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TITLE: Cathepsins B and K: Role in Metastasis of Human Prostate
         Cancer to Human Bone in an In Vivo SCID-hu Mouse Model

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13. ABSTRACT *(Maximum 200 Words)*

Cathepsins K and B have been implicated in bone resorption in several pathologies. Expression of cathepsin B is highly upregulated in human prostate cancer. Cathepsin K, in contrast to cathepsin B, is expressed predominantly in osteoclasts. Cathepsin K has recently been identified in tumor cells within human breast tumor bone metastases and in adjacent osteoclasts, suggesting that cathepsin K may participate in bone resorption by tumor cells. Therefore, we are testing the hypothesis that cathepsins B and K are responsible for osteolysis by human prostate cancers. One goal in this first year was to establish stable GFP-expressing transfectants of the three prostate cancer cell lines in order to facilitate their detection by confocal laser fluorescence microscopy both *in vitro* and *in vivo*. This will also allow us to more readily assess the interaction of the prostate cancer cells with the surrounding tissue and bone matrix. Another goal was to establish selective assays for determining expression of cathepsins B and K at the transcript, protein and activity levels and for following changes in localization of the two enzymes by immunofluorescence staining in vitro and in situ as well as by analysis of secretion in vitro.

14. SUBJECT TERMS proteases, protease inhibitors, metastasis, bone, prostate cancer, osteolysis, cysteine Proteases, collagen I, cathepsin B, cathepsin K

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INTRODUCTION

The research conducted by Sloane and colleagues is designed to determine the roles played by two cysteine proteases in the metastasis of human prostate cancers to bone and in the subsequent bone degradation. The two proteases, cathepsins B and K, were selected for analysis as: 1) cathepsin B has been shown to be upregulated in prostate carcinoma cells using genomic, immunochemical and biochemical techniques, and 2) cathepsin K, which is expressed predominantly by osteoclasts, is involved in osteolytic processes. Cathepsin K is the only known mammalian protease that is capable of completely digesting the organic matrix of bone. Both in vitro and in vivo model systems will be employed for these analyses. These include a SCID-hu animal model that recapitulates the interaction between human prostate cancer cells and human bone and two novel in vitro assays developed in the Sloane laboratory to assess proteolysis by living cells. The research addresses two critical issues for prostate cancer. In terms of cancer biology, the studies examine underlying mechanisms for bone metastasis, i.e., do osteoclasts and osteoclast-associated proteases facilitate bone metastasis of human prostate cancer and are cathepsins B and K causally involved? In terms of therapy, the studies will determine whether the SCID-hu model can be used to screen protease inhibitors as potential therapeutic agents and specifically whether cathepsins B and K are important therapeutic targets for bone metastasis of human prostate cancers.

BODY

Task 1. Characterize PC3, DU145 and LNCaP human prostate cancer cell lines for expression, localization and secretion of cathepsins B and K when grown on human bone explants and in type I collagen gels (months 1-12).

- Develop stable EGFP-expressing variants of PC3, DU145 and LNCaP cell lines (months 1-3).

  Stable GFP-expressing variants of DU145 cells have been established. GFP-expressing variants of PC3 cells were established, but lost GFP expression with time in culture. This was also the case in a second experiment. We have recently transfected two different clones of PC3 cells with two separate GFP-expression plasmids and isolated clones. LnCaP cells grow much more slowly and have proven difficult to transfect so we have decided to delay establishing GFP-expressing LnCaP variants until we have stable GFP-expressing variants for both the DU145 and PC3 cell lines. One of two GFP-expressing DU145 variants is illustrated in Figure 1.

- Grow variants in type I collagen gels and on human bone explants harvest and measure expression of two enzymes (mRNA, protein, activity) (months 3-6).

There are established assays in our laboratory for analysis of cathepsin B expression (mRNA, protein, activity) (Hulkower et al., 2000; Linebaugh et al., 1999; Sloane et al., 1994). However, in order to analyze the expression of cathepsin K (mRNA, protein, activity), we needed to establish assays that would discriminate cathepsin K from other cysteine proteases, including cathepsin B.

mRNA: We initially screened by semi-quantitative RT-PCR for expression of various lysosomal proteases (the aspartic protease cathepsin D, the cysteine endopeptidase legumain and the papain-family cysteine proteases cathepsins B, L, L2 (V), O and K) and cysteine protease inhibitors (stefins A and B, cystatins C and M). The three human prostate cancer cell lines (DU145, LnCaP, PC3) were compared to the human mammary epithelial cell line MCF10A, human mammary carcinoma cell lines (BT20, BT549, MD435), human colon carcinoma cell lines (CaCo2, HT29) and the human glioma cell line U87. The results are illustrated in Figure 2. The levels of expression of cathepsin B and cathepsin K were normalized to expression of ß2-microglobulin (Figure 3). Primers used for the two cathepsins are indicated below. For cathepsin B, we employed 25 cycles of amplification at 60 °C and for cathepsin K, 30 cycles of amplification at 60 °C. Levels of expression of the two enzymes were comparable in the DU145 and LnCaP cell lines, but substantially less in the PC3 cell line.

Cathepsin B forward
    5'-GAA CAA TGG CCA CAG TGT CC-3'
Cathepsin B reverse
    5'-AGG CCC ACG GCA GAT TAG-3'

Cathepsin K forward
    5'-CCA GAC AAC AGA TTT CCA TCA GCA G-3'
Cathepsin K reverse
    5'-GAT-CTC TCT GTA CCC TCT GCA TTT AGC-3'

Protein: Although there are immunoblots in the literature for cathepsin K expression (Tepel et al., 2000), we ran into difficulty with cathepsin K antibodies. This was true in terms of their specificity for cathepsin K as well as in terms of their titer. Commercially available antibodies to cathepsin K were uniformly bad. The only antibody that we have found to be useful for immunoblotting is a polyclonal antibody obtained from Axys Pharmaceutical. Access to this antibody was not easy to obtain as all reagents relating to cathepsin K had

been licensed to Merck due to an industrial collaboration between the two firms on cathepsin K as a therapeutic target for osteoporosis and other diseases involving osteolysis. With the Axys antibody, we have been able to visualize cathepsin K in lysates of cells that express high levels of the enzyme [U87 human glioma cells and 12TT human breast fibroblasts] as well as visualize recombinant cathepsin K (not illustrated); however, this required a 1:1000 dilution of the antibody. This is in contrast to the 1:4000 dilution of our cathepsin B antibody when used for immunoblotting (Mai et al, 2000). Dr. Sloane just returned from a visit to one of the laboratories (University of Bonn, Germany) that has publications using cathepsin K antibodies (Tepel et al., 2000). The antibodies used by this group are no longer working for them so they have tested virtually all of the cathepsin K antibodies presently available, but have not found suitable antibodies. Given the importance of cathepsin K antibodies to our work, we have initiated a collaboration with Dr. Janko Kos of Krka in Slovenia. Dr. Kos has been successful in raising good monoclonal antibodies to a wide variety of cysteine proteases (Premzl et al., 2001). One of his students will spend 3 months working in our laboratory this coming fall.

