Cysteine Cathepsins and Their Extracellular Roles: Shaping the Microenvironment

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Abstract: For a long time, cysteine cathepsins were considered primarily as proteases crucial for nonspecific bulk proteolysis in the endolysosomal system. However, this view has dramatically changed, and cathepsins are now considered key players in many important physiological processes, including in diseases like cancer, rheumatoid arthritis, and various inflammatory diseases. Cathepsins are emerging as important players in the extracellular space, and the paradigm is shifting from the degrading enzymes to the enzymes that can also specifically modify extracellular proteins. In pathological conditions, the activity of cathepsins is often dysregulated, resulting in their overexpression and secretion into the extracellular space. This is typically observed in cancer and inflammation, and cathepsins are therefore considered valuable diagnostic and therapeutic targets. In particular, the investigation of limited proteolysis by cathepsins in the extracellular space is opening numerous possibilities for future break-through discoveries. In this review, we highlight the most important findings that establish cysteine cathepsins as important players in the extracellular space and discuss their roles that reach beyond processing and degradation of extracellular matrix (ECM) components. In addition, we discuss the recent developments in cathepsin research and the new possibilities that are opening in translational medicine.

Keywords: cathepsin; inflammation associated disease; cancer; osteoporosis; extracellular matrix

1. Introduction

Cysteine cathepsins are an important group of proteases that regulate numerous physiological processes and are normally found in high concentrations in endosomes and lysosomes where they are crucial for protein breakdown and major histocompatibility complex (MHC) class II-mediated immune responses [1,2]. There are 11 cathepsins encoded in the human genome (B, C, F, H, K, L, O, S, V, W, and X) [3], and different studies have shown that a number of them have largely overlapping specificities [4–7]. Nevertheless, there are several examples of specific proteolytic functions of cathepsins demonstrating that their roles are not limited to the endolysosomal system. Cathepsins have thus been found in the cytoplasm, cell nucleus, and the extracellular space [8,9], and their extra-lysosomal localization and activity is frequently associated with ongoing pathological processes [10]. Moreover, high cathepsin activities, especially in extracellular spaces, are a hallmark of inflammation that often accompanies different diseases including cancer, arthritis, cardiovascular...
disease, and bone and joint disorders as a consequence of dysregulated localization, activation or transcription, as well as inhibitor imbalance [10–12].

Extracellular cathepsins have been shown to participate in extracellular matrix remodeling by degrading abundant structural components of the extracellular matrix (ECM) (e.g., collagen or elastin), but their extracellular functions go beyond simple proteolysis [13–15]. Accordingly, cathepsins have been found to be involved in the processing of cytokines and chemokines, thereby representing an important bridge between inflammation and diseases like cancer and psoriasis [16–20]. Moreover, other more specific functions of cathepsins were reported recently, and cathepsins were thus found to shed a group of extracellular receptors and cell adhesion molecules, demonstrating that their limited extracellular proteolysis could not only directly impact the cell surface but also influence intracellular signaling pathways (e.g., kinase receptor signaling), thereby contributing to the disease progression [21]. In addition, cathepsin S was identified as the critical sheddase of the membrane-anchored chemokine fractalkine, thereby critically contributing to the neuropathic pain [22,23].

With the emerging novel roles of cathepsins, new possibilities are opening for the development of diagnostic and therapeutic tools that will further improve our understanding of their extracellular roles and support of translational medicine. In this review, we focus on cysteine cathepsins and their roles in limited proteolysis in the extracellular space. We describe their roles in pathologies, highlight their most important disease-specific extracellular substrates, and discuss how the new findings can translate into improved diagnostic and therapeutic tools. We also discuss the future perspectives of cathepsin research that will benefit from the emerging systems biology approaches.

2. Cysteine Cathepsins: Structure, Function, and Regulation

Cysteine cathepsins belong to the papain family of cysteine proteases sharing the typical papain-like fold, which is composed of two domains (L—left domain, R—right domain) that form the active protease with the catalytic Cys-His ion pair located in the active site cleft on opposite sides (Cys25-His159; papain numbering) [24] (Figure 1A). In general, cysteine cathepsins are monomers with a MW in the 20–35 kDa range, with variations being the consequence of different posttranslational modifications (e.g., glycosylation). The only exception is the tetrameric cathepsin C with a MW of 200 kDa. Several cysteine cathepsins, including cathepsins B, C, F, H, L, O, and X, are ubiquitously expressed in human tissues and cells, whereas cathepsins K, S, V, and W have more specific localization due to their more specific functions (Table 1). Cathepsin K is expressed in osteoclasts and synovial fibroblasts, cathepsin S is expressed predominantly in immune cells, cathepsin V in thymus and testes, and cathepsin W in CD 8+ lymphocytes and natural killer (NK) cells [1,3,12]. All cathepsins are synthesized as proenzymes, and, after activation, their activity is kept under tight control by pH, compartmentalization, and by their endogenous protein inhibitors stefins, cystatins, kininogens, thyropins, and serpins, which are important for the fine-tuning of their proteolytic activity [25,26].

