most work has been done on cathepsin K, which was identified as the main bone degrading protease in the osteoclasts and thereby the major target for osteoporosis [6,8–10,14,129,201–203]. Currently, there are two cathepsin K inhibitors in advanced clinical trials for osteoporosis, odanacatib (Merck & Co., Inc., Whitehouse Station, NJ, USA) and ONO-5334 (Ono Pharmaceutical Company, Osaka, Japan).

Odanacatib is a nonbasic nitrile, highly selective for cathepsin K with high metabolic half-life. Furthermore, odanacatib showed significantly higher selectivity in cellular assays than balicatib and relacatib [204,205], and was the reason for the discontinuation of clinical studies with balicatib. The latter was namely found to be lysosomotropic due to its basic character, which resulted in nonselective inhibition of cathepsin S in cellular assays and adverse effects [206]. The results from Phase I and II clinical studies showed that odanacatib is generally well tolerated and without side effects with very favorable pharmacokinetic properties that support once a week oral administration. The studies further showed that based on clinical biomarkers odanacatib decreased bone resorption, whereas bone formation was maintained. In addition, the drug increased estimated bone strength at both the hip and spine [207–210]. Moreover, the first results from a Phase III trial showed a robust antifracture efficacy and a favorable benefit-risk profile in a study that recruited 16,000 women with post-menopausal osteoporosis. Based on these results, the study was closed early for efficacy and Merck is expected to start regulatory filings in the U.S., Europe and Japan. Anyhow, for real market success odanacatib still has to demonstrate substantial advantages in antifracture efficacy or safety compared to the current generic drugs, which are reasonably effective and inexpensive [211]. The first results are encouraging as odanacatib exhibited superior properties in a head-to-head comparison with alendronates in ovariectomized rhesus monkey model [130].

ONO-5334 is a ketone type inhibitor, which showed a statistically significant and persistent increase of trabecular and integral bone mineral density at the spine and the hip without adverse effects in extended Phase II trials over a 24 month period [212]. The drug is currently in Phase III clinical trials [202]. In 2012, Medivir launched Phase I clinical trials for another cathepsin K inhibitor, MIV-711, and the first results presented in October 2013 were encouraging, demonstrating that the drug was well tolerated at the doses where a substantial reduction of bone and cartilage degradation biomarkers was observed in serum and urine (www.medivir.se). In addition to osteoporosis, cathepsin K is also considered a highly relevant target for osteoarthritis and other bone-related disorders including bone metastasis [6,9,83,202,203].

Although cathepsin S is a potent elastase and ECM-degrading enzyme, development of cathepsin S inhibitors is largely focused on inhibiting its critical role in invariant chain processing and MHC II immune response in several inflammation-associated diseases, including rheumatoid arthritis, psoriasis and cancer. Celera was the first company to initiate a Phase I clinical trial for a cathepsin S inhibitor for psoriasis treatment [6,9], which was, however, halted. In 2013, Virobay Inc. (Menlo Park, CA), a spinout of Celera which has a very active cathepsin program, entered Phase I clinical trials for psoriasis (VBY-891; in partnership with LEO Pharmaceuticals) and neuropathic pain and autoimmunity (VBY-036) with the next generation of cathepsin S inhibitors (http://www.virobayinc.com/index.php). However, preclinical work also supports cathepsin S inhibition relevant for preventing ECM degradation, in particular in atherosclerosis [163,165] and autoimmune disorders such as rheumatoid arthritis [213], so we may expect progress also in this direction.

In addition, a selective reversible cathepsin B inhibitor (VBY-376; Virobay Inc.; http://www.virobayinc.com/VBY-376.php) has been tested in Phase I clinical trials for liver fibrosis and the first results are encouraging. However, this is not linked with the role of cathepsin B in ECM degradation, but more with preventing the death of hepatocytes and development of hepatic stellate cells.

All other approaches for targeting cathepsins are currently in preclinical development.

6. Perspectives

Although a major role of cathepsins in the degradation of ECM and diseases linked with it has been found ~30 years ago, it is now clear that the extracellular functions of cathepsins are much more complex and not limited to ECM degradation only. As with metalloproteases [214,215], development of new technologies, in particular proteomic-based, will enable identification of novel physiological substrates of cathepsins that we know surprisingly little about despite very good knowledge about their biochemistry and specificity. Moreover, when these findings will be combined with advanced cellular and *in vivo* studies, we will understand the signaling pathways governed by the cathepsins that will also be of major help in combating diseases, where cathepsins play a major role.

Acknowledgements

The work has been supported by grants from the Slovene Research Agency (P1-0140 and J1-3602) to B.T. We would like to thank Barbara Sobotič and Dušan Turk for help in Figure preparation.

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