Activity: Aibe et al. (1996) and Xia et al. (1999) have demonstrated that the substrate Z-Gly-Pro-Arg-AMC is most efficiently hydrolyzed by cathepsin K and that the relative substrate hydrolysis rates for cathepsins L (0.3%), S (0.03%) and B (8%) were low when comparing k_{cat}/k_m values for those enzymes to that for cathepsin K. In osteoclasts, there is a low abundance of cathepsins L, S, and B as compared to K (<1%), therefore the majority of the hydrolyzing activity for the Z-Gly-Pro-Arg substrate within osteoclasts would be due to the activity of cathepsin K. Unfortunately, this is not the case for us when trying to perform comparative analyzes of the activities of cathepsins B and K in the human prostate cancer cell lines used here. For cathepsin B, we have a highly selective inhibitor, CA074 (Murata et al., 1991) that can be used to eliminate the contribution of cathepsin B to hydrolysis of the Z-Gly-Pro-Arg substrate. We illustrate hydrolysis of the Z-Gly-Pro-Arg-AMC substrate by lysates of PC3, DU145 and U87 cells in Figures 4, 5 and 6, respectively. Note that the contribution of cathepsin K to the hydrolysis of this substrate, i.e., the activity in the presence of CA-074 is substantially more in the U87 cells, i.e., in cells which express high levels of cathepsin K. Also please note that EGFP-expressing transfectants of the DU145 cells have activities against this substrate comparable to that of the parental cells.

One might not expect cathepsin B to exhibit substantial activity against the Z-Gly-Pro-Arg substrate. Nonetheless, the activity could be significantly blocked by CA-074. As further proof that cathepsin B is contributing to hydrolysis of this substrate, we tested the ability of immunopurified recombinant cathepsin B to cleave the substrate (Ren et al., 1996). The enzyme is synthesized as an inactive pro form that must be activated with pepsin to generate mature active enzyme. The inactive procathepsin B was unable to cleave either the selective

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cathepsin B substrate Z-Arg-Arg-AMC or the putative cathepsin K substrate Z-Gly-Pro-Arg-AMC, whereas the pepsin-activated mature cathepsin B cleaved both substrates (Figure 7). Hydrolysis of the Z-Gly-Pro-Arg-AMC substrate was ~50% that of the Z-Arg-Arg-AMC substrate, indicating that it would be difficult to accurately assess cathepsin K activity in cells which express high levels of cathepsin B.

Given the problems with analysis of cathepsin K, we elected to initiate studies comparing cathepsin B expression in cells grown on collagen I gels and plastic. The results are preliminary, but encouraging. mRNA: RT-PCR (Figure 8) indicated that the levels of cathepsin B (Figure 9) and cathepsin K (Figure 10) transcripts were slightly increased in DU145 parental and EGFP-expressing (clone 4) cells grown on collagen I gels. Protein: Levels of cathepsin B protein in lysates of DU145 parental and clone 4 cells were substantially increased in cells grown on collagen I gels (Figure 11). Note the presence of two differentially glycosylated heavy chain forms (25/24 kDa) of mature double chain cathepsin B in all cases. In the cells grown on collagen I, the single chain form of cathepsin B (~31 kDa) was also observed. Activity: Cathepsin B activity was measured against the selective substrate Z-Arg-Arg-AMC and shown to be increased 3-6-fold in DU145 cells grown on collagen I gels (Figure 12). Both the basal and collagen-induced activity could be totally blocked by 10 μM CA-074. Note also the substantially lower basal activity of cathepsin B in PC3 cells. We have measured hydrolysis of the Z-Gly-Pro-Arg-AMC substrate by bone extracts harvested from SCID-hu mice in the absence and presence of PC3 colonies implanted on the bone. The amount of activity was 4-7-fold higher in extracts from PC3-colonized bones, and all of this activity was inhibited by the cathepsin B inhibitor CA-074. In contrast, the activity in the uncolonized bone extract was only partially reduced by CA-074 or the broad spectrum cysteine protease inhibitor E-64. This latter inhibitor should have reduced activity of cathepsin K. Thus, these results suggest that a protease other than a cysteine protease may cleave this substrate in these bone extracts. Nonetheless, these initial in vivo observations substantiate the induction of cathepsin B expression by collagen I.

- Grow variants as above, but process for immunofluorescence localization of enzymes by laser confocal microscopy (months 6-9).

We followed our published protocols (Sloane et al., 1994) to localize cathepsin B in DU145 cells by immunofluorescence (Figure 13). The substantive increases in cathepsin B protein and activity induced by growing the cells on collagen I gels were not obvious in the immunofluorescent images. We will do optical sectioning of the cells and 3-dimensional reconstructions to obtain a better assessment of the amount of cathepsin B staining in cells grown on plastic and collagen I. In previous studies, we have found that antibody

penetration is much less for the cells grown on collagen gels and thus we may need to modify our procedures to account for this (Koblinski et al., submitted).

We also illustrate here the problems that we have encountered with cathepsin K antibodies. In Figure 14 is illustrated staining of DU145 cells using antibodies from Axys and Chemicon. The staining with the Chemicon antibody is diffuse rather than vesicular as one should obtain for a lysosomal protease. These results suggest that the Chemicon antibody is reacting with something other than cathepsin K, resulting in non-specific staining. The results with the Axys antibody are somewhat more promising as the staining is vesicular; however, there is a substantial amount of non-specific staining over the nuclei. We will try different fixatives and protocols in an effort to improve our staining with this antibody.

- Grow variants as above, assay for enzyme protein and activity in conditioned medium (months 3-6).

We analyzed secretion of cathepsin B from DU145 parental cells and clone 4 EGFP-transfectants comparing cells grown on plastic and those grown on collagen I. Protein: Substantially more secretion of procathepsin B and single chain mature cathepsin B was observed from DU145 cells grown on plastic then those grown on collagen I (Figure 11). Activity: Activity assays (Figure 12) confirmed the results obtained by immunoblotting. Procathepsin B secretion from DU145 cells grown on collagen I gels was substantially higher (2.3-8-fold) than in cells grown on plastic. That the secreted enzyme was predominantly proenzyme is evident from the need to incubate the conditioned media with pepsin to obtain cathepsin B activity.

