Cathepsin D in breast cancer: mechanisms and clinical applications, a 1999 overview

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Abstract

A short review of the literature first confirms the clinical value of cathepsin D as a prognostic marker in breast cancer, when using well standardized assays. We then summarize results of studies, mostly performed in our laboratory, aimed at understanding the effect of cathepsin D overexpression on metastasis and the molecular mechanisms involved. Cathepsin D–cDNA transfection increases tumor cell proliferation in vitro and the metastatic potential of 3Y1-Ad12 embryonic rat tumorigenic cells when injected in vivo into nude mice. The mechanism by which cathepsin D increases the incidence of clinical metastasis involves increased cell growth and decreased contact inhibition rather than escape of cancer cells through the basement membrane. Different mechanisms are considered to explain this mitogenic activity. Cathepsin D could act as a protease following its activation at an acidic pH, or as a ligand of different membrane receptors at a more neutral pH. In this case cathepsin D can displace IGFII from the mannose-6-phosphate/IGFII receptor to the IGF1 receptor or activate another membrane receptor to be identified. The nature of the mechanisms involved in vivo may depend on the micro environment of the tumor cells. These studies should guide in the development of new therapies aimed at inhibiting the deleterious effect of overexpressed cathepsin D. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

For more than 10 years overexpression and secretion of cathepsin D (cath-D), in cancer cells, mostly breast cancer, have been associated with increased risk of relapse and metastasis [1–3]. We will review the actual state of the art on the clinical significance and the role and possible mechanisms resulting from cath-D overexpression in mammary cancer.

Cath-D is a lysosomal protease without known endogenous inhibitor and catalytically active at acidic pH values that vary according to substrates, but is mostly found in intracellular vesicles, lysosomes, phagosomes and late endosomes. The biological function of this protease, also secreted as a proenzyme in the milk, is not only to degrade proteins in the lysosomes but also to help in the development of newborns, by protecting them from intestinal necrosis and thymic apoptosis [4]. Studies in estrogen receptor positive breast cancer cell lines revealed that this “housekeeping” enzyme is also highly regulated by estrogens and growth factors [5–7]. The unexpected regulation of procath-D by estrogens in breast cancer cell lines prompted several clinical and basic studies aimed at understanding the significance of this estrogen-induced protein in breast cancer and other solid tumors.

1.1. A marker associated to metastasis risk in breast cancer

The first clinical studies showed that this estrogen induced protein in estrogen receptor positive breast cancer was also overexpressed in estrogen receptor negative breast cancer [6], and was associated with a higher risk of relapse and metastasis but not with hormone dependence and estrogen receptor status [8]. Since then, there have been controversies on the clinical value of this protease [9], mostly based on mixing results of well standardized methodology with various studies using non validated or non quantified methods such as immunohistochemistry [10,11]. It was however remarkable that, measuring the protein by immunoradiometric assay or by its proteolytic activity after activation at an acidic pH, gave the same information [12]. Within the last 10 years, most, if not all, studies using a well standardized methods (ELISA or IRMA) to quantify the enzyme in the cytosolic cell extract performed by laboratories trained with the hormone receptor assays, showed the same poor prognostic value of this marker for levels higher than the median value. These studies have been reviewed in a meta analysis [13]. Recently a large scale study in 2810 node negative patients with median follow-up time of 88 months clearly confirmed that high or moderate cath-D level in a primary tumor had a poor prognostic value on relapse-free and overall survival, independently of the classical prognostic parameters such as histological grade, hormone receptor, and tumor size [14]. In our group, we recently showed that cath-D level in patients was independent of
the hormonal and menopausal status by contrast to the pS2 protein which is more strongly estrogen regulated [15]. Therefore the cut point to discriminate high and low cath-D level should be the same in pre- and post-menopausal patients and whatever the period of menstrual cycle for tumorectomy.

Contrasting with the cytosolic studies, the immunohistological studies are more inconsistent. There are several possible explanations for these discrepancies including different type of antibodies used, absence of quality control and of quantification, absence of evaluation of the secreted proenzyme, etc.. This does not mean that a well standardized methodology will not give important information, particularly when one considers early steps of mammary carcinogenesis where the tissue localisation of the enzyme (tumor, cells endothelial cells, macrophages, fibroblasts, etc.) may be important to specify. This can only be performed in formalin fixed, paraffin embedded tissue sections.

