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Award Number: DAMD17-99-1-9257

TITLE: Procathepsin D Stimulation of Human Breast Cancer Cell Growth

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REPORT DATE: July 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20020401 035

REPORT DOCUMENTATION PAGE	*Form Approved OMB No. 074-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Annual (15 Jun 00 - 14 Jun 01)

4. TITLE AND SUBTITLE
Procathepsin D Stimulation of Human Breast Cancer Cell Growth

5. FUNDING NUMBERS
DAMD17-99-1-9257

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Nine different clones transfected with human pCD cDNA and eight clones transfected with cDNA with deleted APpCD have been established and tested. Our results showed that the individual clones differ in secretion of pCD. These clones not only secrete pCD into the medium, but can also react to the estradiol stimulus by proliferation. Similar results have been obtained when the tumor growth has been evaluated *in vivo* and *in vitro.* Matrigel assay demonstrated that the transfection with pCD with deleted activation peptide did not influence the invasiveness of the clone. These data demonstrate that the proliferation and invasiveness of breast cancer cells closely correlates with production and secretion of pCD and the presence of the APpCD.

After establishing that the fragment 27-44 is the part responsible for the pCD binding to cells, we focused our attention on this particular fragment. We found that the biological activity can be located into the fragment AA 36-44. The cancer cells reacted to the addition of AA 36-44 fragments with the same level of proliferation as to the addition of pCD. When this fragment was used for inhibition of the pCD-FITC binding to the cancer cells, this fragment was comparable to the unlabeled pCD. We prepared a library of synthetic peptides with a single amino acid substitution based on the results mentioned above.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	39
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The long-term objective of this project is to develop a new treatment for breast cancer based on blockade of the autocrine growth factor activity of procathepsin D. Breast cancer cells secrete procathepsin D, the enzymatically inactive form from which the aspartic proteinase cathepsin D is generated by removal of an activation peptide (APpCD). Procathepsin D has been identified as an independent prognostic factor in several forms of cancer, particularly breast cancer. In preliminary experiments, procathepsin D was found to act as a specific autocrine growth factor for breast cancer-derived cells, but not for any other cell type tested. These effects were mediated through a specific receptor expressed on breast cancer cell lines that is distinct from the usually proposed cathepsin D-specific mannose-6-phosphate receptor. The region of procathepsin D responsible for its mitogenic activity was localized to APpCD and amino acids 27-44 of the APpCD sequence. No growth factor activity could be shown with the mature enzyme cathepsin D. The proposed specific aims are based on the central hypothesis that procathepsin D is involved in breast cancer via a specific receptor that mediates autocrine activation for increased metastatic growth. This project proposed the following aims: 1) it is hypothesized that the overproduction of procathepsin D results in an increase in the metastatic potential of breast tumor cells. A low metastatic human breast cancer cell line will be transfected with human procathepsin D cDNA such that the cells will secrete constitutively varying amounts of procathepsin D. The metastatic potential of each transfected cell line will be evaluated both *in vitro* and *in vivo* in relationship to the amount of procathepsin D secretion. 2) Attempts will be made to determine the exact site in procathepsin D responsible for breast cancer cell growth factor activity. Synthetic peptides representing fragments of APpCD will be prepared. Amino acid substitutions in the most active peptide fragment will be used to map the essential amino acid contact sites for the receptor. 3) It is hypothesized that inhibition of the APpCD interaction with its receptor will result in inhibition of cancer cell growth. Peptide analogs will be prepared with D-amino acids to block the growth and malignancy of cancer cells.

BODY

TASK 1

Cells of the MDA-MD-231 cell line were transfected with various human procathepsin D cDNA and tested for secretion of procathepsin D *in vivo*. In addition, the growth pattern *in vivo* has been established. Results showed in Figure 1 showed that the individual clones differ significantly in secretion of procathepsin D. Figure 2 shows that

these clones not only secrete pCD into the medium, but can also react to the estradiol stimulus by production of pCD and subsequent proliferation. These data also demonstrate that the difference in amounts of secreted pCD by these clones closely correspond to their proliferation. Similar results have been obtained when the tumor growth has been evaluated *in vivo* (see Figure 4).

Nine different clones transfected with human pCD cDNA and eight clones transfected with pCD cDNA with deleted APpCD have been established and tested. Matrigel assay demonstrated that the transfection with pCD with deleted activation peptide did not influence the invasiveness of the clone (Figure 4). At the same time, increased secretion of the full pCD significantly increased the invasiveness *in vitro* (Figure 4). Commercial cell invasion assay kit was used; the number of cells was evaluated according to manufacturer's instruction (Chemicon International). Figure 5 (see Appendices) shows the Western blot of the different clones with respect to the pCD secretion.

Based on these data, we believe that the *Task 1* has been successfully achieved.

TASK 2

All necessary synthetic peptides and fragments were synthesized. After establishing that the fragment 27-44 is the part of the activation peptide responsible for the procathepsin D binding to the cancer cells (see Vetvicka et al., Breast Cancer Research and Treatment, 57: 261-269, 1999; Figure 6), we focused our attention on this particular fragment. Subsequent studies of a second set of fragments showed that the biological activity can be located into the smaller fragment AA 36-44. The breast cancer cells reacted to the addition of AA 36-44 fragments with the same level of proliferation as to the addition of AA 27-44 fragment (Figure 7). When this fragment was used for inhibition of the pCD-FITC binding to the cancer cells, this fragment was comparable to the unlabeled pCD.

Subsequently we prepared all monoclonal antibodies against individual fragments and used them in testing of the inhibition of proliferative activity. Figure 8 shows results of such experiment in which the stimulation of breast cancer growth by the most active fragment AA 36-44 was inhibited by anti-AP, anti-AA 27-44 and anti-AA 36-44 antibodies, but not by anti-AA1-27 antibodies (1 ug/ml). Control antibody (anti-human CR3 mAb of the same isotype) showed no inhibition (data not shown).

In order to locate the exact binding site of the APpCD to the cancer cell receptor, we prepared a library of synthetic peptides with a single amino acid substitution based on the results mentioned above.

Peptides	Composition
1	SQAVPAVTE
2	XQAVPAVTE
3	SXAVPAVTE
4	SQXVPAVTE
5	SQAXPAVTE
6	SQAVXAVTE
7	SQAVPXVTE
8	SQAVPAXTE
9	SQAVPAVXE
10	SQAVPAVTX

Peptide #1 corresponds to the peptide 36-44 AA of the activation peptide. X represents a mixture of W, Y, L, P, D, H, R, N, M, and S.

In the first set of experiments, these peptides will be tested for inhibition of binding of procathepsin D-FITC to breast cancer cells using flow cytometry. The peptides which show binding to the receptor will be used in subsequent tests evaluating their growth factor activity. Peptides showing binding activity but not growth factor function will be subsequently tested as possible antagonists of procathepsin D as part of Task 3. All peptides showing significant binding to the cancer cells will be assessed for binding affinity using Scatchard plot analysis. At present, all these peptides have been purchased and the cell surface binding experiments are under way. In addition, monoclonal antibodies against these peptides are being prepared. As all these experiments are straightforward and all necessary peptides are available, we do not anticipate any problems.

Based on these data, we believe that the *Task* 2 will be achieved in next few months.

TASK 3

Anticipating the positive outcome of the experiments mentioned in Task 2, we evaluated the feasibility of the idea of breast cancer growth inhibition using anti-procathepsin D antibodies. First we established human tumors by injecting athymic mice with $5x10^6$ MDA-MB-231 cells directly into mammary fat pads. After two weeks, the mice were checked for tumor development. Mice with palpable breast tumors were then injected intravenously with biodegradable microspheres containing 100 ug of antibodies. Mice were sacrificed at various time intervals and tumor growth was evaluated by size. Mice treated with anti-procathepsin D or anti-fragment 36-44 AA antibodies showed greatly reduced tumor growth. A control antibody (anti-HLA) of the same isotype had no effect on tumor growth. Figure 9 shows results of these experiments. Five mice per group have been used in each interval.

These results strongly demonstrated the feasibility of the whole hypothesis. We expect that the final results from Task 2 will be finished in approximately two months. Based

on those results, we will prepare the analogs of the most active fragment using D-amino acid substitutions. Subsequently, the analogs will be evaluated using the same experimental design as in Figure 9. As all these experiments are straightforward and the technique was already successfully used, we do not anticipate any problems.

KEY RESEARCH ACCOMPLISHMENTS

- ♣ Demonstration that the binding site of the procathepsin D activation peptide is located in the 36 – 44 AA region
- ♣ Establishment of first cell lines transfected with human procathepsin D cDNA
- ♣ Establishment of basic values for invasiveness *in vitro* and *in vivo*
- ♣ Demonstration that invasiveness of breast cancer cells can be blocked by inhibition of procathepsin D secretion

REPORTABLE OUTCOMES

Abstracts:

1. **Vetvicka,V., Vetvickova, J., Fusek, M.:** Anti-procathepsin D activation peptide antibodies inhibit human breast cancer development, **VIIIth International Aspartic Proteinase Conference,** Funchal, Madeira, 1999, S-4-6
2. **Vetvicka,V., Vetvickova, J., Fusek, M., Voburka, Z.:** Blocking of growth factor activity of procathepsin D inhibits human cancer, Faseb J., Abstracts, p. A1000, 2000.
3. **Vetvicka, V., Vetvickova, J., Fusek, M., Voburka, Z.:** Inhibition of breast cancer by the procathepsin D activation peptide, EACR XVI –2000 Meeting, May 2000, Halkidiki, Greece, Abstract page 88.

5. **Vetvicka, V., Vetvickova, J., Voburka, Z., Fusek, M.:** Procathepsin activation peptide and its fragments in regulation of cancer growth, oral presentation, 13th International Symposium on Regulatory Peptides, Cairns, Australia, October 22-26, 2000.
6. **Vetvicka, V., Vetvickova, J., Fusek, M., Voburka, Z.:** Procathepsin D activation peptide and human cancer, AACR Meeting Molecular Biology and New Therapies in the 21st Century, Maui, February 12-16 2001.
7. **Vetvicka, V., Vetvickova J., Voburka, Z., Fusek, M.:** Procathepsin D stimulates the growth of human cancer – effect of cytokines. 92th Annual meeting of American Association of Cancer Research, New Orleans 2001, p. 878

PAPERS

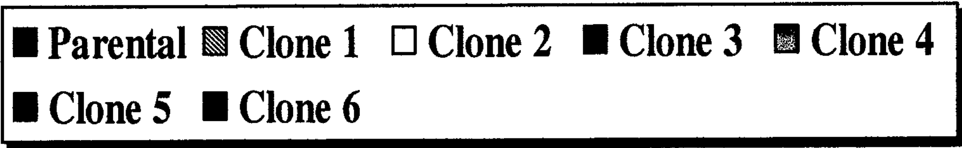
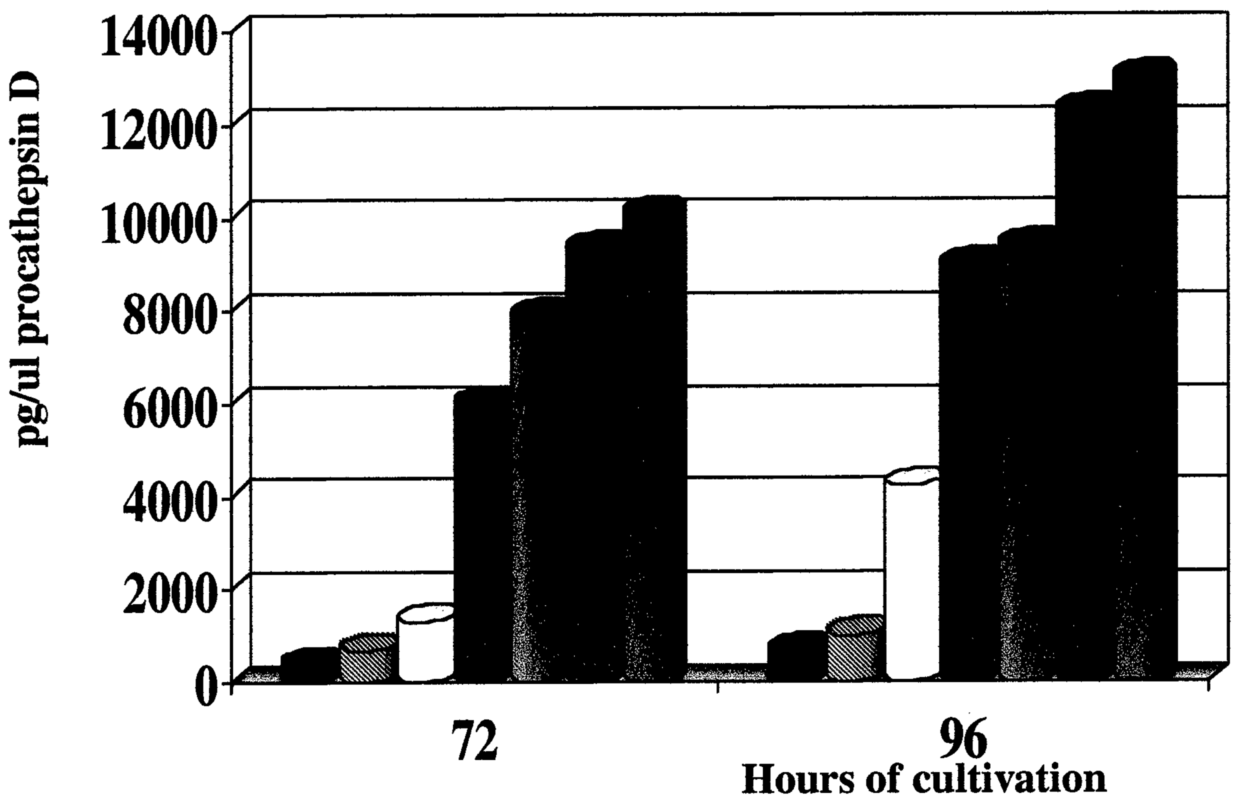
1. **Vetvicka, V., Vetvickova, J., Fusek, M.:** Role of procathepsin D activation peptide in prostate cancer development. Prostate, 44: 1-7, 2000

