

Pathophysiological functions of cathepsin D: targeting its catalytic activity versus its protein binding activity?

Olivier Masson, Anne-Sophie Bach, Danielle Derocq, Christine Prébois, Valérie Laurent-Matha, Sophie Pattingre and Emmanuelle Liaudet-Coopman

IRCM, Institut de Recherche en Cancérologie de Montpellier, Montpellier, F-34298, France; INSERM, U896, Montpellier, F-34298, France; Université Montpellier1, Montpellier, F-34298, France; CRLC Val d'Aurelle Paul Lamarque, Montpellier, F-34298, France. Tel (33) 467 61 24 23; FAX (33) 467 31 37 87; E-mail: e.liaudet@valdorel.fncclcc.fr

Keywords: cathepsin D, protease, cancer, proliferation, apoptosis

Abstract

The lysosomal aspartic protease cathepsin D (cath-D) is over-expressed and hyper-secreted by epithelial breast cancer cells. This protease is an independent marker of poor prognosis in breast cancer as it is correlated with the incidence of clinical metastasis. In normal cells, cath-D is localized in intracellular vesicles (lysosomes and endosomes). In cancer cells, overexpressed cath-D accumulates in cells, where it may affect their degradative capacities, and the pro-enzyme is hypersecreted in the tumor micro-environment. In addition, during apoptosis, lysosomal cath-D is released into the cytosol, where it may interact with and/or cleave pro-apoptotic, anti-apoptotic, or nuclear proteins. Several studies have shown that cath-D affects various different steps in tumor progression and metastasis. Cath-D stimulates cancer cell growth in an autocrine manner, and also cath-D plays a crucial paracrine role in the tumor micro-environment by stimulating fibroblast outgrowth and tumor angiogenesis. A mutant D231N-cath-D, which is devoid of catalytic activity, remained mitogenic, indicating an additional action of cath-D by protein-protein interaction. Targeting cath-D in cancer may require the use of inhibitors of its catalytic activity, but also the development of new tools to inhibit its protein binding functions. Thus, elucidation of the mechanism of action of cath-D is crucial if an appropriate strategy is to be developed to target this protease in cancer. The discovery of new physiological substrates of cath-D using proteomic approaches can be expected to generate new critical targets. The aim of this review is to describe the roles of the cath-D protease in cancer progression and metastasis, as well as its function in apoptosis, and to discuss how it can be targeted in cancer by inhibiting its proteolytic activity and/or its binding protein activity.

1. Introduction

Proteases irreversibly hydrolyze the peptide bond in proteins, which results in an important and irreversible post-translational modification. The human genome encodes over 569 proteolytic enzymes or homologs, which constitute the second largest class of human enzymes. Proteases are assigned to five classes on the basis of the active site residue that executes the nucleophilic attack on the target peptide bond: aspartic, cysteine, serine, threonine and metallo-proteinases. These proteases are implicated in normal physiological processes, but deregulation of their expression and/or enzyme activity in disorders such as cancer has profound consequences.

Different families of proteases have been implicated in motility, invasion, extravasation, proliferation and metastasis: the serine proteases (uPA, uPAR and PAI1) [1], the metalloproteinase family [2], the cysteine cathepsins (cathepsin-B and cathepsin-L) [3], and the aspartic cathepsin-D (cath-D) [4], respectively. Cathepsin proteases are lysosomal hydrolases that degrade proteins at acidic pH in the lysosomes, or extracellularly in the matrix. Cathepsins can be divided into three subgroups, based on their active-site amino acid (*i.e.*, cysteine (B, C, H, F, K, L, O, S, V, X, and W), aspartate (D and E), or serine (A and G) cathepsins). The possible involvement of cysteine and aspartic cathepsins in cancer has been the subject of more debate than that of metalloproteases and serine proteases. This might result from the assumption that only secreted proteases that are proteolytically active at neutral pH play an active role in cancer, whereas cathepsins, which require a more acidic pH to be proteolytically active, are thought to be likely to play only a minor role. However, it has been demonstrated that cathepsins are hypersecreted in cancer, and cath-B and cath-D have been described as being associated with the cell surface [5, 6]. Recent studies using transgenic mouse models have stimulated fresh interest in the fundamental roles of cathepsins in cancer [7-10]. Although historically studies have tended to focus on the role of lysosomal proteases

within the endocytic and lysosomal compartments, recent discoveries have shown that these proteases play a critical role in other intracellular compartments, such as the cytosol [11] or the nucleus [12], and within the extracellular milieu in the tumoral stroma [13]. It has become clear that their pattern of expression and their substrate specificities are more complex than was originally envisaged. In cancer, lysosomal proteases are overexpressed and their cellular localizations are profoundly altered, leading to major changes in their targets and consequently in their biological activities.

In addition, cathepsins, metalloproteases and serine proteases act in a cascade-like manner and as part of a proteolytic pathway rather than simply functioning individually [14]. Elucidating the cascade of enzymatic activities that contribute to overall proteolysis during carcinogenesis may identify rate-limiting steps or pathways that could be targeted by anti-cancer treatments [14]. The proteolytic cascade of activation of the different classes of proteases strongly suggests that anti-cancer strategies intended to target several classes of proteases simultaneously might be more promising than those that target a single protease or class of proteases.

Recent studies have focused on extracellular proteases as primary targets for drug discovery, because of their differential expression in many pathophysiological processes, including cancer, cardiovascular conditions, and inflammatory, pulmonary, and periodontal diseases [3, 15]. Interestingly, new extracellular inhibitors of metalloproteases, serine proteases and cysteine proteases are currently under clinical investigation [15]. The aim of this review is to present the role of the cath-D protease in cancer progression and metastasis, as well as its function in apoptosis, and to discuss how it could be targeted in cancer by inhibiting its proteolytic activity and/or its protein binding activity.

2. Structure and trafficking of cath-D

Cath-D [E.C. 3.4.23.5] is a ubiquitous, lysosomal, aspartic endo-proteinase that requires an acidic pH to be proteolytically active (Figure 1). The human cath-D gene contains 9 exons, and is located on chromosome 11p15 [16]. During its transportation to lysosomes, the 52-kDa human pro-cath-D is proteolytically processed to form a 48-kDa, single-chain, intermediate which is an active enzyme located in the endosomes. Further proteolytic processing yields the mature active lysosomal protease, which is composed of both heavy (34 kDa) and light (14 kDa) chains. Cysteine cathepsins are known to be implicated in cath-D processing [17-19]. The involvement of cath-B and L has been shown more recently [19-21]. The human cath-D catalytic site includes two critical aspartic residues (amino acids 33 and 231) located on the 14-kDa and 34-kDa chains, respectively (Figure 1) [22]. Cath-D, like other aspartic proteases such as renin, chymosin, pepsinogen, has a bilobed structure. The crystal structures of the native and pepstatin-inhibited form of mature human cath-D [22-25] have been shown to have a high degree of tertiary structural similarity with other members of the aspartic protease family (*e.g.* pepsinogen and human immunodeficiency virus protease). The high-resolution structure of pro-cath-D remains to be elucidated. In estrogen receptor (ER)-positive breast cancer cell lines, cath-D is highly up-regulated by estrogens and growth factors (*i.e.* IGF1, EGF, insulin) [26]. In ER-negative breast cancer cell lines, cath-D is constitutively overexpressed. The mechanism for cath-D overexpression in ER-negative breast cancer cells may involve local reorganization of the chromatin structure of the cath-D promoter [27]. Cath-D overexpression leads to the hypersecretion of the 52-kDa, proteolytically-inactive pro-enzyme, and the accumulation of intracellular cath-D [28]. Release of mature cath-D by exocytosis has been observed in specialized cells [29]. Mannose-6-phosphate (M6P) receptors are involved in cath-D lysosomal routing, and in the cellular uptake of secreted pro-cath-D, although cath-D may also be targeted to the lysosomes, and undergo endocytosis independently of M6P receptors (Figure 1) [30, 31]. The LRP1 receptor has been excluded as

a possible receptor mediating the alternative endocytosis of pro-cath-D on the basis of the inability of the protein chaperone RAP, which competes with ligands that bind to the alpha chain of LRP1, to prevent cath-D endocytosis [32]. It has recently been shown that sortilin functions as an alternative sorting receptor to the M6P receptors for cath-D and cath-H [33].

3. The tight control of cath-D expression and catalytic function is fundamental in normal cells

During fetal development, the level of cath-D increases gradually in all tissues, suggesting a gradual maturation of the lysosomal system [34]. A reduction of cath-D expression or catalytic activity leads to devastating neurodegenerative disorders. Cath-D knockout mice die shortly after birth, and display a neuronal accumulation of ceroid lipofuscin, accompanied by neurodegeneration in the retina and central nervous system, and the accumulation of autophagic vacuoles [35-38]. Cath-D-deficient *Drosophila* recapitulates the key features of neuronal ceroid lipofuscinoses (NCLs) [39]. Congenital cath-D mutations leading to reduced expression of cath-D and/or the production of enzymatically-inactive protein result in typical NCL in dogs and humans [40-45]. More recently, cath-D deficiency has been shown to be associated with Parkinson disease [46].

In contrast, an increase in cath-D expression can also lead to fatal disorders. A recent study indicates that increased cardiac cath-D expression and activity induces heart failure. This is attributable to a 16-kDa cath-D-cleaved form of prolactin that mediates postpartum cardiomyopathy [47]. Increased cath-D levels have also been observed in the cerebellum of autistic subjects, suggesting that altered activities of cath-D may play an important role in the pathogenesis of autism [48].

