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Procathepsin D in breast cancer: What do we know? Effects of ribozymes and other inhibitors

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Procathepsin D (pCD) is a major secreted glycoprotein in some human breast and other cancer cell lines. Several groups proposed that pCD served as a growth factor for these cell lines. Secreted pCD has been demonstrated in tissue section, tissue culture supernatants, carcinoma cytosols, and nipple aspirates. Moreover, several clinical studies suggested a potential role for this molecule in metastasis because its concentration in primary tumors correlated with an increased incidence of tumor metastases. In this paper, the effects of pCD were evaluated by proliferation *in vitro* and by mouse studies *in vivo*. Subsequent flow cytometry experiments showed the specificity of pCD binding to cancer cells. Cell cultivation showed that addition of either pCD or its activation peptide stimulates growth of cancer cells. These effects can be inhibited both *in vitro* and *in vivo* by anti-pCD antibodies. In addition, production of pCD can be inhibited by specifically designed ribozymes. This paper is focused on mitogenic effects of pCD, which seem to involve interaction of the activation peptide with as yet unidentified receptor. Different mechanisms by which pCD could promote development and spread of cancer cells are discussed.
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Cathepsin D (CD; E.C.3.4.23.5) is a soluble lysosomal aspartic proteinase. It is synthesized in the endoplasmic reticulum as a *prepro*cathepsin D.[1,2] After removal of the signal prepeptide by signal peptidases and having a mannose-6-phosphate tag, procathepsin D (pCD) is recognized by a mannose-6-phosphate receptor (M6P-R). The M6P-R targets pCD to lysozomes. Upon entering into an acidic lysozome, the single-chain pCD (52 kDa) is activated to CD and subsequently to a mature two-chain CD (31 and 14 kDa, respectively).[3] The two M6P-Rs involved in the lysosomal targeting of pCD are expressed both intracellularly and on the outer cell membrane. The glycosylation followed by phosphorylation is believed to be crucial for normal intracellular trafficking. Under physiologic conditions, pCD is sorted to the lysozomes and neither pCD nor CD is secreted. The fundamental role of CD is to degrade intracellular and internalized proteins.[4] CD has been suggested to take part in antigen processing[5] and in enzymatic generation of peptide hormones. The tissue-specific function of CD seems to be connected to the processing of prolactin. Rat mammary glands use this enzyme for the formation of biologically active fragments of prolactin.[6] CD is functional in a wide variety of tissues during their remodeling or regression and in apoptosis.[7]

In recent years, an increased amount of data has documented an important role of CD and pCD in cancer development.[8–12] Increased levels of CD were first reported in several human neoplastic tissues in the mid-1980s.[13,14] Subsequent studies employing several different approaches such as immunohistochemistry, *in situ* hybridization, cytosolic assay, Northern blot, and Western blot analyses have indicated that in most breast cancer tumors, pCD is overexpressed 2- to 50-fold.[15] The overexpression was demonstrated both at the mRNA and protein levels. Vignon et al[16] first proposed that pCD secreted from breast cancer cell lines might serve as an autocrine growth factor and promote the cancer cell growth. These findings generated intense research in a possible role for CD in neoplastic processes. This research is focused into two major subjects: (a) use of CD (respectively, pCD) levels for diagnostic/predictive purposes; and (b) study of the molecular mechanism of pCD involvement in biological processes.

The synthesis of CD is controlled by steroid hormones. Progesterone and its derivatives increase the rate of uterine CD synthesis,[17] and in breast cancer cell lines, CD expression is regulated by estrogens[18] that interact at the promoter level.[19] In ER^+ cell lines, pCD is secreted only after estrogen stimulation. The estrogen stimulation of pCD secretion from breast cancer cells can be specifically inhibited by a specific estrogen inhibitor 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.[20] Conversely, it is secreted constitutively in

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ER^- cell lines.[21] The mechanism of regulation of expression has been studied extensively and DNA sequences responsible for this regulation have been determined.[19,22]

A strong predictive value was found for CD concentrations in breast cancer as well as many other tumor types.[15,23,24] Subsequently, it has been recommended that levels of CD be monitored as a prognostic indicator in breast cancer and used independent of classical prognostic parameters such as tumor size, histological grade, lymph node status, and steroid receptor expression.[25–27] The significance of pCD and CD levels in other tumors such as endometrial adenocarcinoma,[28,29] colon carcinoma,[30] colorectal carcinoma,[31] oral cancer,[32] laryngeal tumors,[33,34] head and neck squamous cell carcinoma,[35] carcinoma of thyroid tissue,[36] prostate tumors[37,38] melanoma,[8,39] gastric cancer,[40] and ovarian cancer,[41,42] has also been demonstrated, suggesting that this system might be involved in the metastatic growth of numerous other cancer cell types.

The second part of the research, which focused on the mechanism of CD/pCD involvement in breast cancer development, remains unclear. The situation is complicated by the fact that, at any given time, three different components of CD are present in breast tumor tissue: (a) pCD, (b) mature enzyme CD (which is present often in two forms — single chain and double chain), and (c) differently posttranslationally modified pCD. The authors very often do not discriminate between these forms when describing their results. For example, the antibodies that have been used for visualization of CD in tissue samples react with both CD and pCD. From currently available data, it is clear that when using Western blotting, which distinguishes pCD and other forms by molecular weight, most of these molecules are in the form of pCD. It has never been demonstrated that the secreted pCD is converted to the mature enzyme in an extracellular milieu, which is not sufficiently acidic. Despite numerous studies suggesting the involvement of mature CD,[43] this direct proteolytic involvement in the invasiveness of breast cancer cells has never been demonstrated.[44,45] For a review and more details about the role of CD in cancer, see Ref. [46]. For the understanding of pCD/CD role in cancer development, we see as crucial the discrimination between the two forms – pCD and CD.

In this paper, we focused on the mechanism of pCD in the regulation of cancer growth and development by both overview of the latest results achieved by our group and by discussing the significance of the pCD in several types of cancer.

Materials and methods

Chemicals

RPMI 1640 medium, Iscove's modified Dulbecco's medium, HEPES, MTT, E-TOXATE human CD, transferrin, bovine CD, and M6P were obtained from Sigma (St. Louis, MO); fetal calf serum was from Hyclone Laboratories (Logan, UT); complete Freund adjuvant and pig pepsinogen A from Worthington (Freeholf, NJ); and pepstatin A and Protein A Sepharose from Pharmacia LKB Biotechnology (Piscataway, NJ).

Synthetic peptides

The 44-amino-acid-long peptide corresponding to the activation peptide of pCD, the 26-amino-acid-long peptide (1–26 AA), the 22-amino-acid-long peptide (15–36 AA), and the 18-amino-acid-long peptide (27–44 AA) corresponding to the fragment of AP were synthesized in the Institute of Organic Chemistry and Biochemistry (Academy of Sciences of the Czech Republic, Prague, Czech Republic). The purity of the activation peptide and its fragment was controlled using following methods: HPLC, amino acid analysis, and mass spectrometry. The HPLC method showed the purity to be more than 95%. The amino acid analysis confirmed the amino acid composition. Mass spectrometry results were in accordance with the proposed molecular structure. In addition to techniques mentioned above, the purity of peptides was controlled also by N-terminal sequencing using an automated system where the first nine N-terminal amino acids were in agreement with the designed structure.