The majority of cathepsins are potent endopeptidases; nevertheless, some cathepsins have exopeptidase activities due to loops and propeptide regions that limit the accessibility of the active site. Accordingly, cathepsins B and X are carboxypeptidases and cathepsins C and H are aminopeptidases, although cathepsin B can also have endopeptidase activity at a neutral pH [2]. Moreover, cathepsins are not very specific enzymes with highly similar substrate specificities and a moderate preference for cleavage after basic and hydrophobic residues. The only substrate recognition site that actually forms a defined pocket is the S2 site, which, together with the S1 and S1’ sites, seems to be the major substrate recognition site. Several studies using combinatorial peptide libraries and proteomic approaches have demonstrated a strong preference for small hydrophobic amino acid residues (Leu, Val, Ile) in the P2 position, although aromatic amino acid residues (Phe, Tyr) are also accepted (Figure 1B). However, there are a few exceptions. The first is the acceptance of Pro in the P2 position of cathepsin K, which is important for the collagenolytic activity of the latter. The second is the acceptance of Arg in the P2 position of cathepsin B [4–7,24]. The broad specificity of cathepsins is in good agreement with their roles in protein turnover and degradation, including in antigen processing. On the other hand,
such broad substrate specificity combined with the high proteolytic efficiency ensures that even in unfavorable conditions, cathepsins can have major roles not only in protein degradation but also in more subtle protein processing, thereby having more signaling roles. This is supported by numerous examples where several cathepsins were found to cleave their protein substrates at the same or similar cleavage sites, although there were also examples of substrates like collagen, osteocalcin, cytokines, and chemokines, which were only cleaved by a subset of cathepsins [10,14].

Table 1. Cysteine cathepsins. Overview of cysteine cathepsins, their peptidase activities, expression, and gene names according to the HUGO Gene Nomenclature Committee.

| Cysteine Cathepsins. | Gene Name | Peptidase Activity | Expression |
|----------------------|-----------|--------------------|------------|
| Cathepsin B          | CTSB      | Carboxydipeptidase, Endopeptidase | Ubiquitous |
| Cathepsin C          | CTSC      | Aminopeptidase      | Ubiquitous |
| Cathepsin F          | CTSF      | Endopeptidase       | Ubiquitous |
| Cathepsin H          | CTSH      | Aminopeptidase, Endopeptidase | Ubiquitous |
| Cathepsin K          | CTSK      | Endopeptidase       | Osteoclasts [27] |
| Cathepsin L          | CTSL      | Endopeptidase       | Ubiquitous |
| Cathepsin O          | CTSO      | Unknown             | Ubiquitous |
| Cathepsin S          | CTSS      | Endopeptidase       | Antigen-presenting cells (e.g., dendritic cells, B-cells) [28,29] |
| Cathepsin V          | CTV       | Endopeptidase       | Thymus, testis [30,31] |
| Cathepsin W          | CTSW      | Unknown             | Natural killer cells, cytotoxic T cells [32] |
| Cathepsin Z (Cathepsin X) | CTSZ | Carboxymonopeptidase | Ubiquitous |

If not otherwise stated, expression profiles are from ref. [3].

Figure 1. Cysteine cathepsin structures and specificities. (A) Crystal structure of the two-chain form of cathepsin L (PDB 1icf [33]) in standard orientation colored according to surface hydrophobicity (red: most hydrophobic, blue: most hydrophilic). Active site Cys25 is colored in yellow. (B) Substrate specificity of different cysteine cathepsins relative to the cleavage site, which is between the P1 and P1’ residues. S4–S1 and S1’–S2’ represent the substrate binding sites into which the corresponding substrate residues P4–P2’ bind, with P4–P1 designating the substrate residue N-terminals of the cleavage site and P1’–P2’ designating residue C-terminals of the cleavage site, respectively. Amino acid residues of the substrate, which bind to their designated sites, are shown with colored circles, with each color representing a different amino acid class required for the binding of the substrate to the active site.
For their optimal activity, cysteine cathepsins require reducing and mildly acidic conditions and, except cathepsin S, all are irreversibly inactivated at a neutral pH with cathepsin L being the most unstable [34–37]. While these findings originate mostly from in vitro assays, different components in the extracellular milieu and ECM building blocks can stabilize or alter cathepsin activity in the extracellular milieu. A good example is the glycosaminoglycans (GAGs) that can stabilize cathepsins at a neutral pH [38]. Moreover, experiments have shown that GAGs and other negatively charged surfaces enable autocatalytic activation of cathepsins even at neutral pH and possibly contribute to the cathepsin activity in the extracellular space [39]. Nevertheless, effects can be substantially different. Accordingly, the collagenolytic activity of cathepsin K is reduced in case of dermatan sulfate, heparan sulfate, and heparin, but GAGs like keratan and chondroitin sulfates can potentiate it [40]. Another good example is chondroitin sulfate, which inhibits the elastolytic activity of cathepsins V, K, and S [41]. In addition, very potent regulators of cysteine cathepsins are their endogenous inhibitors: cystatins (stefins, cystatins, and kininogens), thyropins, and serpins. They inhibit most of the cathepsins with very high affinities in the nM to pM range. While stefins are essentially cytosolic, the others are primarily extracellular, and their main function is to block the cathepsins escaped into the extracellular milieu, thereby acting as emergency-type inhibitors [1,26,42].

3. Extracellular Cysteine Cathepsin Origins

There is long-lasting evidence that cysteine cathepsins can be present in the extracellular milieu. Under physiological conditions, they are commonly involved in the processes of wound healing, bone remodeling, and prohormone activation [9,43]; however, their extracellular localization is far more common in different pathological conditions [10,12,44]. Prolonged extracellular cathepsin activity upon their secretion is unusual since they have optimal stability under acidic conditions. Nevertheless, the loss of activity at neutral pH can at least partly be delayed by secretion in zymogen form in which they remain relatively stable until their activation [45,46]. Apart from that, cysteine cathepsins can be secreted in an active form and their concentration is sufficient for ECM degradation [47]. An important factor for their secretion is also the type of cell. High levels of cathepsins are most commonly secreted by different immune cells [48], which is in agreement with the fact that cathepsins are elevated in inflammation [49]. In addition, keratinocytes, osteoclasts, smooth muscle cells, and thyroid cells also secrete cathepsins [10]. Nevertheless, the concentration is much lower than in the case of immune cells, where it can reach up to 100 nM in the macrophage secretome [21].