- Grow variants as above, but assess secretion of enzyme activity in a "real-time assay" (months 9-12).

We also analyzed secretion of active cathepsin B from living PC3 and DU145 cells growing on coverslips using our established assay (Linebaugh et al., 1999). A small but measurable amount of active cathepsin B (pericellular) was secreted from all three cell lines (Figure 15). The amount secreted was only 2-5% of that present in the cells (total). Total activity was assessed by lysing the cells with 0.1% Triton X-100. Cathepsin K activity cannot be measured in this assay as the optimal pH for hydrolysis of Z-Gly-Pro-Arg-AMC by cathepsin K is 5.5, i.e., a pH that will kill the cells.

Problems encountered: The postdoctoral fellow (Research Associate) named on the original application elected to return to Italy to assume an Assistant Professor position. We have appended a biosketch for the postdoctoral fellow who assumed this position. Please note that Dr.

Bakhshi was on pregnancy leave followed by medical leave for >4 months of the initial year of this grant. This extended absence affected our ability to complete all of the tasks which we expected to complete in the initial 12 months.

FIGURE LEGENDS:

FIGURE 1
Fluorescent image of GFP-expressing variant of DU145 cells (Clone #4).

FIGURE 2
RT-PCR products for various lysosomal proteases and cysteine protease inhibitors:- Comparison of expression in prostate cancer lines with human mammary epithelial and carcinoma cells, human colon carcinoma cells and a human glioma cell line.

FIGURE 3
Comparison of expression of cathepsin B and K in the above cell lines by semi-quantitative RT-PCR.

FIGURE 4
PC-3 Lysate: Hydrolysis of Z-Gly-Pro-Arg-AMC. PC-3 cells in log growth were lysed in SME (150 mM sucrose, 25mM MES, 1 mM EDTA) pH=6.5 containing 0.1 % Triton X-100. The lysate was stored at –20 °C and prior to assay was thawed, sonicated (2 X 10 sec., 50 watts), and a portion of the lysate sample was assayed according to the procedure of Aibe et. al. (1996) for its ability to hydrolyze the substrate Z-Gly-Pro-Arg-AMC which was present at 100 μM. The cathepsin B inhibitor CA-074 (10 μM) and the general cysteine protease inhibitor E-64 (10 μM) were added and the hydrolytic activity determined in their presence.

FIGURE 5
DU-145 Clones: Hydrolysis of Z-Gly-Pro-Arg-AMC. DU-145 cells and clones (#4 and #6) were harvested and treated as PC-3 cells in Fig. 4. The hydrolytic activity of the lysates were determined in the absence and presence of
10 μM CA-074.

FIGURE 6
U 87 Lysate: Hydrolysis of Z-Gly-Pro-Arg-AMC. U 87 cells were harvested and the lysate assayed for its ability to hydrolyze the substrate as described in Fig. 4. The cathepsin B inhibitor CA-074 (10 μM)was added to the reaction during the measurement of substrate hydrolysis and to an equal volume of lysate at 10 μM concentration for 5 min (23 °C) prior to assesment of hydrolytic activity.

FIGURE 7
Substrate Hydrolysis by Activated rprocat B. Recombinant pro-cat B (rprocat B) was pepsin activated in formate buffer (pH 3.2). A sample (15 μl of 40 μl activated) was assayed for its ability to hydrolyze the substrates Z-Arg-Arg-AMC in the cathepsin B assay (Linebaugh et.al. 1999) and Z-Gly-Pro-Arg-AMC in the cathepsin K assay of Aibe et.al. (1996).

FIGURE 8
RT-PCR products of cathepsins B and K. A comparison of expression in DU145 cells (parental and GFP-variant), grown on plastic and collagen.

FIGURE 9
Comparison of expression of cathepsin B in DU145 cells (parental and GFP-variant), grown on plastic and collagen by semi-quantitative RT-PCR.

FIGURE 10
Comparison of expression of cathepsin K in DU145 cells (parental and GFP-variant), grown on plastic and collagen by semi-quantitative RT-PCR.

FIGURE 11
Western analysis of cathepsin B expression in DU145 cells (parental and GFP-variant), grown on plastic and collagen – overnight conditioned media and cell lysates.

FIGURE 12
Cathepsin B activity in cell lysates (upper panel) and overnight conditioned media (lower panel).

FIGURE 13
Confocal image, superimposed on phase image of cathepsin B expression in GFP-expressing variant of DU145. Comparison of expression of cells grown on plastic versus cells grown on collagen.

FIGURE 14
Confocal image, superimposed on phase image of cathepsin K expression in GFP-expressing variant of DU145 cells. Comparison, using a polyclonal antibody (Axys) and a monoclonal antibody from Chemicon.

