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REVIEW

Procathepsin D and cancer: From molecular biology to clinical applications

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Abstract

Procathepsin D (pCD) is overexpressed and secreted by cells of various tumor types including breast and lung carcinomas. pCD affects multiple features of tumor cells including proliferation, invasion, metastases and apoptosis. Several laboratories have previously shown that the mitogenic effect of pCD on cancer cells is mediated *via* its propeptide part (APpCD). However, the exact mechanism of how pCD affects cancer cells has not been identified. Recent observations have also revealed the possible use of pCD/APpcD as a marker of cancer progression. The purpose of this review is to summarize the three major potentials of pCD-tumor marker, potential drug, and screening agent.

Key words: Procathepsin D; Cancer; Screening; Enzyme; Cancer cells; Stimulation

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MATURE ENZYME CATHEPSIN D

Cathepsin D (CD) is a soluble lysosomal aspartic endopeptidase (EC 3.4.23.5) synthesized in rough endoplasmic reticulum as pre-procathepsin D (Table 1). After removal of signal peptide, the 52 kDa 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 and 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]. An alternate method to target pCD to lysosomes is independent of the M6P tag and is not yet fully understood. However, the role of sphingolipid activator precursor protein prosaposin has been suggested[6-10].

Upon entering the 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. In addition, 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]. In addition, it was proposed that pCD can be converted to enzymatically active pseudo-cathepsin D by autocatalysis. Cysteine proteases and autocatalytic activity of CD is sure to be 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 which is unlike other members of the aspartic endopeptidase family that 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 CD (34+ 14 kDa) 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, and undergoes further maturation[26,27]. CD expression and activity was also detected in the 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].

CATHEPSIN D/PROCATHEPSIN D AND CANCER

Increased levels of CD were first reported in several human neoplastic tissues, more than 20 years ago[33]. Several years later, the first clinical studies found pCD/CD related to metastasis-free survival and disease-free survival in breast cancer patients[34,35]. 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[25,36,37]. Studies dealing with pCD/CD diagnostic and prognostic value in cancer are complicated by the fact that, simultaneously, there are several forms of CD in a cell-inactive precursor pCD, enzymatically active intermediate (single chain) CD and mature (two chains) CD. Moreover, different forms of CD are also present in stromal cells and may result in pCD/CD quantification in tumor tissues and consequently its prognostic significance. Therefore, a standardization of techniques is needed to further evaluate the therapeutic and prognostic significance of pCD/CD expression in solid tumors.

Major studies and one meta-analysis found that pCD/CD level in tumor homogenate measured by either ELISA or IRMA represents an independent prognostic factor[38-40]. In these studies, antibodies that can detect both pCD (52 kDa) and CD (48 and 34+ 14 kDa) were used. Conversely, results of immunohistological (IHC) studies using antibodies specific to either pCD, CD or both are less consistent. This could possibly be due to different tissue fixation techniques, antibodies and semi-quantitative nature of IHC.

The mechanism of pCD mitogenic effect on cancer cells remains unclear. Numerous clinical studies have revealed that the level of pCD/CD represents an independent prognostic factor in a variety of cancers. These include breast and lung carcinomas[41]. It has been demonstrated that pCD/CD affects multiple stages of tumor progression including proliferation, invasion, metastasis, angiogenesis and apoptosis[41-43]. Clearly, prognosis of many types of cancer is significantly worse in cases of high pCD/CD expression and release.

We, addition to others, have shown that secreted pCD binds to surface of breast cancer cells[26,44]. We therefore 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 characterization 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 the breast cancer cell surface[45-48]. Numerous studies have demonstrated that neither binding nor pCD mitogenic potential is blocked by M6P, anti-M6PR antibodies or pCD deglycosylation[44,48-51]. 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*[52]. 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. However, the binding of pCD to M6P/IGF-Ⅱ receptor may decrease its binding capacity to other M6P/IGF-Ⅱ receptor natural ligands (e.g. IGF-Ⅱ, latent TGF-β) and thus perturb their biological functions[48].