Diverse cellular mechanisms and pathways were shown to be involved in the secretion of cysteine cathepsins (Figure 2A), which is often accompanied by acidification of the extracellular milieu. Experiments performed with macrophages showed that acidification can be achieved either by vacuolar-type H⁺ ATPase, which undergoes activation by protein kinase C or serotonin [50–52] or by Na⁺/H⁺ exchanger I, activated following the binding of immunoglobulin E to high-affinity immunoglobulin-ε receptor [53]. Acidification is especially pronounced in the tumor microenvironment, where tumor-associated immune cells secrete large amounts of cathepsins, and their extracellular presence is connected with more aggressive cancers and inflammation [54]. Apart from cancer, acidification was also observed to be present in advanced osteoarthritis [55] and in atherosclerotic plaques [56]. Usually, secretion of cysteine cathepsins is linked with their overexpression, a common consequence of activation of transcription factor EB [57], or signal transducer and activator of transcription (STAT) signaling pathways by activation of STAT3 or STAT6 [58,59]. Elevated expression can also be the result of extracellular stimuli provided by different cytokines and interleukins [60,61]. The main examples of cytokine-triggered overexpression and secretion are cathepsins S and K. Cathepsin S is overexpressed and secreted in its active form by human chondrocytes upon stimulation from pro-inflammatory cytokines interleukin 1α (IL-1α) and tumor necrosis factor α (TNF-α) [60], while regulation of cathepsin K expression is controlled by RANKL (receptor activator of NF-κB ligand; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells) [62]. Another factor that can act as an initiator of the positive feedback loops that
drive the cathepsin secretion are proteolytic products of ECM degradation [63], whereas elevation of intracellular levels of Ca\(^{2+}\), which triggers the fusion of lysosomes with the plasma membrane, enables secretion of cathepsins by vesicular exocytosis [64,65]. In addition, secretion of cathepsins may even result from increased concentrations of reactive oxidative species, which may lead to the permeabilization of the lysosomal membrane and the release of lysosomal proteases into the cytoplasm and further into the extracellular milieu [66,67].

![Figure 2](image.png)

**Figure 2.** Cysteine cathepsin secretion and their extracellular roles. (A) Secretion of extracellular cathepsins is often tightly connected with their overexpression and can be triggered by diverse cell signaling pathways. Overexpressed cysteine cathepsins are usually secreted with vesicular exocytosis. (B) In the extracellular milieu, cysteine cathepsins cleave different targets. Cleavages of cell adhesion molecules (CAM), cell-cell contacts, and proteins of ECM mainly influence cell adhesion and migration. Additionally, proteolytic products of these cleavages can act as signaling molecules and have an impact on cell growth, invasion, and angiogenesis. Other main target of cysteine cathepsins are cell receptors, and their cleavage can result in either constantly triggered signaling, in the case of partial trimming of the receptor, or inhibited signaling, in the case of a complete removal of the extracellular domain. CAM, cell adhesion molecules; ECM, extracellular matrix; IL, interleukin; RANK, receptor activator of NF-κB ligand; STAT, signal transducer and activator of transcription; TFE, transcription factor EB; TNFα, tumor necrosis factor alpha; TNFR1, tumor necrosis factor receptor 1.

Once secreted, cathepsins can either remain bound to the plasma membrane or interact with molecules from the extracellular milieu [15]. In particular, the former may be of special importance as such membrane association may also protect the cathepsins against inactivation in otherwise unfavorable conditions of the extracellular milieu. Cathepsin S was thus shown to associate with the plasma membrane and co-localizes with αvβ3 integrin on the surface of smooth muscle cells present in the vasculature [68], but its exact binding partners remain unknown. Another cathepsin that translocates to the plasma membrane is cathepsin X, which acts as an activator of β2 integrins that are crucial for cell adhesion of dendritic cells and lymphocytes during their maturation [69–71]. Translocation of cysteine cathepsins occurs also in pathological conditions as shown for cathepsin B, which localizes to the cell membrane in cancer cells [72] either by binding to annexin II tetramers [73] or by association to the caveole site [74]. Moreover, its membrane-bound localization was correlated with shortened survival in the case of colorectal cancer [75,76].

4. ECM Proteolysis and the Cathepsins

The ECM that surrounds the cells is composed of the proteins these cells secrete [77]. The main components of ECM are structural proteins, like collagen, elastin, fibronectin and laminins, non-structural matricellular proteins, polysaccharides, mainly glycosaminoglycans and hyaluronan,
and proteoglycans [78,79]. The structure of the ECM is dynamic and depends on the equilibrium between synthesis and degradation [80,81]. Modifications of the ECM are achieved through both enzymatic and non-enzymatic processes, which can influence the stability and function of the ECM [82]. Among these processes, proteolytic processing is one of the most important mechanisms involved in the regulation of the ECM. It is therefore not surprising that a vast number of proteases were found to be involved, with major players being various metalloproteases such as a disintegrin and metalloprotease (ADAMs), matrix metalloprotease (MMPs), meprins and bone morphogenetic protein (BMP) / tolloid metalloproteases [83] and various cysteine proteases [15].

Cysteine cathepsins are the main cysteine proteases participating in the reorganization of ECM and are associated with non-specific degradation of abundant ECM proteins, which can take place both extracellularly and intracellularly following endocytosis [82]. Proteolysis of ECM proteins occurs under normal conditions, as in the case of collagen degradation in bone resorption or elastin degradation in the vascular system, as well as under pathological conditions, since ECM degradation is an influential factor in many diseases including cancer, cardiovascular diseases, and arthritis [14,15,84]. In particular, extracellular proteolysis seems to be of crucial importance, since the extracellular cathepsins can cleave a plethora of structural and functional proteins, thereby affecting not only the structural aspects of ECM but also the associated signaling pathways (Figure 2B). While non-specific and specific proteolysis by cysteine cathepsins are important for ECM remodeling in disease, one has to keep in mind that extracellular substrates of cysteine cathepsins were mostly identified in in vitro studies. Nevertheless, much evidence for their physiological significance and substrates confirming their involvement in disease development and progression has emerged just in the last decade (Table 2), and the next sections will provide a detailed overview of the extracellular roles of cathepsins in different pathologies.