Cell cultivation

For growth experiments, cells were first incubated for 2 days in 0.1% FCS. The cells were harvested by centrifugation and washed six times in Iscove's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics, and 10 μg/mL human transferrin. Cells were seeded in 96-well tissue culture plates at a density of 5×10^4 cells/mL (150 μL/well) in the presence or absence of different concentrations of purified pCD or various substances tested in triplicate wells. After 5 days in culture, the proliferation was evaluated using an MTT assay. The incorporation of MTT was stopped by the addition of 50 μL of 10% SDS in 0.01 N HCl and the OD of the well supernatants was measured 24 hours later at 570 nm using SLT ELISA reader (Tecan, Research Triangle Park, NC). All media were tested for endotoxin contamination and shown to contain <0.1 ng/mL LPS using the *Limulus* lysate test. Steroid-deprived cells were used in all experiments. The levels of pCD in FCS at the concentration used were below detection levels (data not shown).

Human cell lines

Human breast cancer cells lines ZR-75-1 and MCF-7, human B lymphoblastoid cell line Raji, human breast cell line HBL-100, and prostate cancer cell line LNCaP were obtained from the American Tissue Culture Collection (ATCC; Manassas, VA). Breast cancer cell lines MDA-MB-231, MDA-MB-483, and MDA-MB-436 were obtained from Dr Ceriani of the John Muir Cancer and Aging Research Institute (Walnut Creek, CA). The T lymphoblastoid cell line 8402 was obtained from The Tissue Culture Facility of the Lineberger Cancer Research Center of the University of North Carolina-Chapel Hill (Chapel Hill, NC). The cancer cell lines were grown in RPMI 1640 medium with HEPES buffer supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin in plastic disposable tissue culture flasks at 37°C in a 5% CO_2/95% air incubator.

Antibodies

Monoclonal antibodies against activation peptide and its fragments were described previously.[47] IgG was isolated from ascites fluid by 50% ammonium sulfate precipitation followed by Mono-Q anion exchange chromatography. Monoclonal anti-CD antibodies were purchased from Calbiochem-Novabiochem (La Jolla, CA). Control MOPC-21 IgG antibody was purchased from Sigma. Goat anti-human 300 kDa M6P receptor antibody was donated by Dr K von Figura and by Dr T Braulke (Georg-August University, Gottingen, Germany); rabbit antihuman M6P receptor antibody was donated by Dr S Kornfeld (University of Washington, St. Louis, MO). Soluble Ca-independent M6P-IGF II receptor was donated by Dr P Lobel (Center for Advanced Biotechnology and Medicine, Piscataway, NJ).

Isolation of pCD

Human pCD was isolated from the culture supernatant of human breast cancer cell line ZR-75-1 as described earlier.[48] Briefly, a two-step procedure was used. In the first step, immunoaffinity chromatography was used with antiactivation peptide antibodies attached to Protein A Sepharose. In the second step, FPC chromatography, using a Mono-Q column and 20 mM Tris (pH 7.2), was used.

Ribozymes

The RNase H mapping in cell extract was carried out by a slightly modified procedure as described by Scherr and Rossi[49] Approximately 8×10^7 cells were pelleted and washed twice in PBS. The pellets were resuspended in hypotonic swelling buffer (7 mM Tris–HCl, pH 7.5, 7 mM KCl, 1 mM $MgCl_2$, 1 mM β-mercaptoethanol), incubated for 10 minutes on ice, transferred to a Dounce homogenizer, followed by addition of 1/10 of the final volume of neutralizing buffer (21 mM Tris–HCl, pH 7.5, 116 mM KCl, 3.6 mM $MgCl_2$, 6 mM β-mercaptoethanol). The homogenate was centrifuged at $20{,}000\times g$ for 10 minutes at 4°C. The supernatants will be stored at −70°C in hypotonic buffer containing 45% glycerol. The RNase H–mediated cleavage experiments were carried out in a total volume of 30 liters, containing 20 liters of cell extract, 1 mM DTT, 20–40 U RNase inhibitor (Promega), and 50 nM of the various antisense oligodeoxyribonucleotides corresponding to ribozyme target sequences identified by MFOLD program. Sense oligodeoxyribonucleotide will serve as a control. This mixture was incubated for 5–10 minutes at 37°C followed by digestion with DNase I for 45 minutes and phenol extraction and ethanol precipitation. Reverse transcription was performed according to the manufacturer's protocol (Life Technologies, Carlsbad, CA) using 50 ng of random hexamer primer and 10 U of Moloney murine leukemia virus reverse transcriptase.

Different aliquots of the RT reaction were then amplified using pCD primers (5′-CCAGTACTACGGGGAGATTG-3′ and 5′-CCATAGTGGATGTCAAACGA-3′) and β-actin–specific primers (5′-TGCTATCCAGGCTGTGCTAT-3′ and 5′-TTCCAGTTTTTAAATCCTGAGTC-3′) for 25 cycles. Sequences of oligodeoxynucleotides that show the highest potential in reduction of pCD mRNA in cell extracts were chosen as target sites for ribozymes. DNA sequences coding for ribozymes were synthesized by PCR using overlapping oligonucleotide primers. The PCR primers were designed in such a way that the corresponding PCR products will contain the T7 promoter sequence at their 5′ ends. PCR products were fractionated through 3% composite agarose gel (2% NuSieve, 1% Seakem) (FMC, Rockland, ME), electroeluted, and purified with phenol/chloroform. The DNA sequence corresponding to target pCD mRNA sequence cloned to pcDNA3 vector (Invitrogen, Carlsbad, CA) was kindly provided by Dr John Chirgwin.

Cleavage activity of all ribozymes and control ribozymes was tested by *in vitro* cleavage assay. Ribozyme RNA, control RNA, and target pCD RNA were prepared by *in vitro* transcription reaction using RiboScribe RNA Probe Synthesis Kit (Epicentre, Madison, WI) according to the manufacturer's instructions. Cleavage reaction was performed by the method described by Biegelman et al.[50] PCR products were digested with appropriate restriction endonucleases (New England Biolabs, Beverly, MA) and ligated into PCI-neo and pH Apr-1-neo vectors. These vector constructs were transformed to competent *Escherichia coli* DH5 (Gibco, Carlsbad, CA). Large-scale vector DNA with cloned ribozyme and control sequences were then prepared using an EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA). All plasmid constructs were custom-sequenced (Invitrogen) to confirm the sequence and orientation of ribozyme templates.

Subsequently, human breast cancer cell lines were stably transfected by lipofection with either pH Apr-1-neo or pCI-neo vectors with cloned ribozyme and control sequences as mentioned above. The expression of ribozymes in transfected cells was screened as described by Dawson and Marini.[51] The resulting PCR products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The lengths of PCR products were compared with those predicted from vector ribozyme sequences. The expression of target pCD RNA in transfected cells was analysed using both the semiquantitative RT-PCR and Northern blot analysis.

Matrigel assay

For evaluation of the invasion across Matrigel layers, a commercial kit manufactured by Chemicol International (Temecula, CA) has been used according to the manufacturer's instruction.

Animals

Athymic nu/nu BALB/c female mice were purchased from the Jackson Laboratory (Bar Harbor, ME).

