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CATHEPSIN D – MANY FUNCTIONS OF ONE ASPARTIC PROTEASE

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Abstract

For years, it has been held that cathepsin D (CD) is involved in rather nonspecific protein degradation in a strongly acidic milieu of lysosomes. Studies with CD knock-out mice revealed that CD is not necessary for embryonal development but it is indispensable for postnatal tissue homeostasis. Mutation that abolishes CD enzymatic activity causes neuronal ceroid lipofuscinosis (NCL) characterized by severe neurodegeneration, developmental regression, visual loss and epilepsy in both animals and humans.

In the last decade, however, an increasing number of studies demonstrated that enzymatic function of CD is not restricted solely to acidic milieu of lysosomes with important consequences in regulation of apoptosis. In addition to CD enzymatic activity, it has been shown that apoptosis is also regulated by catalytically inactive mutants of CD which suggests that CD interacts with other important molecules and influences cell signaling.

Moreover, procathepsin D, secreted from cancer cells, acts as a mitogen on both cancer and stromal cells and stimulates their pro-invasive and pro-metastatic properties. Numerous studies found that pCD/CD level represents an independent prognostic factor in a variety of cancers and is therefore considered to be a potential target of anti-cancer therapy.

Studies dealing with functions of cathepsin D are complicated by the fact that there are several simultaneous forms of CD in a cell – pCD, intermediate enzymatically active CD and mature heavy and light chain CD. It became evident that these forms may differently regulate the above mentioned processes.

In this article, we review the possible functions of CD and its various forms in cells and organisms during physiological and pathological conditions.

Keywords

Alzheimer's disease; Apoptosis; Atherosclerosis; Aspartic protease; Cancer; Cathepsin D; Knock-out mice; Procathepsin D

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1. Introduction

Cathepsin D (CD) is a soluble lysosomal aspartic endopeptidase (EC 3.4.23.5) synthesized in rough endoplasmic reticulum as preprocathepsin D. After removal of signal peptide, the 52 kDa procathepsin D (pCD) is targeted to intracellular vesicular structures (lysosomes, endosomes, phagosomes) [1,2]. pCD is a glycoprotein with two N-linked oligosaccharides modified with mannose 6-phosphate (M6P) residues at asparagine residues 70 and 199 [3,4]. Lysosomal targeting is mediated by two M6P-receptors (cation-dependent 46 kDa and cation-independent 300 kDa M6PR) [2,5]. Another way to target pCD to lysosomes is independent of the M6P tag and is not yet fully understood but the role of sphingolipid activator precursor protein prosaposin was suggested [6–10].

Upon entering acidic endosomal and lysosomal compartment, the cleavage of the 44 amino acid N-terminal propeptide results in a 48 kDa single chain intermediate active enzyme form. Further proteolytic cleavage that does not result in dissociation of CD globular structure yields the mature active lysosomal protease which is composed of heavy (34 kDa) and light (14 kDa) chains linked by non-covalent interactions [11–13]. Cysteine proteases and autocatalytic activity of CD is most-likely involved in pCD/CD processing [14–17]. Several factors were found to affect CD activation including a lipid second messenger ceramide and prosaposin [10,18].

Under normal physiological conditions, pCD is sorted to the lysosomes and found intracellularly unlike other members of the aspartic endopeptidase family, which are mostly secretory proteins [19]. In some physiological and pathological conditions, pCD/CD escapes normal targeting mechanism and is secreted from the cells. pCD was found in human, bovine and rat milk [20–22], serum [23] and the presence of both pCD and 34 kDa CD was demonstrated in human eccrine sweat and urine [23,24]. pCD is a major secreted protein of numerous types of cancer cells [25]. It has been also shown that secreted pCD can be endocytosed via M6PR or another yet unknown receptor by both cancer cells and fibroblasts. Endocytosed pCD undergoes further maturation into 48 kDa intermediate and 34 kDa and 14 kDa forms [26,27]. CD expression and activity was also detected in extracellular matrix and synovial fluid of cartilage during physiological and pathological conditions [28–31]. pCD and mature CD was also found in macrophage-conditioned media and extracellularly in macrophage-rich regions of atherosclerotic lesions [32]. We have recently demonstrated pCD secretion by human keratinocytes [33].

Cathepsin D has been studied over last three decades, mainly from the perspective of its role in cancer development and as a suggested independent tumor marker. This research has also impacted the specification of CD's physiological role and helped to discover new functionalities of CD. Therefore, it is imperative to combine both functions in order to better understand the therapeutic and diagnostic potential of CD in human oncology.

2. Physiological function

Aspartic proteases form a group of enzymes that consist of two lobes separated by a cleft containing the catalytic site made up of two aspartate residues. Similar to other aspartic proteinases (pepsin, renin, cathepsin E, chymosin, HIV protease), CD accommodates up to 8 amino acid residues in the binding cleft of the active site. There is a preference for hydrophobic residues around the cleaved bond. The specificity toward oligopeptidic substrates is only slightly narrower than the broad specificity of pepsin while renin and HIV protease possess high specificity [34,35].

Numerous physiological functions of CD have been suggested, based on its ability to cleave structural and functional proteins and peptides. These include metabolic degradation of

intracellular proteins, activation and degradation of polypeptide hormones and growth factors, activation of enzymatic precursors, processing of enzyme activators and inhibitors, brain antigen processing and regulation of programmed cell death (Tab 1) [24,32,36–81].