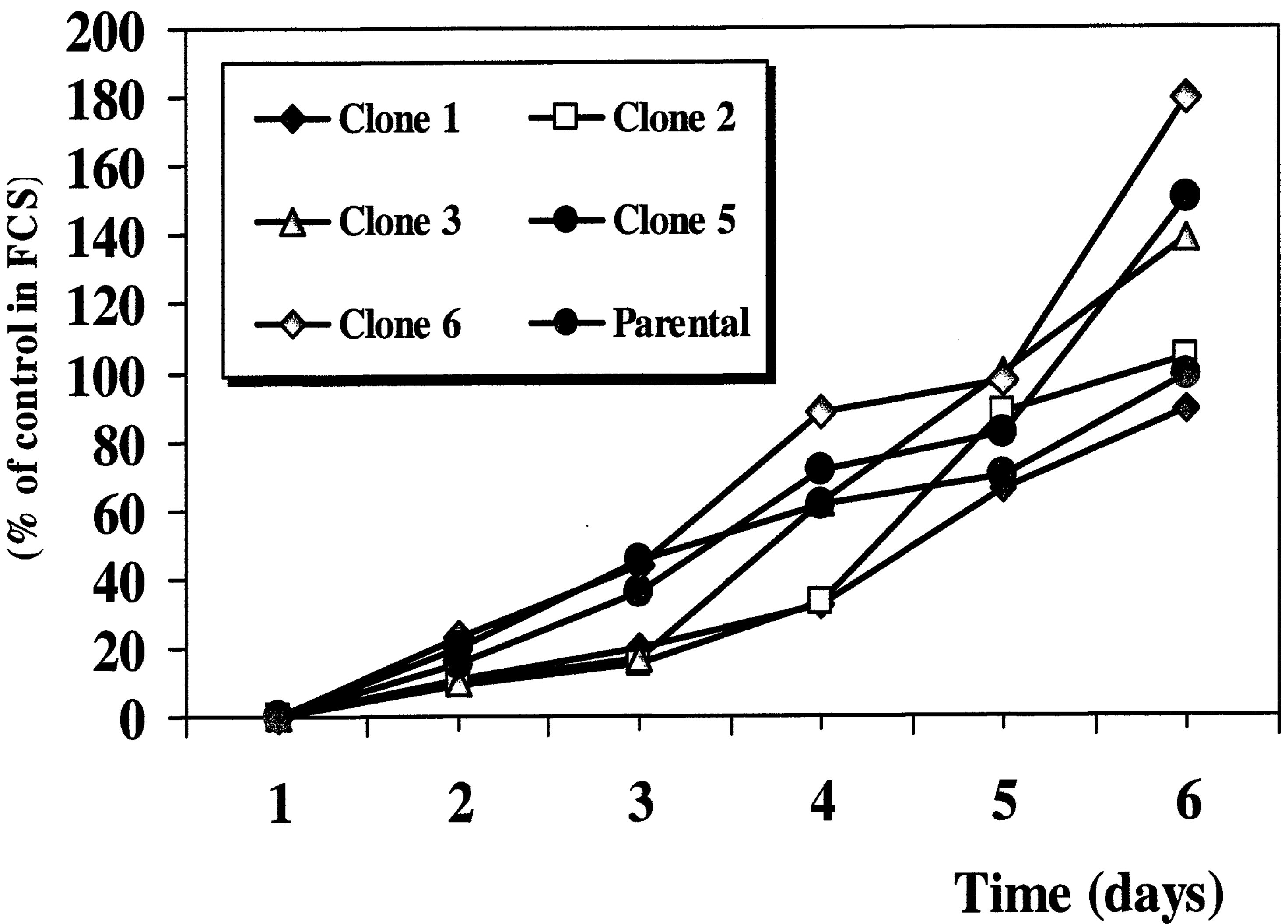
CONCLUSIONS

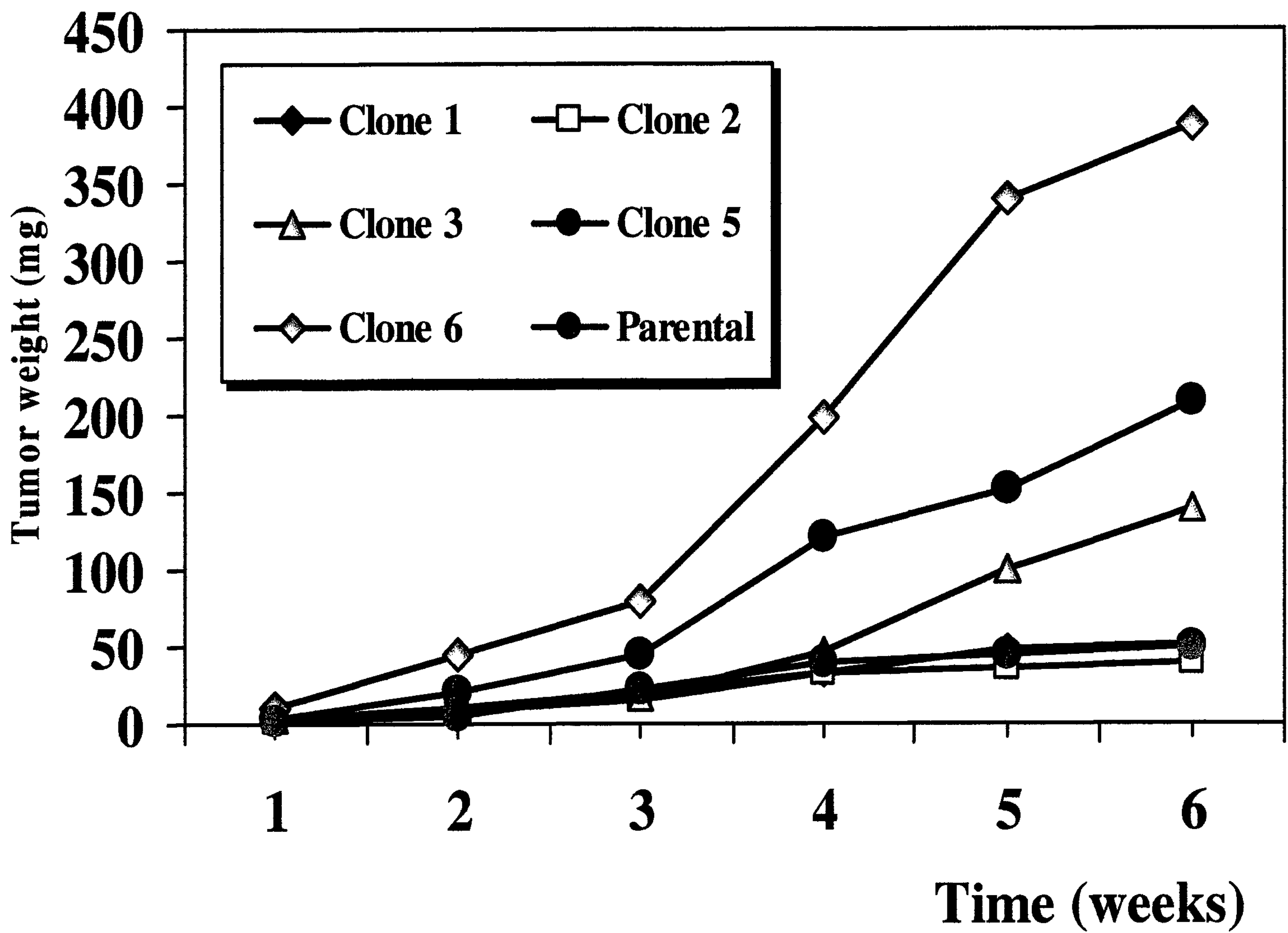
Based on results mentioned above, we have all reasons to believe that this project will be finished in successful and timely manner. Aims summarized in Task 1 have been successfully achieved. Only few experiments remain to be finish in Task 2, and we expect to finish these experiments during next few months. For aims of the Task 3, we already established both the feasibility of the project and the technique of the evaluation of the pCD inhibition *in vivo*. As all these experiments are straightforward and the technique was already successfully used, we do not anticipate any problems and we are convinced that the project will be finished in time.

To summarize, all obtained data support the original hypothesis that procathepsin D significantly stimulates the growth and spreading of breast cancer cells. If this hypothesis is further confirmed by this research project, this project has very significant potential to be developed into preclinical trials leading toward a new, very specific treatment of human breast cancer. In addition, recent observations by our and other groups suggest that the role of procathepsin D in human cancer development is probably even more general than was originally believed. In such a case, this project might be even more important and might provide treatment to several types of cancer.

Appendices



Parental
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
pg/ul procathepsin D
14000
12000
10000
8000
6000
4000
2000
0
72
96
Hours of cultivation


200
180
160
140
120
100
80
60
40
20
0
(% of control in FCS)
Clone 1
Clone 2
Clone 3
Clone 5
Clone 6
Parental
1
2
3
4
5
6
Time (days)


Tumor weight (mg)
450
400
350
300
250
200
150
100
50
0
Clone 1
Clone 2
Clone 3
Clone 5
Clone 6
Parental
1
2
3
4
5
6
Time (weeks)