Table 1 Cellular localization of procathepsin D/cathepsin D

Form	Localization	Process
Pre-procathepsin	Rough endoplasmic reticulum	Synthesis
Procathepsin	Golgi apparatus	Modification of oligosaccharides
Procathepsin	Prelysosomal compartments	Cleavage of the activation peptide
Single chain cathepsin	Lysosomes	Proteolytic processing, triming of oligosaccharides
Mature cathepsin	Lysosomes	

We determined that binding to cancer cells, as well as pCD mitogenic potential, is blocked by antibodies specific for propeptide part of pCD[49,53,54]. 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 (as is the case with other mammalian aspartic proteases) a deep cleft between the two lobes of the active enzyme[55,56]. 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[57,58].

Utilizing synthetic peptides that correspond to different parts of AP, we showed that the region responsible for binding of pCD to cancer cell surface is localized between amino acids 33-44 of the AP[44,54]. 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*[44,49,51-54,59-61]. Although the mitogenic effect of AP was not confirmed by Glondu *et al*[50] under their experimental conditions, Bazzett *et al*[62] independently demonstrated mitogenicity of AP in ovarian cancer cells.

Tumorigenesis is a complex process involving not only growth of the primary tumor cells or tumor stem cells, but also communication with surrounding tissues and cells. In this process, different parts of stromal tissue, including the vasculature, adipocytes, resident immune cells, and fibroblasts, play a role. All these cells are secreting numerous cellular products, including various growth factors and extra-

cellular matrix components. It is likely that tumor fibroblasts originate from normal fibroblasts and that they are very similar to fibroblasts involved in wound healing processes. There is clear evidence that fibroblasts communicate with the primary tumor cells and this communication is critical for development of the disease. There is an extensive *in vitro* and *in vivo* research demonstrating that this communication can promote the growth of cancer cells. For more information about pCD/CD and tumor environment[63].

Secretory proteins of different families play a role in primary tumor growth and metastasis formation and angiogenesis. In addition to this autocrine mitogenic effect, pCD was also found to possess paracrine proliferative properties. Berchem's group found that pCD stimulates not only parent cancer cell proliferation but also tumor angiogenesis by a paracrine mechanism[42]. This possibility was further potentiated by the work of the Liaudet-Coopman group who demonstrated that pCD was able to stimulate proliferation, survival, motility and invasion of fibroblasts[43]. The detailed mechanisms of the proliferative function of pCD remain unknown. In experiments testing the influence of IL-4, IL-10 and IL-13 on growth of several cancer cell lines, we have found that these cytokines had a similar proliferative effect as pCD. The difference between the pCD- and cytokine-induced proliferation lies in fact that the stimulation of proliferation has been observed only in the case of ER+ cell lines. Supposed mechanism of action is pCD-dependent triggering of IL-4, IL-8, IL-10, and IL-13, which subsequently further stimulate cancer cell growth.

In an attempt to better understand the autocrine and paracrine effects of pCD, we tested the possibility of secretion of cytokines upon pCD addition. We demonstrated substantial secretion of cytokines, especially IL-4, IL-8, IL-10, IL-13 and MIP-1β from both cancer cell lines and fibroblasts upon addition of pCD. This secretion was shown to promote the growth of both cancer cells and fibroblasts. As a result of our experiments, we can conclude that pCD secretion observed in many cancer derived cell lines leads to a secretion of cytokines which, in turn, promote the growth of both types of cells. Therefore, a selective inhibition of pCD interaction with a cellular receptor could decrease or halt this process. These data underline pCD as a potential target for cancer therapy.

PCD IN SCREENING

Research performed in our laboratory, in addition to others, has demonstrated the presence of anti-pCD autoantibodies[64]. As these antibodies are specific to pCD only, and do not recognize mature CD[65-67], they represent an ideal target for comparison of the pCD presence and cancer progression.

We hypothesized that the level of anti-pCD autoantibodies correlates with the stage of breast, lung, and prostate cancer and offers development of a cost-effective, non-invasive screening test. We prepared an ELISA assay for evaluation of the presence of anti-AP/pCD antibodies. Attributable due to the low affinity of the antibodies, activation peptide alone is not optimal for evaluation in ELISA or RIA assays. We decided to overcome this potential setback by using a specifically modified synthetic activation peptide as an antigen assay. Employing Multiple Antigen Peptide (MAP), we were able to measure the level of anti-pCD autoantibodies in patient serum.