2.1 CD knock-out mice - overall

Recent advances in understanding the role of CD in organisms were obtained by analysis of CD knock-out mice. These develop normally and do not have any manifest phenotype at the time of birth suggesting that CD is not essential during embryonic development [82]. It is likely that a functional overlap in cathepsins function ensures the proper protein degradation and turnover during embryonic development even in the absence of one single cathepsin [83]. CD-deficient mice, however, developed abnormalities later in life.

Two weeks postnatally, CD-deficient mice exhibited weight loss that is associated with progressive atrophy of intestinal mucosa, followed by massive intestinal necrosis, thromboembolia and profound destruction of lymphoid cells in the spleen and thymus. In addition, near the terminal stage they develop seizures and progressive retinal atrophy, which leads to blindness. Mice died in a state of anorexia at the age of 4 weeks [82,84]. Increased apoptosis observed in the thymus, thalamus and retina suggests that CD is essential for proteolysis of proteins regulating cell growth, tissue homeostasis, remodelling and renewal [84].

2.1.1 Neuronal ceroid lipofuscinosis—Inactivation of CD homologue in *Drosophila* [85] or naturally occurring mutation of CD gene in sheep [86,87] and American bulldogs [88], resulted in progressive neuronal accumulation of autofluorescent storage material lipofuscin (“aging pigment”) and neurodegeneration. Similar to these animal models, human neuronal ceroid lipofuscinosis (NCL) forms are characterized by neurodegeneration, developmental regression, visual loss and epilepsy. It has been shown that mutations in CD that significantly reduced CD activity and/or affect pCD stability, postranslational processing, and intracellular targeting, result in NCL development [89–92]. Phenotype severity in both animals and humans in CD-derived form of NCL appears to depend on residual CD enzymatic activity. It should be noted, however, that only CD knock-out mice, and not other CD-deficient mammals, exhibit devastating pathological changes in the lymphoid organs and intestine. At the present time, it remains unclear whether this visceral pathology reflects species-species differences or whether other molecular lesions occurred during production of CD knock-out mice. However, such pathology in the mice may also arise from deficiency in CD protein which is present in other animal models of NCL. This intriguing possibility could be investigated by producing “knock-in” mice with specific targeted mutations in the CD gene.

2.1.2 Mechanisms of neuronal and retinal cell death in CD knock-out mice—

Although the exact mechanism of neuron death is not entirely clear, a dramatic induction of autophagic stress has been evidenced in the brains of CD-deficient mice. The number of autophagosomes containing ceroid lipofuscin, cathepsin B and subunit c mitochondrial ATP synthase progressively increases in neurons from day 0 to day 20 [92,93]. Altered regulation of pro-survival phosphatidylinositol 3-kinase signaling following autophagic stress has also been observed [94]. Autophagic stress precedes markers of apoptosis suggesting that CD-deficiency-induced lysosome dysfunction may contribute to induction of apoptosis. However, although deficiency in pro-apoptotic protein Bax clearly reduced apoptosis in CD-deficient brain, it did not alter the severity of neurodegeneration, nor attenuates autophagic stress and neuron loss suggesting an importance of caspase-independent neuron death in NCL brains [95].

In retina of CD-deficient mice, classical caspase-dependent apoptotic pathway was activated in a specific population of RPE cells while in other RPE cell populations the role of NO-induced cell death has been suggested [84]. In a transgenic mouse model expressing both catalytically inactive CD and wild-type CD, RPE changes indicating accelerated debris accumulation, RPE atrophy, proliferation and the accumulation of basal laminar and linear deposits were associated with hyperfluorescence, hypopigmentation, decrease in number of photoreceptors and pleomorphism [96].

All these data suggest that CD enzymatic activity is important in postnatal tissue homeostasis including tissue renewal, remodelling and regulation of aging and programmed cell death.

2.1.3 CD role in skin development and function—In addition to the role of CD in brain and retina, altered expression of CD in the skin has been reported during wound healing and psoriasis, a hyperproliferative skin disorder [54,97,98]. CD protein expression and enzymatic activity increases during epidermal differentiation. Pepstatin A, an inhibitor of CD enzymatic activity, prevented an increase in the CD protein expression and activity and significantly delayed permeability barrier repair after experimental disruption. CD belongs to candidate proteases that intracellularly activate a zymogen of transglutaminase 1 (TG-1) into enzymatically active form by proteolytic cleavage [54]. Upon activation, TG-1 cross-links the proteins of cornified envelope, a structure beneath plasma membrane of keratinocytes, during epidermal differentiation [99–101]. CD knock-out mice have reduced TG-1 activity and reduced protein levels of the cornified envelope proteins involucrin, loricrin, filaggrin and keratins K1 and K5. Morphology of stratum corneum in CD-deficient mouse was impaired resembling to the human skin disease lamellar ichthyosis [54]. It should be noted, however, that the exact site of TG-1 cleavage by CD is unknown. In keratinocytes, most of the newly synthesized TG-1 is attached to membrane and therefore the possible mechanism of its cleavage by lysosomal CD remains ambiguous [102]. Moreover, the role of cytoplasmic cathepsin in TG-1 processing and cornified envelope formation has also been demonstrated [103].

Beside the suggested role of CD enzymatic activity during epidermal development, we observed that exogenous addition of enzymatically inactive pCD enhanced proliferation and regeneration of keratinocyte cell line HaCaT *in vitro*. Moreover, substantial secretion of pCD, up-regulated by stress conditions, was also observed in these cells [33]. These data suggest that enzymatically inactive pCD may serve as mitogenic factor for keratinocytes in a manner similar as that for cancer cells (discussed later).