### Table 2. Cathepsins in disease.

| Disease                                      | Cathepsins Involved | Cleaved Targets                                                                 | Selected References |
|----------------------------------------------|---------------------|---------------------------------------------------------------------------------|---------------------|
| Angiogenesis/Leukocyte recruitment           | K, B, L, S         | ELR (glutamate-leucin-arginin motif) chemokines/non-ELR chemokines, CD18        | [16,85]             |
| Cancer                                       | B, K, L, S, X      | Tenascin-C, nidogen-1, fibronectin, osteonectin, laminin, periostin, collagen IV, general degradation | [86–91]            |
| Cardiovascular and kidney diseases (e.g., atherosclerosis, abdominal aortic aneurysm, chronic kidney disease) | K, L, S, V         | Elastin, CX3CL, heparanase, collagen I (catK: Gly61-Lys62, Arg144-Gly145, Glu189-Gly190) | [41,92,93]         |
| Lung fibrosis                                | S                   | Decorin                                                                         | [94]                |
| Neuroinflammation/hyperalgesia               | S                   | CX6,CL1, PAR2                                                                   | [95–97]            |
| Osteoarthritis and rheumatoid arthritis       | K, B, L, S         | Collagen II (catK: Gly61-Lys62, Arg144-Gly145, Glu189-Gly190), aggrecan (catB: Asn341-Phe342, Gly344-Val345, catL: Gly344-Val345) | [98–100]           |
| Osteoporosis                                 | K, B, L, S, H      | Collagen I (catK: Gly61-Lys62, Arg144-Gly145, Glu189-Gly190), osteocalcin (catB: Arg44-Phe45, catL: Gly7-Ala8) | [101–104]          |
| Tuberculosis                                 | K                   | Collagen I (catK: Gly61-Lys62, Arg144-Gly145, Glu189-Gly190)                   | [105]               |

Diseases where cysteine cathepsins are involved in the development and progression of the pathology together with their extracellular substrates. Known cleavage sites of extracellular substrates for each cathepsin are shown in parentheses.

### 4.1. Cathepsins in Cancer

Cysteine cathepsins were historically first linked to extracellular proteolysis in cancer, as first demonstrated for cathepsin B almost 40 years ago [106]. However, despite the use of various in vivo
and in vitro cancer progression models, the exact roles of individual cysteine cathepsins are not completely understood [107–109]. A comprehensive understanding of the functions of cathepsins B, C, H, K, L, S, and X in the extracellular milieu has proved complicated because of their broad substrate specificity [1], their endogenous inhibitors [110], compensatory effects [111,112], effects that are not associated with their proteolytic function [113], their ability to act as tumor suppressors [114], and the various different cell types that comprise many tumors [115].

Cathepsins are released into the tumor microenvironment by different cells including tumor cells, endothelial cells, tumor-associated macrophages (TAM), myoepithelial cells, fibroblasts, and other cells, which infiltrate the tumor site [48,54]. Among these, TAMs are considered to release the largest amount of cathepsins primarily due to stimulation by the IL-4, IL-6, and IL-10 cytokines in the tumor microenvironment [10,48]. Interestingly, the source and role of secreted cathepsins responsible for cancer progression are not universal among different types of cancer, as evident primarily from different mouse in vivo cancer models. In the RIP1-Tag2 model of the pancreatic neuroendocrine cancer, cathepsins B, H, and S, derived from the TAMs, had a predominant, if not exclusive, effect on cancer progression through the reduction of tumor burden, increase in apoptosis, and decrease of angiogenesis [48]. On the other hand, cathepsin L-mediated cancer progression was largely due to its secretion from cancer cells, whereas the effect of cathepsin X was due to its release from both sources. Moreover, cathepsin X was found to partially compensate for the loss of cathepsins B and S, as revealed by simultaneous deletion of the two cathepsin genes. In addition, simultaneous deletion of cathepsins B and S revealed additive effects in early stages, but at late stages, several differences were restored to the wild-type level, although the mechanisms are not known [111,113]. Interestingly, expression of cathepsin C was also increased in RIP1-Tag2 and MMTV-PyMT mammary gland models of cancer but had no functional role in their progression [109,116]. Cathepsin S was also found to have a major role in a syngeneic colorectal carcinoma murine model, where its release from cancer cells, endothelial cells, and TAMs was found to be responsible for the progression of cancer through the promotion of neovascularization and tumor growth [117]. An analogous effect was also observed in vivo in the MMTV-PyMT mammary gland cancer model, where cathepsin B, originating from both tumor cells and macrophages, was suggested to have a major role in the tumor progression and metastasis spread [107,118], which could have been partially compensated by cathepsin X [112]. However, using an orthotopic transplantation of primary mouse PyMT cancer cells overexpressing cathepsin B showed that the enzyme expressed and secreted from tumor, but not stromal, cells increased invasiveness into adjacent tissues by excessive extracellular matrix degradation [119]. Another in vivo mouse model, where the roles of individual cathepsins were systematically investigated, was the K14-HPV16 model of squamous cell carcinoma. In this model, cathepsin C, but not cathepsin B, released from stromal cells was shown to be responsible for cancer development and angiogenesis. Moreover, cathepsin L was found to have a tumor suppressor role in this model, further showing the complexity of cathepsins involvement in tumorigenesis [109].

