Original Article

Procathepsin D Involvement in Chemoresistance of Cancer Cells

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Abstract

Background: The role of pCD in cancer has been studied for a long time. We have focused on the hypothesis that increased expression and/or secretion of pCD in cancer cells causes increased chemoresistance to apoptosis inducing molecules. **Aim:** The aim was to evaluate the effects of pCD expression/release on chemoresistance. **Materials and Methods:** We tested the LC_{50} values for various transfectants of breast cancer cell line MDA-MB-231 as well as effects of exogenous additions of pCD, its mutants, pepstatine, antibodies, and Brefeldin on the resistance. **Results:** We found that pCD levels can be correlated with chemoresistance, the pro-resistant activity seems to be localized outside the cells, proteolytic activity is not involved, and PI3-Akt signaling has an important role in antiapoptotic effects of pCD. **Conclusion:** We can conclude that overexpression of pCD has strong influence on increased resistance of tumor cells. This could, in fact, be an important contribution in the possible use of pCD level determination for prognostic and/or therapeutic purposes.

Keywords: Apoposis, Breast cancer, Cyclophosphamide, Doxorubicine, 5-fluorouracil, Procathepsin D, Resistance

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Introduction

Cathepsin D (CD) is a lysosomal aspartic peptidase involved in physiological as well as pathological processes. The proteolytic activity of CD takes part in lysosomal protein degradation and processing. The importance of CD for normal development of an organism has been shown by several knock-down experiments.[1] Reports on participation of CD in apoptosis are describing both pro- and anti apoptotic properties.[2] CD is synthesized as pre-pro-CD. The presequence is cleaved by signal peptidases. Procathepsin D (pCD) is then activated in an acidic mileu of lysosomes by proteolytic cleaveage of the propeptide. The activated CD then undergoes another proteolytic maturation when the single chain molecule is cleaved to two chain molecule.[3] CD is glycosylated at two positions. The combination of proteolytic processing and glycosylation is the explanation of the microheterogeneity of CD isolates.

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CD is found intracellularily in almost all tissues. The localization in prelysosomal compartments and lysosomes is well documented. The localization in cytosol and nucleus has been also reported.[4] On the other hand, substantial secretion of pCD had been discovered by Rochefort's group almost three decades ago. This secretion was initially observed on cancer-derived cell lines and it was shown that the extracellular pCD has autocrine mitogenic properties unrelated to the proteolytic activity.[5] The reason for secretion seems to be the mis-sorting of pCD due to its abnormally high expression level. The overexpression of pCD is upregulated by the estrogen in these cell lines. Subsequently, pCD was reported to be secreted by keratinocytes suggesting that the secretion might be involved also in some physiological events.[6]

The mechanism of autocrine mitogenic effects of extracellular pCD is not fully known. In our previous research, we suggested that the mitogenic function is related to the extracellular presence of pCD, namely to the structure within the propart of the pCD interacting with a thus far unknown receptor.[7] This interaction leads to

activation of NFκB in MCF-7 cells. Results of Ref.[8] show that pCD/CD interacts by a domain around the connection of heavy and light chains of CD with LPR1 receptor and this interaction leads to promoted growth of fibroblasts.

The overexpression and secretion of pCD from tumor-derived cell lines led to a substantial amount of research focusing on the prognostic value of pCD/CD in cancer as well as on the possibility to use pCD/CD as a therapeutic target. Reports on participation of pCD/CD in the chemoresistance have appeared lately with somehow contradictory conclusions. The work of Ref.[9] using 3Y1-Ad12 cancer cells and etoposid concluded that overexpression of pCD/CD enhances the etoposid-induced apoptosis and decreases the chemoresistance of the cells while concurrently showing that this induction of apoptosis is not connected to the proteolytic activity of CD. Conversely, Sagulenko used neuroblastoma cells and doxorubicin as a chemotherapeutic agent and concluded that the high expression and secretion of pCD leads to chemoresistance to doxorubicin which is brought on by high antiapoptotic signaling caused by pCD.[10] In our present work, we have used cell lines ZR-75-1 and MCF-7 potentiated with or depleted of estrogen and MDA-MB-231 cells which were transfected by either native pCD or pCD mutated at different positions[11] and tested the chemosensitivity of these cell lines to 5-fluorouracil, doxorubicin, and cyclophosphamid. We have asked whether the overexpression of pCD leads to (a) increased resistence; (b) whether the effects are related to proteolytic activity, and/or to the propeptide structure, (c) whether the resistance is consequence of intra- or extracellular effects of pCD; and (d) if the effects can be correlated with apoptosis.