4. Function of cath-D in apoptosis

Cysteine and aspartic cathepsins play key roles in tumor cell death via the mediation of apoptosis [4, 11, 49-51]. The function of cath-D in apoptosis needs further investigation, since this protease has both anti-apoptotic and pro-apoptotic functions.

Anti-apoptotic characteristics of cath-D

Even though cath-D gene expression outlines the areas of physiological cell death during embryo development [52], cath-D deficiency in mice has revealed its anti-apoptotic function under physiological conditions [35-37]. Indeed, cath-D knock-out mice developed apoptosis in the thymus and in the retina [35-37]. Some other studies have also suggested that cath-D may have an anti-apoptotic role in cancer. Our own immunohistochemical studies have revealed that xenografts of cancer cells overexpressing cath-D displayed less tumor apoptosis than mock-transfected cancer cells [53]. More recently, cath-D has been shown to protect human neuroblastoma cells from doxorubicin-induced cell death [54].

Pro-apoptotic characteristics of cath-D

Cath-D is a key mediator of induced-apoptosis, and its proteolytic activity has often been shown to be involved in this event [49, 55-63]. During apoptosis, mature lysosomal cath-D is translocated to the cytosol due to lysosomal membrane permeabilization (LMP) [56-58, 60, 64, 65]. Cytoplasmic cath-D has been shown to cleave Bid to form tBid [66, 67], which triggers the insertion of Bax into the mitochondrial membrane [62, 68], and leads in turn to the mitochondrial release of cytochrome c into the cytosol, and the activation of pro-caspases 9 and 3 [56, 60, 64, 66]. Cath-D is also involved in caspase-independent apoptosis by activating Bax independently of Bid cleavage, and leading in turn to the mitochondrial release of the apoptosis inducing factor (AIF) [68]. More recently, it has been shown that cath-D can also activate pro-caspase 8, initiating neutrophil apoptosis during the resolution of

inflammation [69]. Interestingly, a recent report indicates the presence of mature cath-D in the nucleus during cell death [70], and it has been proposed that nuclear cath-D may mediate the proteolytic activation of endonuclease 23 during cryonecrotic cell death [71].

Since cath-D is one of the lysosomal enzymes that requires a more acidic pH to be proteolytically-active than lysosomal cysteine enzymes, such as cath-B and cath-L, it is open to question whether cytosolic cath-D is able to cleave the substrate(s) implicated in the apoptotic cascade. In some studies, pepstatin A, an inhibitor of the enzyme, partially delayed the apoptosis induced by IFN-gamma and FAS/APO [55], staurosporin [60, 68], TNF-alpha [55, 66, 72], serum deprivation [73], oxidative stress [56, 57, 59], or when pepstatin A was co-micro-injected with cath-D [64]. Other studies indicate that the effect of a mutant cath-D deprived of catalytic activity was indistinguishable from that of the normal enzyme [61, 74]. Furthermore, microinjection of the inactive precursor pro-cath-D into cytosol confirmed that the pro-apoptotic effect of cytosolic cath-D may be also independent of its catalytic activity [75]. In conclusion, cath-D can promote apoptosis by mechanisms that may be dependent on and/or independent of its active site.

5. Roles of cath-D in cancer

Cath-D is an independent marker of a poor prognosis in breast cancer

In the 1990s, several independent clinical studies showed that the cath-D level in primary breast cancer cytosols is an independent prognostic parameter correlated with the incidence of clinical metastasis and shorter survival times [76]. A meta-analysis of studies on node-negative breast cancer [77], as well as a complete study of 2810 patients in Rotterdam [78], indicate that high concentrations of cath-D are an effective marker of aggressiveness. Cath-D is now recognized as an independent marker of poor prognosis in breast cancer associated with metastatic risk [79]. In recent years, independent studies have confirmed the prognostic

value of cath-D in breast cancer [80-88]. The main cath-D producing cells appear to be cancer cells and stromal macrophages [89]. Pro-cath-D is also increased in the plasma of patients with metastatic breast cancer [90-92], indicating that some of the pro-cath-D secreted by tumors can be released into the circulation. Interestingly, proteomic studies have recently confirmed the up-regulation of cath-D in many types of cancer [87, 93, 94].

Cath-D affects multiple steps of cancer progression and metastasis

Cath-D is overexpressed and hypersecreted in a multitude of cancer types (breast cancer, ovarian cancer, endometrial cancer, cancer of the head and neck, bladder cancer, malignant glioma, melanoma). In cancer cells, overexpressed cath-D accumulates in cells where it may affect their degradative capacities, and the pro-enzyme is hypersecreted in the tumor micro-environment (Figure 2). Cath-D hypersecreted by cancer cells may affect stromal cell behavior and/or degrade components of the extracellular matrix, thus modifying the tumor micro-environment (Figure 2).

Several reports have indicated that cath-D stimulates cancer cell proliferation [95-101], and increases the metastatic potential [96, 100, 102-104]. Cath-D stimulates cancer cell growth in an autocrine manner [97, 98, 105-107]. Various different mechanisms have been proposed to explain the mitogenicity of cath-D. Intracellular cath-D stimulates high density cancer cell growth by inactivating secreted growth inhibitors, such as heat shock 70 protein [99, 108]. Secreted pro-cath-D may act as a mitogen by competing with IGF2 for interaction with the M6P moieties of the M6P/IGF2 receptor, displacing IGF2 from the IGF1 receptor, and resulting in the activation of the mitogenic IGF1 receptor pathway [109, 110]. Many publications have suggested that the interaction of a part of the cath-D pro-fragment (amino acids 27 to 44) with an unknown cell surface receptor is implicated in its mitogenic function [97, 101, 107, 111-113]. Alternatively, it has also been suggested that the catalytic activity of

secreted cath-D may be implicated in releasing growth factors, such as FGF2, from the extracellular matrix [114]. Even though, the extracellular pH in tumors is generally more acidic than that in the corresponding normal tissues [115], the question remains as to whether secreted pro-cath-D could be activated extra-cellularly in a sufficiently acidic milieu. We have demonstrated that a mutated ^{D231N}cath-D, which is devoid of proteolytic activity, was still mitogenic for cancer cells both *in vitro*, in three-dimensional (3D) matrices, and in athymic nude mice [53, 105], suggesting that cath-D can also act by protein-protein interaction [116]. Interactions between stromal and epithelial cells are important in cancer progression and metastasis [117-119]. Stromal and tumor cells can exchange numerous tumor-promoting factors, such as growth factors, cytokines, and proteases. The fibroblast is a major cell type of the stromal compartment and, as such, is intimately involved in orchestrating the stromal side of the dialogue in tissue homeostasis. Cath-D is localized on the surface of breast fibroblasts [6], and can be taken up by fibroblasts [30, 120, 121]. Cath-D has been shown to play a crucial paracrine role in the tumor micro-environment by stimulating fibroblast outgrowth and tumor angiogenesis [53, 122], and possibly by inhibiting anti-tumor responses [123]. More recently, endothelial cells have been shown to secrete pro-cath-D *via* the action of inflammatory cytokines [124]. A mutant version of cath-D (D231N) that was devoid of catalytic activity, still proved to be mitogenic for fibroblasts, suggesting a mechanism involving protein-protein interaction [120].

Interestingly, some reports have indicated that the cysteine lysosomal cathepsins, cath-L and cath-F, which lack a signal peptide, localize in the nucleus [125, 126]. Nuclear cath-L proteolytically processes CDP/Cux transcription factor [12, 125] and histone H3 [127, 128], and has important functions in the control of cell transformation [129, 130] as well as in differentiation [127]. It has been shown that translation initiation at downstream AUG sites within cath-L mRNA is the first requirement in the chain of events that leads to the presence

of active cath-L in the nucleus [125]. There is, at present, no clear evidence for the presence of cath-D in the nucleus, and the nuclear function of cath-D in cancer is still unknown. However, our preliminary experiments strongly suggest the presence of cath-D in the nucleus of cancer cells. This may be due to translation initiation at downstream AUG sites within the cath-D mRNA (Figure 2). Indeed, it is worth noting that, like that of cath-L [125], the coding sequence of cath-D contains several AUG codons that are located downstream of the first AUG codons. Alternatively, two recent reports have suggested that cytosolic mature cath-D may reach the nucleus in apoptosis (Figure 2) [70]. One important question concerns the ability of cath-D to act as a functional enzyme at the neutral pH of the nucleus. Since enzymatic activity by cath-D is achieved at acidic pH, we can reasonably assume that cath-D might be only weakly active in the nuclear milieu. However, even limited cath-D activity in the nucleus could be compatible with a role in the proteolytic processing of specific nuclear proteins. In contrast, the optimal activity of cathepsins in the acidic environment of the lysosomes is necessary for the terminal degradation of proteins. Thus, the suboptimal pH that prevails in the nucleus should not be taken as an obstacle, but rather as an important element that enables cath-D to play a role in the limited proteolysis of nuclear proteins. Another possibility is that nuclear cath-D may sequester transcription repressors and/or activators, modulating the composition of the complexes implicated in the tight control of transcription. Our preliminary results indicate that cath-D can indeed interact with a nuclear repressor implicated in cancer. Future studies will clarify whether cath-D participates in the regulation of transcription in cancer by cleaving and/or interacting with nuclear proteins, and thus modulates their activity.