FIGURE 15
Pericellular and Total Cathepsin B in PC-3 and DU-145 cells. Cells in log growth on coverslips (9 X 22 mm) were assayed for pericellular and total cellular cathepsin B activity in the hydrolysis of the substrate Z-Arg-Arg-AMC according to Linebaugh et. al. (1999).
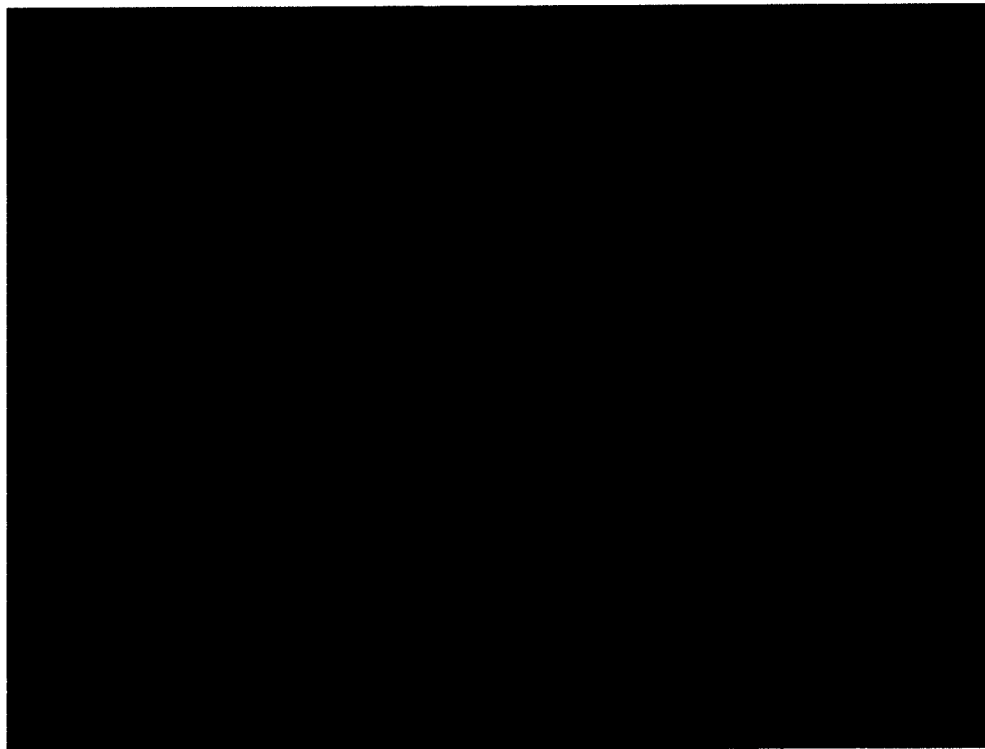
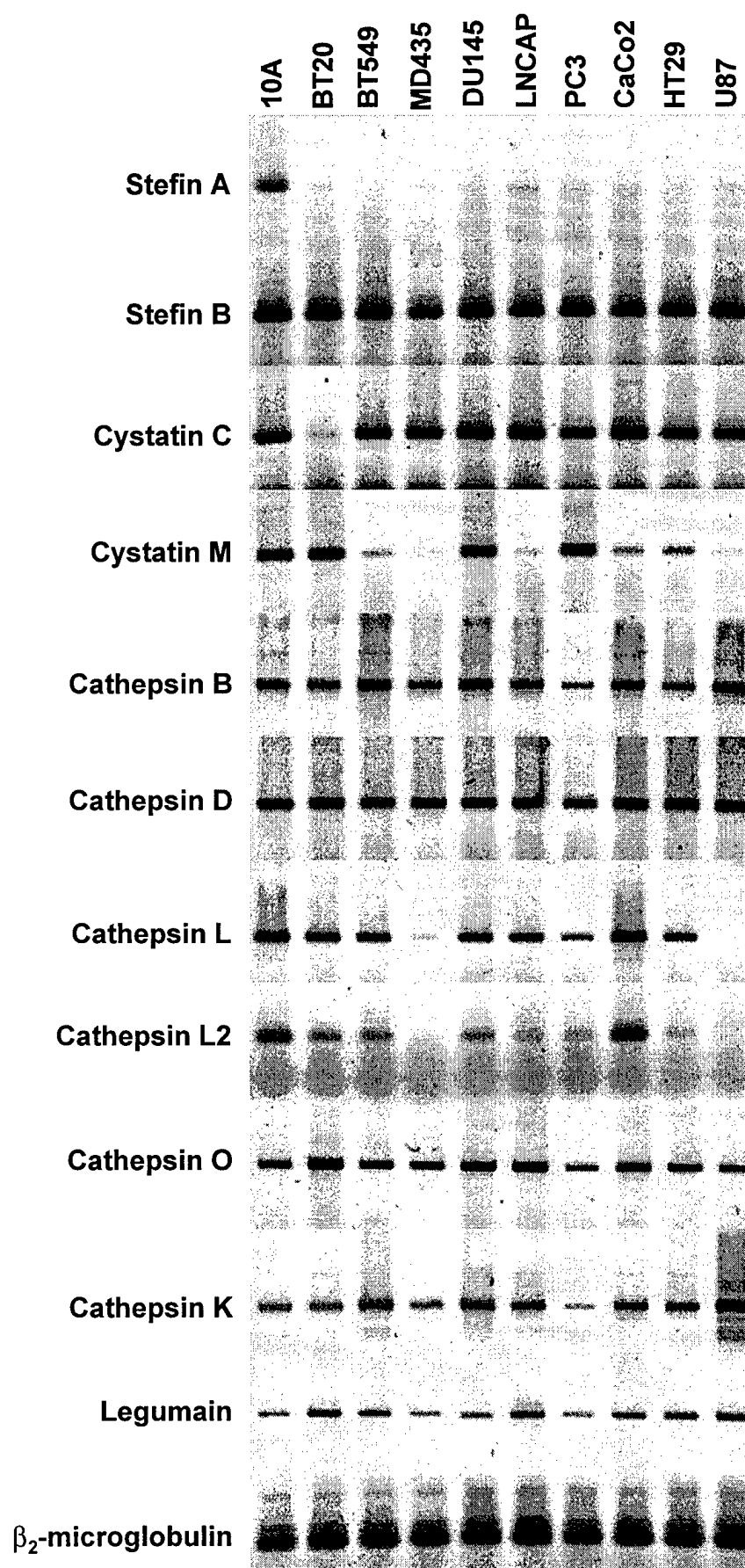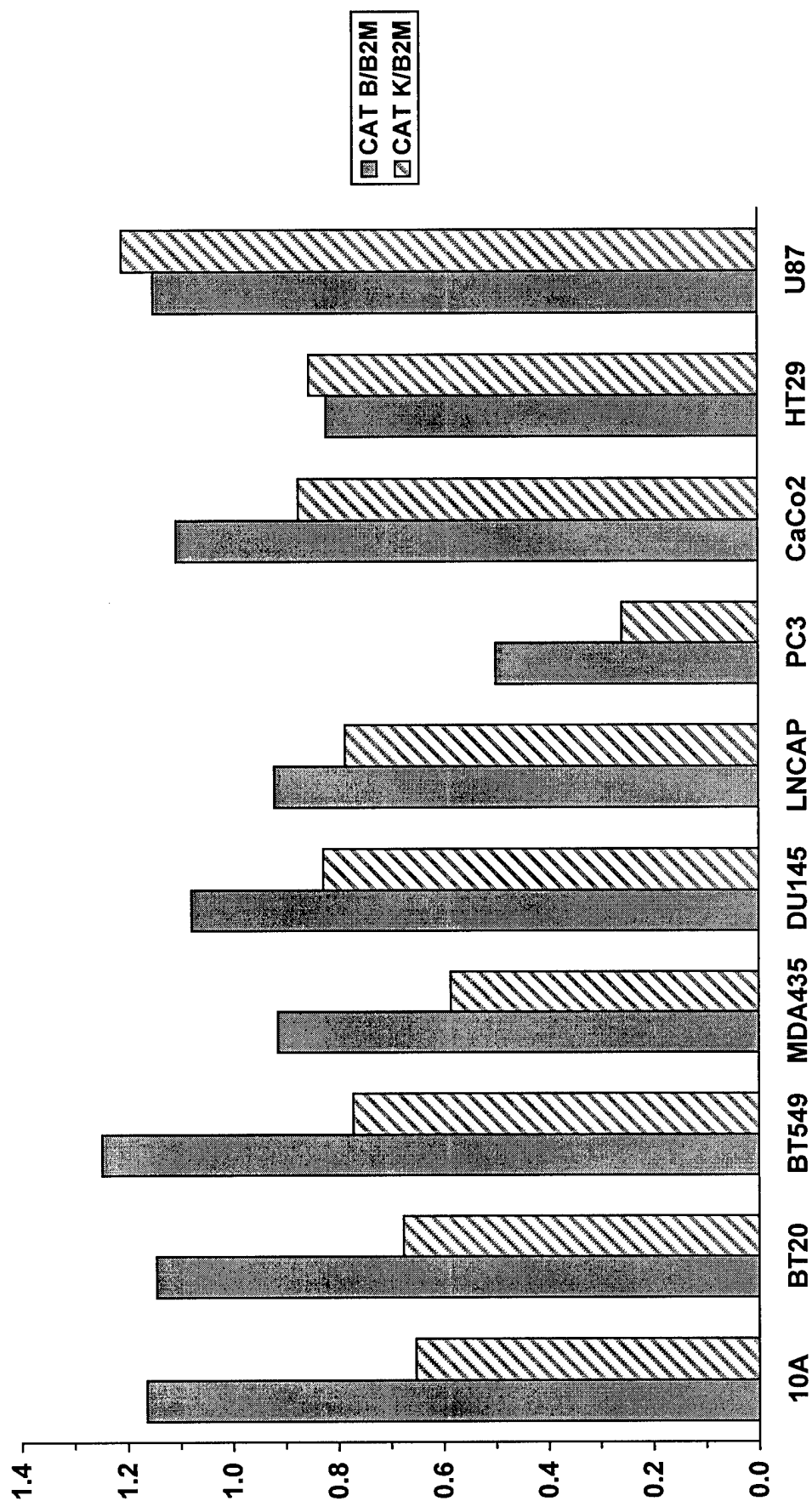DU145 EGFP Clone #4