Materials and Methods

Chemicals

RPMI 1640 medium, Iscoves's modified Dulbecco's medium, HEPES, antibiotics, glutamine, brefeldin, pepstatin A and, 5-fluorouracil, doxorubicin, cyclophosphamid, estrogen, transferrin, trypan blue, propidium iodine, and LY294002 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was procured from Hyclone Laboratories (Logan, UT, USA). Monoclonal anti-AP IgG antibodies were described previously,[11] monoclonal anti-CD antibodies were purchased from Calbiochem (San Diego, CA). Purified pCD and individual mutant proteins were described in details in Ref.[12].

Cells

Breast cancer cell lines MDA-MB-231, ZR-75-1 and MCF-7 were obtained from ATCC (Manassas, VA, USA) and were cultivated in RPMI medium supplemented with 10% FCS. Individual transfected clones were described in full details in Ref.[12].

In vitro cell proliferation assay

Cells were harvested and washed six times in Iscoves's modified Dulbecco's medium with HEPES buffer supplemented with glutamine, antibiotics, 10 µg/ml of human transferrin and 0.1% FCS. The 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 tested substances in triplicate. After 5 days in culture, the proliferation was evaluated using a Biotrak cell proliferation ELISA system version 2 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the instructions given by the manufacturer.

Western blot analysis

The 2×10^6 cells were seeded in the normal growth medium in 25 mm plastic flasks. After 24 hours, the cells were treated with doxorubicin and were allowed to continue to grow for an additional 48 hours. The cells were washed twice with 1X PBS followed by lysis in a Laemmli sample buffer. Equal volumes from each lysates were separated on a 10% SDS-PAGE, transferred onto PVDF membrane and probed with a mouse Phospho-AKT (Ser473) antibody (1:1000; Cell Signaling Technology, Inc., Danvers, MA). The antibody was revealed by a secondary antibody and ECL reaction (Amersham Biosciences, Pittsburg, PA) and the ECL signal documented by exposure of the X-ray film. Blot was reprobed using a rabbit total AKT antibody (1:1000; Cell Signaling Technology, Inc., Danvers, MA) to serve as a normalizing immunosignal.

Apoptosis

Cells were treated with doxorubicin or cultured without the drug for the indicated time and apoptosis was determined by the extent of DNA fragmentation according to the FACS analysis of propidium iodine-stained nuclei.[10] Viability of cells was evaluated by their ability to exclude Trypan blue.

Statistics

Student's *t*-test was used to statistically analyze the data.

Results

Cancer cells expressing estrogen receptor are known to overexpress and secrete pCD upon addition of estrogen to the growth media. The results shown in Table 1 indicate that for all three cytostatics the addition of estrogen increases the resistance (increases the LC_{50}) of both cell lines ZR-75-1 and MCF-7. Simultaneous addition of estrogen and anti-pCD antibodies inhibited the effects of estrogen. These data suggested the correlation between the overexpressed and/or secreted pCD and drug resistance and served as an original proof-of-concept experiment.

Table 1: Sensitivity to chemotherapeutic agents in ZR-75-1 and MCF-7 cells

Cells	Addition	5-FU	Doxorubicin	Cyclophosphamide
ZR-75-1	PBS	3.4±0.4	1.2±0.2	412.7±27.1
	Estrogen	8.5±1.1*	4.7±0.7*	776.4±56.3*
	Estrogen+mAb	3.5±0.4	1.5±0.2	430.0±25.7
MCF-7	PBS	4.6±0.8	2.3±0.5	554.8±35.8
	Estrogen	7.1±1.3*	6.5±1.3*	881.2±78.1*
	Estrogen+mAb	4.4±0.9	2.6±0.6	551.1±40.5