Taken together, CD enzymatic activity may be involved in the processes of epithelial differentiation, epidermal barrier function and wound healing. The possible role of pCD, however, can not be ruled out and needs further investigation.

2.2 Apoptosis

In the classic cell death paradigm, lysosomes were solely considered to be involved in necrotic and autophagic cell death and the role of lysosomal proteases was limited to the nonspecific protein degradation. In the last decade, however, it has become evident that the role of lysosomes in cell death is far more sophisticated [104]. Some models of apoptosis appear to be dependent either on cathepsins or caspases, whereas others rely on both caspases and cathepsins for their initiation and execution. Partial lysosomal permeabilization, with subsequent release of proteolytic enzymes into cytosol, and their active contribution to the apoptosis signaling pathways, has been described in several models of apoptosis [83]. Studies with lysosomotropic detergents indicate that the key factor in determining the type of cell death is the magnitude of lysosomal permeabilization. Whereas partial, selective permeabilization triggers apoptotic-like cell death, massive breakdown of lysosomes results in unregulated necrosis [105–107]. Bidere et al. suggested that the release of proteins from lysosomes may

be size selective [108]. In many instances, lysosomal permeabilization appears to be an early event in the apoptotic cascade, preceding other hallmarks of apoptosis like destabilization of mitochondria, cytochrome c release and caspase activation. Several mechanisms of lysosomal membrane permeabilization were proposed including the involvement of reactive oxygen species [109–111], members of Bcl-2 family [112], and intracellular sphingosine, a biologically active metabolite of ceramide [69,113].

The release of lysosomal proteases may directly or indirectly cause mitochondrial dysfunction. Addition of purified CD to mitochondria *in vitro* results in substantial ROS generation and cytochrome c production [114]. In endothelial cells, hydrogen peroxide cytotoxicity is mediated via CD executed intralysosomal degradation of thioredoxin-1, an essential anti-apoptotic and ROS scavenging protein [70]. Bidere et al. showed that CD triggers Bax activation and relocation to the mitochondria, resulting in release of apoptosis-inducing factor (AIF) during staurosporine induced apoptosis of T lymphocytes [108]. In another model of apoptosis, ectopic expression of CD in CD-deficient fibroblasts results in enhanced TNF-mediated apoptosis presumably by ability of CD to cleave Bcl-2 family member Bid, subsequent release of cytochrome c from mitochondria and activation of caspase-9 and -3. These processes are dependent on endosomal ceramide generated by the endosomal acid sphingomyelinase [69]. Ceramide is a mediator of apoptosis induced by various cytokines, ionizing radiation, serum deprivation, glucocorticoids and anticancer drugs in many cell types [115,116]. In CD-deficient fibroblast model of TNF-mediated apoptosis, endosomal ceramide binds pCD, induces CD maturation and translocation of CD into cytosol [69]. Interestingly, increased ceramide levels in HT-29 colorectal cancer cells, induced by exogenous N-acetylsphingosine (NAS) or by 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol (PDMP), inhibited transport and processing of pCD. Therefore, it has been proposed that compartmentalization influences the consequences of ceramide increase on lysosomal targeting and maturation of pCD [117].

While some studies reported that CD can directly induce apoptosis [118–120], many studies demonstrated that CD is a mediator of apoptosis induced by several stimuli, such as staurosporine, IFN- γ , Fas/CD95/APO-1, TNF, etoposide, 5-fluorouracil, cisplatin [118,121,122], adriamycin [122], resveratrol [123], doxorubicin [124], growth factor deprivation [125], oxidative stress [109,126,127], and sphingosine [113]. These data suggest that CD promotes apoptosis induced by cytotoxic and stress agents. It should be noted, however, that the central role of CD in induced apoptosis has been challenged by other reports [128, 129]. Moreover, anti-apoptotic role of CD has been also described. Exogenously expressed CD significantly inhibited tumor apoptosis in cancer xenografts [130] and transfection of wt or mutant catalytically inactive CD promotes survival of CD-deficient fibroblasts [27].

One of the most discussed problems in studies involving pro-apoptotic role of CD is the question of its possible enzymatic activity in slightly acidic cytosol of pre-apoptotic cells. CD is optimally active against most substrates at pH 3 to 4. However, CD is able to cleave Bid at pH 6.2 and tau protein at pH 7 *in vitro* [57,69]. Bidere et al. found that Bafilomycin A, an inhibitor of lysosomal acidification, did not inhibit CD mediated apoptosis of T lymphocytes induced by staurosporine. This process, however, was inhibited by pepstatin A. Authors suggested that CD substrates with high affinity could stabilize the active conformation of CD at neutral pH in a similar manner as pepstatin A [108]. Moreover, Heinrich et al. found that Bid colocalizes with CD in vesicular endosome-like structures suggesting that cleavage of Bid may not occur in cytosol [69]. In hydrogen peroxide model of apoptosis, thioredoxin-1 translocates to lysosomes and is cleaved by CD at acidic pH [70].

The large number of studies demonstrated the involvement of CD activity in apoptotic process by using pepstatin A. Pepstatin A is a potent but relatively unspecific inhibitor of aspartic proteases, including cathepsins D and E, renin, and pepsin. It is a hexapeptide originally

isolated from cultures of various species of Actinomyces. One of the major problems in using pepstatin is its poor solubility and inefficient membrane penetration. To overcome these problems, various analogs were synthesized to improve its bioavailability and selectivity but thus far these analogs have not seen common use in biological research [131,132]. In addition to its role in inhibiting protease activities, other nonspecific effects of pepstatin A could not be ruled out. It has been shown that higher doses of pepstatin A inhibit MAPK signaling [133]. In a similar fashion, pharmacological inhibitors of caspases have been shown to nonspecifically inhibit cathepsins, raising the possibility that the role of cathepsins may have been overlooked in defining many „caspase-dependent“ processes [121,134]. The design, synthesis and use of new specific CD inhibitors is therefore of great interest and may have important research and therapeutical consequences [19,135].