Figure 1

Figure 2

Cell lines: 10A, BT20, BT549, MD435, DU145, LNCAP, PC3, CaCo2, HT29, U87

Probes: Stefin A, Stefin B, Cystatin C, Cystatin M, Cathepsin B, Cathepsin D, Cathepsin L, Cathepsin L2, Cathepsin O, Cathepsin K, Legumain, β$_2$-microglobulin

Figrue 3

Cathepsin B and K mRNA Levels

Legend: CAT B/B2M, CAT K/B2M

X-axis categories: 10A, BT20, BT549, MDA435, DU145, LNCAP, PC3, CaCo2, HT29, U87

Y-axis: 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4

Figure 4

PC-3 Lysate: Hydrolysis of Z-Gly-Pro-Arg-AMC

pmoles AMC released/min/sample

800
700
600
500
400
300
200
100
0

PC-3

PC-3/CA-074

PC-3/E-64

Cell Lysate and Inhibitors
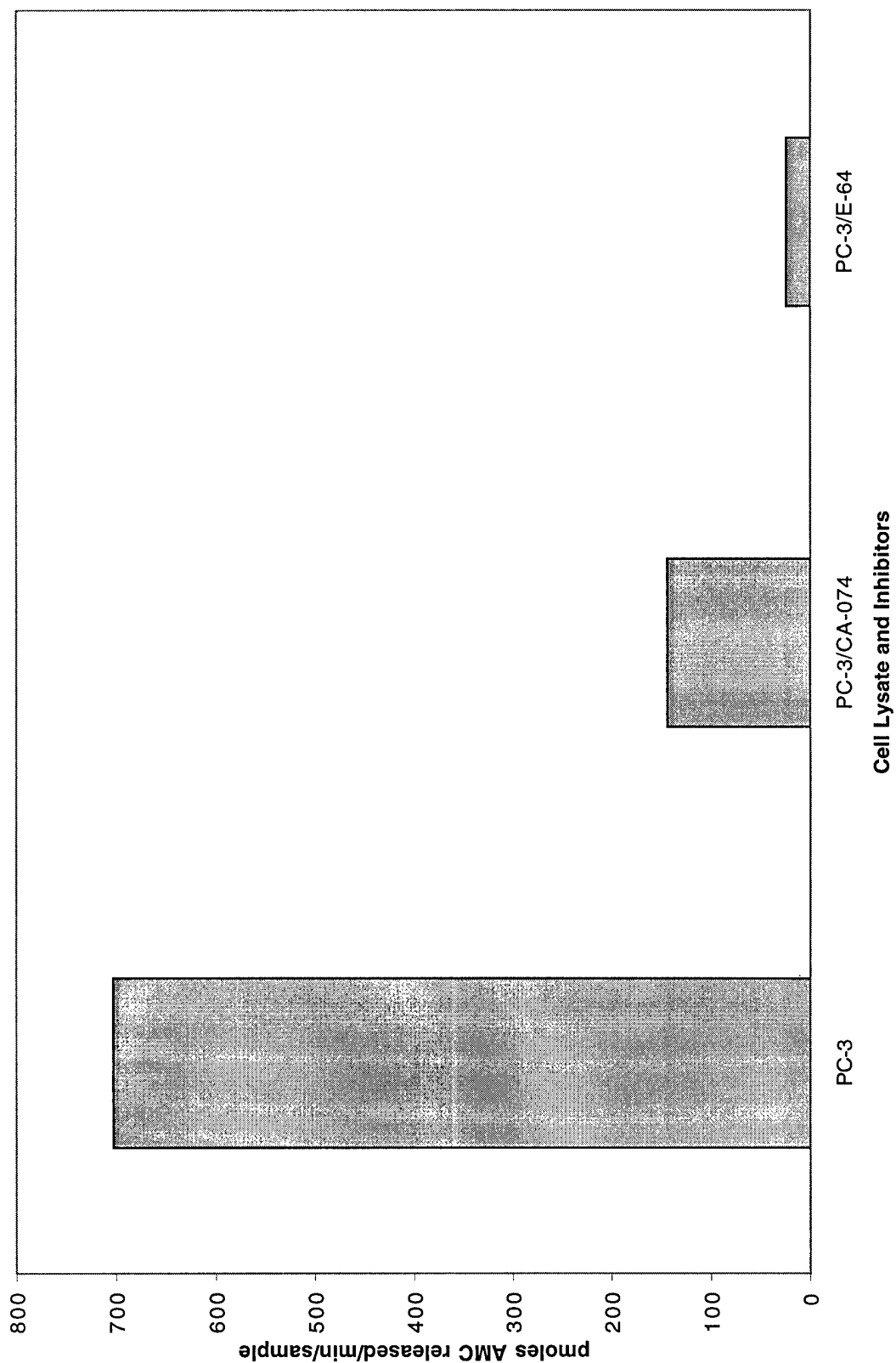
Figure 5

DU-145 Clones: Hydrolysis of Z-Gly-Pro-Arg-AMC

Figure 6

U 87 Lysate: Hydrolysis of Z-Gly-Pro-Arg-AMC

17

Figure 7

Substrate Hydrolysis by Activated rprocatB

pmoles AMC released/min

Z-Arg-Arg-AMC (cat B assay)