Data are shown as LC_{50} (ng/5×10⁴ cells) and represent mean from 3 experiments±SD. *Significant differences between control (PBS) and tested samples at $P \leq 0.05$ levels

Based on these data, we decided to focus on a MDA-MB-231 cell line where numerous well-documented transfected lines allowed detailed evaluation of the possible role of pCD and/or its mutants on resistance to chemotherapeutic drugs. We used the parental cell line MDA-MB-231 and compared its sensitivity to the three cytotoxic agents for following transfectants – stable transfected cell line overproducing native pCD; the cell line transformed with siRNA targeted to silence the production of endogenous pCD; and transfected cell line with vector with no pCD sequence included. Table 2 summarizes the results indicating that the overexpression of pCD considerably increased the resistance of the cell line to all three cytostatics. The same transfection with an empty vector (control) did not change the sensitivity of cells. The silencing of pCD production dramatically decreases the resistance.

Table 2: Sensitivity to chemotherapeutic agents in MDA-MB-231 cells after transfection

Transfection	5-FU	Doxorubicin	Cyclophosphamide
None	50.6±3.6	4.6±0.5	1057.6±232.5
pCD	377.3±11.7*	7.3±0.9*	2007.7±172.4*
Empty vector	45.7±5.1	2.8±0.3*	1028.4±178.9
siRNA	6.0±0.9*	1.3±0.1*	412.5±43.6*

Data are shown as LC_{50} (ng/5×10⁴ cells) and represent mean from three experiments±SD. *Significant differences between control (PBS) and tested samples at $P \leq 0.05$ levels; pCD: Procathepsin D

In order to study the influence of extracellular pCD on the resistance, we supplemented the culture media of parental and individual transfected cell lines with several molecules representing pCD alone or its mutation versions such as pCD mutated in the active site (231 m); and pCD mutated in the propeptide (APM1) or pCD with aborted glycosylation site 199. These results are summarized in Tables 3 and 4. Our experiments showed that the addition of pCD increased the resistance in all tested transformants. The addition of pCD mutated in the active site increased the resistance less then pCD but a clear increase is seen in the cell line with silenced pCD expression. There was practically no influence on resistance observed for the pCDAm1 mutant. The addition of pCD with aborted glycosylation in one of the glycosylation sites had a similar effect as the addition of pCD (data not shown).

The addition of pepstatine A, a selective inhibitor of aspartic peptidases, did not change the resistance profile [Table 5]. The antibodies recognizing the pro-peptide of pCD dramatically decreased the resistance (increased sensitivity) of all of the studied cell lines, while anti-CD antibodies did not change the resistance profile [Table 6]. These experiments also included the addition of Brefeldin – inhibitor of intracellular transport. The addition of Brefeldin had a strong influence on the resistance, decreasing it to low level for all studied mutants and all three cytostatics. In this set of experiments, we also tested the combination of treatment with Brefeldin and pCD mutant 231 m. Interestingly, the addition of this mutant had reverted the influence of Brefeldin [Table 7].

To study the role of pCD on apoptosis in this respect, we evaluated the effects of secreted pCD or individual mutants in our transfected cell lines on the apoptosis. The data summarized in Figure 1 show that pCD expression (normal level, overexpression of pCD) is associated with phosphorylation of protein kinase B (Akt). Inhibition of PI3-Akt signaling by LY294002 restored the sensitivity of cells to doxorubicin, further supporting the possibility that PI3-Akt signaling has an important role in antiapoptotic effects of pCD.