6. Targeting cath-D in cancer

Cathepsins have long been known to play an important role in the progression and metastasis of cancer. Cath-D stimulates cancer cell proliferation, fibroblast outgrowth, tumor angiogenesis, and metastasis. In cancer cells, overexpressed cath-D accumulates in cells where it may affect their degradation capacities, and the pro-enzyme is hypersecreted in the tumor micro-environment (Figure 2). Therefore, inhibiting cath-D action requires the development of inhibitors targeting extracellular cath-D, and/or intracellular cath-D located in different parts of the cell (*e.g.* intracellular vesicles, cytosol, or nucleus).

Inhibitors of cath-D proteolytic activity

In recent years, research interest in the development of potent inhibitors of various aspartic peptidases has arisen, fuelled by the growing evidence of their involvement in human diseases [131], such as that of renin in hypertension [132], γ -secretase in Alzheimer's disease [133], plasmepsins in malaria [134], HIV-1 peptidase in acquired immune deficiency syndrome [135], and secreted aspartic peptidases in *Candida* infections [136]. As opposed to other proteinases (*e.g.* serine proteases, metalloproteinases or cysteine cathepsins), no mammalian endogenous lysosomal or cytoplasmic cath-D inhibitor is known to exist. When released into the plasma, cath-D is inactivated by its interaction with α 2-macroglobulin at a neutral pH, but not at an acidic pH [137, 138]. Since cath-D requires an acidic pH to be proteolytically active, acidic pH may be the physiological regulator of human cath-D activity. In normal cells, cath-D is only active in acidic intracellular vesicles, and therefore uncontrolled proteolysis is avoided. However, no endogenous cath-D inhibitor is known to exist at acidic pH. It is worth noting that, in cancer, cath-D hypersecreted into the acidic extracellular tumor microenvironment may have a profound effect on matrix remodeling or extracellular factor proteolysis. Most exogenous cath-D inhibitors are synthetic compounds: peptides and polypeptides produced by micro-organisms, plants and lower animals [139, 140].

Organic compounds that esterify the carboxyl group of the Asn33 or Asp231 are synthetic cath-D inhibitors. Studies coupling the complementary methods of combinatorial chemistry and structure-based design, yielded low nanomolar inhibitors of cath-D [141-145]. Cath-D activity is inhibited by structural analogs of synthetic substrates in which an L-amino acid has been replaced by a D-amino acid [139]. The cath-D propeptide segment, which is cleaved off during zymogen activation, has been reported to inhibit pro-cath-D by blocking the active site at neutral pH [24, 25, 146, 147]. At high pH, a stable conformational species of cath-D exists in which the active site is blocked [25]. More recently, a pH-dependent conformational change has been shown to be mediated by electrostatic switches [24]. Peptide fragments derived from the propeptide have been shown to display some inhibitory potency against mature cath-D, suggesting that the development of new classes of pro-peptide-derived inhibitors of cath-D may be promising [147]. Pepstatin A, an inhibitor of aspartic proteases produced by a micro-organism, is the most potent polypeptide inhibitor of cath-D so far identified [148]. This is a hexa-peptide containing the unusual amino acid, statin (Sta, (3S,4S)-4-amino-3-hydroxy-6-methylheptanoic acid), and has the sequence Iva-Val-Val-Sta-Ala-Sta. It was originally isolated from cultures of various species of *Actinomyces* due to its ability to inhibit pepsin at picomolar concentrations. It was later found to be a potent inhibitor of nearly all acidic proteases and, as such, has become a valuable research tool. Pepstatin is commonly used to study the role of cath-D in *in-vitro* systems and in cells. Some studies have seemed to show that pepstatin A administered *in vivo* induces a significant reduction in the number of metastases, whereas other studies have not confirmed this effect [149]. Inhibition of cath-D by tripeptides containing statin analogs has also been reported [150]. Cath-D polypeptide inhibitors have also been identified in many plants [139], such as tomato leaves [151] and potato tubers [152]. Cath-D inhibitors are also produced by lower animals, such as equistatin from *Actinia equina* [153, 154] that can also inhibit cysteine cathepsin activity.

Interestingly, it has been shown that deoxyribonucleic acids (DNA fragments) can inhibit cath-D proteolytic activity [155].

Inhibitors of cath-D binding activity

Cath-D can also act by protein-protein interaction. Studies of the role of secreted pro-cath-D as a mitogen through its protein binding activity in cancer suggest the involvement of a part of the cath-D profragment (position 27-44) in an interaction with an unknown cell surface receptor [97, 101, 107, 111-113]. Interestingly, an anti-procath-D antibody directed against peptide 27-44 can reverse the growth of human breast tumors in athymic nude mice [111, 112, 156]. Secreted pro-cath-D may also act as a mitogen via its interaction with the M6P moieties of the M6P/IGF-2 receptor, displacing IGF2 from the IGF1 receptor, and leading to the activation the mitogenic IGF1 receptor pathway [109, 110]. We have demonstrated that a mutant ^{D231N}cath-D that is devoid of proteolytic activity is still mitogenic for cancer cells and fibroblasts both *in vitro* in three dimensional (3D) matrices, and in athymic nude mice [53, 105]. These findings suggest that pro-cath-D may act as an extracellular binding protein by directly or indirectly triggering an as-yet unidentified cell surface receptor. Our unpublished results also indicate that pro-cath-D hypersecreted by cancer cells triggers fibroblast invasive growth in a 3D matrix by interacting with a newly-identified fibroblastic cell surface receptor (submitted). The GST pull-down experiments revealed that this novel cath-D receptor binds the 52-, 34- and 14-kDa cath-D fragments, but only poorly to the 4-kDa cath-D profragment, indicating that the interaction interface spans both 34- and 14-kDa cath-D sub-units (submitted). Taken together, these observations suggest the importance of targeting extracellular pro-cath-D, and open new perspectives for the therapeutic inhibition of protease function in cancer by means other than the use of classical catalytic activity inhibitors. Because of the pleiotrophic action of secreted cath-D as a binding protein, the best strategy

may be to inhibit the extracellular action of pro-cath-D through the use of neutralizing antibodies, rather than by targeting an individual cath-D partner.

Studies in apoptosis also strongly suggest that mature cytosolic cath-D may have an additional role involving protein-protein interaction. So far, no apoptosis-related binding partner of cath-D has yet been identified. The search for cath-D partners using the yeast-two hybrid approach may elucidate the pro-apoptotic function of cath-D independently of its catalytic activity. Our unpublished results using this approach show that cath-D does indeed interact with a pro-apoptotic constituent of the apoptotic pathway. However, it would be premature to envisage blocking the interaction of cath-D using a component of the apoptotic machinery within the cell.

Cath-D substrates in cancer

The discovery of new cath-D physiological substrates is likely to generate new critical targets for cancer therapy. To understand the functions of proteases, it is crucial to identify their substrates. Cath-D cleaves preferentially -Phe-Phe-, -Leu-Tyr-, -Tyr-Leu-, and -Phe-Tyr- bonds in peptide chains containing at least five amino acids at an acidic pH [157]. These peptides contain L-amino acids, and also contain hydrophobic amino acid residues at the site cleaved by cath-D. Recently, proteome-derived database-searchable peptide libraries have been developed to identify endoprotease cleavage sites [158]. This approach may be applicable for cath-D. For a long time the main function of cath-D was thought to be to degrade proteins in lysosomes at an acidic pH. In addition to its established role as a major protein-degrading enzyme in lysosomes and phagosomes, it has been shown that cath-D can also activate precursors of biologically-active proteins, such as prolactin and osteopontin in specialized cells [159-163]. Many cath-D substrates have been reported *in vitro*, but no endogenous substrates of cath-D in cancer have yet been clearly identified. In

proteomics, the set of proteins that can be hydrolyzed by a protease is named its substrate degradome or degradomics [164]. A method termed Terminal Amine Isotopic Labeling of Substrates (TAILS) has recently been developed to identify extracellular and membrane protease substrates using iTRAQ labeling and mass spectrometry [165-167]. This powerful proteomic approach, which permitted the discovery of the MMP-2 substrate degradome [168], can also be applied to the identification of cath-D substrates using cells that do or do not express cath-D.

7. Conclusion

Cath-D is a key protease that affects many fundamental functions in cells. The molecular mechanism by which cath-D affects cancer progression remains largely unknown. Furthermore, we still do not have any specific cath-D inhibitors that could be used to target its action in cancer. Since this protease may also act by protein-protein interaction, it will be crucial to identify its partners in order to develop inhibitors to block its protein binding function.

Acknowledgement

Grant sponsors ‘ANR Jeunes chercheurs Jeunes chercheuses’ ANR-05-JCJC-0215-01, and EU FP6; Grant number: LSHC-CT-2007-037665.