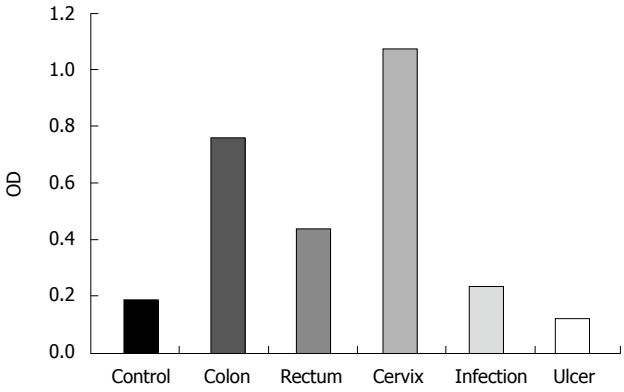

Figure 1 Level of anti-procathepsin D autoantibodies in patients with three types of cancer and two cancer-unrelated diseases evaluated by enzyme-linked immunosorbent assay. Data represent mean value from 5 patients/group.

Since pCD has been found to be involved in numerous types of cancer, one can assume that the specific autoantibodies will also be formed in additional types of cancer. Using a small number of samples, we found the elevated levels of these autoantibodies in lung, prostate, and stomach cancer (unpublished data). Serum samples were mostly from the commercial source ProMedDx, which provides serum samples from over 60 different diseases. Compared to the cancer samples, sera of patients suffering from additional diseases were negative (Figure 1).

We hypothesize that the amount of the APpCD/pCD in the patient's serum will change with the progress of the cancer disease, thus corresponding with the increased number of pCD-releasing cancer cells. This hypothesis configures well with our preliminary findings on breast cancer (Figure 2) and clearly shows higher levels of antibodies in more advanced stages. These preliminary data define the high clinical potential in the evaluation of specific anti-AP autoantibodies.

Based on these data, we prepared a model of a proposed mechanism of pCD action (Figure 3). The overexpressed pCD escapes normal intracellular 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 specific cell surface receptor. This interaction releases a signal resulting 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. In addition, stress affects keratinocytes resulting in increased both cytokine and pCD synthesis and secretion leading to the elevated proliferation of keratinocytes (Figure 4).

OTHER POTENTIAL ROLE OF PCD

In recent decades, there has been focused on pCD's additional contribution toward wound healing, tissue remodeling[68] and programmed cell death-apoptosis[69,70]. Epidermis, the barrier between the body and external environment, is constantly exposed to various environmental and physical stresses. Keratinocytes are elemental cells forming the epidermis and are crucial

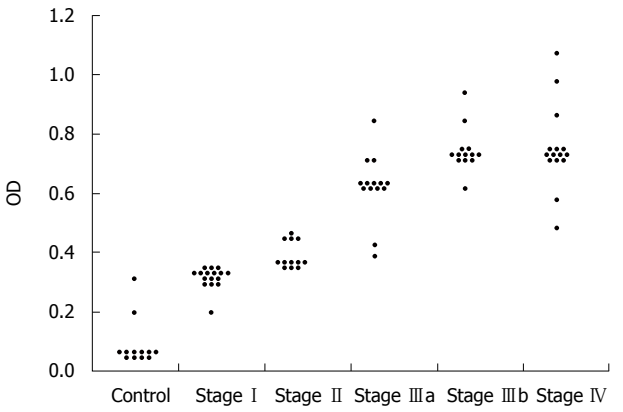

Figure 2 Level of anti-procathepsin D autoantibodies in patients with various stages of breast cancer evaluated by enzyme-linked immunosorbent assay. Average age of patients in each group varied from 31 to 69.