Discussion

Enzymatically inactive proform of CD is known to be multifunctional within and outside the cells.[13] Numerous studies suggested that pCD serves as a major prosurvival factor secreted from cancer cells.[7] The role of pCD/CD in apoptosis is currently unclear, with growing evidence of pCD involvement in programmed cell death.[14] In the present paper, we focused on the potential effects of pCD on chemoresistance and on apoptotic processes triggered by chemotherapy. We have used three different chemotherapeutic substances with different primary cytostatic mechanism of action but with the same apoptotic ending. Metabolites of 5-fluorouracil are incorporated to DNA and RNA

Table 3: Effect of addition of various substances on sensitivity to chemotherapeutic agents in MDA-MB-231 cells after transfection

Transfection	5-FU		Doxorubicine		Cyclophosphamide	
	PBS	pCD	PBS	pCD	PBS	pCD
None	50.6±3.6	90.5±7.9	4.6±0.5	7.3±1.2	1 057.6±232.5	2 208.5±305.4
pCD	377.3±11.7*	452.8±27.9*	7.3±0.9*	8.1±1.3	2 007.7±172.4*	2 419.1±343.0
Empty vector	45.7±5.1	77.8±8.1	2.8±0.3*	6.9±0.8	1 028.4±178.9	1728.6±236.5
siRNA	6.0±0.9*	38.9±3.5*	1.3±0.1*	5.2±0.7	412.5±43.6*	1 682.0±301.5

Data are shown as LC_{50} (ng/5´10^4 cells) and represent mean from three experiments±SD. pCD was used at the 20 ng/ml concentration. *Significant differences between control (PBS) and tested samples at $P \leq 0.05$ levels; pCD: Procathepsin D

Table 4: Effect of addition of pCD mutants on sensitivity to chemotherapeutic agents in MDA-MB-231 cells after transfection

Transfection	5-FU			Doxorubicin			Cyclophosphamide		
	PBS	231m	APM1	PBS	231m	APM1	PBS	231m	APM1
None	50.6±3.6	31.5±4.4	48.2±5.6	4.6±0.5	4.0±0.3	5.5±1.0	1057.6±232.5	784.6±106.1	802.6±101.1
pCD	377.3±11.7*	430.5±71.1*	356.0±35.0*	7.3±0.9*	6.7±1.1*	6.3±0.8	2007.7±172.4*	1526.0±347.8*	1 625.0±237.8*
Empty vector	45.7±5.1	40.1±9.2	30.6±2.6*	2.8±0.3*	3.9±0.3	3.5±0.3*	1028.4±178.9	789.3±182.5	923.7±202.3
siRNA	6.0±0.9*	21.1±4.4*	2.6±0.3*	1.3±0.1*	3.5±0.4	1.2±0.2*	412.5±43.6*	736.4±173.9	453.6±89.5*

Data are shown as LC_{50} (ng/5´10^4 cells) and represent mean from three experiments±SD. Mutant molecules 231 m and APM1 were used at the concentration of 10 ng/ml. *Significant differences between control (PBS) and tested samples at $P \leq 0.05$ levels; pCD: Procathepsin D

Table 5: Effect of Pepstatin A on sensitivity to chemotherapeutic agents in MDA-MB-231 cells after transfection

Transfection	5-FU		Doxorubicine		Cyclophosphamide	
	PBS	Pepstatin A	PBS	Pepstatin A	PBS	Pepstatin A
None	50.6±3.6	53.9±4.1	4.6±0.5	2.8±0.4	1 057.6±232.5	977.2±199.5
pCD	377.3±11.7*	493.9±13.7*	7.3±0.9*	7.1±0.9*	2 007.7±172.4*	2028.1±240.3*
Empty vector	45.7±5.1	42.1±5.0	2.8±0.3*	1.1±0.1*	1 028.4±178.9	1 107.5±99.5
siRNA	6.0±0.9*	3.5±0.2*	1.3±0.1*	1.2±0.2*	412.5±43.6*	390.8±32.6*

Data are shown as LC_{50} (ng/5×10^4 cells) and represent mean from three experiments±SD. Pepstatin A was used at the concentration of 1 µM. *Significant differences between control (PBS) and tested samples at $P \leq 0.05$ levels; pCD: Procathepsin D

Table 6: Effect of antibodies on sensitivity to doxorubicine in MDA-MB-231 cells after transfection

Transfection	PBS	Anti-pCD	Anti-CD
None	4.6±0.5	2.2±0.3	4.1±0.2
pCD	7.3±0.9*	1.7±0.4	6.7±0.4*
Empty vector	2.8±0.3*	2.5±0.5	3.3±0.4
siRNA	1.3±0.1*	2.2±0.5	1.7±0.1*