References

- [1] H.W. Smith, C.J. Marshall, Regulation of cell signalling by uPAR, *Nat Rev Mol Cell Biol* 11 (2010) 23-36.
- [2] R. Roy, J. Yang, M.A. Moses, Matrix metalloproteinases as novel biomarkers and potential therapeutic targets in human cancer, *J Clin Oncol* 27 (2009) 5287-5297.
- [3] C. Palermo, J.A. Joyce, Cysteine cathepsin proteases as pharmacological targets in cancer, *Trends Pharmacol Sci* 29 (2008) 22-28.
- [4] E. Liaudet-Coopman, M. Beaujouin, D. Derocq, M. Garcia, M. Glondu-Lassis, V. Laurent-Matha, C. Prebois, H. Rochefort, F. Vignon, Cathepsin D: newly discovered functions of a long-standing aspartic protease in cancer and apoptosis, *Cancer Lett* 237 (2006) 167-179.
- [5] M.M. Mohamed, B.F. Sloane, Cysteine cathepsins: multifunctional enzymes in cancer, *Nat Rev Cancer* 6 (2006) 764-775.
- [6] J.E. Koblinski, J. Dosesu, M. Sameni, K. Moin, K. Clark, B.F. Sloane, Interaction of human breast fibroblasts with collagen I increases secretion of procathepsin B, *J Biol Chem* 277 (2002) 32220-32227.
- [7] J.A. Joyce, A. Baruch, K. Chehade, N. Meyer-Morse, E. Giraudo, F.Y. Tsai, D.C. Greenbaum, J.H. Hager, M. Bogyo, D. Hanahan, Cathepsin cysteine proteases are effectors of invasive growth and angiogenesis during multistage tumorigenesis, *Cancer Cell* 5 (2004) 443-453.
- [8] J.A. Joyce, D. Hanahan, Multiple roles for cysteine cathepsins in cancer, *Cell Cycle* 3 (2004) 1516-1619.
- [9] V. Gocheva, J.A. Joyce, Cysteine cathepsins and the cutting edge of cancer invasion, *Cell Cycle* 6 (2007) 60-64.
- [10] V. Gocheva, W. Zeng, D. Ke, D. Klimstra, T. Reinheckel, C. Peters, D. Hanahan, J.A. Joyce, Distinct roles for cysteine cathepsin genes in multistage tumorigenesis, *Genes Dev* 20 (2006) 543-556.
- [11] S. Ivanova, U. Repnik, L. Bojic, A. Petelin, V. Turk, B. Turk, Lysosomes in apoptosis, *Methods Enzymol* 442 (2008) 183-199.
- [12] H.A. Chapman, Cathepsins as transcriptional activators?, *Dev Cell* 6 (2004) 610-611.
- [13] M.M. Mueller, N.E. Fusenig, Tumor-stroma interactions directing phenotype and progression of epithelial skin tumor cells, *Differentiation* 70 (2002) 486-497.
- [14] N.I. Affara, P. Andreu, L.M. Coussens, Delineating protease functions during cancer development, *Methods Mol Biol* 539 (2009) 1-32.
- [15] M. Cudic, G.B. Fields, Extracellular proteases as targets for drug development, *Curr Protein Pept Sci* 10 (2009) 297-307.
- [16] P. Augereau, M. Garcia, M.G. Mattei, V. Cavailles, F. Depadova, D. Derocq, F. Capony, P. Ferrara, H. Rochefort, Cloning and sequencing of the 52K cathepsin D complementary deoxyribonucleic acid of MCF7 breast cancer cells and mapping on chromosome 11, *Mol Endocrinol* 2 (1988) 186-192.
- [17] A.M. Samarel, A.G. Ferguson, R.S. Decker, M. Lesch, Effects of cysteine protease inhibitors on rabbit cathepsin D maturation, *Am J Physiol* 257 (1989) C1069-1079.
- [18] V. Gieselmann, A. Hasilik, K. von Figura, Processing of human cathepsin D in lysosomes in vitro, *J Biol Chem* 260 (1985) 3215-3220.

- [19] V. Laurent-Matha, D. Derocq, C. Prebois, N. Katunuma, E. Liaudet-Coopman, Processing of human cathepsin D is independent of its catalytic function and auto-activation: involvement of cathepsins L and B, *J Biochem* 139 (2006) 363-371.
- [20] U. Felbor, B. Kessler, W. Mothes, H.H. Goebel, H.L. Ploegh, R.T. Bronson, B.R. Olsen, Neuronal loss and brain atrophy in mice lacking cathepsins B and L, *Proc Natl Acad Sci U S A* 99 (2002) 7883-7888.
- [21] A. Wille, A. Gerber, A. Heimbürg, A. Reisenauer, C. Peters, P. Saftig, T. Reinheckel, T. Welte, F. Buhling, Cathepsin L is involved in cathepsin D processing and regulation of apoptosis in A549 human lung epithelial cells, *Biol Chem* 385 (2004) 665-670.
- [22] P. Metcalf, M. Fusek, Two crystal structures for cathepsin D: the lysosomal targeting signal and active site, *Embo J* 12 (1993) 1293-1302.
- [23] E.T. Baldwin, T.N. Bhat, S. Gulnik, M.V. Hosur, R.C. Sowder, 2nd, R.E. Cachau, J. Collins, A.M. Silva, J.W. Erickson, Crystal structures of native and inhibited forms of human cathepsin D: implications for lysosomal targeting and drug design, *Proc Natl Acad Sci U S A* 90 (1993) 6796-6800.
- [24] N.E. Goldfarb, M.T. Lam, A.K. Bose, A.M. Patel, A.J. Duckworth, B.M. Dunn, Electrostatic switches that mediate the pH-dependent conformational change of "short" recombinant human pseudocathepsin D, *Biochemistry* 44 (2005) 15725-15733.
- [25] A.Y. Lee, S.V. Gulnik, J.W. Erickson, Conformational switching in an aspartic proteinase, *Nat Struct Biol* 5 (1998) 866-871.
- [26] V. Cavailles, P. Augereau, M. Garcia, H. Rochefort, Estrogens and growth factors induce the mRNA of the 52K-pro-cathepsin-D secreted by breast cancer cells, *Nucleic Acids Res* 16 (1988) 1903-1919.
- [27] C. Giamarchi, M. Solanas, C. Chailleux, P. Augereau, F. Vignon, H. Rochefort, H. Richard-Foy, Chromatin structure of the regulatory regions of pS2 and cathepsin D genes in hormone-dependent and -independent breast cancer cell lines, *Oncogene* 18 (1999) 533-541.
- [28] F. Capony, C. Rougeot, P. Montcourrier, V. Cavailles, G. Salazar, H. Rochefort, Increased secretion, altered processing, and glycosylation of pro-cathepsin D in human mammary cancer cells, *Cancer Res* 49 (1989) 3904-3909.
- [29] R. Castino, S. Delpal, E. Bouguyon, M. Demoz, C. Isidoro, M. Ollivier-Bousquet, Prolactin promotes the secretion of active cathepsin D at the basal side of rat mammary acini, *Endocrinology* 149 (2008) 4095-4105.
- [30] V. Laurent-Matha, M.R. Farnoud, A. Lucas, C. Rougeot, M. Garcia, H. Rochefort, Endocytosis of pro-cathepsin D into breast cancer cells is mostly independent of mannose-6-phosphate receptors, *J Cell Sci* 111 (Pt 17) (1998) 2539-2549.
- [31] F. Capony, T. Bräulke, C. Rougeot, S. Roux, P. Montcourrier, H. Rochefort, Specific mannose-6-phosphate receptor-independent sorting of pro-cathepsin D in breast cancer cells, *Exp Cell Res* 215 (1994) 154-163.
- [32] V. Laurent-Matha, A. Lucas, S. Huttler, K. Sandhoff, M. Garcia, H. Rochefort, Procathepsin D interacts with prosaposin in cancer cells but its internalization is not mediated by LDL receptor-related protein, *Exp Cell Res* 277 (2002) 210-219.
- [33] M. Canuel, A. Korkidakis, K. Konnyu, C.R. Morales, Sortilin mediates the lysosomal targeting of cathepsins D and H, *Biochem Biophys Res Commun* 373 (2008) 292-297.
- [34] T. Kageyama, M. Tatematsu, M. Ichinose, N. Yahagi, K. Miki, A. Moriyama, S. Yonezawa, Development-dependent expression of cathepsins d and e in various rat tissues, with special reference to the high expression of cathepsin e in fetal liver, *Zoolog Sci* 15 (1998) 517-523.
- [35] P. Saftig, M. Hetman, W. Schmahl, K. Weber, L. Heine, H. Mossmann, A. Koster, B. Hess, M. Evers, K. von Figura, et al., Mice deficient for the lysosomal proteinase cathepsin D

exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells, *Embo J* 14 (1995) 3599-3608.