There are also numerous cellular studies available where secretion of cathepsins was shown to be an important factor in changing cellular properties possibly leading to tumor progression, although these studies were mostly focused on tumor cell lines but not on macrophages or related immune cells. Among the cathepsins, cathepsin B was most often associated with changes of cellular properties, although there were also numerous reports about cathepsins L, X, and K. Cathepsins B and X were thus shown to be important in the epithelial to mesenchymal transition in two breast cancer cell lines, MCF7 and MDA-MB-231, which express low and high amounts of cathepsins, respectively. Upregulation of cathepsins B and X in MCF7 cells decreased E-cadherin, a marker for epithelial cells, which was mainly attributed to cathepsin B [120]. Similar observations involving cathepsin X were also reported in hepatocellular cancer where its overexpression increased invasiveness and motility in a matrix-coated transwell model and a wound healing assay [121], although the enzyme seems to be primarily expressed in the immune cells [70]. Release of cathepsins B and L was recently reported in different melanoma cell lines, where Abl/Arg nonreceptor tyrosine kinases were shown
to play an important role in cathepsin B and L expression and their release from the cells [122]. Tumor progression and increased cell invasiveness following cathepsin B release are also characteristic for pancreatic ductal adenocarcinoma [123], esophageal adenocarcinoma [124], and glioma, where cathepsins K and X were also found to be involved [125–127]. Recently, a mechanism of cathepsin B release followed by enhanced lung cancer cell migration was proposed [128], indicating a possible role in the progression of lung cancer. However, the role of cathepsins in cancer is not only extracellular but also has an important intracellular component [48,129]. An example of this is the leakage of cathepsin B to the cytoplasm following chemotherapy-induced lysosomal membrane permeabilization in myeloid-derived suppressor cells (MDSC). Upon release, cathepsin B was found to interact with NLRP3, leading to a non-proteolytic activation of the inflammasome and pro-IL-1β processing. The released IL-1β enhanced IL-17 production in CD4+ T cells, which resulted in angiogenesis and tumor relapse [130].

Unfortunately, there are almost no in vivo studies on the role of cysteine cathepsin inhibitors that could shed some more light on the possible imbalance between the endogenous inhibitors and the target proteases as one of the reasons for tumor progression, as suggested some time ago [54]. The first study revealed that genetic ablation of cystatin C, major extracellular cathepsin inhibitor, in the pancreatic neuroendocrine tumor model (Rip-Tag2) resulted in an increased size of islet cell carcinomas and angiogenic islets, which was linked to deregulated cathepsin S activity leading to increased endostatin generation [91]. The other two studies available included genetic ablation of stefin B, the major intracellular cathepsin inhibitor [131], and of cystatin C [132], in the mammary gland PyMT mouse model. However, contrary to all expectation, tumors were smaller in both inhibitor knock-out models despite the increased cathepsin activity in the tumors. While in the case of stefin B this was linked to increased sensitivity to lysosomal cathepsin-mediated cell death and oxidative stress, the potential link in case of cystatin C were the 14-3-3 proteins, but the evidence is not entirely conclusive. This further supports the idea of differential and context-dependent roles of cathepsins and their inhibitors in cancer.

Nevertheless, it seems that the major role of cathepsins in cancer is in extracellular matrix degradation [87], shedding of receptors and adhesion molecules [21,85], activation of cytokines and growth factors [48], and cleavage of proteins forming cell-cell junctions [133]. In addition, cathepsins were also suggested to be involved in the activation of other tumor-associated proteases, although the evidence is primarily based on in vitro studies. An example is cathepsin B, which was suggested to be involved in the activation of urokinase-type plasminogen activator (uPA), which influences uPA/uPAR signaling, thereby possibly affecting cell migration, whereas cathepsin L was suggested to be involved in the activation of MMP1 and MMP3 [13,54].

Despite many phenotypic changes following cathepsin ablation or inhibition in in vivo and in vitro tumor models, the exact roles, besides the degradation of extracellular matrix, are less-well known. This is supported by a recent proteomic study where individual genetic ablation of cathepsins B, H, L, S, and X in the Rip-Tag2 pancreatic tumor model was shown to have a predominant effect on the degradation of the extracellular matrix, with very few limited proteolysis events detected [87]. Nevertheless, limited proteolysis of E-cadherin, an important adhesion molecule and marker for epithelial phenotype, by cathepsins B, L, and S was shown to potentially drive invasiveness of cancer cells in the same RIP1-Tag2 model [116]. There are several other substrates of individual cathepsins identified in different cancer models, but their importance for cancer progression is unclear. Among these targets is CD18, which was found to be cleaved by cathepsin B released from adhering leukocytes following physiological levels of shear stress [134] and may have a role in leukocyte recruitment in angiogenic vessels [85]. Another set of substrates of extracellular cathepsins associated with angiogenesis are basement membrane proteins, which are cleaved during basement membrane degradation, in mother vessels formation, following vascular endothelial growth factor A (VEGF-A) stimulation [135]. One of these is nidogen-1, which was found to be degraded by cathepsin S in patients with non-small cell lung cancer [136]. In addition, cathepsins B, L, and S were reported to
Cleave laminin, with cathepsin S generating a fragment with pro-angiogenic effects [91]. Cathepsins B, L, and S can also cleave fibronectin, the results of which are still poorly understood [15,88]. Another such substrate is tenasin-C, which was found to be cleaved by cathepsin B, resulting in a pro-angiogenic effect in glioma [86]. An important cathepsin substrate also seems to be collagen XVIII, which can serve as a source of endostatin that can be generated by cathepsins L and S, thereby affecting angiogenesis [137,138]. Finally, cathepsin K was demonstrated to cleave peristin, which may be linked to breast cancer bone metastasis. The decrease of C-terminal intact peristin was namely shown to be a marker for osteolytic lesions in breast cancer bone metastasis and could be used to detect bone relapse in patients [89]. Cathepsin K cleavage of osteonectin was also implicated in experimental prostate to bone tumor metastasis, where both the protease and substrate expression levels were higher. Additionally, high levels of released inflammatory cytokines suggested that cathepsin K and/or osteonectin may regulate their release [90]. Some additional cathepsin ECM substrates linked to cancer have also been described elsewhere [15,82]. A more detailed overview on the roles of cysteine cathepsins in cancer development and progression can be found elsewhere [48].