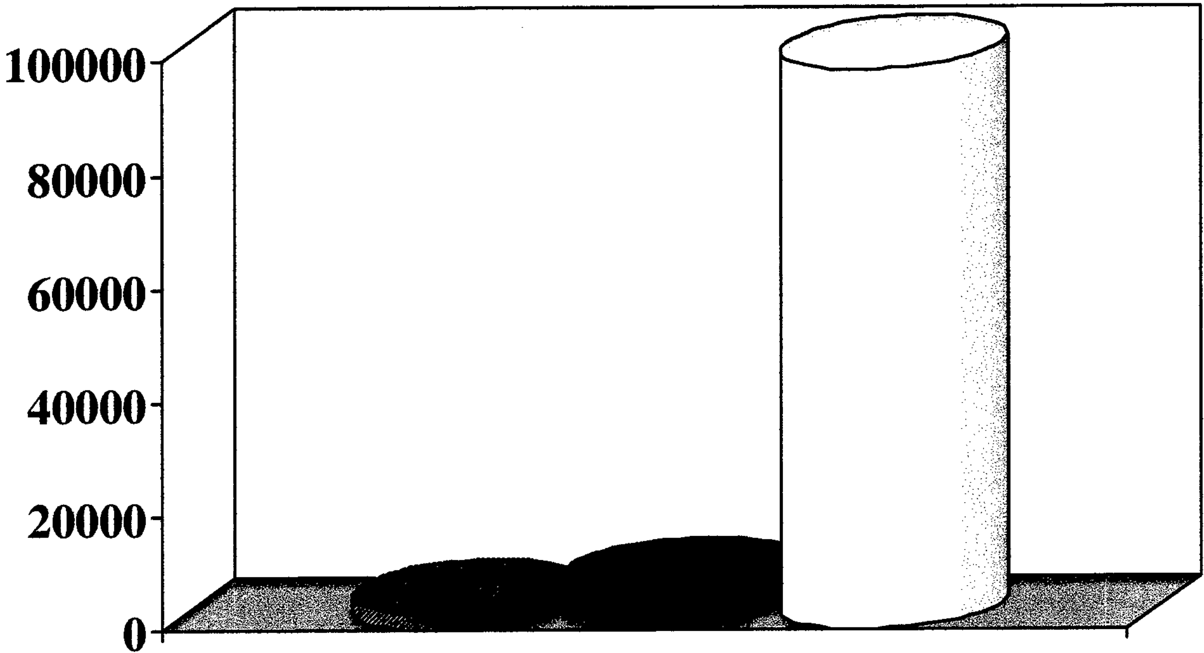
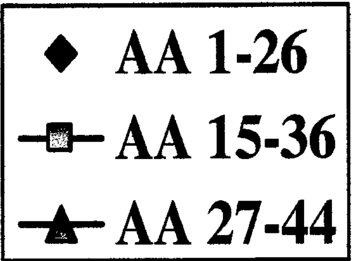
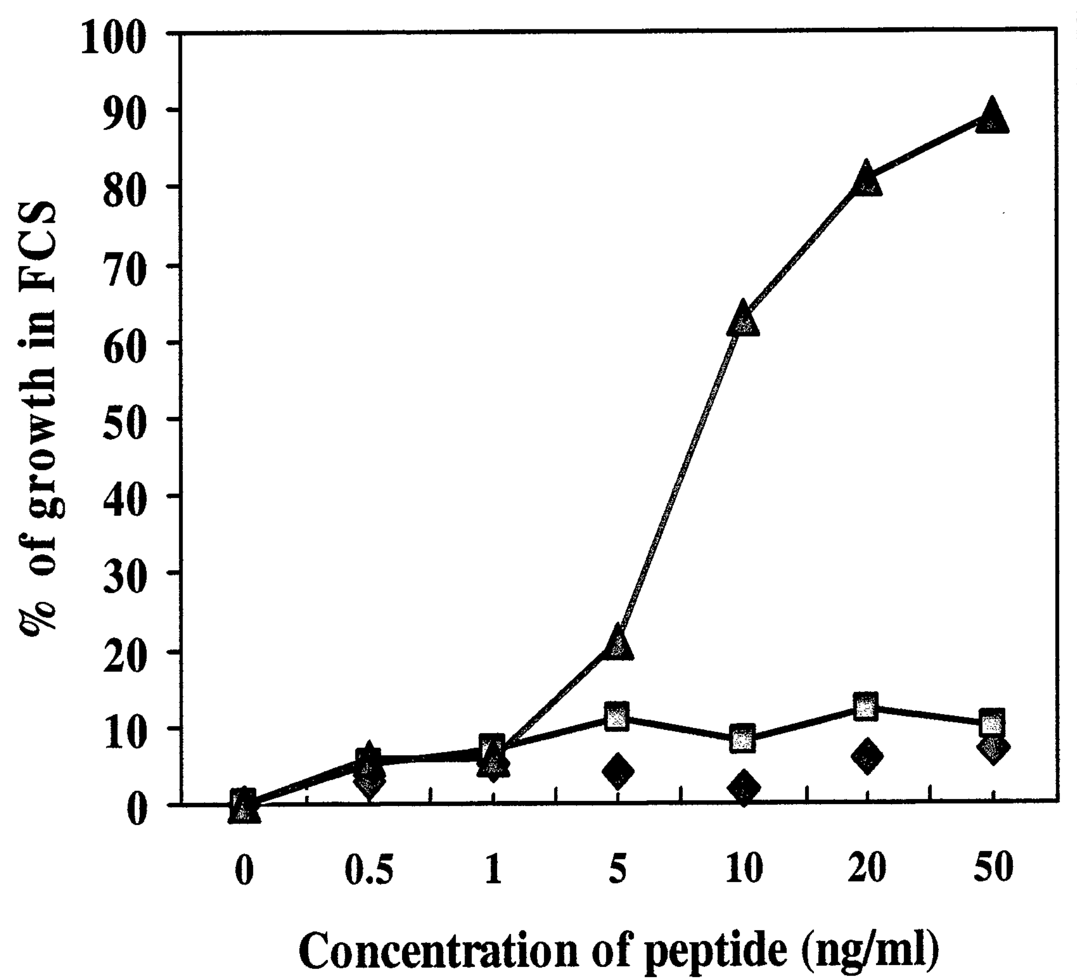
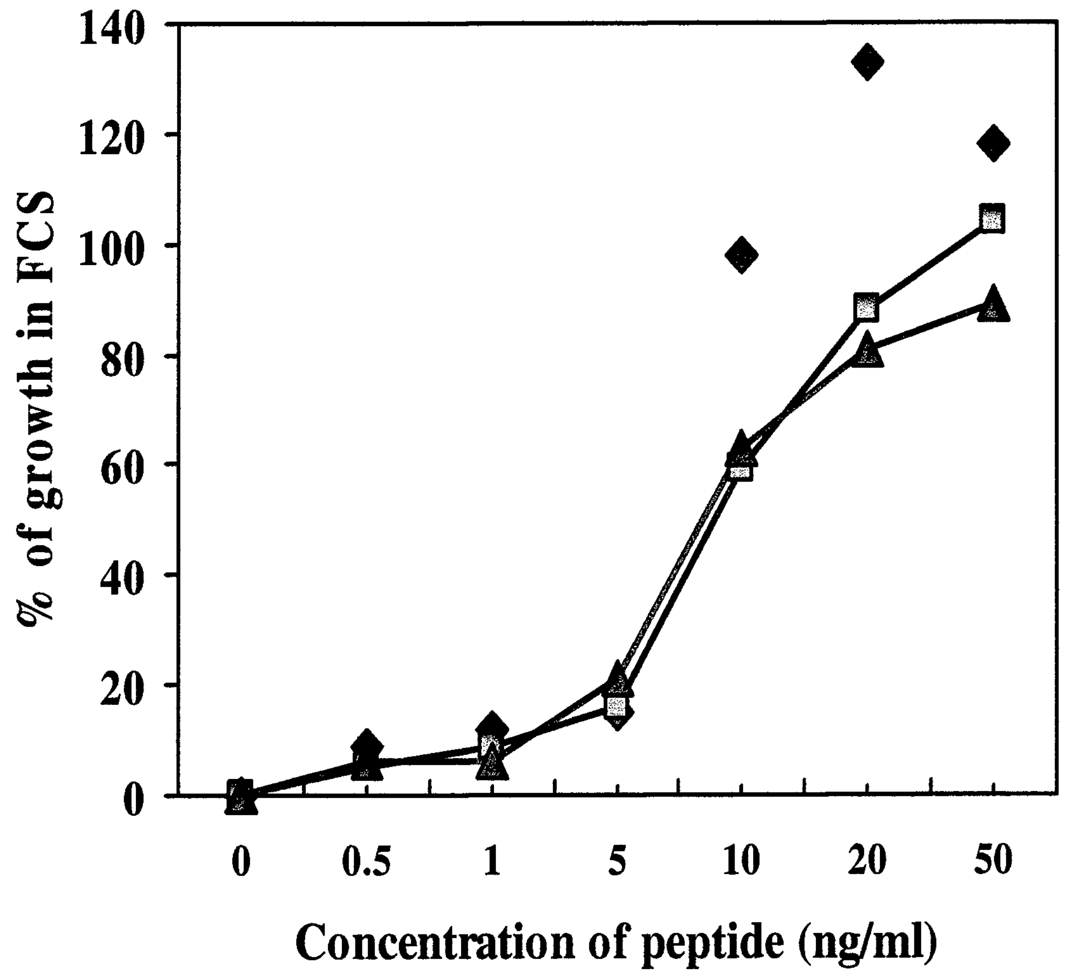
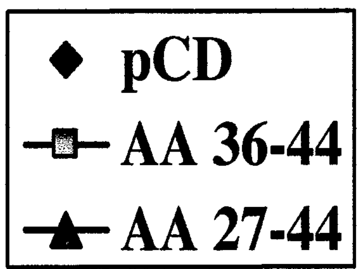
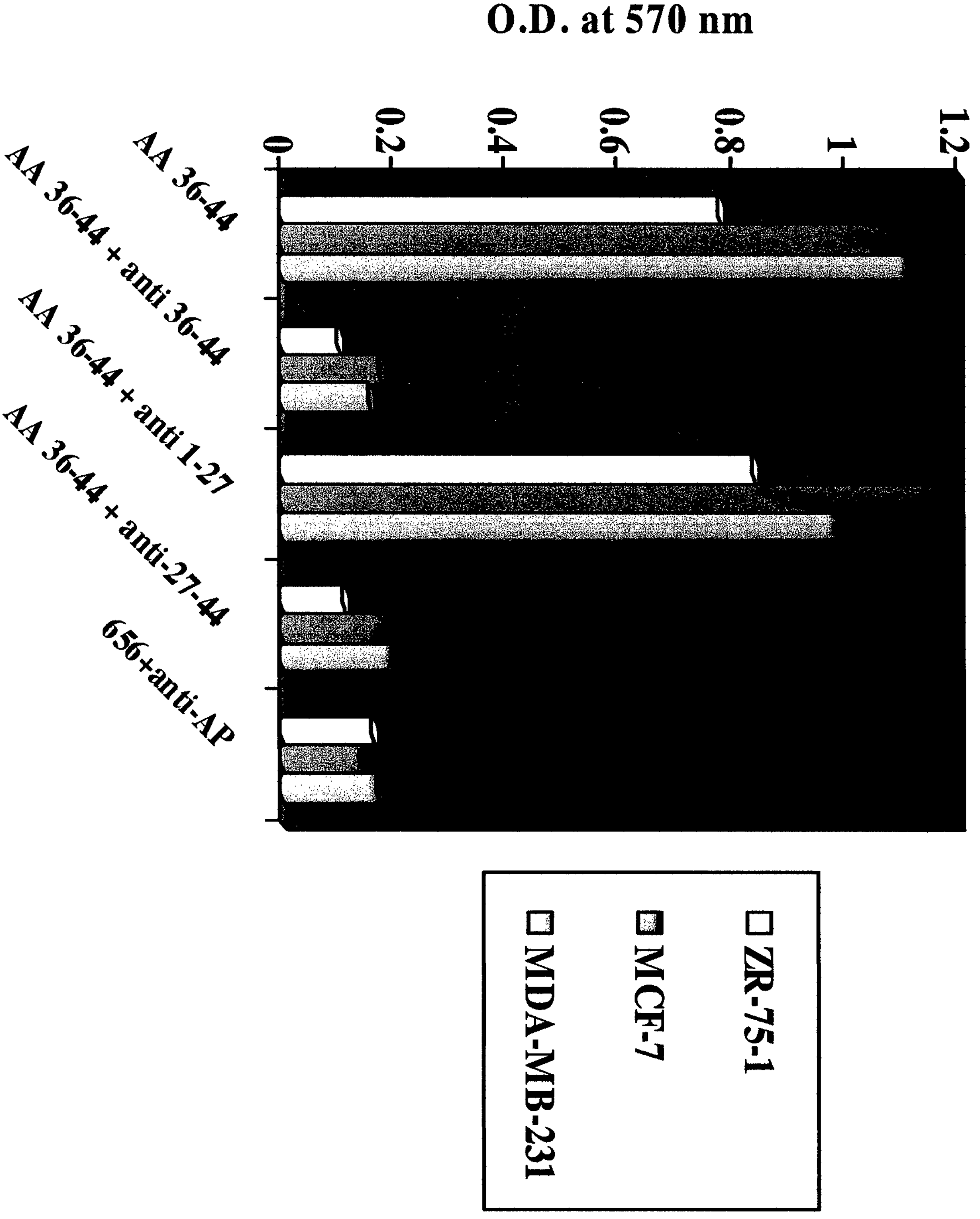