no pro cat B act pro cat B

Z-Gly-Pro-Arg-AMC (cat K assay)

no pro cat B act pro cat B

18
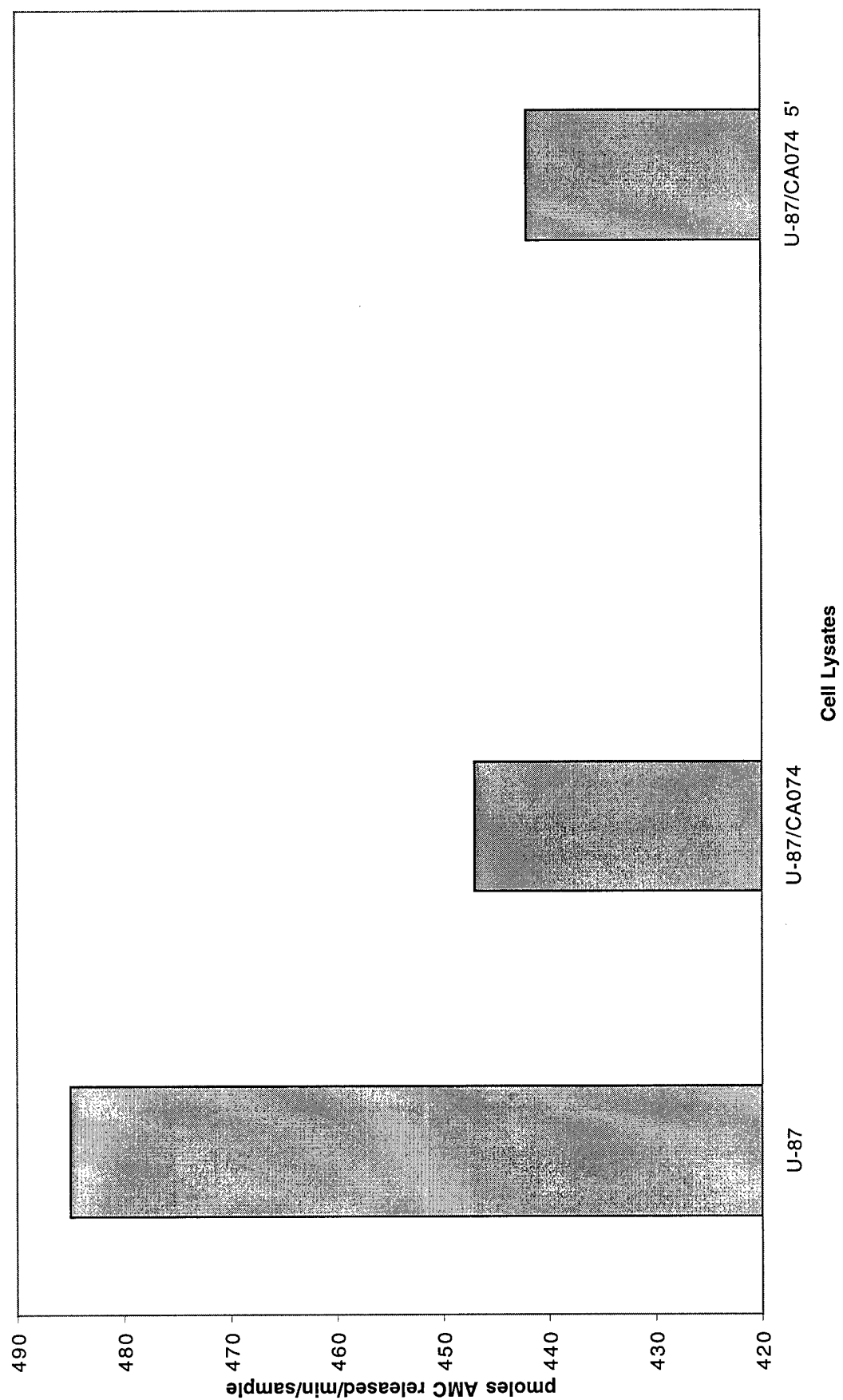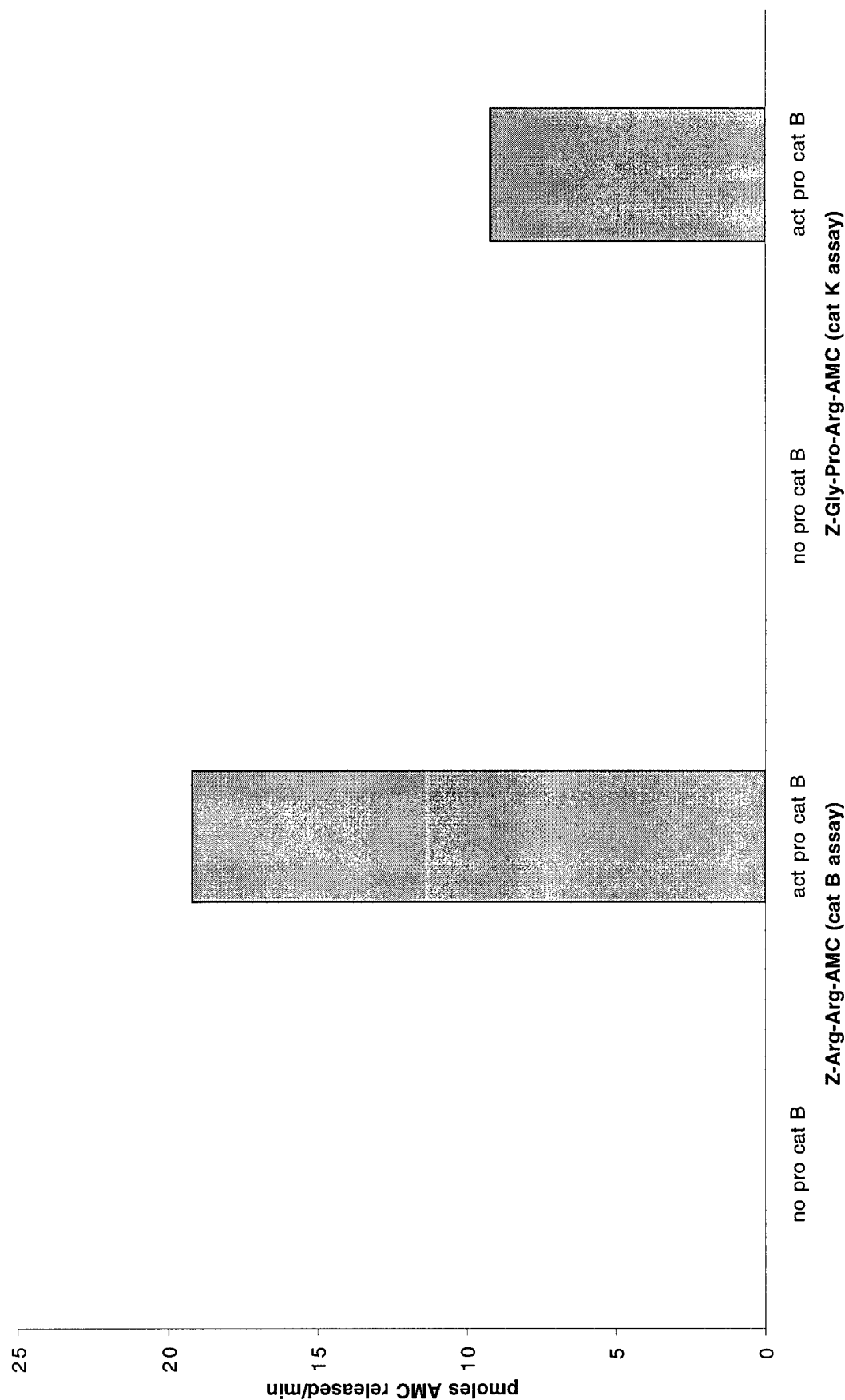
Figure 8