4.2. Cysteine Cathepsins and Tissue Remodeling

The role of cathepsins in bone and cartilage processing is extremely well described, with cathepsin K being the most studied. The main source of cathepsin K in these processes are osteoclasts. Cathepsin K is one of the few proteases capable of cleaving the collagen triple helix in the polyproline region, and its activity differs from metalloproteases. It has multiple cleavage sites in the polyproline region of both collagens I and II [102,139] and can also cleave collagen in the telopeptide region [101]. Collagenolytic activity of cathepsin K is strong in the acidic environment at the site where osteoclasts attach to the bone surface and is crucial for normal bone homeostasis [14]. It has been shown that inhibition of cathepsin K can result in an altered structure of the bone, leading to changed crystallinity and crystal structure [140]. Another factor that influences cathepsin K activity is the presence of GAGs, since its collagenolytic activity is completely dependent on the formation of an oligomeric complex between cathepsin K and GAGs [141,142]. Aging-associated changes of collagen fibers were also suggested to influence the collagenolytic activity of cathepsin K. Accumulation of advanced glycation end-products (AGEs) and mineralization were thus shown to reduce cathepsin K collagenolytic activity, while removal of GAGs completely blocked it [143]. Pathological collagen degradation is connected with cathepsin K overexpression and unbalanced osteoclast/osteoblast activation, leading to collagen I degradation and bone loss as seen in osteoporosis [144]. While cathepsin K is the most important cathepsin in bone and cartilage remodeling, one must keep in mind that in vitro studies have identified cathepsins B, L, H, and S as potentially being involved in bone remodeling since they can cleave osteocalcin [103], a biomarker of bone degradation in osteoporosis [145].

Other pathological conditions with similar causes are osteoarthritis and rheumatoid arthritis, where cathepsin K degrades collagen II from the N-terminus, which leads to cartilage erosion [99,102]. Moreover, the low pH, as observed in advanced osteoarthritis, favors cathepsin K as a major collagenase in these diseases [55]. In addition, cathepsin K can cleave the proteoglycan aggrecan, which is also cleaved by cathepsins B, L, and S [98,100], and ECM bone protein osteonectin [104], further supporting its crucial role in osteoarthritis. However, in vivo studies in mouse models showed that disease progression was substantially diminished in cathepsin S-deficient animals in collagen-induced arthritis and in cathepsin L-deficient animals in antigen-induced arthritis [29], suggesting that other cathepsins are also involved in the arthritis progression. Moreover, substantial levels of cathepsins B, L, and S were identified in synovial fluids of patients with rheumatoid arthritis and osteoarthritis, with higher levels detected in rheumatoid arthritis patients, suggesting their involvement in inflammation and cartilage destruction [146–150]. However, despite high serum levels of cathepsins S and L in rheumatoid arthritis patients, they did not correlate with the severity of the disease, arguing against their use as disease biomarkers [151]. Anyhow, substantial differences in the serum levels of these two cathepsins were observed between the rheumatoid arthritis patients with and without autoantibodies,
suggesting specific roles of cathepsins S and L in disease development and progression in seropositive patients [150].

Cysteine cathepsin-mediated ECM remodeling is also important in the cardiovascular system, where cathepsins K, S, and V can cleave elastin, with cathepsin V displaying the highest elastolytic activity [84]. This is a result of two unique exosites present in its structure, which stabilizes the elastin-cathepsin V complex [92]. Since elastin is responsible for tissue stability against stretching forces, its fragmentation decreases the elasticity of the blood vessels and can lead to their rupture [152]. Excessive elastin degradation was observed in cardiovascular diseases, atherosclerosis and abdominal aortic aneurism, kidney diseases, and during aging [15,84], as upon reaching adulthood, elastin is not synthesized de novo anymore, and consequently its degradation is irreversible [153]. Besides cathepsin S, cathepsin K and V also exhibit elastinolytic activity important in cardiovascular pathology, and their ablation in mice showed decreased signs of atherosclerotic pathology [154–156]. Elastin degradation by cysteine cathepsins occurs mainly extracellularly, with only one-third of elastin being degraded intracellularly [41]. However, not all functions of extracellular cysteine cathepsins in the cardiovascular system are harmful. A recent study has shown that cathepsin K has a pivotal role in cardiac remodeling following myocardial infarction, since sufficient collagen degradation is needed to reduce cardiac fibrosis [93]. However, processing of membrane-bound chemokine fractalkine (CX3CL1 or (C-X3-C motif) ligand 1) by cathepsin S homes additional inflammatory cells to the site of atherogenesis, thereby sustaining the inflammation and progressing the pathology [157]. The ECM degradation capability of cathepsin B is also considered to be a marker of the disease, whereas cathepsin X has a role in homing the inflammatory cells, especially T-cells, to the atheroma site [158].