To conclude, this rapid overview shows a large agreement on the prognostic value of cath-D assay which should stimulate large scale randomized prospective studies aimed to evaluate the predictive value of this protease in node negative patients and its benefit for treating the higher risk patients with conventional adjuvant chemotherapy [16]. We will now review results mostly from our laboratory focussed on the biological significance of cath-D overexpression in metastasis. Is it causal or only associated as one of the consequences? If it is responsible, what is the mechanism by which cath-D could facilitate the growth and invasiveness of tumor cells?

1.2. Induced cath-D overexpression increases experimental metastasis via its mitogenic activity

To assess the role of cath-D in cancer metastasis, we stably transfected an expression vector of human procath-D or a control vector alone into a rat tumorigenic cell line (3Y1-Ad12) which does not secrete cath-D in vitro. The metastatic potential of cath-D-expressing clones was compared to that of control clones in athymic mice [17,18]. Four cath-D clones isolated from two independent transfection experiments displayed a higher metastatic potential than four corresponding control clones. The incidence and size of gross liver metastases were significantly increased in mice injected with cath-D clones. This was the first experimental evidence that cath-D overexpression decreases the time required for clinical metastases to appear and reproduce the clinical observation associated to the natural overexpression of cath-D in breast cancer.

Recently, using a retroviral approach, cath-D-overexpression was also found to be involved in two other cell model by increasing NIH3T3 soft agar colony formation and the progression of LNCAP human prostate cancer [19]. A protease can stimulate metastasis by several mechanisms, and at different steps of the metastatic cascade. Cath-D, contrary to other proteases, seems to be more
a mitogen than a protease, allowing cancer cells to cross the basement membrane and invade connective tissue or the blood system. A 2-fold increase of cell growth by purified procath-D was initially shown in MCF7 cells cultured in monolayers [20] suggesting that the proenzyme could be acting as an autocrine mitogen. However, another laboratory did not detect this mitogenic activity [21] which appeared to be dependant on the mode of protein purification. Following transfection of cath-D cDNA into rat tumor cells the stimulation of cell growth in vitro was confirmed [17]. The level reached at confluence being more stimulated than the growth rate [17] and cath-D appeared to decrease cell–cell contact inhibition [22]. Recently we found that the mitogenic activity of cath-D was more dramatic when tested in a three dimensional matrix (type 1 collagen or matrigel) than on plastic (E. Liaudet et al., submitted for publication).

Currently, there is no evidence of increased invasion through an extracellular matrix such as matrigel ([23] and M. Garcia and D. Deroq, unpublished). Therefore, the increased number and size of liver metastasis described in the rat tumor model appears to be the result of increased colonization and growth in distant site rather than an increased invasiveness through a basement membrane.

1.3. Cath-D action via its proteolytic activity

Overall, the overexpressed and secreted enzyme could act as a protease after its activation or as a ligand on membrane receptors before its activation. The action could take place extracellularly as suggested by the high hypersecretion of this proenzyme, or intracellularly following or not its endocytosis via membrane receptors. At present, it is difficult to exclude any of these mechanisms: cath-D precursor being abnormally secreted in cancer cells, the first mechanism coming to mind is that of a protease digesting basement membrane and facilitating by this way tumor invasion and metastasis. The major problem however is that a pH sufficiently acidic (<5.5) to activate the proenzyme is required. This pH may be rarely found in extracellular milieu, even though this pH may vary according to the nature of the substrate and of the neighbour partners such as membrane lipids. A series of circumstantial evidence, however, is in favor of an activation and action of cath-D as an enzyme. Maturation occurs in vivo in primary breast cancers since separate assays of procath-D and total cath-D, using different monoclonal antibodies, showed that the tumor contained only 4 to 6% of procath-D [24], while metastatic breast cancer cell lines secrete in vitro up to 50% of the precursor [25]. Moreover the assay of total cath-D includes the mature form which has more prognostic value than the procath-D assay [24].

In the rat tumor model, we tried to inhibit cath-D secretion and maturation by insertion mutagenesis of a KDEL endoplasmic reticulum retention signal at the
C-terminal of the coding sequence [18]. Mice injected with six different KDEL clones developed metastases with the same incidence as those injected with control clones. We verified that a control KDAS peptide inserted at the same position did not affect cath-D maturation and its metastatic effect. The metastatic potential of cath-D was totally inactivated by modification of two aminoacids (KDEL vs. KDAS) in the protein structure. This strongly supports a direct and specific effect of cath-D on the metastatic process and excludes artefacts due to transfection or the selection procedure. The major consequence of procath-D retention in the endoplasmic reticulum was to prevent both its intracellular maturation and its stimulatory effect on experimental metastasis, suggesting its role as a protease. However, the result of this study did not exclude a potential role of procath-D as a ligand for transducing signals via a membrane receptor, since the addition of the KDEL signal might have altered its interaction on a receptor. The mechanism of cath-D action as a protease and the nature of the substrate would obviously depend upon the site of activation and action of the enzyme.