RT-PCR of DU-145 cells

ß-2 Microglobulin   Cathepsin B   Cathepsin K
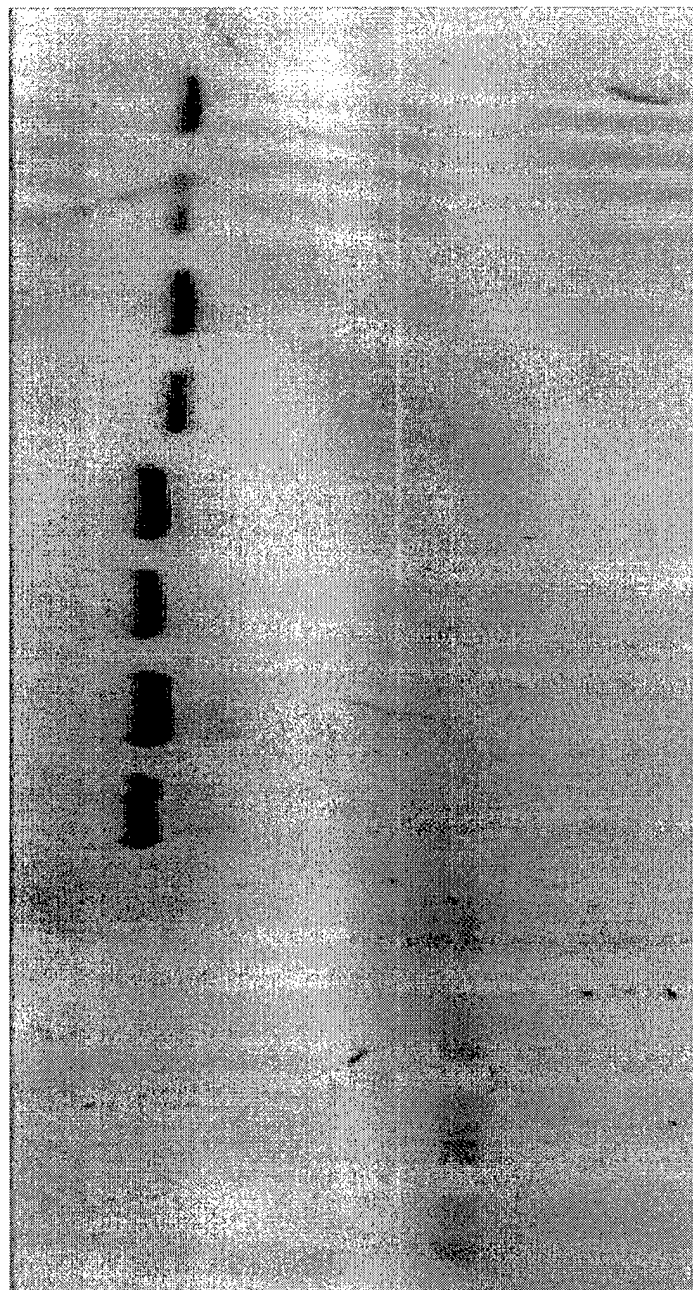
1. DU (P) - on plastic
2. DU (P) - on collagen