Parental
Clone -44
Clone AP
No. of cells
100000
80000
60000
40000
20000
0

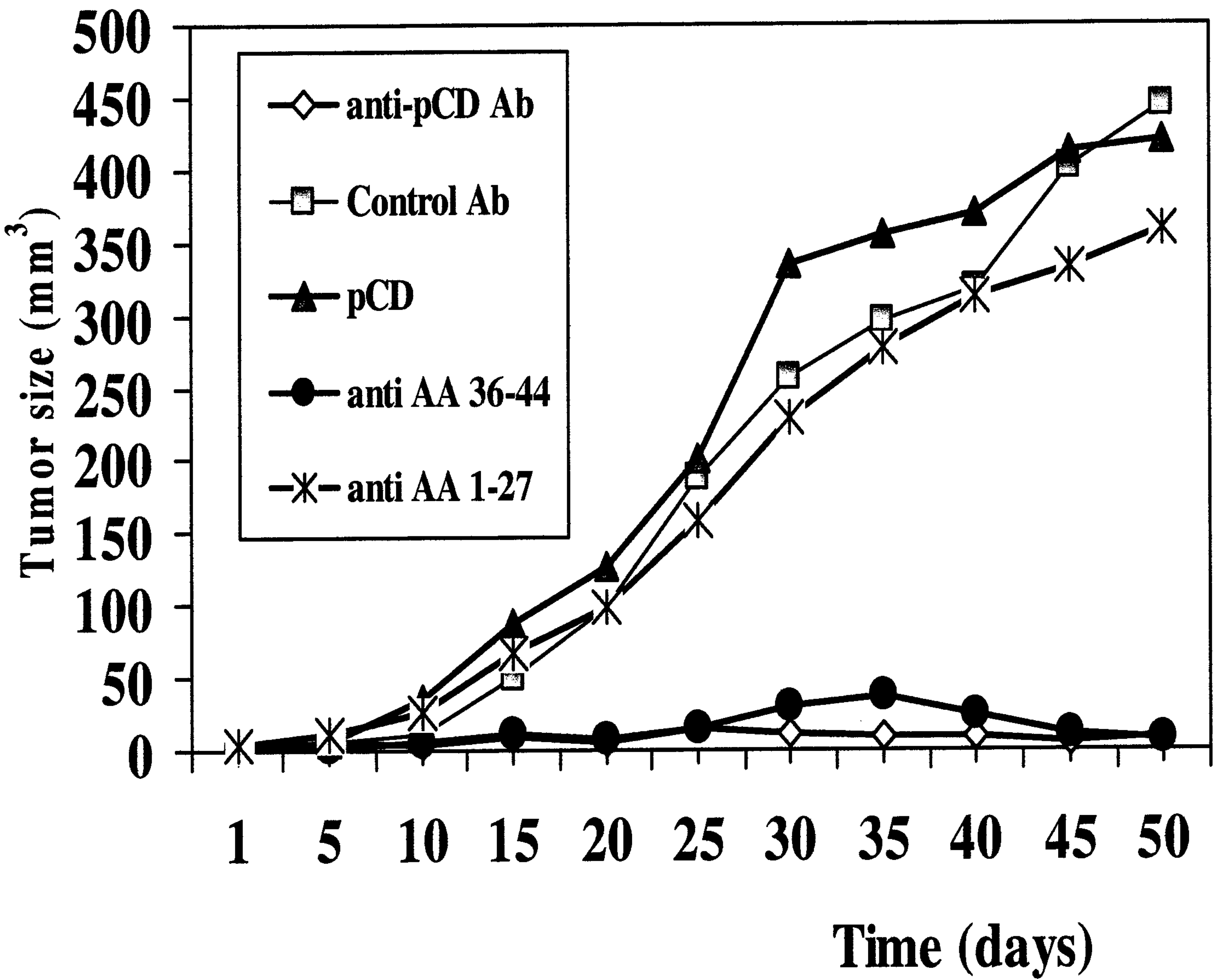
Figure 5


AA 1-26
AA 15-36
AA 27-44


% of growth in FCS
100
90
80
70
60
50
40
30
20
10
0
0
0.5
1
5
10
20
50
Concentration of peptide (ng/ml)


140
120
100
80
60
40
20
0
% of growth in FCS
0
0.5
1
5
10
20
50
Concentration of peptide (ng/ml)

pCD
AA 36-44
AA 27-44


O.D. at 570 nm
0
0.2
0.4
0.6
0.8
1
1.2
AA 36-44
AA 36-44 + anti 36-44
AA 36-44 + anti 1-27
AA 36-44 + anti-27-44
656+anti-AP
ZR-75-1
MCF-7
MDA-MB-231



Tumor size (mm^3)
500
450
400
350
300
250
200
150
100
50
0
anti-pCD Ab
Control Ab
pCD
anti AA 36-44
anti AA 1-27
1
5
10
15
20
25
30
35
40
45
50
Time (days)

Legend to the Figures

Figure 1

Comparison of the procathepsin D secretion in tissue culture supernatants of six cell clones transfected with cathepsin D cDNA. Total nine different clones have been established from parental cell line MDA-MB-231. Using an ELISA assay employing anti-procathepsin D antibodies, we compared the levels of procathepsin D secreted into tissue culture supernatants of transfected and parental cells.

Figure 2

Proliferation of individual clones in serum-free medium containing estradiol. Clones correspond to the clones used in Figure 1. Growth in presence of FCS represent 100%.

Figure 3

In vivo growth of individual clones (see Figure 1) in athymic nude mice. Human tumors were generated by injecting athymic mice with $5x10^6$ cells directly into mammary fat pads. Mice were sacrificed at various time intervals and tumor size was evaluated by weight.

Figure 4

In vitro invasion of transfected cells using a Matrigel membrane. Cells from serum-free cultures were tested for their ability to transmigrate a Matrigel membrane toward a 1% serum stimulus. In addition to a parental control (MDA-MB-231 cells), the transfected clones with the highest production of pCD (clone AP) and pCD with deleted APpCD (clone -44) were used. Commercial cell invasion assay kit was used, the number of cells was evaluated according to manufacturer's instruction (Chemicon International).

Figure 5

Western blot of tissue culture media from individual clones of MDA-MB-231 cell line transfected with pCD cDNA. 5x concentrated samples and 12% gel were used, commercial anti-pCD mAb was used for detection. Samples from left to right: parental cell line, Clone 1, Clone 2, Clone 4, Clone 5, Clone 6, and Standards.

Figure 6

The effect of various synthetic fragments of the activation peptide on proliferation of the breast cancer cell line MDA-MB-231 growth in serum-free conditions.

Figure 7

Subsequent evaluation of a second set of fragments showed that the biological activity can be traced into the fragment AA 36-44. The conditions were identical to those described in Figure 6. Procathepsin D was used as a positive control.

Figure 8

Stimulation of breast cancer growth by the most active fragment AA 36-44 and its inhibition by various anti-fragment monoclonal antibody (1 ug/ml).

Figure 9

Inhibition of breast cancer growth in athymic nude mice by treatment with anti-procathepsin D or anti-activation peptide fragment antibodies. A control antibody (anti-HLA) of the same isotype had no effect on tumor growth. Human tumors were generated by injecting athymic mice with $5x10^6$ MDA-MB-231 cells directly into mammary fat pads. After two weeks, the mice were checked for tumor development. Mice with palpable breast tumors were then injected intravenously with biodegradable microspheres containing 100 ug of antibodies. Mice were sacrificed at various time intervals and tumor growth was evaluated by size. Mice treated with anti-procathepsin D or anti-fragment 36-44 AA antibodies showed greatly reduced tumor growth.

AACR American Association for Cancer Research

92nd Annual Meeting

March 24-28, 2001 New Orleans, LA

Volume 42 March 2001

Proceedings

Differential Secretion of Cytokines and Several Proteases in Pgp-Overexpressing MDR and Sensitive MCF-7 Cells. Laurent Mougel, Richard Le Naour, Moncef Guenounou, and Jean Claude Jardillier. *Université de Reims, Reims, France.*

Multidrug resistance (MDR) is a major problem occurring during cancer treatment. One of the major reasons for MDR is the overexpression of *mdr1* gene encoding a P-170 membrane glycoprotein (Pgp) which acts as an efflux pump for the drugs. On an other hand, the potential of the cancer cells to be sensitive or not to the action of immunocompetent cells depends on their secretion of cytokines and/or the activation of cytokines by proteases. We present here the first experimental data on comparative quantitative analysis of secretion of several cytokines and of different proteases within MCF-7 breast cancer MDR and their corresponding sensitive cells cultured in 3D-spheroids. The levels of expression of the cytokines IL-1α and β, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, MCP-I, and MIP-Iα, potentially involved in the immunosentitivity of cells, have been quantified. We have found that the MDR cells express IL-1α, IL-6, MCP-I, MIP-Iα and IL-8 cytokins, whereas only MCP-I, MIP-Iα, and IL-8 cytokins were found to be detectable in the corresponding sensitive cells. The MDR cells exhibit much higher level of activity of the collagenase IV, cathepsin D and elastase, compared with the sensitive cells. We have further tested the effect of anti-Pgp specific antibodies (mAbs), able to block the Pgp-mediated drug efflux from the cancer cells, on the cytokines secretion profiles and proteases levels of activities. No any effects of mAbs have been detected in these experiments. In the aggregate, our results show different immunoreactive capacity of the Pgp-overexpressing MDR cells compared to the sensitive ones and the absence of correlation between the Pgp drug efflux activity and the MDR secretion profile of MCF-7 cancer cells.

#4710 Procathepsin D Stimulates the Growth of Human Cancer—Effect of Cytokines. Vaclav Vetvicka, Jana Vetvickova, Zdenek Voburka, and Martin Fusek. *Institute of Organic Chemistry and Biochemistry, Prague, Czech Republic, and University of Louisville, Louisville, KY.*

Oversecretion of enzymatically inactive 52 kDa procathepsin D (pCD) from human cancer cells is well documented and procathepsin D levels are considered to be a prognostic factor. Our previous findings demonstrated that procathepsin D serves as an autocrine mitogen. Furthermore we found that the mitogenic activity of procathepsin D is mediated by a receptor different from the currently suggested M6P-R as this reaction was not inhibited by anti-M6P-R or soluble M6P-R. The influence of procathepsin D on tumor cell growth was demonstrated both in vitro using breast and prostate cancer cell lines and in vivo on human breast and prostate tumors in athymic nude mice. Furthermore, these effects seem to be mediated by a structure within the activation peptide of procathepsin D. Based on our experiments, the growth factor activity of procathepsin D can be localized in position 27-44 (and possibly 36-44) of the activation peptide and involves interaction with a new cell surface receptor different from all known M6P receptors. Using monoclonal antibodies raised against individual fragments of the activation peptide we demonstrated strong inhibition of both estradiol- and activation peptide-derived stimulation of breast cancer cell proliferation. In addition, using an in vivo model of human breast cancer, we showed that injection of tumor-bearing mice with biodegradable microspheres containing anti-fragment or anti-activation peptide antibodies inhibited the growth of breast cancer. In addition, IL-4, IL-10 and IL-13 stimulated secretion of procathepsin D in dose dependent manner, addition of anti-procathepsin D antibodies inhibited the interleukin-stimulated cell proliferation. Based on presented experiments, we propose that the interaction of procathepsin D activation peptide with a new surface receptor is mediated by a sequence 36-44 plus close vicinity and leads in certain types of tumors to a potentiated growth and higher motility. The activation peptide is a new potential target for suppression of growth and spreading of several types of tumors including breast tumors. Blocking of the autocrine growth factor activity of activation peptide by peptide analogs might specifically inhibit cancer development. Supported in parts by the U.S. Army Breast Cancer Program grant, American Cancer Society grant and Phi Beta Psi Sorority grant.

#4711 Cytokine Combinations Enhance the Cytotoxic Ability of Human Umbilical Cord Blood Cells Against Breast Cancer Cells. Shantaram S. Joshi, Todd Lovgren, Peng Wang, Cody Evans, and Charles A. Kuszynski. *University of Nebraska Medical Center, Omaha, NE.*

We have previously demonstrated that Interleukin-2 (IL-2) activated mononuclear cells (MNC) from umbilical cord blood (UCBC) have significant ($p<0.001$) cytotoxicity against human leukemic and breast cancer cells in vitro and in vivo compared to MNC from blood stem cell apheresis harvest. In order to further improve the antitumor cytotoxic ability of UCBC, IL-2 was combined with GM-CSF, G-CSF or M-CSF in vitro for 72 hours. The cytotoxicity of these activated cells were then tested against MDA-231 human breast cancer cells. Immunophentoypes of activated cells were determined by flow cytometry and specific

compared to IL-2 or each cytokines alone. This increase be due to: 1) increases in CD56 positive cytotoxic cells; 2 accessory cell populations such as CD86+ dendritic ce helper cytokine INF-Γ gene expression; 4) increases in p Thus, our results suggest the combination of IL-2 w activate cord blood MNC to increase cytotoxicity again cells better than IL-2 alone. (This was supported by the Cancer Foundation).