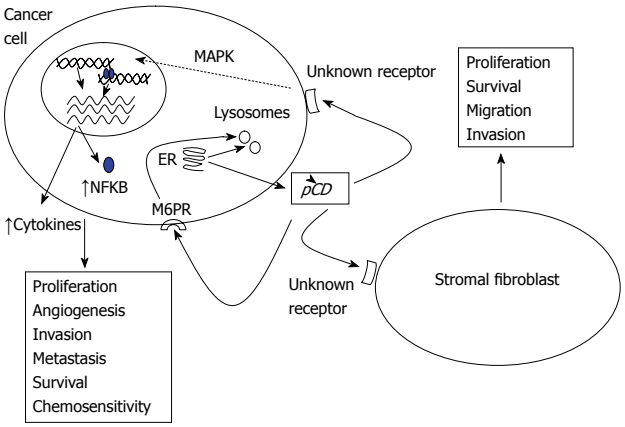

Figure 3 Proposed mechanism of procathepsin D/cathepsin D function in cancer progression. The over-expressed procathepsin D (pCD) escapes the physiological intracellular targeting pathways and is secreted by the cancer cells. Small part of secreted pCD is bound and internalized *via* M6PR pathway, the rest by a yet unidentified receptor. The receptor-pCD interaction activates mitogen-activated protein kinases (MAPK) pathway with subsequent changes in expression of numerous cancer-promoting genes including NFKB2 and some cytokines. Interaction of pCD with endothelial and stromal cells is also involved.

for normal regeneration and healing. Skin healing is dependent upon several processes that comprise inflammation, protein synthesis, matrix deposition, migration and subsequent proliferation of keratinocytes[71,72]. Keratinocytes are known to secrete numerous proteins that include proteolytic enzymes such as matrix metalloproteinases[73], interstitial collagenase[74] and cathepsin B[75]. During the wound healing process, these proteolytic enzymes might play a role in motility of keratinocytes by remodeling of extracellular matrix for migration of keratinocytes to peripheral layers of epidermis. When Katz *et al*[76] studied proteins secreted by cultured human epidermal keratinocytes, they found that CD was one of them.

In skin, increased levels of the mature form of CD has been shown in basal keratinocytes during hyperproliferative skin disorders such as psoriasis[77]. In addition, the involvement of different isoforms of CD in the epidermal cell differentiation was also suggested. The presence of pCD was shown in the spinous layer. These forms were present in stratum corneum, where they played a role in epidermal desquamation[78,79]. Although, the role of CD in epidermal differentiation has been defined, the presence of pCD at different stages of differentiation is still unclear. Moreover, many of these studies were performed using cell lysates where all the isoforms are present, making clear definition of the roles played by individual isoforms virtually impossible.

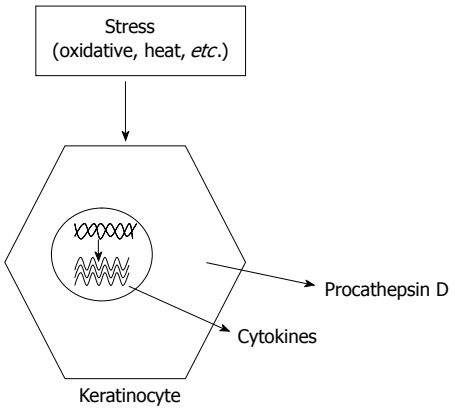

Figure 4 Stress affects keratinocytes resulting in increased both cytokine and procathepsin D synthesis and secretion leading to the elevated proliferation of keratinocytes.

Therefore, we tested the hypothesis that the secretion of pCD from cells is one of the normal physiological features in the skin. Initially, we began by demonstrating the secretion of pCD *via* the human keratinocytes cell line HaCaT. Subsequent experiments showed that the exogenous addition of purified pCD enhanced the proliferation of HaCaT cells. The proliferative effect of pCD was inhibited by monoclonal antibody against the activation peptide (AP) of pCD. Treatment of HaCaT cells with pCD or AP led to the secretion of a set of cytokines that may promote the growth of cells in a paracrine manner. The role of secreted pCD and its mechanism of action were further studied in a scratch wound model. The presence of pCD and AP enhanced the regeneration of monolayer. Simultaneously, this effect was reversed by the addition of anti-AP antibodies. Expression and secretion of pCD was upregulated in HaCaT cells exposed to various stress conditions. Taken together, our results strongly suggest that the secretion of pCD is not only linked to cancer cells but also plays an essential role in the normal physiological conditions such as wound healing and tissue remodeling[80]. However, current knowledge does not support the possible connection between pCD function in cancer and wound healing. This proteinase (both in enzymatically active and inactive state) just has several biological functions, including both pathological and physiological ones.

CONCLUSION

While many functions of pCD/CD in the physiological and pathological processes could be attributed to its enzymatic activity, it is clearly established that some of its functions in the organism are independent of its protease activity and rely on the ability of pCD to interact with other important molecules. It appears to be inevitable that the search for pCD-interacting partners should be conducted to explore the mechanism of pCD actions.

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