In recent years, some authors reported that wild type as well as mutant catalytically inactive CD can induce apoptosis. Microinjection of wt or mutant CD induces apoptosis in human fibroblasts that was not inhibited by pepstatin A [120]. Both wild-type and mutant CD strongly enhances apoptotic response to etoposide, doxorubicin and 5-fluorouracil in rat tumor cell line [124]. These data highlight the possibility that CD pro-apoptotic effect may be mediated by interaction with some member(s) of apoptotic machinery.

In conclusion, it appears that CD triggers apoptosis via multiple molecular pathways which often integrate with traditional mediators of apoptosis, like cyt c, caspases and Bcl-2 family members. Some pathways may rely on CD enzymatic activity while others on pCD/CD interacting capacity (Fig 1).

3. Pathological function

In addition to its physiological function, numerous studies described the importance of pCD/CD in pathological processes with a majority of studies dealing with its role in cancer. Before we review the function of pCD/CD in cancer, we will briefly summarize possible connections to other pathologies that are currently discussed in CD-related literature.

3.1 Alzheimer's disease

The pathologic hallmarks of Alzheimer's disease (AD) are plaques of extracellular amyloid-beta protein and intraneuronal neurofibrillary tangles of hyperphosphorylated tau proteins, both of which accumulate in brain regions mediating memory and cognition [136]. The association between CD and AD has been suggested. In AD, senile plaques and tangles show abundant CD immunoreactivity. The CD level also increases in cerebrospinal fluid [137–141]. CD has been implicated in the processing of amyloid precursor protein (APP) [56], apolipoprotein E (apoE) [61] and the tau protein [57], which are important factors of AD pathogenesis [136, 142]. However, the study of CD-knockout mice revealed that CD is not essential for APP processing [143]. It is yet to be determined whether the processing of apolipoprotein E and tau by CD in the brain is pathologically relevant.

A CD Ala38Val polymorphism that has been linked to altered intracellular routing and maturation of the proenzyme [144,145] has been associated with AD [146–148], the level of beta-amyloid and tau [149–151] but there was significant heterogeneity in the results of various studies. According to the results of two recent meta-analyses, we may conclude that the impact of this CD polymorphism on the risk of AD development is rather small on a population level [152,153].

3.2 Atherosclerosis

Atherogenesis is characterised by the accumulation of low-density lipoprotein (LDL)-derived lipids in the ECM of the arterial intima, recruitment of macrophages, and their transformation

into lipid-laden foam cells [154]. *In vivo* and *in vitro* results suggest that lysosomal enzymes, including pCD, are released from monocyte-derived macrophages in atherosclerotic lesions [32,155]. Macrophages are able to acidify their environment by proton pumps and secretion of lactic acid [156,157]. Therefore, macrophage pericellular environment may be sufficiently acidic for CD activation.

CD enzymatic activity induces hydrolytic modification of apolipoprotein B-100-containing lipoproteins, including LDL [32,74,158,159]. These modifications render the LDL particles unstable and induce their fusion and extracellular accumulation in the arterial intima. Macrophages and smooth muscle cells take up the modified LDL avidly and are transformed into foam cells [32,155].

Abundant apoptotic and necrotic cells exist in human atherosclerotic lesions [160]. LDL oxidation is considered to be an important step in atherogenesis. oxLDL contributes to atherosclerosis by causing cell death [161,162]. After its uptake into macrophage lysosomes by receptor-mediated endocytosis, oxLDL are poorly degraded and induce synthesis of CD, destabilization of lysosomes and relocation of CD into cytosol. The release of lysosomal enzymes to the cytosol may subsequently induce cell death [163–165].

Recently, it has been shown that CD expression is reduced in macrophages of low HDL-cholesterol subjects and the role of CD in intracellular metabolism and transport of phospholipids and cholesterol was suggested [166].

3.3 Cancer

The cathepsin D is in respect to cancer studied in these related parallel approaches – i) the possible use of its level/activity as marker of cancer status and prognosis; ii) its functional role in cancer progression including mitogenic functions, influence on invasion, metastasis, angiogenesis, apoptosis and consequently - iii) the use of cathepsin D as a target of cancer therapy.

3.3.1. Cathepsin D as a tumor marker—Increased levels of CD were first reported in several human neoplastic tissues more than 20 years ago [167]. Several years later, the first clinical studies found pCD/CD related to metastasis-free survival and disease-free survival in breast cancer patients [168,169]. Since then, numerous clinical studies reported an association between pCD/CD level and tumor size, tumor grade, tumor aggressiveness, incidence of metastasis, prognosis, and a degree of chemoresistance in variety of solid tumors including neuroblastoma, glioma, melanoma, endometrial and ovarian tumors, colorectal carcinoma, head and neck tumors, thyroid tumors, pancreatic tumors, lung carcinoma, liver tumors, bladder carcinoma, prostate tumors and gastric carcinoma [25,170,171]. It should be noted, however, that conflicting results were obtained which may reflect the different methodology used for pCD/CD quantification, patients and diagnosis selection, and the length of follow-up period [25,172,173]. Studies dealing with pCD/CD diagnostic and prognostic value in cancer are complicated by the fact that there are several forms of CD in a cell at the same time – pCD, intermediate enzymatically active CD and mature heavy and light chain CD (Fig 2). Moreover, different forms of CD are also present in stromal cells and may therefore affect pCD/CD quantification in tumor tissues and consequently its prognostic significance. A standardization of techniques is therefore needed for further evaluation of therapeutic and prognostic significance of pCD/CD expression in solid tumors.