Cysteine cathepsins B and L have an important role in neural tissue remodeling, especially in axon growth, but exact mechanisms remain mostly unknown [159,160]. Nevertheless, both can cleave perlecan, and this cleavage generates a C-terminal fragment with neuroprotective roles [161]. Another role of cathepsin B is the degradation of chondroitin sulfate proteoglycans (CSPGs) [160], which have inhibitory effects on axon growth and regeneration transmitted by receptor protein tyrosine phosphatase σ (RPTKσ) [162]. A recent study found that regulation of cathepsin B expression and secretion by intracellular sigma peptide (ISP)-modified RPTKσ signaling can possibly lead to axon overgrowth in a CSPG-rich environment [160]. Another study also identified cathepsin B as a myokine that is systemically secreted during running and can cross the blood-brain barrier. This running-induced, systemically secreted cathepsin B induced neurogenesis, improved spatial memory, and also influenced plasticity, cell survival, differentiation, and neuronal migration [163]. In addition, in vitro experiments have also identified extracellular cathepsin L as a potential stimulus of axonal growth [159].

Cysteine cathepsins also participate in lung pathologies [164]. In silicosis, cysteine cathepsins B, H, K, L, and S were found in large excess compared to their inhibitors in the patient's bronchoalveolar lavage fluid (BALF) and are likely involved in the breakdown and remodeling of the ECM [165]. Recently, cathepsin K overexpression and release was also found in lymphangioleiomyomatosis (LAM), a rare nodule-forming disease. Interestingly, cathepsin K was overexpressed and released from fibroblasts associated with LAM cells similar to tumor cell–stroma interaction and could be the driving factor behind matrix degradation and cytokine processing [166]. In the lung, cysteine cathepsin activity is involved in tuberculosis where cathepsin K is one of the collagenolytic proteases that degrade the ECM and cause the formation of lung cavities [105,167]. On the other hand, cathepsin K overexpression and extracellular activity warranted protection against bleomycin-induced pulmonary fibrosis. Additionally, cathepsin K knock-out mice deposited more extracellular matrix and had decreased collagenolytic activity, which points to an important role of cathepsin K in lung collagenolytic activity [168,169]. The same model also provided evidence for cathepsin B overexpression and presence in BALF [170]. Furthermore, cathepsin S was shown to cleave decorin and produce a fragment, which can be robustly detected in the serum of fibrosis and cancer patients [94]. Finally, pharmacological inhibition of cathepsin S was shown to substantially reduce a cystic fibrosis-like disease in a mouse.
model, possibly via a link to protease-activated receptor 2 (PAR2) [171], which was previously reported to be a cathepsin S substrate [172].

More examples of cathepsin-mediated tissue remodeling are scattered throughout tissue types. In the case of pre-adipocyte cells, cleavage of fibronectin by cathepsin S was suggested as a possible mechanism for their differentiation [173]. Recent in silico and in vitro studies reported that cathepsins K, L, and S could cleave fibrinogen and fibrin, resulting in fragments that differ from the ones produced by plasmin, which opened the door to further investigate their roles in vascular homeostasis, especially in coagulation-associated diseases [174,175]. Moreover, cathepsin K was recently demonstrated to have an essential role in skeletal muscle remodeling, dysfunction, and fibrosis following injury [176]. The roles of cysteine cathepsins in tissue remodeling are diverse and can be both detrimental and beneficial; therefore, cysteine cathepsins are emerging as possible targets in therapeutic strategies.

4.3. Cysteine Cathepsins in Inflammation

Cysteine cathepsin secretion often accompanies different inflammation-driven pathologies where they are released from recruited immune cells or aberrantly expressed or processed in the inflamed tissue. Because of this general mechanism, there are numerous organ systems or tissues where cathepsins can be detected extracellularly during inflammation, and their extracellular localization has been implicated in different aspects of the immune cell physiology [10]. Extracellular cathepsins, in particular cathepsins L, S, and K, were shown to process and activate the glutamate-leucin-arginin motif (ELR) and inactivate the non-ELR CXC (N-terminal Cys-X-Cys motif) chemokines, thereby regulating chemotaxis and angiogenesis [16]. In the case of cathepsin S, its extracellular localization can influence macrophage and monocyte migration through the basal membrane [154]. The arginin-glycin-aspartate (RGD) motif in procathepsin X is responsible for its binding to integrin $\alpha_v\beta_3$, thus modulating the binding of cells to the ECM components [177]. In intestinal goblet cells, cathepsin K is highly expressed and released and provides an antimicrobial effect. As a result, cathepsin K-deficient mice exhibit more severe colitis and have an altered microbial community [178]. During inflammation, a crosstalk is established with the coagulation cascade where fibrin, which can be cleaved by cathepsins K, L, and S in vitro, plays an important role [179].

Moreover, it seems there is also a connection between the nervous and immune systems, where cathepsins were suggested to play a role [180]. At this neuroimmune interface, cathepsin S has been shown to cleave PAR2 in a different pattern as previously described for serine proteases, inducing hyperalgesia [96]. Additionally, cathepsin S cleavage of CX3CL1 from neurons results in microglial stimulation, which is critical in chronic pain maintenance [181]. Furthermore, microglia cells were shown to secrete cathepsin B, S, and X, which are considered important in inflammation-induced neurodegeneration [182].

Cysteine cathepsins also play a role in both acute and chronic phases of kidney disease, albeit there are currently more known intracellular functions compared to extracellular ones [183]. Nevertheless, cathepsin L-mediated heparanase activation was shown to play a role in the pathogenesis of diabetic nephropathy, causing proteinuria and renal damage [97]. Finally, cathepsin S elastolytic activity was also implicated in the occurrence of calcifications during the pathogenesis of chronic kidney disease [184].