Tumor cell growth in mice

Human breast tumors were generated by injecting athymic nude mice with 5×10^5 MDA-MB-231 cells subcutaneously. After 10–14 days, the mice were checked for tumor development and only mice with palpable tumors were subsequently injected intravenously with biodegradable gelatin microspheres containing 100 μg of either antifragment mAbs or irrelevant antihuman HLA IgG_1 mAb. Mice

were sacrificed at various intervals and tumor size was evaluated by weighing of the tumors. Eight weeks after the initial injection of breast cancer cells (left), mice from the anti-AA 27–44 mAb–treated group were secondarily challenged with prostate cancer cells. Again, the mice were divided into several groups and treated by mAb. The original groups consisted of 10 mice per group, the second part of the experiment used three mice per group. Mice were sacrificed at various time intervals and tumor size was evaluated by weight. Biodegradable gelatin microspheres were prepared as described by Golumbek et al.[52]

In immunization experiments, mice were immunized with 25 μg of activation peptide in complete Freund adjuvant (four immunizations in 6-week interval). After checking the level of antiactivation peptide antibodies by ELISA (results not shown), mice were injected with human tumor cells as described above. The mice were sacrificed at various intervals as described.

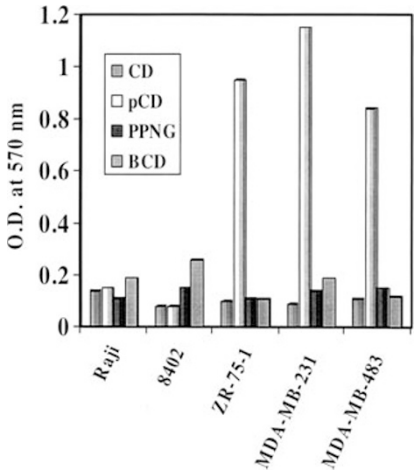

Figure 2 Growth of human cell lines in serum-free medium containing 2 nM human cathepsin D (CD), 2 nM pCD, 20 nM pepsinogen (PPNG), or 2 nM bovine cathepsin D (BCD).

Results

Because only the enzymatically inactive pCD is secreted from cancer cells, we assumed possible relationship between procathepsin and the development of breast cancer. These studies demonstrated that pCD had an autocrine growth factor activity on breast cancer cells that was mediated by a specific, so-far-unknown receptor, which seems to recognize structure within the activation peptide of pCD (27–44 AA sequence[47] and probably even 36–44 AA).

pCD was tested by cultivation of tumor cell lines from different tissue origins in serum-free medium. Cell growth was monitored by an MTT assay at 570 nm (Fig 1). pCD only had mitogenic activity for mammary tumor cell lines. The growth of other cell lines was not sustained with pCD in serum-free medium. Additional tumors not responding to pCD include U937, HeLa, and HepG2 lines.[53] We have also compared the effects of pCD to the effects of insulin-like growth factor II (IGF II), a known mitogen for many human cell lines. The results showed that the growth potentiation effects of IGF II on breast cancer cells are identical to those of pCD, but IGF II stimulated the proliferation of all types of cancer cells we tested.[53] Additional experiments were focused on potentially comparing the effects of pCD to the mature human and bovine CD or pepsinogen. No proliferative activity was observed for any of these control molecules (Fig 2).

Experiments were then performed to determine whether the mitogenic activity could be mediated by the M6P-R or a yet different receptor. First, high concentrations of M6P known to inhibit the M6P-R–mediated interaction and internalization of pCD[54] were used as a specific inhibitor of the M6P-R. The mitogenic function of pCD was not blocked by high concentrations of M6P.[55] Figure 3 further demonstrates that the growth factor activity of pCD was not blocked by either of two types of anti–M6P-R antibodies (either calcium-dependent 46-kDa or calcium-independent 300-kDa receptor) nor by competing soluble M6P-R. Finally, there was very little loss of activity when deglycosylated pCD was used, indicating that pCD has growth factor

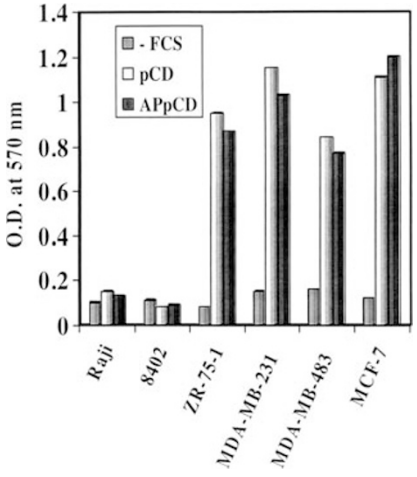

Figure 1 Growth of human cell lines in serum-free medium containing either 40 ng/mL pCD or 50 ng/mL activation peptide (APpCD).

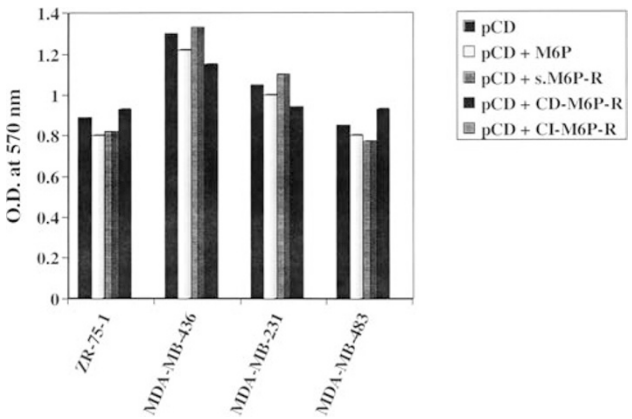

Figure 3 Effect of various potential inhibitors on pCD-mediated growth of human cell lines. The concentrations were used as follows: 40 ng/mL pCD; 10 mM M6P; 1 μg/mL soluble M6P receptor (s.M6P-R); 1 μg/mL anti–M6P-R antibodies.

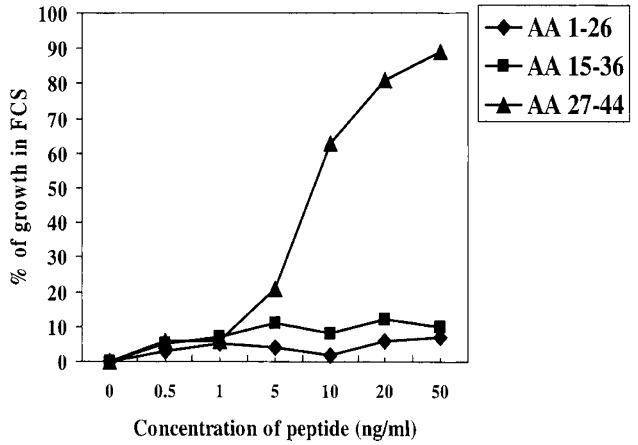

Figure 4 Effect of various concentrations of three synthetic peptides representing fragments of activation peptide on the growth of human breast cancer cell line ZR-75-1 in serum-free medium.

activity that is not mediated by an interaction of M6P with the M6P-R.