A majority of clinical studies focused on the role of pCD/CD as a tumor marker were published on breast cancer. Large studies and one meta-analysis found that pCD/CD level in tumor homogenate measured by either ELISA or IRMA represents an independent prognostic factor [174–177]. In these studies, antibodies that can detect both pCD (52 kDa) and CD (48 and 34

kDa) were used. On the contrary, results of immunohistological (IHC) studies using antibodies specific to either pCD, CD or both are less consistent. This could possibly be explained by the use of different tissue fixation and antibodies, semi-quantitative nature of IHC and under-estimation of secreted pCD amount.

According to recent recommendation of American Society of Clinical Oncology, data published thus far are insufficient to use cathepsin D measurement for management of patients with breast cancer [178].

3.3.2 Functional role of cathepsin D in cancer progression

3.3.2.1 Cancer cell proliferation: The mitogenic effect of secreted pCD on breast cancer cells was first proposed by Vignon et al. in 1986 [179]. Since that time, numerous studies clearly demonstrated that pCD secreted from cancer cells serve as an autocrine growth factor for breast [180,181], prostate [182,183], ovarian [184] and lung cancer cells [185,186]. Breast cancer cells with down-regulated expression of pCD by either antisense gene transfer [187], RNA interference [188] or ribozymes [189] displayed reduced growth *in vitro* and *in vivo*. Tumor growth was also inhibited by anti-pCD antibodies *in vivo* and *in vitro* [180,183,190,191].

Earlier studies suggested that CD may stimulate cancer growth via its enzymatic activity. Several growth factors, growth factor receptors and extracellular matrix (ECM) components have been found among CD substrates (Tab 1). Moreover, CD digests various chemokines and may therefore attenuate anti-tumoral immune response [50]. The extracellular pH of tumors is moderately acidic [192,193] and the secreted pCD could therefore be converted to the active enzyme in an extracellular milieu. However, we showed that the growth promoting effect of pCD on cancer cells is not inhibited by pepstatin A [180,183]. Recent studies clearly demonstrated that enzymatically inactive pCD mutants stimulate growth of cancer cells *in vitro* and *in vivo* in the same manner as wild type pCD [130,194].

The mechanism of pCD mitogenic effect on cancer cells remains unclear. We, as well as others, have shown that secreted pCD binds to surface of breast cancer cells [26,190]. Thus we hypothesize that pCD binds to a cell surface receptor with signaling properties. Despite a significant effort, the suggested pCD receptor has not been identified as yet and its molecular characterisation remains elusive.

Until now, the only receptors with known pCD/CD binding capacity are M6P receptors that recognize M6P tag on numerous glycoproteins. It has been shown that pCD secreted by cancer cells is highly glycosylated and is able to bind to M6P/IGF-II receptor (cation-independent M6PR) on breast cancer cell surface [195–198]. Numerous studies demonstrated that neither binding nor pCD mitogenic potential is blocked by M6P, anti-M6PR antibodies or pCD deglycosylation [180,190,194,199]. Moreover, we recently showed that mutation in one or both glycosylation sites of pCD only slightly lower pCD mitogenic and pro-invasive activity *in vivo* and *in vitro* [200]. These results indicate that the sugar moieties are not important in the tumor-promoting effect of pCD and that M6P receptors are not involved in mediating pCD mitogenicity. The binding of pCD to M6P/IGF-II receptor may, however, decrease its binding capacity for other M6P/IGF-II receptor natural ligands (e.g. IGF-II, latent TGF- β) and thus perturb their biological functions [198].

We found that binding to cancer cells as well as pCD mitogenic potential is blocked by antibodies specific for propeptide part of pCD [180,183,191]. The propeptide (also called activation peptide – AP) of pCD serves at neutral or basic pH to block the access of substrates to the active site. The active site of CD forms a deep cleft between the two lobes of the active enzyme [201,202]. According to the 3D model of the pCD structure constructed by *in silico* homology molecular modeling using known coordinates of pCD and pepsinogen, the AP forms

a loop where most of the N- terminal half is making electrostatic bonds with the active site aspartates and most of the C-terminal part of AP is on the surface of the molecule of pCD suggesting that the C-terminal part can interact with other molecules [145,203].

Using synthetic peptides corresponding to different parts of AP, we showed that the region responsible for binding of pCD to cancer cell surface is localised between amino acids 33–44 of the AP [190,191]. In numerous experiments using synthetic AP, anti-AP antibodies or mutant pCD with deleted AP, we demonstrated that AP itself stimulates growth of breast, prostate and lung cancer cells *in vitro* and *in vivo* [180,182,183,185,186,190,191,199,200]. Although the mitogenic effect of AP was not confirmed by Glondou et al. under their experimental conditions [194], Bazzet et al. independently demonstrated mitogenicity of AP in ovarian cancer cells [184].

Recently, the growth-promoting effect of pCD was also described for fibroblasts [27] and keratinocytes [33]. While in the latter case the role of pCD in wound healing has been suggested, the role of pCD as paracrine communicator between cancer cells and stromal fibroblasts might be extremely important for cancer development. Laurent-Matha et al. described that pCD secreted from cancer cells stimulates fibroblasts proliferation, survival, motility and invasive capacity, accompanied by activation of the ras-MAPK pathway [27]. pCD secreted from cancer cells might be therefore crucial for fibroblast invasive outgrowth and further dissemination of cancer.