- [36] M. Koike, H. Nakanishi, P. Saftig, J. Ezaki, K. Isahara, Y. Ohsawa, W. Schulz-Schaeffer, T. Watanabe, S. Waguri, S. Kametaka, M. Shibata, K. Yamamoto, E. Kominami, C. Peters, K. von Figura, Y. Uchiyama, Cathepsin D deficiency induces lysosomal storage with ceroid lipofuscin in mouse CNS neurons, *J Neurosci* 20 (2000) 6898-6906.
- [37] M. Koike, M. Shibata, Y. Ohsawa, H. Nakanishi, T. Koga, S. Kametaka, S. Waguri, T. Momoi, E. Kominami, C. Peters, K. Figura, P. Saftig, Y. Uchiyama, Involvement of two different cell death pathways in retinal atrophy of cathepsin D-deficient mice, *Mol Cell Neurosci* 22 (2003) 146-161.
- [38] K.C. Walls, B.J. Klocke, P. Saftig, M. Shibata, Y. Uchiyama, K.A. Roth, J.J. Shacka, Altered regulation of phosphatidylinositol 3-kinase signaling in cathepsin D-deficient brain, *Autophagy* 3 (2007) 222-229.
- [39] L. Myllykangas, J. Tyynela, A. Page-McCaw, G.M. Rubin, M.J. Haltia, M.B. Feany, Cathepsin D-deficient *Drosophila* recapitulate the key features of neuronal ceroid lipofuscinoses, *Neurobiol Dis* 19 (2005) 194-199.
- [40] J. Tyynela, I. Sohar, D.E. Sleat, R.M. Gin, R.J. Donnelly, M. Baumann, M. Haltia, P. Lobel, A mutation in the ovine cathepsin D gene causes a congenital lysosomal storage disease with profound neurodegeneration, *Embo J* 19 (2000) 2786-2792.
- [41] T. Awano, M.L. Katz, D.P. O'Brien, J.F. Taylor, J. Evans, S. Khan, I. Sohar, P. Lobel, G.S. Johnson, A mutation in the cathepsin D gene (CTSD) in American Bulldogs with neuronal ceroid lipofuscinosis, *Mol Genet Metab* 87 (2006) 341-348.
- [42] E. Siintola, S. Partanen, P. Stromme, A. Haapanen, M. Haltia, J. Maehlen, A.E. Lehesjoki, J. Tyynela, Cathepsin D deficiency underlies congenital human neuronal ceroid-lipofuscinosis, *Brain* 129 (2006) 1438-1445.
- [43] R. Steinfeld, K. Reinhardt, K. Schreiber, M. Hillebrand, R. Kraetzner, W. Bruck, P. Saftig, J. Gartner, Cathepsin D deficiency is associated with a human neurodegenerative disorder, *Am J Hum Genet* 78 (2006) 988-998.
- [44] J. Tyynela, I. Sohar, D.E. Sleat, R.M. Gin, R.J. Donnelly, M. Baumann, M. Haltia, P. Lobel, Congenital ovine neuronal ceroid lipofuscinosis--a cathepsin D deficiency with increased levels of the inactive enzyme, *Eur J Paediatr Neurol* 5 Suppl A (2001) 43-45.
- [45] K. Fritchie, E. Siintola, D. Armao, A.E. Lehesjoki, T. Marino, C. Powell, M. Tennison, J.M. Booker, S. Koch, S. Partanen, K. Suzuki, J. Tyynela, L.B. Thorne, Novel mutation and the first prenatal screening of cathepsin D deficiency (CLN10), *Acta Neuropathol* 117 (2009) 201-208.
- [46] V. Cullen, M. Lindfors, J. Ng, A. Paetau, E. Swinton, P. Kolodziej, H. Boston, P. Saftig, J. Woulfe, M.B. Feany, L. Myllykangas, M.G. Schlossmacher, J. Tyynela, Cathepsin D expression level affects alpha-synuclein processing, aggregation, and toxicity in vivo, *Mol Brain* 2 (2009) 5.
- [47] D. Hilfiker-Kleiner, K. Kaminski, E. Podewski, T. Bonda, A. Schaefer, K. Sliwa, O. Forster, A. Quint, U. Landmesser, C. Doerries, M. Luchtefeld, V. Poli, M.D. Schneider, J.L. Balligand, F. Desjardins, A. Ansari, I. Struman, N.Q. Nguyen, N.H. Zschemisch, G. Klein, G. Heusch, R. Schulz, A. Hilfiker, H. Drexler, A cathepsin D-cleaved 16 kDa form of prolactin mediates postpartum cardiomyopathy, *Cell* 128 (2007) 589-600.
- [48] A.M. Sheikh, X. Li, G. Wen, Z. Tauqeer, W.T. Brown, M. Malik, Cathepsin D and apoptosis related proteins are elevated in the brain of autistic subjects, *Neuroscience* 165 (2010) 363-370.
- [49] L. Emert-Sedlak, S. Shangary, A. Rabinovitz, M.B. Miranda, S.M. Delach, D.E. Johnson, Involvement of cathepsin D in chemotherapy-induced cytochrome c release, caspase activation, and cell death, *Mol Cancer Ther* 4 (2005) 733-742.

- [50] O. Vasiljeva, B. Turk, Dual contrasting roles of cysteine cathepsins in cancer progression: apoptosis versus tumour invasion, *Biochimie* 90 (2008) 380-386.
- [51] A. Minarowska, L. Minarowski, A. Karwowska, M. Gacko, Regulatory role of cathepsin D in apoptosis, *Folia Histochem Cytobiol* 45 (2007) 159-163.
- [52] V. Zuzarte-Luis, J.A. Montero, N. Torre-Perez, J.A. Garcia-Porrero, J.M. Hurle, Cathepsin D gene expression outlines the areas of physiological cell death during embryonic development, *Dev Dyn* 236 (2007) 880-885.
- [53] G. Berchem, M. Glondou, M. Gleizes, J.P. Brouillet, F. Vignon, M. Garcia, E. Liaudet-Coopman, Cathepsin-D affects multiple tumor progression steps in vivo: proliferation, angiogenesis and apoptosis, *Oncogene* 21 (2002) 5951-5955.
- [54] V. Sagulenko, D. Muth, E. Sagulenko, T. Paffhausen, M. Schwab, F. Westermann, Cathepsin D protects human neuroblastoma cells from doxorubicin-induced cell death, *Carcinogenesis* 29 (2008) 1869-1877.
- [55] L.P. Deiss, H. Galinka, H. Berissi, O. Cohen, A. Kimchi, Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha, *Embo J* 15 (1996) 3861-3870.
- [56] K. Ollinger, Inhibition of cathepsin D prevents free-radical-induced apoptosis in rat cardiomyocytes, *Arch Biochem Biophys* 373 (2000) 346-351.
- [57] K. Kagedal, U. Johansson, K. Ollinger, The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress, *Faseb J* 15 (2001) 1592-1594.
- [58] K. Roberg, K. Ollinger, Oxidative stress causes relocation of the lysosomal enzyme cathepsin D with ensuing apoptosis in neonatal rat cardiomyocytes, *Am J Pathol* 152 (1998) 1151-1156.
- [59] K. Takuma, M. Kiri, K. Mori, E. Lee, R. Enomoto, A. Baba, T. Matsuda, Roles of cathepsins in reperfusion-induced apoptosis in cultured astrocytes, *Neurochem Int* 42 (2003) 153-159.
- [60] A.C. Johansson, H. Steen, K. Ollinger, K. Roberg, Cathepsin D mediates cytochrome c release and caspase activation in human fibroblast apoptosis induced by staurosporine, *Cell Death Differ* 10 (2003) 1253-1259.
- [61] M. Beaujouin, S. Baghdiguian, M. Glondou-Lassis, G. Berchem, E. Liaudet-Coopman, Overexpression of both catalytically active and -inactive cathepsin D by cancer cells enhances apoptosis-dependent chemo-sensitivity, *Oncogene* 25 (2006) 1967-1973.
- [62] Y. Gan, X. Zhao, J. Hu, Z.G. Wang, X.T. Zhao, HCCS1 overexpression induces apoptosis via cathepsin D and intracellular calcium, and HCCS1 disruption in mice causes placental abnormality, *Cell Death Differ* 15 (2008) 1481-1490.
- [63] Y. Miura, Y. Sakurai, M. Hayakawa, Y. Shimada, H. Zempel, Y. Sato, S. Hisanaga, T. Endo, Translocation of lysosomal cathepsin D caused by oxidative stress or proteasome inhibition in primary cultured neurons and astrocytes, *Biol Pharm Bull* 33 (2010) 22-28.
- [64] K. Roberg, K. Kagedal, K. Ollinger, Microinjection of cathepsin d induces caspase-dependent apoptosis in fibroblasts, *Am J Pathol* 161 (2002) 89-96.
- [65] K. Roberg, U. Johansson, K. Ollinger, Lysosomal release of cathepsin D precedes relocation of cytochrome c and loss of mitochondrial transmembrane potential during apoptosis induced by oxidative stress, *Free Radic Biol Med* 27 (1999) 1228-1237.
- [66] M. Heinrich, J. Neumeyer, M. Jakob, C. Hallas, V. Tchikov, S. Winoto-Morbach, M. Wickel, W. Schneider-Brachert, A. Trauzold, A. Hethke, S. Schutze, Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation, *Cell Death Differ* 11 (2004) 550-563.
- [67] R. Blomgran, L. Zheng, O. Stendahl, Cathepsin-cleaved Bid promotes apoptosis in human neutrophils via oxidative stress-induced lysosomal membrane permeabilization, *J Leukoc Biol* 81 (2007) 1213-1223.