At this stage, all the experimental evidence indicated that a pivotal role in the mitogenic activity is included in the activation peptide of the pCD. The mitogenic function of pCD was further tested by using activation peptide alone or various synthetic peptides based on modeled 3D structure of pCD.[48] All these results suggested that the mitogenic function of activation peptide was mediated through a specific receptor expressed on all eight human breast cancer cell lines examined. Scatchard plot analysis revealed that the number of binding sites was 1.83×10^4 per cell, with a K_d of 0.61 nM[56] Next we prepared 10 synthetic peptides representing several parts of the activation peptide and compared their mitogenic activity. Our data indicated that the binding site of APpCD is located somewhere between amino acid positions 27 and 44[47,56] All peptides representing first 1–27 AA of the APpCD peptide were not effective (Fig 4); AA 27–44 peptide was effective at similar doses to those found with the APpCD. Subsequent experiments demonstrated that the active part of the APpCD can be located to the smaller peptide AA 36–44 (data not shown). This information should be very useful as we screen for potential pCD receptors. In addition, significant inhibition of breast cancer growth *in vitro* was achieved by anti–AA 27–44 fragment antibodies[47] After determining that the growth-stimulating effects of pCD and its activation peptide may be mediated through a new, previously unknown receptor, we investigated if the activation peptide is similar to other known proteins. When searching for the receptor structure in PIR and SWISSPROT databases, using the Fasta program against all known human proteins, only significant similarities to other human aspartic proteinases were found. No additional similarity was considered to be significant.

Subsequent experiments demonstrated that prostate cancer cells PC3, DU145, and LNCaP[38,57] and ovarian cancer cells UL-1 and SKOV-3[58] are similarly sensitive to the pCD treatment. Furthermore, this mitogenic activity was blocked by antibodies to APpCD. Finally, there was no mitogenic activity detected when a peptide of the same size but with a scrambled sequence was used (data not shown).

Next, the growth-promoting activity of pCD was demonstrated in an *in vivo* animal model in which anti-pCD antibodies were shown to reverse breast cancer development. Human breast tumors were generated by injecting athymic nude mice with 5×10^6 MDA-MB-231 cells (which lack the estrogen receptor) directly into mammary fat pads. After 2 weeks, the mice were checked for tumor development. Mice with palpable breast tumors were then injected intravenously with biodegradable gelatin microspheres containing 100 μg of either pCD, anti-pCD mAb, antifragment 1–27 mAb, antifragment 36–44 mAb, or irrelevant anti-HLA mAb (control Ab) or the same isotype. Mice were sacrificed at various time intervals and tumor size was evaluated by weight. Mice treated with either anti-pCD or antifragment 36–44 antibodies showed greatly reduced tumor growth[47] pCD alone increased the tumor growth only slightly over the control group, no doubt due to a short *in vivo* lifespan of pCD. Subsequent study measured the survival rate of mice treated with either antifragment 27–44 antibodies or antihuman factor I mAb after challenge with MDA-MB-231 cells. Again, antibodies were coupled with gelatin microspheres. Ten days after subcutaneous injection of tumor cells, the mice were given the antibody. This treatment was repeated on day 45 after original challenge with tumor cells (Fig 5).

To examine the feasibility of our proposal even further, we tested the effects of antifragment antibody treatment on mice challenged with secondary cancer (Fig 6). The treatment started 10 days after first tumor cells injection. Eight weeks after the initial injection of breast cancer cells (left), mice from the treated group were secondarily challenged with prostate cancer cells. Again, the mice were divided into several groups and treated by mAbs (right). The original groups consists of 10 mice per group; the second part of the experiment used three mice per group. A control mAb (anti-HLA) of the same isotype or mAb against AA 1–26 fragment had no effect. The results of all animal experiments showed the feasibility of developing agents that block the mitogenic activity of pCD in human breast tumors. Similarly, we have used the complete activation peptide

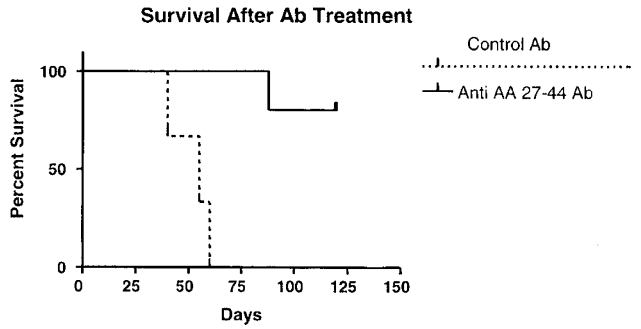

Figure 5 Survival of groups of six mice treated with anti–AA 27–44 mAb or antihuman factor I mAb after challenge with the MDA-MB-231 cells. Ten days after subcutaneous injection with breast cancer cells, the mice were given treatment by intraperitoneal injection. The treatment was repeated on day 45 after original challenge.

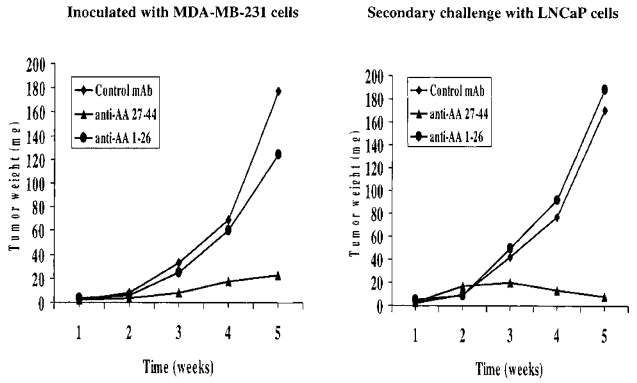

Figure 6 Inhibition of cancer growth in athymic mice by treatment with antifragment mAb. A control mAb (anti-HLA) of the same isotype or mAb against AA 1–26 fragment had no effect. The treatment started 10 days after tumor cells injection. Eight weeks after the initial injection of breast cancer cells (left), mice from the treated group were secondarily challenged with prostate cancer cells. Again, the mice were divided into several groups and treated by mAb (right). The original groups consist of 10 mice per group; the second part of the experiment used three mice per group.

for immunization of athymic mice prior the tumor cell implantation (see Fig 7). The results of immunized and control animals were analyzed in the same way as in previous experiments.

In the final experiment, we focused our attention on possible inhibition of pCD synthesis using specifically designed ribozymes. One of the problems with the design of ribozymes is the identification of potential cleavage sites on the target RNA because RNA folds into complex secondary structures, which interfere with binding of the ribozyme. Efficient ribozyme cleavage sites on long substrates cannot be easily predicted. We used MFOLD Program version 3.1. for prediction of the most stable secondary structure of pCD mRNA using energy minimization method.[59] Ribozyme target sites that are part of, or in close proximity to, open loop regions and that contain NUH sequence motif (N is any nucleotide and H is C, U, or A) were selected. The preference was given for GUH sites (especially GUC sites) because the highest ribozyme cleavage efficiency has been shown for these sites.[60] Seven hammerhead ribozymes were designed. These were tested using the same program to determine whether their sequences are able to fold into a typical hammerhead ribozyme structure. Finally, we tested the selected ribozyme sequences for specificity against other known human sequences using the BLAST program.[61]

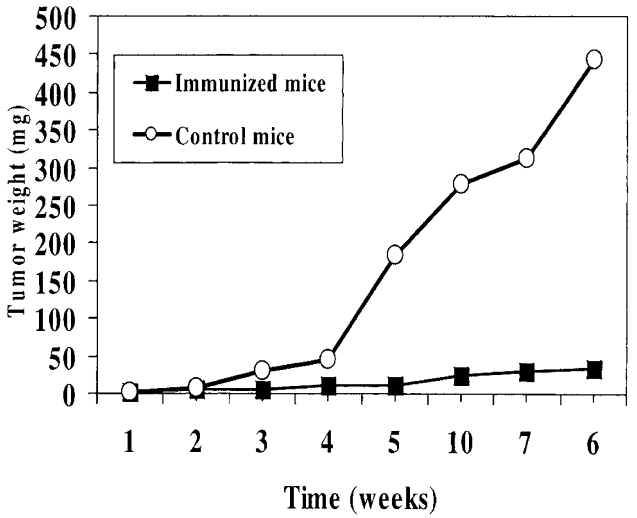

Figure 7 Athymic nude mice were immunized by activation peptide as described in *Materials and methods*. Injection with cancer cells and subsequent evaluation of mice were identical to that in Figure 6. Day of cancer cells application is considered as day 0.