3.3.2.2 Invasion and metastasis: In addition to the mitogenic effects of pCD on cancer cells, numerous studies demonstrated the involvement of pCD in cancer invasion and metastasis. The direct role of pCD in cancer metastasis was first demonstrated in rat tumor cells in which over-expression of pCD increased their metastatic potential [204]. Glondou et al. described that down-regulation of pCD expression by antisense gene transfer inhibits lung metastasis of breast cancer cells MDA-MB-231 but no effect on invasion *in vitro* was observed [187]. On the contrary, in a similar experiment, Tedone et al. found that down-regulation of pCD expression reduced invasion of breast cancer MCF-7 cells *in vitro* [205]. Sivaparvathi et al. observed that anti-CD antibody inhibited glioblastoma cell invasion through Matrigel [206]. In our experimental conditions, genetic manipulation with the amount of pCD secreted by breast and lung cancer cells strongly influences their ability to cross Matrigel membrane *in vitro* as well as their metastatic potential *in vivo* [186,188,189,199]. Invasive capacity *in vitro* was also stimulated by overexpression of pCD in CD-deficient fibroblasts [27].

3.3.2.3 Angiogenesis: Several authors suggested that CD is involved in regulation of blood vessel formation, especially in solid tumors. Stromal CD expression correlated with microvessel density in ovarian tumors [207]. Significant association between CD expression of host stromal cells and vascular density was described in breast cancer tumours [208].

Angiogenesis is controlled by the balance between positive and negative angiogenic factors. It has been shown that CD may influence the production and degradation of both activators and inhibitors *in vitro*. Briozzo et al. observed that CD facilitates the release of pro-angiogenic bFGF from ECM [209]. This observation was supported by the finding that CD mediates proteolysis of FGF in conditioned media from breast cancer MCF-7 cells [37]. Moreover, it has been described *in vitro* that CD is able to activate/inactivate cryptic anti-angiogenic factors including angiostatin, 16K prolactin (PRL) and endostatin [38–41,210]. It is unclear, however, whether the above mentioned findings are of any physiological relevance in tumors *in vivo*. Tumors secrete numerous proteases of several classes that can process multiple substrates. Each substrate can usually be cleaved by numerous proteases and substrates cleaved by one protease can have distinct biological activities. Development of *in vivo* imaging strategies for

monitoring CD proteolytic activity and identification of its physiological substrates is therefore necessary for evaluation CD enzymatic activity contribution to cancer progression *in vivo*.

Similarly to the role of pCD in cancer cell proliferation and apoptosis, pCD may influence angiogenesis also by its signaling properties. In agreement with this hypothesis, Berchem et al demonstrated that pCD stimulated tumor angiogenesis in tumor xenografts in athymic nude mice independently on its enzymatic activity [130]. Moreover, we recently observed that several regulators of angiogenesis are differentially expressed upon treatment of breast cancer cells with AP [211].

3.3.2.4 Mechanism of pCD action on cancer cells: To gain insight into the possible mechanism of pCD action, we identified several differentially expressed genes involved in cell cycle progression, cell survival, cell adhesion/angiogenesis, invasion and metastasis upon treatment of breast cancer cells with AP. Differential regulation of some of these genes, including NFKB2 and Cdc42, was confirmed in pCD and AP treated breast and lung cancer cells [186,211]. Moreover, differential expression of these genes was also observed in breast cancer cells with down-regulated expression of pCD by RNA interference as compared to its wild-type counterparts [188].

Recently, we described enhanced secretion of various cytokines from breast cancer and fibroblast cell lines upon pCD or AP treatment [212,213]. Moreover, inhibition of particular cytokine by specific antibody lowered the mitogenic effect of the remaining cytokines and a mixture of all antibodies almost totally abolished the mitogenic effect of pCD or AP [212]. We therefore concluded that the pCD secretion observed in many cancer-derived cell lines leads to a secretion of cytokines that promote the growth of both types of cells.

Based on above mentioned data, we may update a proposed model of mechanism of pCD action [211,214]. The over-expressed pCD escapes normal targeting pathways and is secreted out of the cancer cells. Subsequently, pCD interacts with surrounding proteins and is recognized via its AP part by a yet unidentified cell surface receptor. This interaction releases a signal (e.g. activation of MAPK pathway – [27,188]) that results in differential expression of cancer promoting genes including various cytokines that in turn stimulate tumor growth. pCD secreted by cancer cells is also captured by stromal cells and promote fibroblasts proliferation, motility and invasion that results in cancer progression [27] (Fig 3). In a similar manner, secreted pCD may affect angiogenesis via its action on endothelial cells [215]. As a mutant catalytically inactive pCD is also effective in stimulation of cancer growth and progression, it seems that CD activity is not necessary. The supporting role of CD activity in cancer progression, however, cannot be definitively ruled out. Intraperitoneal administration of pepstatin A significantly reduce the number of lung or liver metastases in mice transplanted with Lewis lung carcinoma, MCa mammary carcinoma or M5076 ovarian reticulum cell sarcoma [216–218].