#4712 The Use of a Chip-Based Protein Array for t Associated Cytokines. Pamela E. Bechtel, Leslie Hens yer. *Motorola BioChip System, Tempe, AZ.*

The development of malignancy is characterized by r alterations with in the cell. These alterations result in escape normal cell cycle checkpoints and confer a grow One of the biggest obstacles in the fight against canc malignant cells at an early stage and to determine whic potential for metastasis. Changes in the levels of pro-i genic cytokines have been associated with the ability of and metastasize. We are developing a novel protein ar quantify levels of cancer associated cytokines in cells different cytokines (VEGF, IL 13, TNFa, Leptin, Oncc Thrombopoiten (TPO), IL 2, IL 6 and IL 12) were dispe Linkä three dimensional coated slides. Sandwich-type i performed using purified antigens and biotintylated ant was detected by a streptavidin-alexaTM conjugate, scar 532 nm. The cytokines were first examined individually to of our assay. The results indicate that VEGF, IL 13, TGFE IL 12 can be detected at nanomolar concentrations anc and OSM can be detected at picomolar concentrations. examined for specificity. Antibodies against each cytol each array, and assays were performed using a mixture tylated antibodies. The results indicate TNFalpha, Leptir 6 and IL 12. There was a low that there is no cross rea level of cross reactivity observed with OSM. Our resul protein array technology has the capability to detect canc This technology has the potential to be incorporated i which will not only detect the onset of cancer, but will fa ization for patient prognosis. By providing rapid, low-volu bioarrays should become invaluable in disease detectic monitoring of patient treatment.

#4713 Beneficial Effect of Oral Treatment with P Patients with Myelofibrosis. Lucia Desser, Adriena Sa and Irene Herbacek. *Institute of Cancer Research, Univ Austria, Institute of Hematology - University of Bratislav public, and Institute of Hematology, University of Brat Republic.*

Myelofibrosis (MF) is characterised by an increased de matrix proteins (ECM) in the bone marrow stroma as a myeloid disorders. Overproduction of growth factors like factor and Transforming growth factor-β (TGF-β) are i induce the production of ECM in bone marrow (BM) str enhanced fibroblast proliferation as seen in BM fibrosis. T the organisms by endocytosis, when bound to activated Endogenous proteases, but also exogenous supplied pro Chymotrypsin, Bromelain and Papain are capable of act TGF-β1 irreversibly. Subsequently the α2M + protease be quickly removed via endocytosis. According to in vitr lytic enzymes have a TGF-β scavenging capacity, as fou cells and tumor associated macrophages, both at mR animal models with induced nephrosclerosis or glomeru with proteolytic enzymes reduces mRNA for TGF-β in tu TGF-β in urine. We investigated the effect of therapy w (daily 2x3 tablets) in patients with MF (primary MF n=3 This therapy improved clinical outcome (1/13 remission; progression) over a period up to 6 years. These results a histopathological examination of the bone marrow. In 7 TGF-β concentration in serum over a period of 1 to 2 ye significant ($p<0,001$) TGF-β elevation in patients with M In 6/7 patients a reduction of TGF-β concentration to cor Our results demonstrate the potential benefit of a the enzymes in the inhibition of fibrosis and immunosuppre

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Immunology 2000

Seattle, Washington

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ABSTRACTS

Abstracts 34.1-200.30

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The American Association of Immunologists and the Clinical Immunology Society Joint Annual Meeting

Official Publication of the Federation of American Societies for Experimental Biology
Volume 14, Number 6, April 20, 2000

53.1

BLOCKING OF GROWTH FACTOR ACTIVITY OF PROCATHEPSIN D INHIBITS HUMAN CANCER

[1]V.Vetvicka, [1]J.Vetvickova, [2]M.Fusek, [2]Z.Voburka. [1]University of Louisville, Dept. of Pathol., Louisville, KY 40292, and [2]Inst. Organic Chem. and Biochem., Prague, Czech Republic

Oversecretion of 52 kDa procathepsin D (pCD) from human cancer cells of various origin is well established. Our previous findings demonstrated that pCD serves as an autocrine mitogen for breast cancer cells via interaction with a new receptor. The influence of pCD on tumor cell growth was demonstrated both *in vitro* using breast and prostate cancer cell lines and *in vivo* on human breast and prostate tumors in mice. Furthermore, these effects seem to be mediated by a structure within the activation peptide (AP) of pCD. Based on our experiments, the growth factor activity of pCD can be localized in position 36-44 of the AP. Using mAbs raised against individual fragments of the AP we demonstrated strong inhibition of an AP-derived stimulation of cancer cell proliferation. In addition, using an *in vivo* model of human breast and prostate cancer, we showed that injection of tumor-bearing mice with biodegradable microspheres containing anti-fragment or anti-AP antibodies inhibited the growth of cancer. Using a library of synthetic peptides, we analyzed the interaction of AP with its receptor. Based on presented experiments, we propose that the interaction of pCD activation peptide with an unknown surface receptor is mediated by a sequence 36-44 plus close vicinity and leads in certain types of tumors to a potentiated growth and higher motility. The AP is a new potential target for suppression of growth of several types of tumors including breast and prostate tumors. This research was supported by a grant from the U.S. Army Breast Cancer Program.

53.2

A Natural Killer Cell-dependent Antitumor Re Anti-idiotype-interleukin 2 Fusion Protein

1P.Y. Wu, 1, 2S.J. Liu, 1C.N. Lee, and 1Mi-Hua Tao. Sciences, Academia Sinica, Taipei 115, Taiwan 2Gradu Sciences, National Defense Medical Center, Taipei 106.

Combination of IL-2 with an anti-Id Ab was shown to tic effect to treat B-cell lymphoma. To further improv therapy, a monoclonal anti-Id Ab, S5A8, specifically r lymphoma 38C13, was genetically modified to contain utilize the unique targeting ability of Abs to direct IL-2 Id-IL-2 fusion protein contains mouse-human chimeric attached to the carboxy-terminal end of each heavy anti-Id-IL-2 fusion protein retained the IL-2 biological tentiate tumor cell lysis in vitro. In vivo studies showe protein was very proficient in inhibiting 38C13 tumor g a combined therapy with anti-Id Ab and IL-2. The effe the treatment effect of anti-Id-IL-2 were delineated by u vivo-depleted immunocompetent mice. We found that tein was completely effective in the T- and B-cell-deficie NK-cell-deficient C3H/beige mice. In vivo-depleted ex depletion of NK cells but not T cells abrogated the ther IL-2 fusion protein. These results demonstrate that th represents a potent reagent for treatment of B-cell ly activity is largely dependent on NK cells.

53.3

CHARACTERIZATION OF CELL LINES DERIVED FROM A MURINE T CELL LEUKEMIA

S. E. Hajos, G. Ernst, E. Caldas Lopes, P. Aulicino, M. Scolnik and E. Alvarez. Facultad de Farmacia y Bioquimica. IDEHU, UBA-CONICET. Buenos Aires, ARGENTINA.

Various cell lines from a murine T cell leukemia were established. LBL was obtained by culture in RPMI-C medium. After 80 in vitro passages two sublines originated, one growing as packed clusters of floating cells (LBLc), the other comprised of adherent monolayers (LBLa). LBL-V and LBL-D are cell lines resistant to Vincristine (VCR) or Doxorubicine (DOX) selected in increasing concentrations (5-160 ng/ml) of the drugs. LBR- is the control cell line obtained in the absence of drugs, similar to LBL. Phenotypic characterization carried out by flow cytometry showed that all cell lines maintained the pattern of parental leukemic cells although some of them down-regulated expression of adhesion molecules. Cell lines expressed CD24+, CD25+, CD8+, $CD4^{lo}$ and $CD44^{hi}$ $CD18^{hi}$ (LBL, LBLa, LBR-), $CD44^{hi}$ $CD18^{lo}$ (LBR-V), $CD44^{lo}CD18^{lo}$ (LBLc,LBR-D). CsA (1ug/ml) induced apoptosis (evaluated by DNA fragmentation and cell staining) in all cell lines while FK506 (10ug/ml) failed. CsA and PSC833 produced reversal of drug resistance in LBR-V and LBR-D lines. Both these agents as well as Verapamil blocked Daunorubicin efflux from resistant cells and improved cell kill as they were combined with VCR or DOX. We conclude that down regulation of CD44 and CD18 could be involved in the delay of tumor growth and invasive capacity previously demonstrated for LBLc, LBR-V and LBR-D cell lines. Besides, CsA and PSC833 could be used to improve therapeutic approaches in this experimental model. Financial Support: UBA, CONICET, Roemmers Foundation. Drugs were provided by Gador and Novartis.

53.4

Induction of Immunity against Tumor Antigen of MUC1 cDNA

Keiichi Kontani, Satoru Sawai, Minako Fujita, Jun Ha Shozo Fujino. Second Department of Surgery, Shiga U Otsu, Japan

DNA vaccines have been reported to be beneficial for tumor antigen expression and for eliciting a strong anti this study, we attempted to induce anti-MUC1 immun DNA encoding MUC1 mucin. This glycoprotein is high cancer cells and can be recognized by cytotoxic T lymp murines. BALB/c mice were vaccinated with expressi cDNA. In 8 of the 11 mice vaccinated with MUC1 cDNA to MUC1 core peptides by ELISA. In contrast, all of t control vector did not acquire anti-MUC1 humoral i is useful for the development of a DNA vaccine targ immunotherapy.

PROCEEDINGS

Molecular Biology and New Therapeutic Strategies: Cancer Research in the 21st Century

5th Joint Conference of the American Association for Cancer Research
and the Japanese Cancer Association

February 12-16, 2001
Maui Marriott Resort
Maui, Hawaii

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Tokyo, Japan

PROCATHEPSIN D ACTIVATION PEPTIDE AND HUMAN CANCER

Vaclav Vetvicka[1], Jana Vetvickova[1], Martin Fusek[2], Zdenek Voburka[2]
[1]University of Louisville, Department of Pathology, Louisville, KY 40292 and [2]Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague, Czech Republic

Oversecretion of procathepsin D from human cancer cells is well established. Our previous findings demonstrated that procathepsin D serves as an autocrine mitogen for breast and prostate cancer cells. Furthermore, we showed that the mitogenic activity of procathepsin D is mediated by a novel receptor different from the currently suggested mannose-6-phosphate receptor (M6P-R). The effects of procathepsin D on breast and prostate tumor cell growth was demonstrated both *in vitro* using cell cultures and *in vivo* using athymic nude mice. Additional experiments showed that the activation peptide is the molecule responsible for growth factor activity of procathepsin D. In next set of experiments we used monoclonal antibodies raised against individual fragments of activation peptide and tried to localize the amino acid sequence of the activation peptide responsible for its biological activities. Based on our experiments, the growth factor activity of procathepsin D can be localized in position 36-44 of the activation peptide and involves interaction with a new cell surface receptor different from all known M6P receptors. Using a library of synthetic peptides, we analyzed the interaction of activation peptide with its receptor. Based on these results, a precise localization of the binding site is proposed. In additional set of experiments we studied the question of the influence of several cytokines on growth of breast cancer cells. IL-4, IL-10 and IL-13 are known to stimulate secretion of procathepsin D in various cell types. All three cytokines stimulated breast cancer growth in a dose-dependent manner. Using specific anti-cytokine receptor antibodies or anti-activation factor antibodies, we demonstrated strong inhibition of an cytokine-derived stimulation of cancer cell proliferation. The activation peptide is a new highly promising target for suppression of growth and spreading of several types of tumors including breast tumors.

Supported by the U.S. Army Breast Cancer Program Grant and by the American Cancer Society Grant.