Designed ribozyme sequences:

RZ385: 5′-GUGCUGCUgAUGAGUCCGUGAGGAC-GAAACUUGUCGCUGU-3′
RZ763: 5′-UUGUAACUgAUGAGUCCGUGAGGAC-GAAACUUGGAGUCUG-3′
RZ774: 5′-ACAGAGCUgAUGAGUCCGUGAGGAC-GAAACCCUUGUAAUAC-3′
RZ644: 5′-GUCGAACUgAUGAGUCCGUGAGGAC-GAAACGGGCAGCAC-3′
RZ1127: 5′-UGGCGGCUgAUGAGUCCGUGAGGAC-GAAAUGUCCAUGC-3′
RZ94: 5′-CGGAUGCUgAUGAGUCCGUGAGGAC-GAAACGUGAACUUG-3′
RZ1254: 5′-CGCGCGCUgAUGAGUCCGUGAGGAC-GAAACGCCUUGGGAA-3′.

Ribozymes were numbered according to the position of their cleavage site in pCD mRNA sequence. Ribozyme RZ94 was designed to target pCD mRNA in AP sequence. Sequences of antisense and control sense oligodeoxyribonucleotides corresponding to ribozyme target sequences identified by MFOLD program were as follows:

antisense RZ385: 5′-GTGCTGGACTTGTCGCTGT-3′
antisense RZ763: 5′-TTGTAATACTTGGAGTCTG-3′
antisense RZ774: 5′-ACAGAGAACCCTTGTAATAC-3′
antisense RZ644: 5′-GTCGAAGACGGGCAGCAC-3′
antisense RZ1127: 5′-TGGCGGGATGTCCATGC-3′
antisense RZ94: 5′-CGGATGGACGTGAACTTG-3′
antisense RZ1254: 5′-CGCGCGGACGCCUUGGGAA-3′
sense control: 5′-ACAGCGACAAGTCCAGCAC-3′.

For evaluation of the invasion across Matrigel layer, we used a commercial kit. The results summarized in Table 1

Table 1 Invasiveness through Matrigel membrane

	Transfected with plasmid coding			
Cell line	Original line	Control	pCD	RZ644 ribozyme
HBL-100	–	–	++	–
ZR-75-1	+	–	+++	–

The kit was used according to manufacturer's instruction. Each experiment was done in triplicate.

clearly show that transfection with pCD cDNA increased the invasion in both control (previously negative cell line) and in breast cancer cell line ZR-75-1. In addition, transfection of these lines with specific ribozyme RZ644 resulted in inhibition of invasion. Transfection with control sense resulted in no changes (data not shown).

Discussion

Secreted pCD was demonstrated by immunofluorescence in tissue section, and by ELISA in tissue culture supernatants, carcinoma cytosols, saliva,[62] or nipple aspirates of both normal and carcinoma tissues (for review, see Ref. [46]). Moreover, several clinical studies suggested a potential role for this molecule in metastasis because its concentration in primary tumors correlated with an increased incidence of tumor metastases.[14,15,63] In nude mice, it was shown that rat tumor cells were converted from low to high metastatic potential by transfection with the cDNA for human CD.[64,65] indicating the role of pCD in metastases. The major question remains: What mechanism is employed in the cancer-promoting activity of pCD?

In the initial experiments, Rochefort and Capony[66] hypothesized that hypersecretion of pCD caused and/or regulated by estrogens is followed by maturation at the unknown site, which suggests the role of mature CD instead of zymogen pCD. This possibility was further increased by experiments employing rat cancer cell line transfected with human CD cDNA. Clones transfected with regular CD cDNA developed larger metastases than clones transfected with vectors where KDEL signal retained pCD in the endoplasmic reticulum.[67] However, the addition of the KDEL signal inhibited maturation of pCD, not the secretion of it. Thus, the conclusion of these experiments remains controversial. Later reviews discussed the possible dual role of (pro)cathepsin D in cancer development — acting as a protease after its activation and/or as a ligand on membrane receptor before activation.[10,12]

Secreted pCD might be activated by acidic extracellular conditions and subsequently degrade growth inhibitors and extracellular matrix, or liberate some growth factors. The possibility of the extracellular matrix degradation seems to be supported by findings of Briozzo et al,[68] but their experiments showing liberation of FGF_2 from extracellular matrix are rather difficult to interpret. An important observation, using a mutated CD with abolished proteolytic activity, showed stimulation of cancer cell proliferation both *in vivo* and *in vitro*.[69] These experiments clearly indicated that proteolytic activity is not involved in the ''cancer-promoting'' or mitogenic function of pCD in certain cancer tissues.

When the proteolytic activity is ruled out as the mechanism of mitogenicity, the second possibility is that pCD is (as a whole molecule) directly involved in cancer cell growth and/or differentiation. To test this, it was necessary to demonstrate specific binding of pCD. Hence, at least two types of receptors are known to interact with pCD. The first type is represented by two different M6P/IGF II receptors (for review, see Ref. [10]). These two receptors involved in the lysosomal targeting of pCD are expressed both intracellularly and on the outer cell membrane. Despite the fact that intracellular targeting of pCD through M6P is well established,[70–72] studies indicating an alternative targeting of pCD, both intra- and extracellularly, are common[53,73–77] and represent a second known interaction of pCD with a receptor. This independence on established M6P-R binding is even more pronounced in breast cancer cells. Rochefort et al analyzed pCD uptake in two human breast cancer cell lines and showed that the internalization is independent on M6P. Additional experiments showed the presence of saturable, M6P-independent binding sites.[78] The proofs of the new pathway of pCD interaction with cell membrane were further strengthened by the finding that the binding of FITC-labeled pCD to cancer cells can be inhibited by unlabeled procathepsin but only marginally by either M6P, anti–M6P-R antibodies, or by soluble M6P-R.[56,79] In addition, the biological activities of pCD cannot be blocked by anti–M6P-R antibodies.