Data are shown as LC_{50} (ng/5×10^4 cells) and represent mean from three experiments±SD. Antibodies were used at the 1 µg/ml concentration. *Significant differences between control (PBS) and tested samples at $P \leq 0.05$ levels; pCD: Procathepsin D

and arrest the cell cycle.[15] Doxorubicin acts by a complex mechanism which is mainly accounted to his intercalation into the DNA double strand. The complex then inhibits the topoisomerse leading to termination of transcription.[16] Formation of metabolite phosphoramide mustard of cyclophosphamid leads to DNA cross-linking and ultimately to apoptosis of the cell.[17]

The initial experiments with MCF-7 and ZR-75-1 cell lines [Table 1] included supplementing the growth media with estrogen which promotes the production of pCD.[18] Results have indicated that the production of pCD might have influence on the resistance to the three tested substances even though we cannot exclude other pro-growth effects of estrogen which are not related to pCD production. On the other hand, the decrease of resistance caused by simultaneous addition of estrogen and antibodies specific for the propeptide of pCD support the hypothesis of influence of pCD on the chemoresistance. We used these data as a proof of concept and focused further on a well-established panel of stable transfected MDA-MB-231 cells.

From the second set of experiments [Table 2], it seems obvious that the pCD expression level correlates with the resistance to tested cytostatic. The control cells (empty vector) have not shown any changes in the resistance and at the same time excluded the possibility that the changes in resistance are caused by the antibiotic resistance or some other effects of the transformation. Another supporting fact indicating a positive influence of pCD overexpression on the resistance is based on results of

Table 7: Effect of Brefeldin on sensitivity to chemotherapeutic agents in MDA-MB-231 cells after transfection

Transfection	5-FU			Doxorubicin			Cyclophosphamide		
	PBS	Brefeldin A	Brefeldin A+231m	PBS	Brefeldin A	Brefeldin A+231m	PBS	Brefeldin A	Brefeldin A+231m
None	50.6±3.6	8.1±1.1	45.1±4.5	4.6±0.5	1.9±0.2	3.5±0.4	1 057.6±232.5	633.2±12.8	1 102.5±141.6
pCD	377.3±11.7*	22.9±6.6*	340.0±12.5*	7.3±0.9*	1.7±0.2	3.0±0.5	2 007.7±172.4*	692.0±28.9	1 478.8±129.9
Empty vector	45.7±5.1	11.6±2.8	34.6±3.3	2.8±0.3*	2.3±0.3	2.4±0.1*	1028.4±178.9	711.7±19.5	1 076.1±100.3
siRNA	6.0±0.9*	14.5±4.3*	12.4±1.2*	1.3±0.1*	2.2±0.2	1.8±0.2*	412.5±43.6*	620.5±23.6	1 029.4±97.1

Data are shown as LC_{50} (ng/5×10⁴ cells) and represent mean from three experiments±SD. Brefeldin A was used at the 2.5 µg/ml concentration, 231 m mutant at 10 ng/ml concentration. *Significant differences between control (PBS) and tested samples at $P \leq 0.05$ levels; pCD: Procathepsin D