EUROPEAN ASSOCIATION
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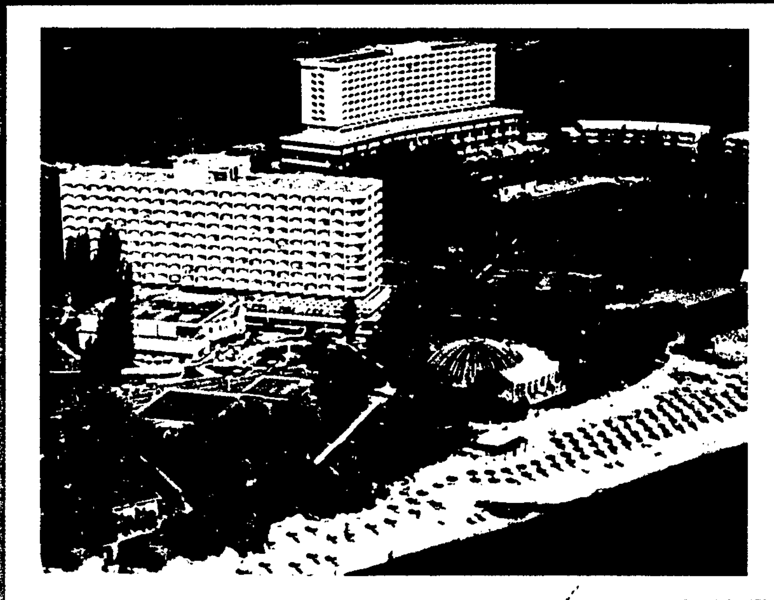
PROGRAM & PROCEEDINGS BOOK



30 May – 3 June 2000

Athos – Pallini Hotel (Kallithea)
Halkidiki – Hellas

The meeting has been supported also by:

General Secretariat for Research and Technology
(EPET II and XVI General Directorate of European Union)
and
Prefecture of Halkidiki

WS26

INHIBITION OF BREAST CANCER BY THE PROCATHEPSIN D ACTIVATION PEPTIDE

V. Vetvicka[1], J. Vetvickova[1], M. Fusek[2], Z. Voburka[2]
[1]University of Louisville, Department of Pathology, Louisville, KY 40292, USA and [2]Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague, Czech Republic

Our previous findings demonstrated that of 52 kDa procathepsin D (pCD) serves as an autocrine mitogen for breast cancer cells. Furthermore we found that the mitogenic activity of pCD is mediated by a receptor different from the currently suggested M6P-R. The influence of pCD on tumor cell growth was demonstrated *in vitro* using breast and prostate cancer cell lines. Furthermore, these effects seem to be mediated by a structure within the activation peptide of pCD. Based on our experiments, the growth factor activity of pCD can be localized in position 27-44 (and possibly 36-44) of the activation peptide. Using monoclonal antibodies raised against individual fragments of the activation peptide we demonstrated strong inhibition of an activation peptide-derived stimulation of cancer cell proliferation. In addition, using an *in vivo* model of human breast and prostate cancer, we showed that injection of tumor-bearing mice with biodegradable microspheres containing anti-fragment or anti-activation peptide antibodies inhibited the growth of cancer. The antibody-mediated protection from an induced cancer was almost complete for the whole tested interval. Using a library of synthetic peptides, we analyzed the interaction of activation peptide with its receptor. Based on presented experiments, we propose that the interaction of pCD activation peptide with an unknown surface receptor is mediated by a sequence 36-44 plus close vicinity and leads in certain types of tumors to a potentiated growth and higher motility. The activation peptide is a new potential target for suppression of growth of tumors.
Supported by the U.S. Army Breast Cancer Program grant.

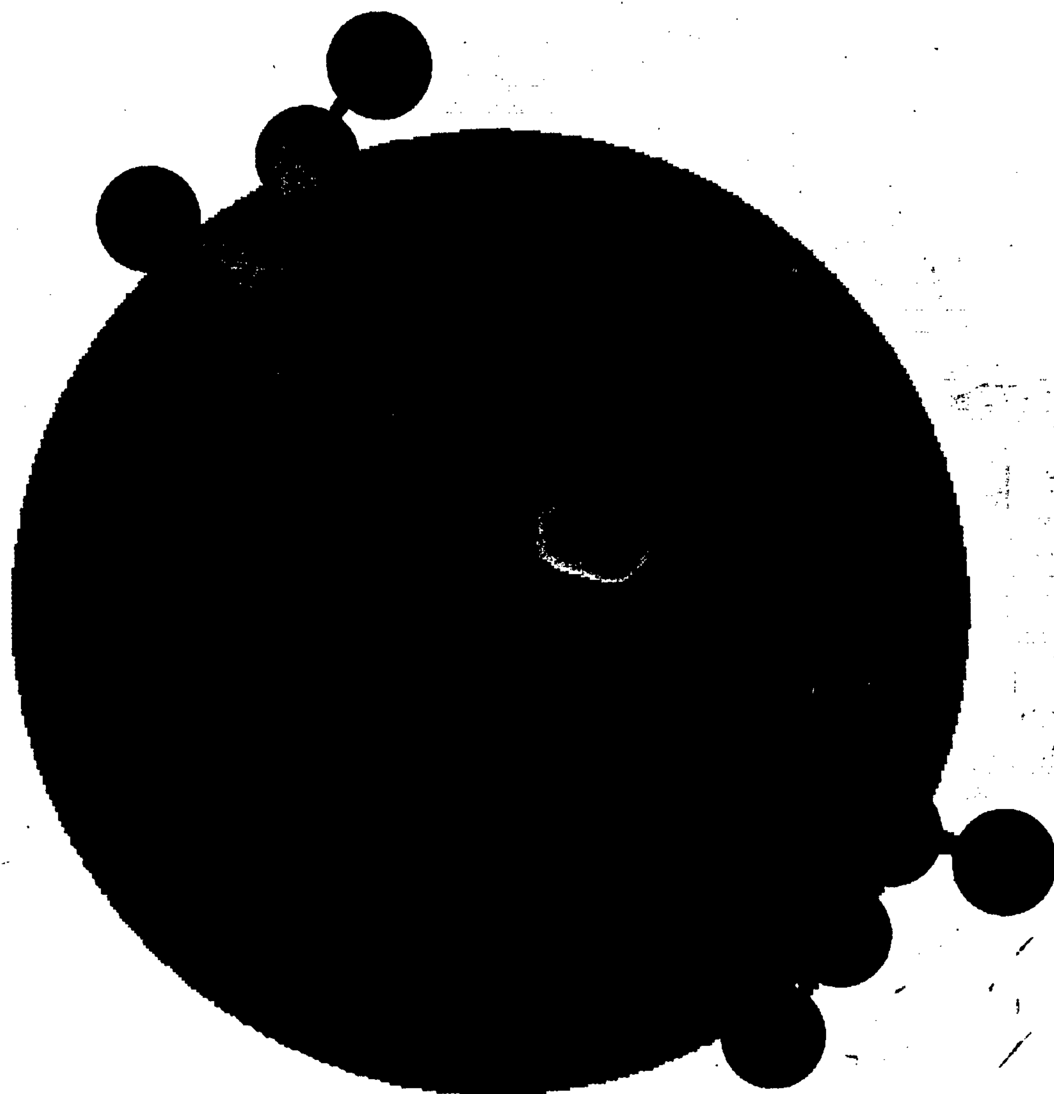
WS27

PREVENTION OF SPONTANEOUS MAMMARY CARCINOMA BY VACCINATION WITH MYCOBACTERIUM TUBERCULOSIS Ag38 GENE-TRANSDUCED TUMOR CELLS AND IL-12

L. Sfondrini, M. Rodolfo, M. Singh, M. I. Colnaghi, S. Menard and A. Balsari
Molecular Targeting Unit [L.S., M.I.C., S.M.], Melanoma Genetic Unit [M.R.], Department of Experimental Oncology, Istituto Nazionale Tumori, Milan, Italy; GBF-National Research Center for Biotechnology, Braunschweig, Germany [M.S.]; Institute of Pathology, University of Milan, Italy [A.B.];

An approach to stimulating an immune response against tumors is to transduce tumor cells with bacterial genes, which represent a "danger signal" and can induce a wide immune response. Mycobacterium tuberculosis genes and their encoded proteins play a pivotal role in linking innate and cell-mediated adaptive immunity and represent ideal candidates as immune adjuvants for tumor vaccines. The efficacy of a cancer vaccine, obtained by transduction of a mammary tumor cell line with the M. tuberculosis Ag38 gene, was investigated in female mice transgenically expressing the rat HER-2/neu protooncogene. These mice spontaneously develop stochastic mammary tumors after a long latency period. The onset of spontaneous mammary tumors was significantly delayed in mice vaccinated with Ag38-transduced cells, but not in mice vaccinated with non-transduced cells as compared with untreated mice. Protection from spontaneous tumor development was increased when mice were vaccinated with the mycobacterium-gene-transduced vaccine plus a systemic administration of IL-12 at a low dose. Mice vaccinated with non-transduced cells plus IL-12 developed tumors, with only a slight delay in tumor appearance as compared with the control group. Lymphocytes obtained from lymph nodes of mice vaccinated with transduced cells secreted high levels of IFN-γ. $CD3^+$ $CD8^+$ spleen cells derived from these mice responded to the tumor with IFN-γ production.
These data indicate the efficacy of a short-term protocol of vaccinations exploiting the adjuvant potency of a M.tuberculosis gene and low doses of IL-12 in a model of stochastic development of mammary tumors. This adjuvant approach may represent a promising immunotherapeutic strategy for cancer immunization.

VIII th INTERNATIONAL ASPARTIC PROTEINASE CONFERENCE



Sep 7-12, 1999

Funchal, Madeira, Portugal

ANTI-PROCATHEPSIN D ACTIVATION PEPTIDE ANTIBODIES INHIBIT HUMAN BREAST CANCER DEVELOPMENT

Vaclav Vetvicka[1], Jana Vetvickova[1], Martin Fusek[2]

[1] University of Louisville, Department of Pathology, Louisville, KY 40292, USA
[2] Institute of Organic Chemistry and Biochemistry, Academy of Sciences, Prague, Czech Republic

Oversecretion of enzymatically inactive 52 kDa procathepsin D (pCD) from human breast cancer cells is well documented and procathepsin D levels are considered to be a prognostic factor. Our previous findings demonstrated that procathepsin D serves as an autocrine mitogen. Furthermore we found that the mitogenic activity of procathepsin D is mediated by a receptor different from the currently suggested M6P-R. Using flow cytometry analysis we found that no previously known surface antigens or soluble M6P-R or anti-M6P-R antibodies were able to inhibit the specific binding of pCD-FITC. Similarly, none of these substances inhibited growth factor activity of pCD. The influence of procathepsin D on tumor cell growth was demonstrated both *in vitro* using breast cancer cell lines and *in vivo* on human breast tumors in athymic nude mice. Furthermore, these effects seem to be mediated by a structure within the activation peptide of procathepsin D. Based on our experiments, the growth factor activity of procathepsin D can be localized in position 27-44 (and possibly 36-44) of the activation peptide and involves interaction with a new cell surface receptor different from all known M6P receptors. Using monoclonal antibodies raised against individual fragments of the activation peptide we demonstrated strong inhibition of both estradiol- and activation peptide-derived stimulation of breast cancer cell proliferation. In addition, using an *in vivo* model of human breast cancer, we showed that injection of tumor-bearing mice with biodegradable microspheres containing anti-fragment or anti-activation peptide antibodies inhibited the growth of breast cancer. The antibody-mediated protection from an induced breast cancer was almost complete for the whole tested interval of 8 weeks.

Based on presented experiments, we propose that the interaction of procathepsin D activation peptide with an unknown surface receptor is mediated by a sequence 36-44 plus close vicinity and leads in certain types of tumors to a potentiated growth and higher motility. The activation peptide is a new potential target for suppression of growth and spreading of several types of tumors including breast tumors. Blocking of the autocrine growth factor activity of activation peptide by peptide analogs might specifically inhibit breast cancer development

REGPEP 2000

13th International Symposium on Regulatory Peptides

CAIRNS, AUSTRALIA

OCTOBER 22-26, 2000

FINAL PROGRAM

THURSDAY 26 OCTOBER

8:30am – 10:00am Plenary Symposium Hall A

Chairs: Jens Holst and Margaret Morris

8:30 Neuroendocrine Control of Obesity
Roger Cone

9:00 CART peptides in feeding, reward and reinforcement
Mike Kuhar

9:30 Role and significance of orexin in feeding
Hiroshi Yamashita

10:00am – 10:30am Morning Tea

10:30am – 12:30pm Concurrent Sessions

Oral 11 Cancer and Clinical Hall A

Chairs: Juanita Merchant and John Hickman

10:30 Expression of peptide receptors by tumours: implications for diagnosis and therapy
Jean Claude Reubi

11:05 #0026 Procathepsin D activation peptide and its fragments in regulation of cancer growth
Vetvicka, Vaclav*; Vetvickova, Jana; Voburka, Zdenek; Fusek, Martin*

11:20 #0049 Tumor targeting with DOTA-somatostatin analogs: superiority of Ga-labeled peptides
Froidevaux, Sylvie*; Christe, Martine; Sumanovski, Lazar; Heppeler, Axel; Maecke, Helmuth R; Beglinger, Christoph; Eberle, Alex N*

11:35 #0093 The somatostatin analogue PTR-3173, a backbone cyclic peptide with effect on growth hormone but not insulin secretion
Janson, Eva T*; Afargan, Michel; Gelerman, Garry; Rosenfeld, Rakefet ; Ziv, Offer; Karpov, Olga; Bracha, Moshe; Öberg, Kjell*