After establishing the interaction of pCD with a putative cell surface receptor, we focused on experiments testing the hypothesis that structure responsible for mitogenicity of pCD is contained within the APpCD. An important condition for such a function of the APpCD — the localization of the propeptide on a surface of the pCD molecule — is fulfilled.[48]

The effects of various concentrations of either pCD or APpCD on proliferation of various breast cancer cells lines were described.[53,56] When growth factor activity of these molecules was evaluated, irrelevant synthetic peptides with similar purity and molecular weight (peptide representing the intracellular part of CR3 receptor and pig pepsinogen A) were used.[47] The optimal concentration of APpCD was found to be around 50 ng/mL, a dose slightly higher than required for the parent pCD molecule. The differences in effective molar concentration between APpCD and pCD can be explained on the base of the 3D structure of CD and a model of 3D structure of pCD.[48] It is clear that the conformation of the activation peptide in the pCD is stabilized by numerous noncovalent bonds as well as a covalent attachment of APpCD to the N-terminus of a mature molecule and, therefore, the activity of the peptide is less effective.

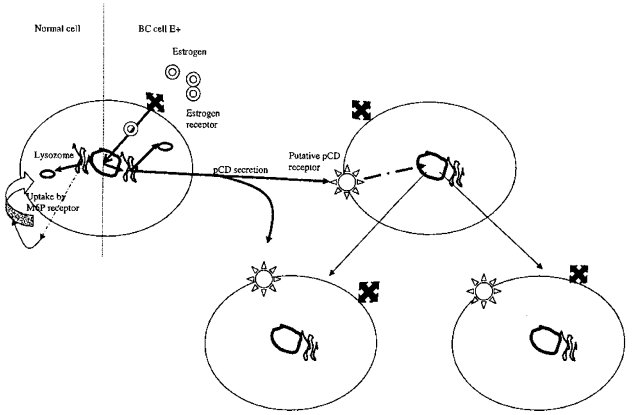

Figure 8 Hypothetical mechanism of pCD involvement in cancer development and progression.

The question of whether the pCD found in cytosols of human breast tumors originates from disintegrating cells or from active secretion has not been adequately addressed. Using technique originally developed for staining intracellular cytokines, we showed not only the presence of pCD inside tumor cells, but also a significant increase of pCD staining when cells were incubated with the protein transport inhibitor brefeldin A.[56] Identical results were obtained when monensin, instead of brefeldin A, was used. The specificity of this secretion was shown by double staining with anti–MUCIN-1 mAb, which specifically stained breast cancer cells. Eight different tumors were tested with identical results. These results clearly indicate that pCD is actively secreted by breast cancer cells.

The role of pCD has recently been demonstrated by using a retroviral approach. Overexpression of pCD increased colony formation in NIH3T3 cells and progression of prostate cancer.[80] Thus, studies in this and other laboratories appear to implicate pCD in growth and/or metastasis of breast cancer. However, few tumor cell lines have been investigated in this regard and many important questions remain with respect to mechanisms. For example, no quantitative information is available to assess a potential correlation between levels of pCD expression and aggressive tumor behavior. Carefully blinded studies will assess transfectants for growth in serum-free medium and invasion in culture, as well as growth and metastasis *in vivo*. In contrast to our results, Liaudet-Coopman et al[69] have not been able, under their experimental conditions, to confirm the role of the APpCD in mitogenesis. The experiments with mutated pCD have also concluded that the mitogenic function was independent on M6P, suggesting the possibility of an involvement of yet additional surface structure on pCD and additional unknown receptor. Nevertheless, Altschul et al[61] have been able to show similar behavior of the APpCD in the case of endometrial tumor–derived cell lines.

Based on the results shown in numerous papers,[47,55–57,79] as well as the data of Chinni et al,[41] we suggest the following mechanism (Fig 8): In normal cells, pCD is targeted to lysozomes using an M6P tag and, parallel to that, by a yet unknown ligand–receptor interaction. The small part of pCD that eludes this targeting and might be secreted is recaptured through M6P receptors expressed on the cell surface. In breast cancer cells, overexpression of pCD induced by estrogens occurs, with subsequent secretion of the major part of the ''extra'' pCD. Due to massive overexpression, saturation of M6P receptors occurs and the recapturing is abolished. The remaining extracellular pCD interacts with additional cancer cells in close vicinity of the secreting cells through putative activation peptide receptor. The interaction is performed by APpCD of pCD with an unknown receptor. This interaction results in higher proliferation of cancer cells.

When antibodies specifically recognizing pCD have been used, two groups reported findings of elevated concentration of pCD in serum of metastatic breast cancer patients,[81,82] suggesting that, at least in final stages of cancer, pCD is released into the bloodstream. It is important that the activation peptide sequence is recognized by the immune system as a non–self-structure and triggers the production of specific antiactivation peptide antibodies.[41,83]

Preliminary experiments using specifically designed ribozymes demonstrated not only that we can influence the synthesis of pCD in cancer cells, but also their characteristics such as invasion across Matrigel layers (Table 1).

Finally, our hypothesis accenting the role of the APpCD in tumor cell growth explains all the experimental data we have gathered. Such hypothesis also offers interesting possibilities for therapeutic use. Direct experiments using antibodies blocking the putative interaction of AP with the receptor in animal model are described in this paper. Blocking of this interaction has caused fundamental changes in growth of carcinoma in experimental animals. The last set of our experiments, using active immunization of mice by the activation peptide with subsequent transfer of the carcinoma, shows possible consequences of this hypothesis. Again, there is considerably stronger suppression of carcinoma growth in the animals that were immunized compared to those without pretreatment.

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References

1. Erickson AH, Blobel G. Early events in the biosynthesis of the lysosomal enzyme cathepsin D. *J Biol Chem*. 1979;254:11771–11774.
2. Kornfeld R, Kornfeld S. Assembly of asparagine linked oligosaccharides. *Annu Rev Biochem*. 1985;54:631–664.
3. Tang J, Wong RNS. Evolution in the structure and function of aspartic proteases. *J Cell Biochem*. 1987;33:53–63.
4. Dingle JT, Poole AR, Lazarus GS, et al. Immunoinhibition of intracellular protein digestion in macrophages. *J Exp Med*. 1973;137:1124–1141.
5. Williams KP, Smith JA. Isolation of membrane-associated cathepsin D like enzyme from the model antigen presenting cell, A20, and its ability to generate antigenic fragments from protein antigen in a cell free system. *Arch Biochem Biophys*. 1993;305:298–306.
6. Baldocchi RA, Nicoll CS. Processing of rat prolactin by rat tissue explants and serum *in vitro*. *Endocrinology*. 1992;130:1653–1659.
7. Loscalzo J. The macrophage and fibrinolysis. *Semin Thromb Haemost*. 1996;22:503–506.
8. Podhajcer OL, Bover L, Bravo AL, et al. Expression of cathepsin D in primary and metastatic human melanoma and dysplastic nevi. *J Invest Dermatol*. 1995;104:340–344.
9. Bazzett LB, Watkins CS, et al. Modulation of proliferation and chemosensitivity by procathepsin D and its peptides in ovarian cancer. *Gynecol Oncol*. 1999;74:181–187.
10. Rochefort H. Cathepsin D in cancer metastasis: a protease and a ligand. *APMIS*. 1999;107:86–95.
11. Lorenzo K, Ton P, Clark JL, et al. Invasive properties of murine squamous carcinoma cells: secretion of matrix-degrading