5. Extracellular Cathepsins and Their Translation into Clinical Applications

Elevated activity of cysteine cathepsins in the extracellular space is now widely recognized as an important hallmark of developing or ongoing disease. Therefore, cathepsins are getting increasing attention in the development of novel therapeutic and diagnostic tools (Figure 3). In particular, therapeutic inhibition of cathepsins was the driving force in the field since the discovery that cathepsin K has a crucial role in bone resorption and thus in osteoporosis [37,104,185,186], and that cathepsin S is the key enzyme in the MHC II-mediated immune response [29,31]. A number of small-molecule inhibitors of cathepsins were developed, and despite several inhibitors of cathepsin K and S showing
good initial results for treatment of osteoporosis, aortic aneurysm, arthritis, and neuropathic pain, none has entered clinical use so far, and very few are in clinical trials at the moment [10,84]. There are several reasons for this, with perhaps the best-known being the on-target toxicity revealed in the case of cathepsin K inhibitors, which became evident after long-term treatment. This was demonstrated in the case of Odanacatib (Merck), a non-basic nitrile, which was very successful in preclinical stages and even successfully concluded phase III clinical trials, but prolonged investigations of the stroke-related side effects lead to its discontinuation [84,187,188]. However, the problem was already at least partially raised with Balicatib (Novartis), the first cathepsin K inhibitors that entered clinical trials for osteoporosis treatment and was discontinued after Phase II [188]. In order to overcome the problem, research went in the direction of exosite inhibitors of cathepsin K that would only block collagen degradation, whereas cytokine processing, such as that of TGF-β, which is active site-driven, would be unchanged. A good progress in this area was demonstrated with tanshinones, a group of so-called ectosteric inhibitors targeting the cathepsin K exosite originating from plants, which already showed good results in preclinical studies [189,190]. Currently, a lot of hope is also in the new inhibitors of cathepsin S for neuropathic pain treatment, but the outcome of the clinical studies remains to be seen [10]. Another strategy is the use of inhibitory antibodies, which demonstrated good potential for cathepsin S inhibition, resulting in reduced tumor growth and improved chemotherapeutic efficiency [191,192], and further developments are expected to be seen in this area as well.

However, the high extracellular cathepsin levels secreted from the immune cells in various inflammation-associated diseases including many cancers opened the door for in vivo diagnostic applications. Different activity-based probes (ABPs) with fluorescent tags or internally quenched substrates that start to emit the reporter signals after cathepsin cleavage were used to detect pathologic cathepsin activity [193]. There are several examples where probes were used to detect inflammation [194], visualization of cancer cells [195], and lung fibrosis [196]. Good results were also achieved with fluorogenic substrates in preclinical imaging of cathepsins, especially with the application of the reverse-design principle where medicinal chemistry-optimized small molecule inhibitors were converted to fluorescent activatable substrates [197]. A cathepsin S-selective lipidated fluorescent substrate based on this principle was successfully used for in vivo imaging of mammary gland mouse tumors [198]. Another set of tools that can be used for labeling cathepsins are designed ankyrin repeat proteins (DARPins). A cathepsin B-selective DARPin with high affinity was successfully used for in vivo imaging in two mouse models of mammary gland cancer [199]. While selective tools perform well at the preclinical level, pan-cathepsin probes were much more successful in image-guided surgery applications to visualize the tumor tissue and thus increase the chances of its complete removal [200], leading to the first compounds being evaluated in clinical trials [10].

Concepts for targeted drug delivery can also largely benefit from the elevated activity of cysteine cathepsins in disease. First, cathepsins can be used as drug activators. Accordingly, several drugs are synthesized as prodrugs or antibody-drug-conjugates (ADCs) and become active only after cathepsin cleavage. This concept has been successfully used in oncology with a good example being ADCETRIS®, which is already clinically approved [10], while several other prodrugs are at different stages of development [201,202]. Second, targeting extracellular or membrane-associated cathepsins also emerged as a promising drug delivery strategy. The power of this concept was demonstrated when cancer cell membrane-associated cathepsin B was targeted by liposomes with a selective cathepsin B inhibitor as a targeting moiety, and the system demonstrated improved selectivity and targeting efficiency [203]. However, while the majority of research has been focused on how to exploit the extracellular presence of cysteine cathepsins for medical applications, the extracellular substrate pool itself represents a major source of potential future therapeutic targets, targeting moieties, and biomarkers that will likely result in the development of new diagnostic and therapeutic strategies with major potential for future clinical applications [204].
Figure 3. Extracellular cathepsins as diagnostic targets, prodrug activators, and targets for targeted drug delivery. The high levels of cathepsins in the ECM can be utilized in different imaging and targeting techniques. (A) Fluorescent substrates, substrate-based probes, and activity-based probes are the most commonly used tools for in vivo and in vitro imaging. (B) Extracellular cathepsins can be also used for targeted drug delivery and for prodrug activation. (C) Since many cathepsins are also overexpressed and active in different cancers, they can be targeted directly using their inhibitors, designed ankyrin repeat proteins (DARPins), or antibodies. These molecules can also be conjugated and, as such, used for drug delivery and tumor visualization.

6. Concluding Remarks and Future Perspectives

Recent findings on the roles of cysteine cathepsins in the extracellular space have substantially improved our understanding of these important proteases and of their normal and pathological proteolysis in the ECM. While cathepsins are widely recognized as important diagnostic and therapeutic targets largely for the diseases that involve ECM remodeling, their multifunctional roles pose a problem in the design of successful tools for their therapeutic targeting as demonstrated by the systematic failure of a number of cathepsin inhibitors in clinical trials. Therefore, we believe
that in the future, the clinical paradigm on cysteine cathepsins will shift from targets for therapeutic intervention to diagnostic targets and targets for image-guided surgery, targets for targeted drug delivery, modifiers of the cancer cell surfaceome, and generators of cancer cell-specific fingerprints with biomarker potential, thereby leading to the development of new cathepsin-based diagnostic and therapeutic applications.

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