1.3.1. Intracellular maturation and action

Cath-D is acting in acidic intracellular vesicles, not only lysosomes, but also phagosomes and late endosomes. In the prelysosomal compartments, it has the potential to activate growth factors or their receptors and to degrade or inactivate growth inhibitors. Acidic milieu has been previously described in prelysosomal compartments such as late endosomes [26]. Using fluorescent dyes, Montcourrier et al. described large intracellular acidic vesicles (or LAVs) of $\geq 5 \mu m$ in diameter. These LAVs were more frequently found in vitro in breast cancer cells than in normal mammary cells and contained high levels of cath-D but no procath-D [27]. LAVs were not restricted to cell lines, since they were also found in primary cultures of pleural effusions from breast cancers. Vesicles with the same diameter and highly concentrated in cath-D have also been observed in vivo by immunohistochemistry in paraffin sections of breast cancer biopsies [11].

To determine the significance of these acidic intracellular compartments (LAVs), we quantified LAV-positive cells in MDA-MB231 breast cancer cells before and after migration through Matrigel, a classical in vitro model for studying invasion through an extracellular matrix [28]. We found more LAV-positive cells after migration through Matrigel than before migration. These vesicles contained phagocytosed extracellular material such as latex beads or pieces of extracellular matrix. The fact that breast cancer cells are fully able to phagocytose extracellular material, including extracellular matrix, and digest this material within heterophagosomes is unusual and might facilitate development of cancer cell colonies in distal parenchyma. A correlation between the invasive ability of different cancer cell lines and their ability to phagocytose the extracellular...
lar matrix was found [29]. Cath-D in these vesicles is able to digest intracellularly many types of proteins, including proteins of the engulfed extracellular matrix, thus providing nutrients, aminoacids and space for invasive breast cancer cells.

Intracellular maturation of pro-cath-D into 48 and 34 kDa active enzymes was also shown using pulse chase experiments [38]. Such maturation was not only observed in the high density fractions containing lysosomes but also in light density fractions containing endosomes, plasma membrane and phagosomes. This maturation was inhibited by NH₄Cl treatment of MCF7 cells, confirming the acidic pH requirement.

Among the possible substrates degraded intracellularly, cath-D could inactivate growth inhibitors. In the rat tumor model system, stable cath-D transfected clones in low serum conditions reached 2- to 4.5-fold higher density at confluence than control clones [17]. Control cells reaching saturation density released an inhibitory activity that was able to prevent growth of control or cath-D clones. By contrast, the production of this growth inhibitory activity was markedly reduced in cath-D clones [22]. Therefore, cath-D overexpression appears to increase cell density by inhibiting the activity or secretion of growth inhibitors released by confluent cells. Intracellular maturation of the proenzyme seems to be necessary since neutralization of acidic compartments by chloroquine or ammonium chloride prevented both cath-D maturation and its mitogenic effect.

1.3.2. Extracellular activation of the secreted pro-cath-D

Secreted pro-cath-D could also be activated extracellularly in sufficiently acidic milieu. The extracellular pH in tumors is generally more acidic than in corresponding normal tissues as shown by non-invasive methods in patients [30]. Breast cancer cells, like macrophages [31], have a high potential to liberate protons in the extracellular milieu through lactic acid production and a functional H⁺/ATPase pump at the plasma membrane level. They are able to decrease the pH down to 5.5 when it is measured under MCF7 cell monolayers and the acidifying potential of the invasive MDA-MB231 breast cancer cells is more important than in estrogen-responsive MCF7 cells [32]. When secreted in the extracellular matrix, pro-cath-D following activation, as other proteases, could degrade growth inhibitors or liberate growth factors and angiogenic factors.

When the MCF7 human breast cancer cells were cultured on extracellular matrix secreted by bovine endothelial cells, a more physiological system than monolayer culture on plastic., they were able to digest this matrix and liberate biologically-active ¹²⁵I FGF₂ which had been preincorporated into the matrix. This FGF₂ endocytosis was inhibited by pepstatin A, indicating that cath-D can be activated to degrade extracellular matrix and liberate FGF₂ [33]. Conse-
quently, liberated FGF might have mitogenic activity on cancer cells and/or increase angiogenesis by stimulating the growth of surrounding endothelial cells. This mechanism, may be decisive in allowing micrometastasis growth at a distance as long as a protease like cath-D is overproduced by migrating cancer cells.