cathepsins is attributable to a deficiency in the mannose 6-phosphate/insulin-like growth factor II receptor. *Cancer Res*. 2000;60:4070–4076.
12. Rochefort H, Garcia M, Glondu M. Cathepsin D in breast cancer: mechanisms and clinical applications, a 1999 review. *Clin Chim Acta*. 2000;291:157–170.
13. Reid WA, Vallor MJ, Kay J. Immunolocalization of cathepsin D in normal and neoplastic tissues. *J Clin Pathol*. 1986;39: 1323–1330.
14. Spyratos F, Brouillet JP, Defrenne A, et al. Cathepsin D: an independent prognostic factor for metastasis of breast cancer. *Lancet*. 1989;ii:1115–1118.
15. Rochefort H. Biological and clinical significance of cathepsin D in breast cancer. *Acta Oncol*. 1992;31:125–130.
16. Vignon F, Capony F, Chambon M, et al. Autocrine growth stimulation of the MCF 7 breast cancer cells by the estrogen-regulated 52 K protein. *Endocrinology*. 1986;118:1537–1545.
17. Elangovan S, Moulton BC. Progesterone and estrogen control of rates of synthesis of uterine cathepsin D. *J Biol Chem*. 1980;255:7474–7479.
18. Westley B, Rochefort H. A secretion of glycoprotein induced by estrogens in human breast cancer cell lines. *Cell*. 1980;20: 352–362.
19. Cavailles V, Augreau P, Rochefort H. Cathepsin D gene is controlled by a mixed promoter, and estrogens stimulate only TATA-dependent transcription in breast cancer cells. *Proc Natl Acad Sci USA*. 1993;90:203–207.
20. Krishnan V, Narasimhan T, Safe S. Development of gel staining techniques for detecting the secretion of procathepsin D (52-kDa protein) in MCF-7 human breast cancer cells. *Anal Biochem*. 1992;204:137–142.
21. Rochefort H, Capony F, Garcia M, et al. Estrogen-induced lysosomal proteases secreted by breast cancer cells: a role in carcinogenesis? *J Cell Biochem*. 1987;35:17–29.
22. Augereau P, Miralles F, Cavailles V. Characterization of the proximal estrogen-responsive element of human cathepsin D gene. *Mol Endocrinol*. 1994;8:693–703.
23. Leto G, Gebbia N, Rausa L, et al. Cathepsin D in the malignant progression of neoplastic diseases. *Anticancer Res*. 1992;12: 235–240.
24. Rochefort H. Cathepsin D in breast cancer: a tissue marker associated with metastasis. *Eur J Cancer*. 1992;28A:1780–1783.
25. Kute TE, Shao ZM, Sugg NK, et al. Cathepsin D as a prognostic indicator for node-negative breast cancer patients using both immunoassays and enzymatic assays. *Cancer Res*. 1992;52:5198–5203.
26. Pujol P, Maudelonde T, Daures JP, et al. A prospective study of the prognostic value of cathepsin D levels in breast cancer cytosol. *Cancer*. 1993;71:2006–2012.
27. Schultz DC, Bazel S, Wright LM. Western blotting and enzymatic activity analysis of cathepsin D in breast tissue and sera of patients with breast cancer and benign breast disease and of normal controls. *Cancer Res*. 1994;54:48–54.
28. Nazeer T, Church K, Amato C, et al. Comparative quantitative immunohistochemical and immunoradiometric determinations of cathepsin D in endometrial adenocarcinoma: predictors of tumor aggressiveness. *Mod Pathol*. 1994;7:469–474.
29. Inoue Y, Abe K, Obata K, et al. Immunohistochemical studies concerning cathepsin D in endometrial carcinomas. *J Obstet Gynaecol Res*. 1999;25:345–352.
30. Huet G, Zerimech F, Dieu MC, et al. The state of differentiation of HT-29 colon carcinoma cells alters the secretion of cathepsin D and of plasminogen activator. *Int J Cancer*. 1994;57:875–882.
31. Oh-e H, Tanaka S, Kitadai Y, et al. Cathepsin D expression as a possible predictor of lymph node metastasis in submucosal colorectal cancer. *Eur J Cancer*. 2001;37:180–188.
32. Vigneswaran N, Zhao W, Dassanayake A, et al. Variable expression of cathepsin B and D correlates with highly invasive and metastatic phenotype of oral cancer. *Hum Pathol*. 2000;31: 931–937.
33. Ferrandina G, Scambia G, Panici P, et al. Cathepsin D in primary squamous laryngeal tumors: correlation with clinicopathological parameters and receptor status. *Cancer Lett*. 1992; 67:133–138.
34. Goussia A, Ioachim E, Peschos D, et al. Immunohistochemical expression of cathepsin D in laryngeal epithelial lesions: correlation with CD44 expression, p53 and Rb status and proliferation associated indices. *Anticancer Res*. 1999;19: 3055–3060.
35. Gandour-Edwards R, Trock B, Donald PJ. Predictive value of cathepsin D for cervical lymph node metastasis in head and neck squamous cell carcinoma. *Head Neck*. 1999;21:718–722.
36. Metaye T, Millet C, Kraimps JL, et al. Estrogen receptors and cathepsin D in human thyroid tissue. *Cancer*. 1993;72:1991–1996.
37. Chambon M, Rebillard X, Rochefort H, et al. Cathepsin D cytosolic assay and immunohistochemical quantification in human prostate tumors. *Prostate*. 1994;24:320–325.
38. Vetvicka V, Vetvickova J, Fusek M. Effect of procathepsin D and its activation peptide on prostate cancer cells. *Cancer Lett*. 1998;129:55–59.
39. Frohlich E, Schlagenhauff B, Mohrle M, et al. Activity, expression, and transcription rate of the cathepsins B, D, H, and L in cutaneous malignant melanoma. *Cancer*. 2001;91: 972–982.
40. Ikeguchi M, Fukuda K, Oka SI, et al. Micro-lymph node metastasis and its correlation with cathepsin D expression in early gastric cancer. *J Surg Oncol*. 2001;77:188–194.
41. Chinni SR, Gercel-Taylor C, Conner GE, et al. Cathepsin D antigenic epitopes identified by the humoral responses if ovarian cancer patients. *Cancer Immunol Immunother*. 1998; 46:48–54.
42. Baekelandt H, Holm R, Trope CG, et al. The significance of metastasis-related factors cathepsin D and nm23 in advanced ovarian cancer. *Ann Oncol*. 1999;10:1335–1341.
43. Tedone T, Correale M, Barbadossa G, et al. Release of the aspartyl protease cathepsin D is associated with and facilitates human breast cancer cell invasion. *FASEB J*. 1997;11:785–792.
44. Johnson MD, Torri JA, Lippman ME, et al. The role of cathepsin D in the invasiveness of human breast cancer cells. *Cancer Res*. 1993;53:873–877.
45. Lah TT, Calaf G, Kalman E, et al. Cathepsins D, B and L in breast carcinoma and in transformed human breast epithelial cells (HBEC). *Biol Chem Hoppe-Seyler*. 1995;376:357–363.
46. Fusek M, Vetvicka V. Aspartic Proteinases: Physiology and Pathology. Boca Raton: CRC Press; 1995.
47. Vetvicka V, Vetvickova J, Fusek M. Anti-human procathepsin D activation peptide antibodies inhibit breast cancer development. *Breast Cancer Res Treat*. 1999;57:261–269.
48. Koelsch G, Metcalf P, Vetvicka V, et al. Human procathepsin D: three-dimensional model and isolation. In: Takahaski K, ed. *Aspartic Proteinases: Structure, Function, Biology, and Biomedical Implications*. New York: Plenum; 1995:273–278.
49. Scherr M, Rossi JJ. Rapid determination and quantitation of the accessibility to native RNAs by antisense oligodeoxynucleotides in murine cell extracts. *Nucleic Acids Res*. 1998;26: 5079–5085.