- [68] N. Bidere, H.K. Lorenzo, S. Carmona, M. Laforge, F. Harper, C. Dumont, A. Senik, Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis, *J Biol Chem* 278 (2003) 31401-31411.
- [69] S. Conus, R. Perozzo, T. Reinheckel, C. Peters, L. Scapozza, S. Yousefi, H.U. Simon, Caspase-8 is activated by cathepsin D initiating neutrophil apoptosis during the resolution of inflammation, *J Exp Med* 205 (2008) 685-698.
- [70] S. Zhao, E.R. Aviles, Jr., D.G. Fujikawa, Nuclear translocation of mitochondrial cytochrome c, lysosomal cathepsins B and D, and three other death-promoting proteins within the first 60 minutes of generalized seizures, *J Neurosci Res* (2010 (in press)).
- [71] N. Grdovic, M. Vidakovic, M. Mihailovic, S. Dinic, A. Uskokovic, J. Arambasic, G. Poznanovic, Proteolytic events in cryonecrotic cell death: Proteolytic activation of endonuclease P23, *Cryobiology* (2010 (in press)).
- [72] M. Demoz, R. Castino, P. Cesaro, F.M. Baccino, G. Bonelli, C. Isidoro, Endosomal-lysosomal proteolysis mediates death signalling by TNFalpha, not by etoposide, in L929 fibrosarcoma cells: evidence for an active role of cathepsin D, *Biol Chem* 383 (2002) 1237-1248.
- [73] M. Shibata, S. Kanamori, K. Isahara, Y. Ohsawa, A. Konishi, S. Kametaka, T. Watanabe, S. Ebisu, K. Ishido, E. Kominami, Y. Uchiyama, Participation of cathepsins B and D in apoptosis of PC12 cells following serum deprivation, *Biochem Biophys Res Commun* 251 (1998) 199-203.
- [74] C. Tardy, J. Tyynela, A. Hasilik, T. Levade, N. Andrieu-Abadie, Stress-induced apoptosis is impaired in cells with a lysosomal targeting defect but is not affected in cells synthesizing a catalytically inactive cathepsin D, *Cell Death Differ* 10 (2003) 1090-1100.
- [75] O. Schestkova, D. Geisel, R. Jacob, A. Hasilik, The catalytically inactive precursor of cathepsin D induces apoptosis in human fibroblasts and HeLa cells, *J Cell Biochem* 101 (2007) 1558-1566.
- [76] B.R. Westley, F.E. May, Prognostic value of cathepsin D in breast cancer, *Br J Cancer* 79 (1999) 189-190.
- [77] G. Ferrandina, G. Scambia, F. Bardelli, P. Benedetti Panici, S. Mancuso, A. Messori, Relationship between cathepsin-D content and disease-free survival in node-negative breast cancer patients: a meta-analysis, *Br J Cancer* 76 (1997) 661-666.
- [78] J.A. Foekens, M.P. Look, J. Bolt-de Vries, M.E. Meijer-van Gelder, W.L. van Putten, J.G. Klijn, Cathepsin-D in primary breast cancer: prognostic evaluation involving 2810 patients, *Br J Cancer* 79 (1999) 300-307.
- [79] P.L. Fitzgibbons, D.L. Page, D. Weaver, A.D. Thor, D.C. Allred, G.M. Clark, S.G. Ruby, F. O'Malley, J.F. Simpson, J.L. Connolly, D.F. Hayes, S.B. Edge, A. Lichter, S.J. Schnitt, Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999, *Arch Pathol Lab Med* 124 (2000) 966-978.
- [80] N. Harbeck, U. Alt, U. Berger, R. Kates, A. Kruger, C. Thomssen, F. Janicke, H. Graeff, M. Schmitt, Long-term follow-up confirms prognostic impact of PAI-1 and cathepsin D and L in primary breast cancer, *Int J Biol Markers* 15 (2000) 79-83.
- [81] S. Greco, S. Marsigliante, G. Leo, C. Storelli, Co-expression of thymidine kinase and cathepsin D in 200 primary breast carcinomas, *Cancer Lett* 160 (2000) 13-19.
- [82] Z. Gaci, M.H. Bouin-Pineau, M. Gaci, A. Daban, P. Ingrand, T. Metaye, Prognostic impact of cathepsin D and c-erbB-2 oncoprotein in a subgroup of node-negative breast cancer patients with low histological grade tumors, *Int J Oncol* 18 (2001) 793-800.
- [83] N. Bossard, F. Descotes, A.G. Bremond, Y. Bobin, P. De Saint Hilaire, F. Golfier, A. Awada, P.M. Mathevet, L. Berrerd, Y. Barbier, J. Esteve, Keeping data continuous when

analyzing the prognostic impact of a tumor marker: an example with cathepsin D in breast cancer, *Breast Cancer Res Treat* 82 (2003) 47-59.

[84] T.E. Kute, G.B. Russell, N. Zbieranski, R. Long, S. Johnston, H. Williams, C. Stackhouse, L. Wilkins, I. Evans, P. Berry, K. Rimmer, E. Tucker, Prognostic markers in node-negative breast cancer: a prospective study, *Cytometry B Clin Cytom* 59 (2004) 24-31.

[85] J. Rodriguez, J. Vazquez, M.D. Corte, M. Lamelas, M. Bongera, M.G. Corte, A. Alvarez, M. Allende, L. Gonzalez, M. Sanchez, M. Vijande, J. Garcia Muniz, F. Vizoso, Clinical significance of cathepsin D concentration in tumor cytosol of primary breast cancer, *Int J Biol Markers* 20 (2005) 103-111.

[86] J.T. Anim, B. John, S.S. Abdulsathar, A. Prasad, T. Saji, N. Akhtar, V. Ali, M. Al-Saleh, Relationship between the expression of various markers and prognostic factors in breast cancer, *Acta Histochem* 107 (2005) 87-93.

[87] A.L. Cheng, W.G. Huang, Z.C. Chen, P.F. Zhang, M.Y. Li, F. Li, J.L. Li, C. Li, H. Yi, F. Peng, C.J. Duan, Z.Q. Xiao, Identifying cathepsin D as a biomarker for differentiation and prognosis of nasopharyngeal carcinoma by laser capture microdissection and proteomic analysis, *J Proteome Res* 7 (2008) 2415-2426.

[88] N.S. Vasudev, S. Sim, D.A. Cairns, R.E. Ferguson, R.A. Craven, A. Stanley, J. Cartledge, D. Thompson, P.J. Selby, R.E. Banks, Pre-operative urinary cathepsin D is associated with survival in patients with renal cell carcinoma, *Br J Cancer* 101 (2009) 1175-1182.

[89] P. Roger, P. Montcourrier, T. Maudelonde, J.P. Brouillet, A. Pages, F. Laffargue, H. Rochefort, Cathepsin D immunostaining in paraffin-embedded breast cancer cells and macrophages: correlation with cytosolic assay, *Hum Pathol* 25 (1994) 863-871.

[90] J.P. Brouillet, F. Dufour, G. Lemamy, M. Garcia, N. Schlup, J. Grenier, J.C. Mani, H. Rochefort, Increased cathepsin D level in the serum of patients with metastatic breast carcinoma detected with a specific pro-cathepsin D immunoassay, *Cancer* 79 (1997) 2132-2136.

[91] S.D. Szajda, J. Snarska, A. Jankowska, W. Roszkowska-Jakimiec, Z. Puchalski, K. Zwierz, Cathepsin D and carcino-embryonic antigen in serum, urine and tissues of colon adenocarcinoma patients, *Hepatogastroenterology* 55 (2008) 388-393.

[92] S.D. Szajda, J. Snarska, W. Roszkowska-Jakimiec, N. Waszkiewicz, K. Siedlecka, K. Zwierz, A. Krupkowska, [Activity of cathepsin D in the blood serum and urine of patients with cancer of the stomach, pancreas and liver], *Pol Arch Med Wewn* 116 (2006) 1150-1154.

[93] I. Selicharova, M. Sanda, J. Mladkova, S.S. Ohri, A. Vashishta, M. Fusek, J. Jiracek, V. Vetvicka, 2-DE analysis of breast cancer cell lines 1833 and 4175 with distinct metastatic organ-specific potentials: comparison with parental cell line MDA-MB-231, *Oncol Rep* 19 (2008) 1237-1244.

[94] U. Roth, H. Razawi, J. Hommer, K. Engelmann, T. Schwientek, S. Muller, S.E. Baldus, G. Patsos, A.P. Corfield, C. Paraskeva, F.G. Hanisch, Differential expression proteomics of human colorectal cancer based on a syngeneic cellular model for the progression of adenoma to carcinoma, *Proteomics* 10 (2010) 194-202.

[95] F. Vignon, F. Capony, M. Chambon, G. Freiss, M. Garcia, H. Rochefort, Autocrine growth stimulation of the MCF 7 breast cancer cells by the estrogen-regulated 52 K protein, *Endocrinology* 118 (1986) 1537-1545.

[96] M. Garcia, D. Derocq, P. Pujol, H. Rochefort, Overexpression of transfected cathepsin D in transformed cells increases their malignant phenotype and metastatic potency, *Oncogene* 5 (1990) 1809-1814.

[97] M. Fusek, V. Vetvicka, Mitogenic function of human procathepsin D: the role of the propeptide, *Biochem J* 303 (Pt 3) (1994) 775-780.