11:50 #0072 Processing-independent quantitation of human chromogranin A in plasma: a promising tool for early diagnosis of carcinoid tumors, other neuroendocrine tumors and SCLC
Børglum Jensen, Tine D*; Hilsted, Linda; Rehfeld, Jens*

12:05 #0116 KGF induction in vitamin D treated cancer cells
Lyakhovich, Alex*; Arksenov, Nickolai; Pennanen, Pasi; Manninen, Tommi; Syvälä, Heimo; Ahonen, Merja H; Ylikomi, Timo; Tuohimaa, Pentti*

12:20 General discussion

The Prostate 44:1–7 (2000)

Role of Procathepsin D Activation Peptide in Prostate Cancer Growth

Vaclav Vetvicka,[1]* Jana Vetvickova,[1] and Martin Fusek[2]

[1]Department of Pathology, School of Medicine, University of Louisville, Louisville, Kentucky
[2]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic

BACKGROUND. Enzymatically inactive procathepsin D secreted from cancer cells has been confirmed to play a role in breast cancer development. We focused on prostate cancer and the role of activation peptide in mitogenic activity.
METHODS. Synthetic peptides and monoclonal antibodies raised against individual fragments of activation peptide were employed. Cell proliferation was measured by MTT (3-[4,5-dimethylthiatol-2-yl]-2,5-diphenyl tetrazolium bromide) assay or by in vivo growth in nude mice.
RESULTS. We demonstrated that the growth factor activity of activation peptide is localized in amino-acid region 27–44. In addition, both anti-activation peptide and anti-27–44 peptide antibodies administered in vivo inhibited the growth of human prostate tumors in mice.
CONCLUSIONS. Based on these data, we hypothesize that the interaction of procathepsin D activation peptide with an unknown receptor is mediated by amino-acid sequence 27–44. This interaction leads in certain types of tumor to a proliferation and higher motility. Blocking of this interaction by antibodies or antagonists might be a valuable tool in prostate cancer inhibition. *Prostate 44:1–7, 2000.* © 2000 Wiley-Liss, Inc.

KEY WORDS: **procathepsin; prostate cancer; proliferation; growth factors; activation peptide**

INTRODUCTION

Cathepsin D (CD) and its enzymatically inactive zymogen procathepsin D (pCD) were originally established as prognostic markers in breast cancer [1]. In the last decade, increasing amounts of data have documented the direct involvement of CD and pCD in the growth of numerous types of cancer, including breast cancer [2–4], colorectal cancer [5], endometrial adenocarcinoma [6], ovarian cancer [7], hepatoma [8], and prostate cancer [9,10], suggesting that this system might be involved in general mechanisms of metastatic growth.

Our work on procathepsin D in human cancer originally showed that this enzymatically inactive precursor acts as an autocrine growth factor specific for breast cancer-derived cells [3,4]. Subsequent experiments demonstrated that the growth of breast cancer cells in vivo could be inhibited by anti-pCD antibodies [11]. The growth factor function of pCD was found to be mediated through a specific receptor expressed on all eight human breast cancer cell lines examined. This receptor was found to be distinct from the well-known mannose-6-phosphate receptor, which has been shown to function as the CD receptor on most other systems. The parts of the procathepsin D molecule responsible for its mitogenic activity were localized near amino acids 27–44 of the activation peptide (APpCD) sequence [11]. In all our experiments, the mature enzyme CD had no role in metastasis.

Based on the currently suggested possible general role of pCD in cancer development, the last few years witnessed numerous studies searching for the involvement of CD and pCD in prostate cancer. At present, the role of pCD/CD in prostate cancer is unclear.

Grant sponsor: Phi Beta Psi Sorority; Grant sponsor: University of Louisville.

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Received 12 May 1999; Accepted 16 February 2000

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Some studies found that the levels of CD correlate with the pathologic state, and some suggested that CD is a useful marker of prostate cancer progression [9]. Experiments using procathepsin D or its activation peptide showed that proliferation of all three human prostate cancer-derived cell lines was potentiated by the addition of either pCD or APpCD to the cultivation medium. In addition, prostate cancer cells were found to respond by proliferation to a number of paracrine factors including bombesin, somatostatin, or vasoactive intestinal peptide [12].

Even estrogen receptor-negative breast cancer cell lines are responsive to pCD [4], and probably there is no correlation with estrogen receptor status [13]. Recent papers showing the possible involvement of surrounding noncancerogenous cells in the secretion of pCD seem to suggest that in vivo, the presence of even a small population of pCD-secreting cells might have a dramatic effect on the proliferation of cells in surrounding tissues [14].

Based on the these data, we decided to evaluate the hypothesis that a small 27–44 amino-acid fragment of the APpCD can serve as a growth factor for human prostate cancer cells, and that the anti-AP antibodies can inhibit the growth of prostate cancer in vivo. In conclusion, we propose that pCD via its activation peptide regulates the growth of prostate cancer by stimulation of cell proliferation, probably through specific interaction with a new, as yet unspecified receptor. Blocking of this interaction of pCD with its corresponding receptor might inhibit the development of prostate cancer. In addition, rapid screening for the receptor involved in pCD binding or for anti-AP antibodies in the blood of prostate cancer patients might be a future prognostic factor.

MATERIALS AND METHODS

Chemicals

RPMI 1640 medium, Iscove's modified Dulbecco's medium, HEPES, (N–[2–Hydroxyethyl]piperazine–N'–[2–ethanesulfuric acid], MTT, E-TOXATE human cathepsin D, transferrin, glutaraldehyde, chondroitin sulfate, and porcine gelatin were obtained from Sigma Chemical Co. (St. Louis, MO); fetal calf serum was from Hyclone Laboratories (Logan, UT); pepstatin A and Protein A Sepharose were from Pharmacia (Pharmacia LKB Biotechnology, Piscataway, NJ), and pig pepsinogen A was from Worthington (Freehold, NJ).

Activation Peptide

The 44-amino-acid-long peptide corresponding to the activation peptide of pCD, the 28-amino-acid-long peptide (1–27 AA), and the 18-amino-acid-long peptide (27–44 AA) corresponding to the fragment of AP were synthesized at 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 (High Pressure Liquid Chromatography) 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 the 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 of human transferrin [4]. 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, 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 optical density (O.D.) of the well supernatants was measured 24 hr later at 570 nm, using the SLT ELISA reader (Tecan, Research Triangle Park, NC). All media were tested for endotoxin contamination and shown to contain <0.1 ng/ml Lipopolysaccharide (LPS) using the *Limulus* lysate test. Steroid-deprived cells were used in all experiments. The levels of procathepsin D in FCS at the concentrations used were below detection levels (data not shown). Similarly, the levels of estrogens in FCS were below 1 pg/ml. We repeated our experiments, using both charcoal-treated FCS and medium without phenol red, with identical results.

Human Cell Lines

Prostate cancer cell lines PC3, DU145, and LNCaP and breast cancer cell line ZR-75-1 were obtained from the American Tissue Culture Collection (ATCC, Manassas, VA). The cancer cell lines were grown in RPMI 1640 medium with HEPES buffer supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in plastic disposable tissue culture flasks at 37°C in a 5% CO_2/95% air incubator.

Isolation of Procathepsin D

Human procathepsin D was isolated from the culture supernatant of human breast cancer cell line ZR-75-1, as described earlier [15]. Briefly, a two-step procedure was used. In the first step, immunoaffinity chromatography was used, with anti-activation peptide antibodies attached to Protein A Sepharose. In the second step, FPC (Fast Protein Chromatography) chromatography using a Mono-Q column (Pharmacia LKB Biotechnology) and 20 mM TRIS (pH 7.2) was used.

Animals

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

Antibodies

Anti-human factor I OX-21 IgG_1 mAb was described elsewhere [16]. Anti-human cathepsin D IgG_{2a} mAb was purchased from Calbiochem (La Jolla, CA). The anti-27–44 AA fragment of the activation peptide was described previously [17]. Each mAb (except anti-cathepsin D) was isolated from ascites fluid by 50% ammonium sulfate precipitation followed by Mono-Q anion exchange chromatography.

Preparation of Biodegradable Microspheres

Biodegradable gelatin microspheres were prepared as described [11,18]. Briefly, 5% gelatin solution was mixed with mAbs diluted in 0.2% chondroitin sulfate at room temperature. Coacervation was achieved by rapidly adding 5 mg of gelatin solution to 5 ml of the stirred chodroitin sulfate solution. The microspheres were cross-linked by the addition of 50 μl of 25% glutaraldehyde solution. After 30 min of incubation, 6 ml of ethanolamined-HCl were added. The microspheres were then washed six times with phosphate-buffered saline.

Tumor Cell Growth in Mice

Human prostate tumors were generated by injecting athymic nude mice with 5×10^5 LNCaP 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 anti-pCD mAb, anti-fragment mAbs, or irrelevant anti-human factor I IgG_1 mAb (three injections 2 weeks apart). Mice were sacrificed at various time intervals, and tumor size was evaluated by weighing the tumors.

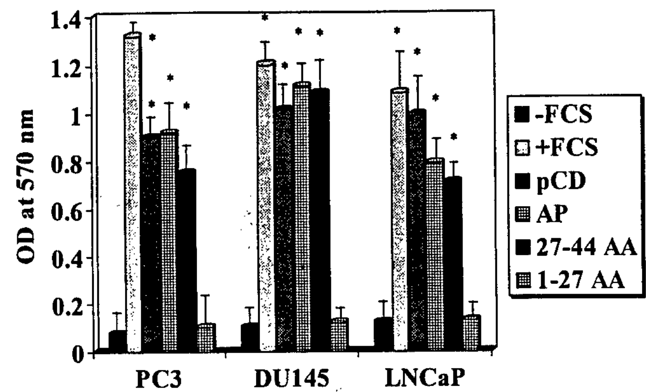

Fig. 1. Growth of human prostate cancer cell lines in serum-free medium containing 50 ng/ml of activation peptide (AP), 10 ng/ml of procathepsin D (pCD), 50 ng/ml of 1–27 AA fragment (1–27 AA), or 50 ng/ml of 27–44 AA fragment (27–44 AA) of the activation peptide. The results from growth in the serum-free medium (−FCS) and in control medium supplemented with FCS (+FCS) are given for comparison. The results represent the mean ± SD of three independent experiments. *Significant differences from control (−FCS) values at $P \leq 0.05$.

Statistical Evaluation

Statistical significance of the differences of the means was evaluated by Student's *t*-test.

RESULTS

Cell Culture

The results showing the effect of 50 ng/ml of activation peptide, 10 ng/ml of procathepsin D, or 50 ng/ml of 27–44 AA fragment of the activation peptide on the proliferation of three different prostate cancer-derived cell lines are shown in Figure 1. Data presented in Figure 1 were obtained after 5 days of incubation instead of the 7 days usually used, in case of more slowly growing breast cancer-derived cells. Proliferation was measured by the incorporation of MTT by cells. The data show that the proliferation of cells was increased in the same way by pCD, activation peptide, or a 27–44 AA fragment of the activation peptide. The proliferative activity of these molecules for prostate cancer cells was dose-responsive, with maximum proliferative activity at a concentration of 10 ng/ml (data not shown). Our previous experiments showed that this dose was the optimal dose both on breast cancer cells [4] and in a prostate cancer cell model [10].

Determination of Possible Involvement of Cathepsin D

Experiments using the enzymatically inactive zymogen of CD always raise the possibility that the en-

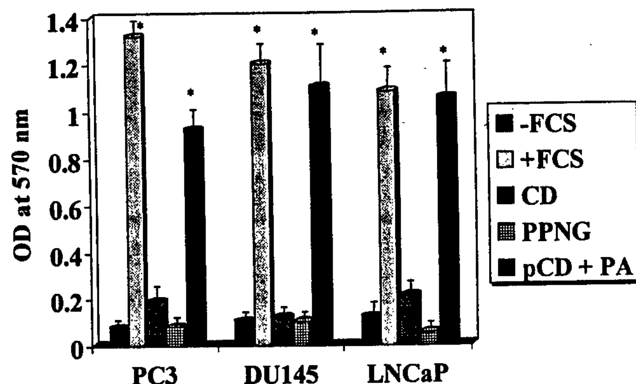

Fig. 2. Growth of human prostate cancer cell lines in serum-free medium is not supported by 2 nM human cathepsin D (CD) or 20 nM pig pepsinogen A (PPNG). Similarly, growth-stimulating activity of procathepsin D (pCD) was not inhibited by 1 μM pepstatin A (PA). The results from growth in serum-free medium (−FCS) and in control medium supplemented with FCS (+FCS) are given for comparison. The results represent the mean ± SD of three experiments. *Significant differences from control (−FCS) values at $P \leq 0.05$.