50. Biegelman L, McSwiggen JA, Draper KG, et al. Chemical modification of hammerhead ribozymes. *J Biol Chem*. 1995; 270:2507–25780.
51. Dawson PA, Marini JC. Hammerhead ribozymes selectively suppress mutant type I collagen mRNA in osteogenesis imperfecta fibroblasts. *Nucleic Acids Res*. 2000;28:4013–4020.
52. Golumbek PT, Azhari R, Jaffee EM, et al. Controlled release, biodegradable cytokine depots: a new approach in cancer vaccine design. *Cancer Res*. 1993;53:5841–5844.
53. Vetvicka V, Vetvickova J, Fusek M. Effect of human procathepsin D on proliferation of human cell lines. *Cancer Lett*. 1994;79:131–135.
54. Mathieu M, Rochefort H, Barenton B, et al. Interaction of cathepsin D and insulin-like growth factor II (IGF-II) on the IGF-II/mannose-6-phosphate receptor in human breast cancer cells and possible consequences on mitogenic activity of IGF-II. *Mol Endocrinol*. 1990;4:1327–1335.
55. Vetvicka V, Fusek M. Activation of peripheral blood neutrophils and lymphocytes by human procathepsin D and insulin-like growth factor II. *Cell Immunol*. 1994;156:332–341.
56. Vetvicka V, Vetvickova J, Hilgert I, et al. Analysis of the interaction of procathepsin D activation peptide with breast cancer cells. *Int J Cancer*. 1997;73:402–409.
57. Vetvicka V, Vetvickova J, Fusek M. Role of procathepsin D activation peptide in prostate cancer growth. *Prostate*. 2000; 44:1–7.
58. Bazzett LB, Watkins CS, Gercel-Taylor C, et al. Modulation of proliferation and chemosensitivity by procathepsin D and its peptides in ovarian cancer. *Gynecol Oncol*. 1999;74:181–187.
59. Zuker M, Mathews DH, Turner DH. Algorithms and thermodynamics for RNA secondary structure prediction: a practical guide. In: Barciszewski J, Clark BFC, eds. *RNA Biochemistry and Biotechnology*. NATO ASI Series, vols. 11–43. Dordrecht: Kluwer Academic Publishing; 1999.
60. Shimayama T, Nishikawa S, Taira K. Generality of the NUX rule — kinetic analysis of the results of systematic mutations in the trinucleotide at the cleavage site of hammerhead ribozymes. *Biochemistry*. 1995;34:3649–3654.
61. Altschul SF, Gish W, Miller W, et al. Basic local alignment search tool. *J Mol Biol*. 1990;215:403–410.
62. Streckfus C, Bigler L, Tucci M, et al. A preliminary study of CA15-3, c-erbB-2, epidermal growth factor receptor, cathepsin-D, and p53 in saliva among women with breast carcinoma. *Cancer Invest*. 2000;18:101–109.
63. Thorpe SM, Rochefort H, Garcia M, et al. Association between high concentration of 52,000 MW cathepsin D and poor prognosis in primary human breast cancer. *Cancer Res*. 1989;49: 6008–6014.
64. Garcia D, Dercoq Q, Pujol P, et al. Overexpression of transfected cathepsin D in transformed cells increases their malignant phenotype and metastatic potency. *Oncogene*. 1990;5: 1809–1814.
65. Liaudet E, Garcia M, Rochefort H. Cathepsin D maturation and its stimulatory effect on metastasis are prevented by addition of KDEL retention signal. *Oncogene*. 1994;9:1145–1154.
66. Rochefort H, Capony F. Alterations of cathepsin D in human breast cancer. In: Bond JS, Barrett AJ, eds. *Proteolysis and Protein Turnover*. London: Portland Press; 1993:233–238.
67. Rochefort H. Oestrogens, proteases and breast cancer. From cell lines to clinical applications. *Eur J Cancer*. 1994;30A: 1583–1586.
68. Briozzo P, Badet J, Capony F, et al. MCF7 mammary cancer cells respond to bFGF and internalize it following its release from extracellular matrix: a permissive role of cathepsin D. *Exp Cell Res*. 1991;194:252–259.
69. Liaudet-Coopman E, Glondu M, Coopman P, et al. A mutated cathepsin-D devoid of its catalytic activity stimulates the growth of cancer cells. 92nd Annual Meeting of the American Association for Cancer Research, New Orleans, vol. 3826, 2001.
70. Von Figura K, Hasilik A. Lysosomal enzymes and their receptors. *Annu Rev Biochem*. 1986;55:167–193.
71. Von Figura K. Molecular recognition and targeting of lysosomal proteins. *Curr Opin Cell Biol*. 1991;3:642–646.
72. Kornfeld S, Mellman I. The biogenesis of lysosomes. *Annu Rev Cell Biol*. 1986;55:167–193.
73. Capony F, Braulke T, Tougeot C, et al. Specific mannose-6-phosphate receptor-independent sorting of procathepsin D in breast cancer cells. *Exp Cell Res*. 1994;215:154–163.
74. Diment S, Leech MS, Stahl PD. Cathepsin D is membrane-associated in macrophage endosomes. *J Biol Chem*. 1998;263: 6901–6907.
75. Rijnbout S, Aerts HMFG, Geuze HJ, et al. Identification of subcellular compartments involved in biosynthetic processing of cathepsin D. *J Biol Chem*. 1991;266:4862–4868.
76. Isidoro C, Demoz M, De Stefanis D, et al. Differential targeting and processing of procathepsin D in normal and transformed murine 3T3 fibroblasts. *Int J Cancer*. 1997;70:310–314.
77. Isidoro C, Baccino FM, Hasilik A. Human and hamster procathepsin D, although equally tagged with mannose-6-phosphate, are differentially targeted to lysosomes in transfected BHK cells. *Cell Tissue Res*. 1998;292:303–310.
78. Laurent-Matha V, Farnoud MR, Lucas A, et al. Endocytosis of pro-cathepsin D into breast cancer cells is mostly independent of mannose-6-phosphate receptors. *J Cell Sci*. 1998;111:2539–2549.
79. Fusek M, Vetvicka V. Mitogenic function of human procathepsin D: the role of the propeptide. *Biochem J*. 1994;303: 775–780.
80. Wu X, Craft N, Raitano A, et al. Functional expression cloning identifies cathepsin D as a candidate for prostate cancer progression. 89th Annual Meeting of the American Association for Cancer Research, New Orleans, vol. 878, 1998.
81. Jarosz DE, Hamer PJ, Tenney DY, et al. Elevated levels of pro-cathepsin D in the plasma of breast cancer patients. *Int J Oncol*. 1995;6:859–865.
82. Brouillet JP, Dufour F, Lemamy G, et al. Increased cathepsin D level in the serum of patients with metastatic breast carcinoma detected wit ha specific procathepsin D immunoassay. *Cancer*. 1997;79:2132–2136.
83. Taylor DD. Tumor-reactive immunoglobulins in ovarian cancer: diagnostic and therapeutic significance? *Oncol Rep*. 1998;5:1519–1524. (Review).