A proteolytic cascade might also be triggered since cath-D has been shown in vitro to activate procath-B [34] and cath-B to activate the prourokinase plasminogen activator [35]. Many other substrates have been proposed on the basis of in vitro experiments [36,37] and one major challenge is to define the substrate that is involved in vivo in the human tumor.

1.4. Procath-D interaction and action on membrane receptors

One of the characteristics of procath-D and also of cysteiny1 procathepsins in cancer cells is their increased secretion described in cell lines [38], but also in primary culture of pleural effusion [39]. The mechanism of this secretion is intriguing since several receptors can transport cath-D to lysosomes as well as its significance in term of action. Moreover following its secretion, procath-D can be endocytosed by the same cancer cells, or by the adjacent stromal cells according to an autocrine, or paracrine mechanism [40].

At least, two types of receptors are able to interact with and transport procath-D. They can be discriminated by the effect of mannose-6-phosphate (Man-6-P)-acting as a competitor.

1.4.1. Man-6-P receptors

As do other lysosomal enzymes, procath-D bears Man-6-P signals on its $N$-glycosylated chains allowing its interaction with two Man-6-P receptors (275 and 46 kDa) which normally transport hydrolases from the trans-Golgi network to lysosomes [41].

The respective roles of these two receptors have been clarified by gene disruption experiments in mice. The 275 kDa Man-6-P/IGFII receptor can mediate endocytosis of secreted lysosomal enzymes and IGFII, whereas the small receptor is inefficient in endocytosis. Moreover, this receptor is multifunctional since in addition to binding lysosomal enzymes with Man-6-P signals, it also binds and inactivates IGFII [41] a major mitogen involved in tumor growth, it activates pro TGFβ a growth inhibitor and interacts with retinoids and urokinase receptor.

We first studied the interaction of procath-D with this receptor in an attempt to explain the secretion and dysrouting of procath-D and other cathepsins. In vitro procath-D appears to interact normally with the Man-6-P/IGFII receptor in
cancer cells, its affinity being similar to that in normal cells [42] and MCF7 cells synthesized normally Man-6-P signals on procath-D N-glycosylated chains [43,44]. However, in vivo in breast and ovarian cancer cell lines, this receptor appears to be non or poorly functional to transport procath-D to the lysosomes, intracellularly or following its secretion. The group of Jirtle has described in hepatocarcinomas loss of heterozygocity (LOH) in 6q 27 corresponding to the Man-6-P/IGFII receptor locus and mutations on the remaining allele [45] and thus suggested that this receptor could behave as a tumor supressor gene. In ovarian and breast carcinomas, LOH is also frequent in the Man-6-P/IGFII receptor locus [46]. Functional mutations on this receptor inactivating IGFII or Man-6-P binding or generating inactive or truncated protein could also be involved to explain cath-D dysrouting. Contrary to hepatocarcinoma, however, we found very rare missense mutations on the Man-6-P/IGFII receptor gene which might explain the Man-6-P independent traffick of procath-D [47], both in MDA-MB231 and MCF7 cells. Biallelic alterations do not appear to be frequent in breast and ovarian cancers in this study [47]. However, this does not eliminate the hypothesis that the Man-6-P/IGFII receptor behaves as a tumor supressor gene [48]. We propose that mono allelic alteration by LOH or mutation, associated to the frequent overexpression in these cancers of the Man-6-P/IGFII receptor ligands (cathepsins and IGFII) may be sufficient to saturate receptor sites and displace these ligands to mitogenic receptors, thus facilitating tumor progression. Downregulation of this receptor by estradiol was also proposed to facilitate receptor saturation by its natural ligands [49]. Therefore overexpression of cath-D might facilitate displacement of cathepsins in the secretory pathway and of IGFII on the IGF1 mitogenic receptor by preventing IGFII degradation in lysosomes [41]. This last mechanism demonstrated in vitro on cell lines is a possible explanation of the cath-D mitogenic activity which in this case will be acting as a competing binding protein efficient at neutral pH and able to activate indirectly a mitogenic plasma membrane receptor.

Alternatively, an excess of IGFII precursor could also displace cathepsins toward the secretory pathway [50] even though the two ligands bind to different domains of the receptor.