- [98] V. Vetvicka, J. Vektvickova, M. Fusek, Effect of human procathepsin D on proliferation of human cell lines, *Cancer Lett* 79 (1994) 131-135.
- [99] E. Liaudet, D. Derocq, H. Rochefort, M. Garcia, Transfected cathepsin D stimulates high density cancer cell growth by inactivating secreted growth inhibitors, *Cell Growth Differ* 6 (1995) 1045-1052.
- [100] S.S. Ohri, A. Vashishta, M. Proctor, M. Fusek, V. Vetvicka, Depletion of procathepsin D gene expression by RNA interference: a potential therapeutic target for breast cancer, *Cancer Biol Ther* 6 (2007) 1081-1087.
- [101] S.S. Ohri, A. Vashishta, M. Proctor, M. Fusek, V. Vetvicka, The propeptide of cathepsin D increases proliferation, invasion and metastasis of breast cancer cells, *Int J Oncol* 32 (2008) 491-498.
- [102] E. Liaudet, M. Garcia, H. Rochefort, Cathepsin D maturation and its stimulatory effect on metastasis are prevented by addition of KDEL retention signal, *Oncogene* 9 (1994) 1145-1154.
- [103] M. Glondou, E. Liaudet-Coopman, D. Derocq, N. Platet, H. Rochefort, M. Garcia, Down-regulation of cathepsin-D expression by antisense gene transfer inhibits tumor growth and experimental lung metastasis of human breast cancer cells, *Oncogene* 21 (2002) 5127-5134.
- [104] A. Vashishta, S.S. Ohri, M. Proctor, M. Fusek, V. Vetvicka, Ribozyme-targeting procathepsin D and its effect on invasion and growth of breast cancer cells: an implication in breast cancer therapy, *Int J Oncol* 30 (2007) 1223-1230.
- [105] M. Glondou, P. Coopman, V. Laurent-Matha, M. Garcia, H. Rochefort, E. Liaudet-Coopman, A mutated cathepsin-D devoid of its catalytic activity stimulates the growth of cancer cells, *Oncogene* 20 (2001) 6920-6929.
- [106] V. Vetvicka, J. Vetvickova, M. Fusek, Effect of procathepsin D and its activation peptide on prostate cancer cells, *Cancer Lett* 129 (1998) 55-59.
- [107] V. Vetvicka, J. Vetvickova, P. Benes, Role of enzymatically inactive procathepsin D in lung cancer, *Anticancer Res* 24 (2004) 2739-2743.
- [108] P. Nirde, D. Derocq, M. Maynadier, M. Chambon, I. Basile, M. Gary-Bobo, M. Garcia, Heat shock cognate 70 protein secretion as a new growth arrest signal for cancer cells, *Oncogene* 29 (2010) 117-127.
- [109] M. Mathieu, H. Rochefort, B. Barenton, C. Prebois, F. Vignon, Interactions of cathepsin-D and insulin-like growth factor-II (IGF-II) on the IGF-II/mannose-6-phosphate receptor in human breast cancer cells and possible consequences on mitogenic activity of IGF-II, *Mol Endocrinol* 4 (1990) 1327-1335.
- [110] J.S. Faridi, S. Mohan, D.D. De Leon, Modulation of cathepsin D routing by IGF-II involves IGF-II binding to IGF-II/M6P receptor in MCF-7 breast cancer cells, *Growth Factors* 22 (2004) 169-177.
- [111] V. Vetvicka, P. Benes, M. Fusek, Procathepsin D in breast cancer: what do we know? Effects of ribozymes and other inhibitors, *Cancer Gene Ther* 9 (2002) 854-863.
- [112] V. Vetvicka, J. Vetvickova, M. Fusek, Anti-human procathepsin D activation peptide antibodies inhibit breast cancer development, *Breast Cancer Res Treat* 57 (1999) 261-269.
- [113] A. Vashishta, S.S. Ohri, M. Proctor, M. Fusek, V. Vetvicka, Role of activation peptide of procathepsin D in proliferation and invasion of lung cancer cells, *Anticancer Res* 26 (2006) 4163-4170.
- [114] P. Briozzo, J. Badet, F. Capony, I. Pieri, P. Montcourrier, D. Barritault, H. Rochefort, MCF7 mammary cancer cells respond to bFGF and internalize it following its release from extracellular matrix: a permissive role of cathepsin D, *Exp Cell Res* 194 (1991) 252-259.
- [115] J.R. Griffiths, Are cancer cells acidic?, *Br J Cancer* 64 (1991) 425-427.

- [116] H. Rochefort, E. Liaudet-Coopman, Cathepsin D in cancer metastasis: a protease and a ligand, *Apmis* 107 (1999) 86-95.
- [117] M.M. Mueller, N.E. Fusenig, Friends or foes - bipolar effects of the tumour stroma in cancer, *Nat Rev Cancer* 4 (2004) 839-849.
- [118] L.A. Liotta, E.C. Kohn, The microenvironment of the tumour-host interface, *Nature* 411 (2001) 375-379.
- [119] R. Masson, O. Lefebvre, A. Noel, M.E. Fahime, M.P. Chenard, C. Wendling, F. Kebers, M. LeMeur, A. Dierich, J.M. Foidart, P. Basset, M.C. Rio, In vivo evidence that the stromelysin-3 metalloproteinase contributes in a paracrine manner to epithelial cell malignancy, *J Cell Biol* 140 (1998) 1535-1541.
- [120] V. Laurent-Matha, S. Maruani-Herrmann, C. Prebois, M. Beaujouin, M. Glondou, A. Noel, M.L. Alvarez-Gonzalez, S. Blacher, P. Coopman, S. Baghdiguian, C. Gilles, J. Loncarek, G. Freiss, F. Vignon, E. Liaudet-Coopman, Catalytically inactive human cathepsin D triggers fibroblast invasive growth, *J Cell Biol* 168 (2005) 489-499.
- [121] N. Heylen, L.M. Vincent, V. Devos, V. Dubois, C. Remacle, A. Trouet, Fibroblasts capture cathepsin D secreted by breast cancer cells: possible role in the regulation of the invasive process, *Int J Oncol* 20 (2002) 761-767.
- [122] L. Hu, J.M. Roth, P. Brooks, J. Luty, S. Karparkin, Thrombin up-regulates cathepsin D which enhances angiogenesis, growth, and metastasis, *Cancer Res* 68 (2008) 4666-4673.
- [123] M. Wolf, I. Clark-Lewis, C. Buri, H. Langen, M. Lis, L. Mazzucchelli, Cathepsin D specifically cleaves the chemokines macrophage inflammatory protein-1 alpha, macrophage inflammatory protein-1 beta, and SLC that are expressed in human breast cancer, *Am J Pathol* 162 (2003) 1183-1190.
- [124] S. Erdmann, A. Ricken, K. Hummitzsch, C. Merkwitz, N. Schliebe, F. Gaunitz, R. Strotmann, K. Spaniel-Borowski, Inflammatory cytokines increase extracellular procathepsin D in permanent and primary endothelial cell cultures, *Eur J Cell Biol* 87 (2008) 311-323.
- [125] B. Goulet, A. Baruch, N.S. Moon, M. Poirier, L.L. Sansregret, A. Erickson, M. Bogyo, A. Nepveu, A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor, *Mol Cell* 14 (2004) 207-219.
- [126] G. Maubach, M.C. Lim, L. Zhuo, Nuclear cathepsin F regulates activation markers in rat hepatic stellate cells, *Mol Biol Cell* 19 (2008) 4238-4248.
- [127] E.M. Duncan, T.L. Muratore-Schroeder, R.G. Cook, B.A. Garcia, J. Shabanowitz, D.F. Hunt, C.D. Allis, Cathepsin L proteolytically processes histone H3 during mouse embryonic stem cell differentiation, *Cell* 135 (2008) 284-294.
- [128] S. Ceru, S. Konjar, K. Maher, U. Repnik, I. Krizaj, M. Bencina, M. Renko, A. Nepveu, E. Zerovnik, B. Turk, N. Kopitar-Jerala, Stefin B interacts with histones and cathepsin L in the nucleus, *J Biol Chem* 285 (2010) 10078-10086.
- [129] B. Goulet, L. Sansregret, L. Leduy, M. Bogyo, E. Weber, S.S. Chauhan, A. Nepveu, Increased expression and activity of nuclear cathepsin L in cancer cells suggests a novel mechanism of cell transformation, *Mol Cancer Res* 5 (2007) 899-907.
- [130] S. Sullivan, M. Tosetto, D. Kevans, A. Coss, L. Wang, D. O'Donoghue, J. Hyland, K. Sheahan, H. Mulcahy, J. O'Sullivan, Localization of nuclear cathepsin L and its association with disease progression and poor outcome in colorectal cancer, *Int J Cancer* 125 (2009) 54-61.
- [131] C. Dash, A. Kulkarni, B. Dunn, M. Rao, Aspartic peptidase inhibitors: implications in drug development, *Crit Rev Biochem Mol Biol* 38 (2003) 89-119.
- [132] G. Riccioni, N. Vitulano, N. D'Orazio, F. Bellocchi, Aliskiren, the first approved renin inhibitor: Clinical application and safety in the treatment of hypertension, *Adv Ther* 26 (2009) 700-710.