3.3.3 pCD as a target of cancer therapy—The experimental and clinical findings suggesting an important role of pCD in tumor progression imply that the modulation of its biological activity may represent an efficient tool of cancer therapy. We have previously observed a reduced breast and prostate cancer tumor growth in mice treated with anti-pCD or anti-AP antibodies [183,190,191]. Another possibility is a gene therapy-based inhibition of pCD synthesis. We and others showed that a decrease of pCD expression using antisense, ribozyme or RNA interference approaches, results in suppression of breast cancer tumor growth and metastasis *in vitro* and *in vivo* [187–189]. Animal studies showing that immunization with AP resulted in significant suppression of implanted cancer growth suggest that inhibition of pCD biological activity may be used in cancer prevention [199]. Interestingly, human milk contains relatively high concentration of pCD [20]. The possible significance of

breast-feeding for the risk of cancer development of both mother and child with respect to pCD immunogenicity has been discussed elsewhere [214].

Many lysosomal enzymes, including CD, are overexpressed in cancer cells. Tumor cell death might be caspase independent, due to acquired defects in the classic caspase-dependent pathways of apoptosis. Enhancing lysosomal cell death pathways therefore represents potential therapeutic strategy in various cancers [83]. For the destabilization of the lysosomal membrane, two methods that have therapeutic potential are available: the formation of reactive oxygen species (ROS) by irradiation or by enzymatic reactions and the lysosomal membrane permeabilization by lysosomotropic compounds. While irradiation is commonly used in clinical practice, the sensitization of tumour cells to anticancer drugs by lysosomotropic compounds may represent a new possibility of cancer treatment [219].

4. Summary

In recent years, studies have demonstrated the usefulness of experimental models with mutated catalytically inactive CD to distinguish between CD enzymatic function and pCD/CD non-enzymatic function. Results obtained with these models were a significant supplement to previous data obtained from analysis of CD knock-out mice and from use of unspecific aspartic protease inhibitor pepstatin A. It can be concluded that:

- A. Primary biological function of enzymatically active CD is protein degradation in an acidic milieu of lysosomes. Failure of this function resulted in accumulation of lipofuscin in variety of cell types, neurodegeneration, developmental regression and visual loss.
- B. Active forms of CD can be released together with other proteases from lysosomes into cytosol, in response to apoptotic stimuli and contribute to cell death. The role of cytoplasmic CD in apoptosis may be dependent on, but also independent of, its enzymatic activity.
- C. Enzymatically inactive pCD can be secreted by various cell types including macrophages, keratinocytes, mammary epithelial cells and cancer cells of different origin. Secreted pCD acts as paracrine and autocrine mitogen on cancer cells, fibroblasts and keratinocytes.
- D. Active forms of CD were found extracellularly in human eccrine sweat, urine, seminal fluid, and macrophage-conditioned media. To our knowledge, active secretion of these forms has never been demonstrated. We therefore presume that active CDs are produced by proteolytic processing of secreted pCD. In solid tumors *in vivo*, the existence of active CD forms in extracellular space is assumed (low pH, release from necrotic cells) but has never been directly evidenced. Although cleavage of various substrates by extracellular CD was demonstrated *in vitro*, the functional role of CD enzymatic activity *in vivo* remains to be confirmed.

5. Future prospects

While many functions of CD in the physiological and pathological processes could be attributed to its enzymatic activity, it is evident that some of its functions in the organism are independent on its protease activity and rely on the ability of pCD/CD to interact with other important molecules (Tab 2) [27,33,120,124,130,186,194,212,220]. It seems inevitable that searching for pCD/CD-interacting partners should be conducted in the next years to explore the mechanism of pCD/CD actions.

The design and synthesis of specific CD inhibitors is also of great interest and may have important research and therapeutical consequences. The extensive pharmaceutical experience

with HIV protease inhibitors suggests that CD-selective activity inhibitors could be developed. CD enzymatic activity can be blocked with either substrate peptidomimetics or antibodies against the active site cleft. Antibodies against the propeptide can be used in experiments to block mitogenic function of extracellular pCD.

Lack of standardization in measurement techniques for many of the markers, including pCD/CD, limits their current clinical usefulness in oncology [25,173]. The standardization in pCD/CD measurement is therefore strongly recommended to allow both correct interpretation of obtained data and comparison of clinical study results and their subsequent meta-analysis.

In cancer studies, the failure to identify the putative propeptide receptor, the specific substrates for CD enzymatic activity and the lack of standardization in pCD/CD measurement continue to be disappointments to the field and make understanding of the protein both as marker and functional molecule elusive.

Biographies

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Dr. Vaclav Vetvicka is a Professor of Pathology in the Department of Pathology and Laboratory Medicine, University of Louisville, Kentucky. Dr. Vetvicka graduated from Charles University in Prague, Czech Republic with an advanced degree in biology and obtained his Ph.D. from the Czechoslovak Academy of Sciences. His postgraduate training included a period at the Oklahoma Medical Research Foundation, Oklahoma City, OK, and the Institute of Microbiology, Prague, Czech Republic. Dr. Vetvicka's primary areas of research interest are in aspartic proteases in cancer development and natural immunomodulators. Dr. Vetvicka is author and co-author of more than 190 peer-reviewed publication and seven books.

Dr. Martin Fusek is an assistant professor at the Institute of Chemical Technology and an external scientist at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Czech Republic in Prague. He completed his PhD in the Department of Biochemistry at the Institute of Organic Chemistry and Biochemistry in Prague. Subsequent to this, he spent several years as a postdoctoral fellow in the laboratory of Professor Jordan Tang and Dr. Steven Foundling at the Oklahoma Medical Research Foundation in the United States and in the laboratory of Dr. Peter Metcalf at the European Molecular Biology Laboratory in Germany. In addition to his research, he works in the commercial sphere of Life Sciences. His major field of interest includes the role of aspartic proteinases in human physiology and pathology.