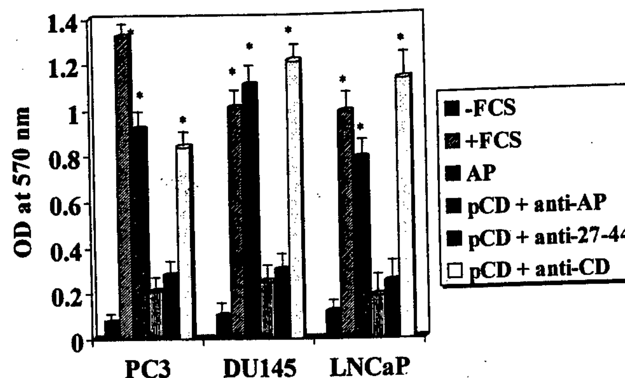

Fig. 3. Stimulation of human prostate cancer cell line growth in serum-free media and its inhibition by various monoclonal antibodies. Procathepsin D (10 ng/ml) was inhibited by 1 μg/ml of anti-activation peptide IgG (anti-AP), anti-27–44 AA fragment of activation peptide IgG (anti-27–44), or anti-human cathepsin D IgG (anti-CD). The results from growth in serum-free medium (−FCS) and in control medium supplemented with FCS (+FCS) are given for comparison. The results represent the mean ± SD of three experiments. *Significant differences from control (−FCS) values at $P \leq 0.05$.

zymatically fully active CD might be formed during the duration of the experiment. We decided to address this possibility by using the mature human CD instead of pCD (Fig. 2). However, CD failed to stimulate the proliferation of prostate cancer cells. In addition, we used pepstatin A, a strong inhibitor of cathepsin D, with K_i at the picomolar level [19], to test the possible involvement of the proteolytic activity of CD on the observed mitogenic function. All these experiments showed that the enzymatic activity of CD had very little influence on the growth-factor activity of pCD. Similarly, no proliferative activity was observed for pig pepsinogen A as a control molecule. Pepsinogen was included as a control protein with a similar overall structure.

27–44 Amino-Acid Fragment of Activation Peptide

Despite these demonstrations that the proteolytic activity of the mature enzyme is not involved in the described effects, we decided to use only synthetic peptides in all subsequent experiments. As shown in Figure 1, there are no significant differences between the activity of the complete molecule of pCD or its activation peptide. Even a fragment of the activation peptide showed a similar level of activity. A strong inhibition of the mitogenic activity of activation peptide was observed when monoclonal antibodies raised against the activation peptide of pCD or against the 27–44 AA fragment of the activation peptide were used (Fig. 3). The specificity of these two antibodies used in this experiment was described previously [17]; briefly, anti-pCD antibodies recognize epitopes contained within the activation peptide only, while anti-CD antibodies interact with epitopes of the mature enzyme and not with the activation peptide. Similar to our observation in a breast cancer model, this inhibition of the mitogenic effect observed for anti-AP antibodies was specific for pCD, and these antibodies had no effect on the function of other known mitogens [3].

In Vivo Treatment

The previously mentioned experiments were followed by a final set of experiments where we focused on the growth-stimulating activity of pCD in an in vivo model in which both anti-activation peptide and anti-27–44 AA antibodies were shown to reverse cancer development. Cells of the human prostate cancer cell line LNCaP were injected s.c. into athymic nude mice. After evaluation for development of tumor mass, mice were injected with biodegradable gelatin microspheres containing 100 μg of antibodies (day 0). Preliminary experiments using both breast cancer and prostate cancer cells revealed that 100 μg of antibodies per injection is the optimal dose. No significant difference between 100, 200, and 300 μg of antibodies was observed (unpublished results). Four mice per experimental group were killed at each time interval, and the tumor mass was carefully extracted and cleaned of all surrounding tissue. Inhibition and regression of tumor size were determined by weight. The first tumor mass was palpable approximately 10 days after the initial injection. However, at this time period the ac-

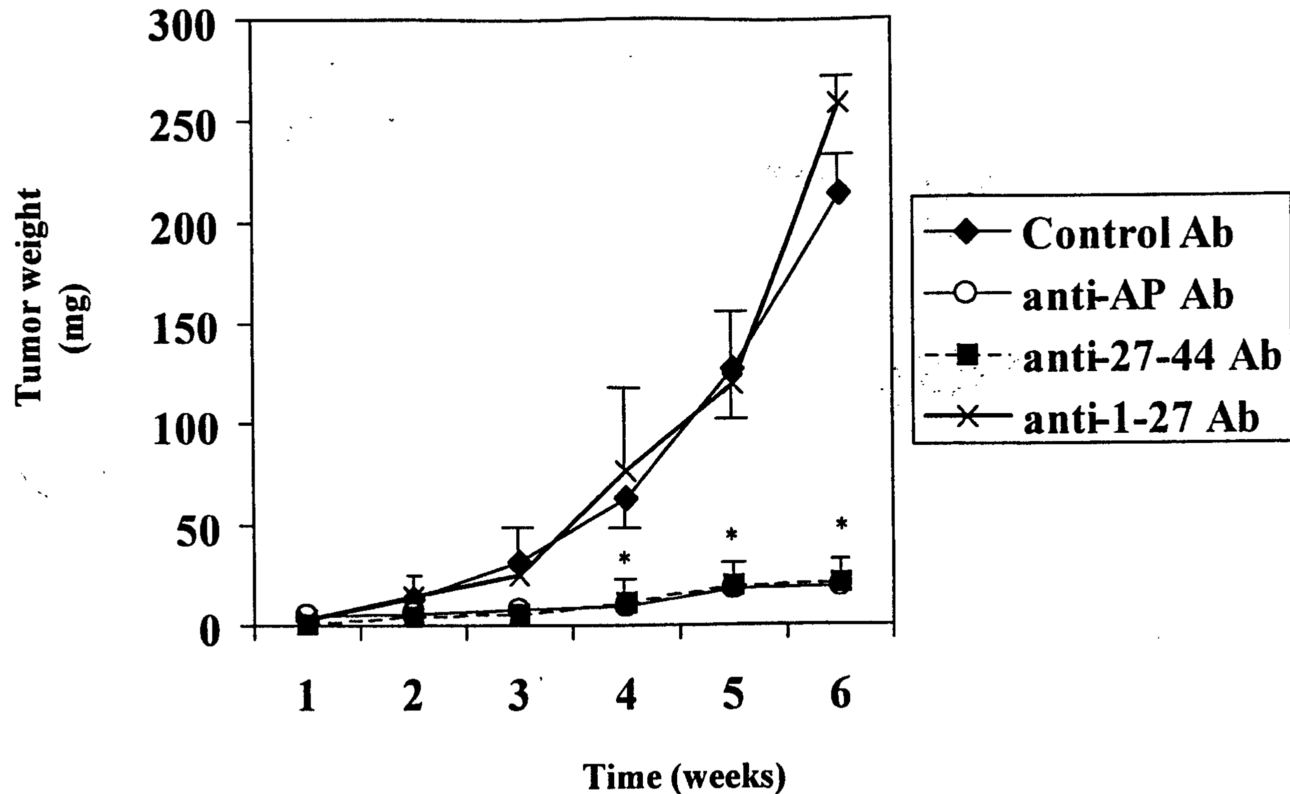

Fig. 4. Inhibition of LNCaP cell tumor growth in athymic mice by treatment with anti-activation peptide (anti-AP Ab), anti-1–27 AA fragment (anti-1–27 Ab), anti-27–44 AA fragment (anti-27–44 Ab), or control anti-factor I (Control Ab) antibodies. See Materials and Methods for experimental details. Results represent mean ± SD of 4 mice per time interval. *Significant differences from control (Control Ab) values at $P \leq 0.05$.

tual weight of tumors is very low, and most of the mass is formed by small granulomas. Figure 4 shows that mice treated with either anti-activation peptide or anti-27–44 AA antibodies had greatly reduced tumor growth. On the other hand, irrelevant anti-factor I antibodies or the antibodies raised against 1–27 AA fragment of the activation peptide (previously shown to be without any significant biological activity [11,17]) had no effect on cancer growth. The antibody-mediated protection from an induced breast cancer was almost complete for the whole tested interval of 6 weeks.

DISCUSSION

The role of cathepsin D and its enzymatically inactive precursor procathepsin D in cancer development has recently attracted considerable attention. Besides their more common involvement in human breast cancer [11,20–23], the significance of procathepsin D and cathepsin D levels in various tumors such as endometrial adenocarcinoma [6], prostate cancer [24], colon carcinoma [25], laryngeal tumors [26], ovarian cancer [7], and carcinoma of thyroid tissue [27] has been demonstrated, suggesting that this system might be involved in the metastatic growth of several other cancer cell types. Numerous studies have demonstrated that this proenzyme is overexpressed and secreted from several types of cancer (such as breast, melanoma, and ovary). In addition, elevated anti-pCD antibodies have been found in plasma of breast [28] or ovarian [7] cancer patients. Numerous papers showed both the in vitro effects of pCD on cancer cell proliferation [3,10,29,30] as well as effects in vivo [11]. Furthermore, induced overexpression of pCD significantly stimulated metastasis in experimental models of mice injected with a rat cancer cell line transformed with human procathepsin D cDNA [30,31].

The synthetic peptides were prepared with the highest possible purity. Nevertheless, the possibility that some of the observed biological effects might be caused by some impurities cannot be completely overlooked. On the other hand, we used extremely low concentrations; thus, it is not probable that the potential impurities might be active in 1 ng/ml concentrations. In addition, we used procathepsin D isolated from the tissue culture supernatant by means of affinity chromatography with virtually identical results.

Furthermore, we believe that the use of synthetic peptides eliminates the effects of possible contaminants much more completely than when biologically isolated material is used.

This paper focused on the hypothesis that the mitogenic growth factor activity of pCD is mediated by its activation peptide [11]. This possibility is generally supported by several functions which the activation peptide was found to play within the family of aspartic proteinases. For example, in the case of pCD it is speculated that a structure within the activation peptide is involved in targeting to lysosomes (in addition to classical mannose-6-phosphate pathways) [32,33]. No inhibition of activation peptide binding or activities by either mannose-6-phosphate, anti-mannose-6-phosphate receptor antibodies, or soluble mannose-6-phosphate receptor was found [11]. As direct mitogenic action has been already demonstrated, the aim here was to determine which parts of the pCD are responsible for receptor activation. Direct mitogenic action has been already demonstrated using several models, including breast and prostate cancer [3,4,10]. In addition, the activation peptide of the pCD molecule was found to be responsible for growth factor activity [11]. Therefore, the aim here was to determine if the activation peptide is similarly involved in prostate cancer proliferation. Additional attention was focused on the question of which amino-acid residues in the activation peptide might be responsible for activation of the receptor involved in this reaction. Finally, to check directly the possibility that pCD is involved significantly in the progression of prostate cancer in vivo, we injected athymic nude mice with a prostate cancer cell suspension. Two weeks later, the mice were treated with biodegradable microspheres containing anti-activation peptide or anti-27–44 AA fragment antibodies. From the results we could conclude that the prolonged administration of these antibodies protected nude mice for up to 6 weeks (the entire tested interval). Previous results showed that elevated secretion of pCD increased the metastatic potential of transfected cells [34]. Our experiments demonstrate even more directly the involvement of pCD in prostate cancer development, and identify the activation peptide as a new potential target for suppression of human prostate cancer.

CONCLUSIONS

The presented data confirm our previous observation [10] that human prostate cancer cells respond to both pCD and its activation peptide by a significant increase of proliferation. The contributions of various enzymes and their zymogens to prostate cancer development were recently the subject of numerous papers [35,36]. In addition, functional expression cloning identified CD as a candidate gene for prostate cancer progression [37]. The data presented in our paper seem to be in agreement with the so-called Fusek and Vetvicka model [38], where pCD does not act as the inactive precursor of the enzyme, but as a ligand for triggering a transmembrane receptor. We propose that the mechanism of the mitogenic function of pCD relies on a specific structure of the activation peptide, most probably inside its 27–44 AA fragment, and its interaction with a cell membrane receptor present on the surface of at least breast and prostate cancer cells. The experiments attempting to isolate and characterize this new receptor are currently in progress.

ACKNOWLEDGMENTS

The authors thank Drs. Marketa Rinnova and Zdenek Voburka of the Institute of Organic Chemistry and Biochemistry (Academy of Science of the Czech Republic, Prague, Czech Republic) for the synthesis and characterization of synthetic peptides and for help with procathepsin D isolation.

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