- [133] C. Bjorklund, S. Oscarson, K. Benkestock, N. Borkakoti, K. Jansson, J. Lindberg, L. Vrang, A. Hallberg, A. Rosenquist, B. Samuelsson, Design and synthesis of potent and selective BACE-1 inhibitors, *J Med Chem* 53 (2010) 1458-1464.
- [134] P.O. Johansson, Y. Chen, A.K. Belfrage, M.J. Blackman, I. Kvarnstrom, K. Jansson, L. Vrang, E. Hamelink, A. Hallberg, A. Rosenquist, B. Samuelsson, Design and synthesis of potent inhibitors of the malaria aspartyl proteases plasmepsin I and II. Use of solid-phase synthesis to explore novel statine motifs, *J Med Chem* 47 (2004) 3353-3366.
- [135] E. Lefebvre, C.A. Schiffer, Resilience to resistance of HIV-1 protease inhibitors: profile of darunavir, *AIDS Rev* 10 (2008) 131-142.
- [136] K.E. Estes, S.R. Penzak, K.A. Calis, T.J. Walsh, Pharmacology and antifungal properties of anidulafungin, a new echinocandin, *Pharmacotherapy* 29 (2009) 17-30.
- [137] S.L. Gonias, S.V. Pizzo, Conformation and protease binding activity of binary and ternary human alpha 2-macroglobulin-protease complexes, *J Biol Chem* 258 (1983) 14682-14685.
- [138] A.J. Barrett, P.M. Starkey, The interaction of alpha 2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism, *Biochem J* 133 (1973) 709-724.
- [139] M. Gacko, A. Minarowska, A. Karwowska, L. Minarowski, Cathepsin D inhibitors, *Folia Histochem Cytobiol* 45 (2007) 291-313.
- [140] A. Minarowska, A. Karwowska, M. Gacko, Quantitative determination and localization of cathepsin D and its inhibitors, *Folia Histochem Cytobiol* 47 (2009) 153-177.
- [141] E.K. Kick, D.C. Roe, A.G. Skillman, G. Liu, T.J. Ewing, Y. Sun, I.D. Kuntz, J.A. Ellman, Structure-based design and combinatorial chemistry yield low nanomolar inhibitors of cathepsin D, *Chem Biol* 4 (1997) 297-307.
- [142] J.A. Ellman, Combinatorial methods to engineer small molecules for functional genomics, *Ernst Schering Res Found Workshop* (2000) 183-204.
- [143] X. Bi, T.S. Haque, J. Zhou, A.G. Skillman, B. Lin, C.E. Lee, I.D. Kuntz, J.A. Ellman, G. Lynch, Novel cathepsin D inhibitors block the formation of hyperphosphorylated tau fragments in hippocampus, *J Neurochem* 74 (2000) 1469-1477.
- [144] W.J. Wood, A.W. Patterson, H. Tsuruoka, R.K. Jain, J.A. Ellman, Substrate activity screening: a fragment-based method for the rapid identification of nonpeptidic protease inhibitors, *J Am Chem Soc* 127 (2005) 15521-15527.
- [145] V. Srivastava, H.O. Saxena, K. Shanker, J.K. Kumar, S. Luqman, M.M. Gupta, S.P. Khanuja, A.S. Negi, Synthesis of gallic acid based naphthophenone fatty acid amides as cathepsin D inhibitors, *Bioorg Med Chem Lett* 16 (2006) 4603-4608.
- [146] M. Fusek, M. Mares, J. Vagner, Z. Voburka, M. Baudys, Inhibition of aspartic proteinases by propeptide peptides of human procathepsin D and chicken pepsinogen, *FEBS Lett* 287 (1991) 160-162.
- [147] M. Masa, L. Maresova, J. Vondrasek, M. Horn, J. Jezek, M. Mares, Cathepsin D propeptide: mechanism and regulation of its interaction with the catalytic core, *Biochemistry* 45 (2006) 15474-15482.
- [148] C.G. Knight, A.J. Barrett, Interaction of human cathepsin D with the inhibitor pepstatin, *Biochem J* 155 (1976) 117-125.
- [149] F.M. Tumminello, G. Leto, N. Gebbia, L. Rausa, R.J. Bernacki, Evaluation of antitumor and antimetastatic activity of pepstatin A in some experimental tumor models, *J Chemother* 1 (1989) 1135-1138.
- [150] M. Bessodes, K. Antonakis, J. Herscovici, M. Garcia, H. Rochefort, F. Capony, Y. Lelievre, D. Scherman, Inhibition of cathepsin D by tripeptides containing statine analogs, *Biochem Pharmacol* 58 (1999) 329-333.

- [151] P. Lison, I. Rodrigo, V. Conejero, A novel function for the cathepsin D inhibitor in tomato, *Plant Physiol* 142 (2006) 1329-1339.
- [152] S.A. Cater, W.E. Lees, J. Hill, J. Brzin, J. Kay, L.H. Phylip, Aspartic proteinase inhibitors from tomato and potato are more potent against yeast proteinase A than cathepsin D, *Biochim Biophys Acta* 1596 (2002) 76-82.
- [153] B. Lenarcic, V. Turk, Thyroglobulin type-1 domains in equistatin inhibit both papain-like cysteine proteinases and cathepsin D, *J Biol Chem* 274 (1999) 563-566.
- [154] B. Strukelj, B. Lenarcic, K. Gruden, J. Pungercar, B. Rogelj, V. Turk, D. Bosch, M.A. Jongsma, Equistatin, a protease inhibitor from the sea anemone *actinia equina*, is composed of three structural and functional domains, *Biochem Biophys Res Commun* 269 (2000) 732-736.
- [155] M. Shibata, M. Koike, S. Waguri, G. Zhang, T. Koga, Y. Uchiyama, Cathepsin D is specifically inhibited by deoxyribonucleic acids, *FEBS Lett* 517 (2002) 281-284.
- [156] V. Vetvicka, J. Vetvickova, I. Hilgert, Z. Voburka, M. Fusek, Analysis of the interaction of procathepsin D activation peptide with breast cancer cells, *Int J Cancer* 73 (1997) 403-409.
- [157] B.M. Dunn, S. Hung, The two sides of enzyme-substrate specificity: lessons from the aspartic proteinases, *Biochim Biophys Acta* 1477 (2000) 231-240.
- [158] O. Schilling, C.M. Overall, Proteome-derived, database-searchable peptide libraries for identifying protease cleavage sites, *Nat Biotechnol* 26 (2008) 685-694.
- [159] M. Lkhider, R. Castino, E. Bouguyon, C. Isidoro, M. Ollivier-Bousquet, Cathepsin D released by lactating rat mammary epithelial cells is involved in prolactin cleavage under physiological conditions, *J Cell Sci* 117 (2004) 5155-5164.
- [160] D. Piwnica, I. Fernandez, N. Binart, P. Touraine, P.A. Kelly, V. Goffin, A new mechanism for prolactin processing into 16K PRL by secreted cathepsin D, *Mol Endocrinol* 20 (2006) 3263-3278.
- [161] D. Seveler, P. Jiang, S.H. Yen, Cathepsin D is the main lysosomal enzyme involved in the degradation of alpha-synuclein and generation of its carboxy-terminally truncated species, *Biochemistry* 47 (2008) 9678-9687.
- [162] M. Sakaguchi, H. Murata, H. Sonogawa, Y. Sakaguchi, J. Futami, M. Kitazoe, H. Yamada, N.H. Huh, Truncation of annexin A1 is a regulatory lever for linking epidermal growth factor signaling with cytosolic phospholipase A2 in normal and malignant squamous epithelial cells, *J Biol Chem* 282 (2007) 35679-35686.
- [163] B. Christensen, L. Schack, E. Klaning, E.S. Sorensen, Osteopontin is cleaved at multiple sites close to its integrin-binding motifs in milk and is a novel substrate for plasmin and cathepsin D, *J Biol Chem* 285 (2010) 7929-7937.
- [164] O. Schilling, C.M. Overall, Proteomic discovery of protease substrates, *Curr Opin Chem Biol* 11 (2007) 36-45.
- [165] C.M. Overall, C.P. Blobel, In search of partners: linking extracellular proteases to substrates, *Nat Rev Mol Cell Biol* 8 (2007) 245-257.
- [166] A. Doucet, C.M. Overall, Protease proteomics: revealing protease in vivo functions using systems biology approaches, *Mol Aspects Med* 29 (2008) 339-358.
- [167] G.S. Butler, R.A. Dean, D. Smith, C.M. Overall, Membrane protease degradomics: proteomic identification and quantification of cell surface protease substrates, *Methods Mol Biol* 528 (2009) 159-176.
- [168] R.A. Dean, C.M. Overall, Proteomics discovery of metalloproteinase substrates in the cellular context by iTRAQ labeling reveals a diverse MMP-2 substrate degradome, *Mol Cell Proteomics* 6 (2007) 611-623.
- [169] P.L. Faust, S. Kornfeld, J.M. Chirgwin, Cloning and sequence analysis of cDNA for human cathepsin D, *Proc Natl Acad Sci U S A* 82 (1985) 4910-4914.

Figure legends

Figure 1. Schematic representation of the human cath-D 52 kDa pro-cath-D sequence

The locations of the 4-kDa cath-D pro-fragment, 14-kDa light, and of the 34-kDa heavy mature chains are indicated. The intermediate 48-kDa form (not shown) corresponds to non-cleaved 14 + 34 kDa chains. According to [169], 1 corresponds to the first amino acid in mature cath-D. The positions of the 2 aspartic acids of the catalytic site are shown, as are the 2 glycosylated chains carrying M6P motifs that are recognized by the M6P receptors. K, kilodalton.

Figure 2. Localization of cath-D in cancer cells

In cancer cells, overexpressed cath-D accumulates in cells where it may affect their degradative capacities. The pro-enzyme is also hypersecreted in the tumor micro-environment. Cath-D hypersecreted by cancer cells may be captured back by both cancer and stromal cells, thus affecting the tumor micro-environment (modulation of stromal cell behavior and/or of components of the extracellular matrix). After lysosomal membrane permeabilization (LMP) during apoptosis, lysosomal mature 34-kDa cath-D released into the cytosol may interact with and/or degrade pro-apoptotic or anti-apoptotic proteins. A cytoplasmic form of cath-D may also be involved in the regulation of transcription in cancer by interacting with nuclear proteins and modulating their activity. K, kilodalton.