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Figure 1. The role of pCD/CD in apoptosis

After the induction of apoptosis, selective permeabilization of lysosomal membrane results in the release of mature CD into cytosol. This leads to mitochondrial dysfunction. The release of CD may cause mitochondrial dysfunction either directly [114]; by ability of CD to cleave Bcl-2 family member Bid, subsequent formation of active Bax conformation and insertion of active Bax in the outer mitochondrial membrane [69]; or by interaction with unknown member of apoptotic machinery where CD enzymatic activity is not involved [124]. CD-induced mitochondrial dysfunction results in release of cyt c from mitochondria followed by activation of caspase-9 and -3 [69,114]. Alternatively, CD may trigger Bax activation via Bid-independent pathway resulting in release of apoptosis-inducing factor (AIF) and caspase-independent apoptosis [108]. In cancer cells, secreted pCD is recognized by a yet unidentified cell surface receptor. This interaction activates MAPK signaling pathway [27,188] leading to differential expression of important regulators of apoptosis [211] and may therefore affect sensitivity of cancer cells to chemotherapeutics.

Figure 2. Forms of CD in tumors

CD is synthesized in rough endoplasmic reticulum (ER) as preprocathepsin D. In ER/Golgi pathway, signal peptide sequence is cleaved off and procathepsin D (pCD) is glycosylated at asparagine residues. Subsequently, procathepsin D is targeted to intracellular vesicular structures (lysosomes, endosomes). Upon entering these acidic compartments, the cleavage of propeptide (AP) results in a single chain intermediate enzymatically active form. Further proteolytic processing yields the mature CD which is composed of linked heavy and light chains [1–4]. Enzymatically inactive pCD is secreted by cancer cells into extracellular space [25]. It was proposed that pCD can be converted in acidic extracellular milieu to enzymatically active pseudocathepsin D (form that retains residues 27–44 of the propeptide) by autocatalytic processing [17]. Pseudocathepsin D can be hypothetically processed by other proteases to single chain CD or further to mature two-chain CD. In tumors, active forms of CD can also be released from necrotic cells. The existence of particular active CD forms in extracellular space of solid tumors in vivo is therefore assumed but has never been directly evidenced.

Figure 3. Proposed mechanism of pCD/CD function in cancer progression

The over-expressed pCD escapes normal targeting pathways and is secreted by the cancer cells. A small portion of secreted pCD is endocytosed via M6PR pathway, while majority of secreted pCD is recognized by a yet unidentified cell surface receptor [26,27,180,190]. This interaction activates MAPK signaling pathway [27,188] that results in differential expression of cancer promoting genes including various cytokines and NFKB2 [211–213]. This, in turn, stimulates tumor growth and invasion. pCD secreted by cancer cells is also captured by stromal cells and promotes fibroblast proliferation, motility and invasion that results in cancer progression [27]. Moreover, secreted pCD may also enhance angiogenesis by stimulation of endothelial cells [215] or by enzymatic processing of angiogenic factors (after pCD → CD activation in acidic extracellular milieu) [37–40].

Table 1

Possible biological functions of CD based on its ability to cleave target substrates.

Biological function	Target protein	Reference
General protein degradation and turnover	Many (including ↓)	[36]
Activation and degradation of polypeptide hormones, chemokines, growth factors and their receptors	FGF	[37]
	Plasminogen	[38]
	Prolactin	[39,40]
	Endostatin	[41]
	Osteocalcin	[42]
	Thyroglobulin	[43]
	Insulin	[44]
	Glucagon	[45]
	IL-1	[46]
	IGFBP	[47]
	Androgen receptor	[48]
	Parathyroid hormone	[49]
	MIP-1 alpha	[50]
	MIP-1 beta	[50]
	SLC	[50]
Activation of enzymatic precursors	Cathepsin B	[51,52]
	Cathepsin L	[51,53]
	Transglutaminase 1	[54]
Brain antigen processing	Amyloid beta A4 protein	[55,56]
	Tau	[57]
	Tubulin	[58]
	Myelin basic protein	[59]
	Mhtt	[60]
	Apolipoprotein E	[61]
	Alpha-synuclein	[62]
Degradation of cytoskeletal proteins	Neurofilaments	[63]
	Actin	[64,65]
	Myosin	[64,66]
	Tropomyosin	[65]
Monocyte-mediated fibrinolysis	Fibrinogen	[67,68]
	Fibrin	[67,68]
Regulation of apoptosis	Bid	[69]
	Thiredoxin-1	[70]
Processing of enzyme activators and inhibitors	Prosaposin	[71]
	Alpha1-antichymotrypsin	[72]
	Kallistatin	[72]
	Cystatin C	[73]

Biological function	Target protein	Reference
	Kininogen	[73]
LDL cholesterol hydrolysis	Apolipoprotein B	[32,74]
ECM protein degradation	Proteoglycans	[75,76]
	Collagens	[77,78]
	Fibronectin	[79]
Others	Hemoglobin	[80]
	Connectin	[81]
	Dermcidin	[24]

Table 2

Possible biological functions of pCD/CD independent on its enzymatic activities

Biological function	Reference
Activation of neutrophils and leukocytes	[220]
Regulation of apoptosis	[27,120,124]
Mitogenicity – cancer cells, fibroblasts, keratinocytes	[27,33,194,212]
Wound healing	[33]
Tumor angiogenesis	[130]
Tumor invasion – cancer cells, fibroblasts	[27,186]