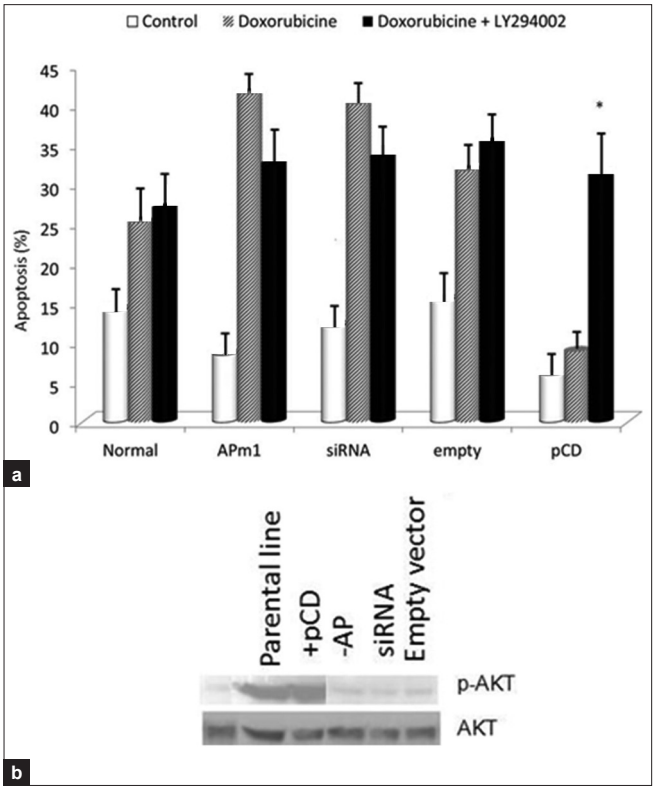

Figure 1: (a) The effect of doxorubicin on MDA-MB-231 cells by assessing the levels of phosphorylation of AKT at Ser473. Equal amount of cell lysates were on a western blot using the antibody against phosphor-AKT and AKT. Data represent mean from 3 experiments±SD. *Represents significant differences between doxorubicin and doxorubicin+LY292002 samples at $P \leq 0.05$ levels. (b) Inhibition of PI3-Akt blocks pCD-induced anti-apoptosis-induced by doxorubicin. Mean from 3 experiments±SD. The AP sample represents transfected cells with deleted activation peptide

silencing the pCD production in the parent cell line—the silencing dramatically increased the sensitivity to all three cytostatics. The overexpression of pCD mutated at the glycosylation site (199) had the same effects as overexpression of pCD, suggesting that gylcosylation does not influence the pCD-induced resistance (data not shown).

We can conclude that the over-expression of pCD correlates with increased resistance of used cell lines to the three cytostatics. Interesting results have been observed with expression of mutant APM1. This mutant (point mutations introduced the the propeptide region which aborts the mitogenic effects of pCD[19] has shown decreased resistance compared to both parental cell line and pCD overexpressing transfectant. This could suggest that the propeptide structure is involved in the resistance (data not shown).

In the experiments where different substances were added to the set of cell lines treated with cytostatics, we have focused mainly on the question of whether the effects of pCD are located extracellulary. First, the addition of pCD to the media considerably increased the chemoresistance of tested cell lines [Table 3]. Suplementing the media with mutant 231 m (aborted proteolytic function of pCD) had similar effect as the pCD. On the other hand, addition of APM1 had almost no effect on the resistance [Table 4]. Addition of pepstatin A caused no drop in the resistance of parent or transformed cell lines, suggesting that the extracellular proteolytic activity is not involved in the resistance [Table 5]. On the other hand, antibodies specifically recognizing the pro-part of pCD did inhibit the effect of pCD [Table 6] very successfully.

Based on this observation, we can conclude that the resistance seems to involve the extracellular functions of pCD. This can be documented by the activity of antibodies (which are acting extracellulary) and by the increased resistence when external pCD is added to the media of native cells or cells transfected with siRNA inhibiting the translation of pCD. The experiment testing the effects of Brefeldin also supports these conclusions.

Last, we showed that pCD expression in breast cancer cells is associated with phosphorylation of protein kinase B (Akt). Further experiments demonstrated that inhibition of PI3-Akt signaling by LY294002 restored the sensitivity of cells to doxorubicin, further supporting the possibility that PI3-Akt signaling has an important role in anti-apoptotic effects of pCD found originally in neuroblastoma cells.[10]

From the previous discussion we can conclude that over-expression of pCD has strong influence on increased resistance of tumor cells. This could, in fact, be an important contribution in the possible use of pCD level determination for prognostic and/or therapeutic purposes. Presented data suggested that the possible use of pCD determination might be prediction of resistence and help in selection of optimal chemotherapy. Another conclusion, based upon our experiments, is that the pro-resistance activity of pCD might be connected to its extracellular localization/secretion of pCD and that the mitogenic effect of pCD stimulates the cell growth and helps the cells to overcome the apoptotic effects of cytostatics probably via the PI3-Akt pathway.

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