To conclude, a decrease of available binding sites of the Man-6-P/IGFII receptor could facilitate carcinogenesis by several mechanisms supporting the tumor supressor hypothesis for this receptor. In invasive breast and ovarian cancers however, biallelic alterations appear to be rare compared to other solid tumors, but receptor saturation due to an unbalance between low level of the receptor protein in cancer cells and high level of its major ligands IGFII and cathepsins appears to be frequent and may be sufficient to alter the functions of this receptor. In agreement with this statement we recently found by immunohistochemistry a decrease of Man-6-P/IGFII receptor protein in breast cancer tissue compared to normal glands located at the periphery. This decrease was more important in node positive breast cancer [51].
1.4.2. The search for man-6-P independent receptor(s)

Several studies indicate the presence of alternative targeting of procath-D to lysosomes in macrophages [52], HepG2 cells [26], and breast and ovarian cancer cells [53]. This alternative routing appeared to be more important in cancer cells than in normal cells since, in MCF7 and MDA-MB231 cells, procath-D secretion and its routing to lysosomes were totally insensitive to NH$_4$Cl treatment of cells [53]. By increasing the pH in sorting endosomes, this weak base prevented dissociation of the Man-6-P receptors–procath-D complex and subsequent Man-6-P receptor recycling. In normal fibroblasts and mammary cells, newly synthesized procath-D normally sorted by the Man-6-P/IGFII receptor was secreted after NH$_4$Cl treatment but not accumulated in cells. This resistance to NH$_4$Cl appears to be procath-D specific, since in the same cell line other enzymes such as β-hexosaminidase, α-glucosidase and arylsulfatase respond to NH$_4$Cl treatment [53]. In NH$_4$Cl-resistant MCF7 cells, procath-D was found to accumulate intracellularly and to be targeted to high density lysosomal particles. The molecule responsible for this alternative targeting has not been identified. It has been proposed that a procath-D–prosaposine interaction may be involved [26,54]. The sequence conferring Man-6-P independent targeting in I Cell disease may be located in the C-terminal part of the molecule [55].

The same insensitivity to Man-6-P has also been observed for procath-D endocytosis into these two breast cancer cell lines, contrary to normal fibroblasts [40]. However, we ignore whether this Man-6-P independent endocytosis is cell or cancer specific. Recently a Man-6-P independent trafficking to lysosomes has been shown to be cell specific since present in liver cells and thymocytes, but not in fibroblasts [56]. These alternative receptors may be involved not only in trafficking, but also and more interestingly in mediating the mitogenic activity of procath-D. The group of Fusek has described in the ZR75 breast cancer cells that cell surface sites may trigger mitogenic activity when binding procath-D or the profragment alone [57,58]. In this case, cath-D might not be acting as a protease, but as a ligand. In support to this, we found at the surface of MDA-MB231 cells, high affinity saturable sites binding procath-D in the presence of Man-6-P. These sites may be involved in the endocytosis of procath-D and its mitogenic activity [40]. Therefore, procath-D may not be acting as an enzyme precursor but as a ligand for triggering a transmembrane receptor. This receptor has not yet be identified but its identification or isolation is of considerable interest to clarify the mechanisms involved and to develop new therapeutical approches.

2. Conclusions

Cath-D overexpression in some cancer (breast, melanoma, ovary, etc.) is not only correlated with, but also facilitates, the development of clinical metastasis
as demonstrated in a rat tumor cell system by transfecting human cath-D gene. Most studies indicate that cath-D overexpression decreases the relapse free and overall survival by stimulating the growth of micrometastasis at distant sites, rather than in stimulating local invasion by digestion of basement membrane. The mechanism of this mitogenic activity may not only involve proteolysis of critical substrate but also interaction of the proenzyme on cell surface receptors. These two mechanisms are not exclusive and according to the conditions observed locally in vivo in the tumor (particularly pH and oxygenation), the importance of one mechanism or the other could predominate.

The concept of a protease acting as a mitogen is not new, its action as a ligand triggering more or less directly a membrane receptor is more original since a proteolytic cleavage of the receptor is often involved, as in the case of the mitogenic thrombin receptor [59]. The consequences of a better understanding of the mechanism of cath-D action in breast cancer are considerable. First it might be extended to other solid tumors overexpressing cath-D, and to other cathepsins such as cath-L, cath-B etc., which are also overexpressed in cancers. Second and more importantly, it will help to define new therapies of cancer targeted on cath-D overexpression and aimed at inhibiting the growth of micrometastasis into clinical metastases. The rational development of such therapy might complement other biological therapies targeted to molecules altered in cancers and could be guided by the current assay of cath-D in cytosolic tumor extracts.

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References


[34] Van der Stappen JWJ, Williams AC, Maciewicz RA, Paraskeva C. Activation of cathepsin B, secreted by a colorectal cancer cell line requires low pH and is mediated by cathepsin D. Int J Cancer 1996;67:547–54.