3. DU (#4) - on plastic
4. DU(#4) - on collagen

Figure 9

RT-PCR of Cathepsin B in DU145 cells
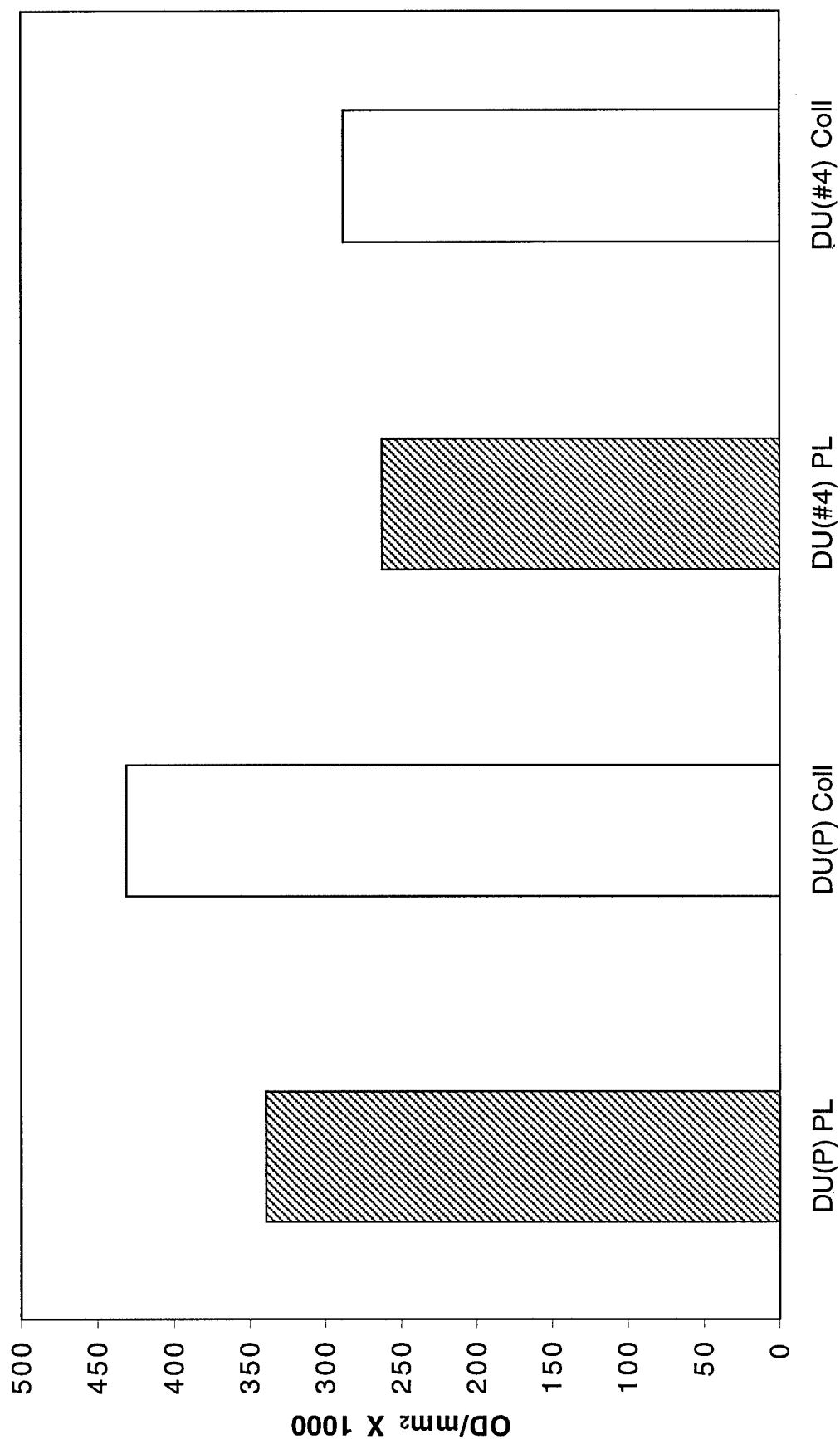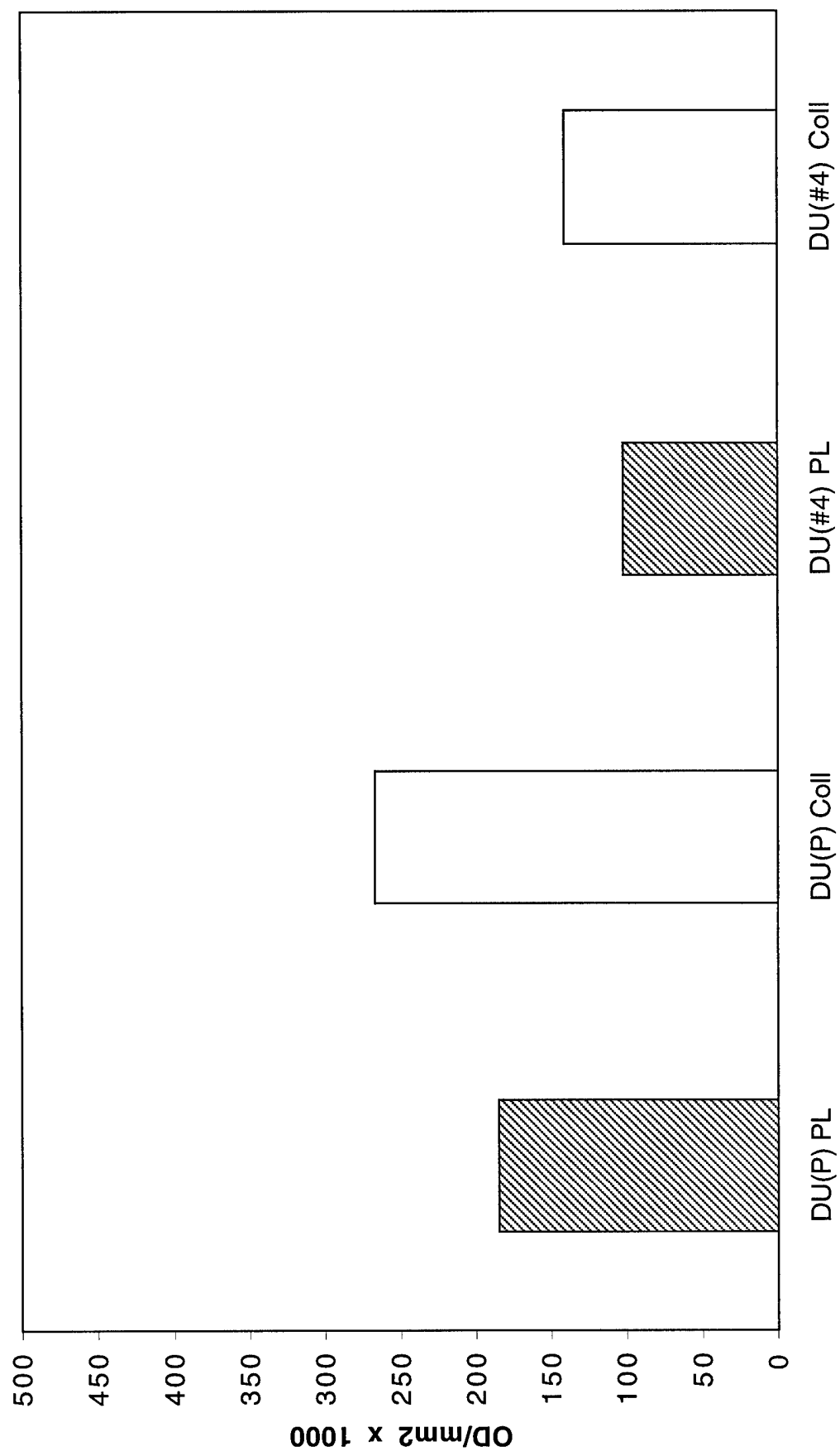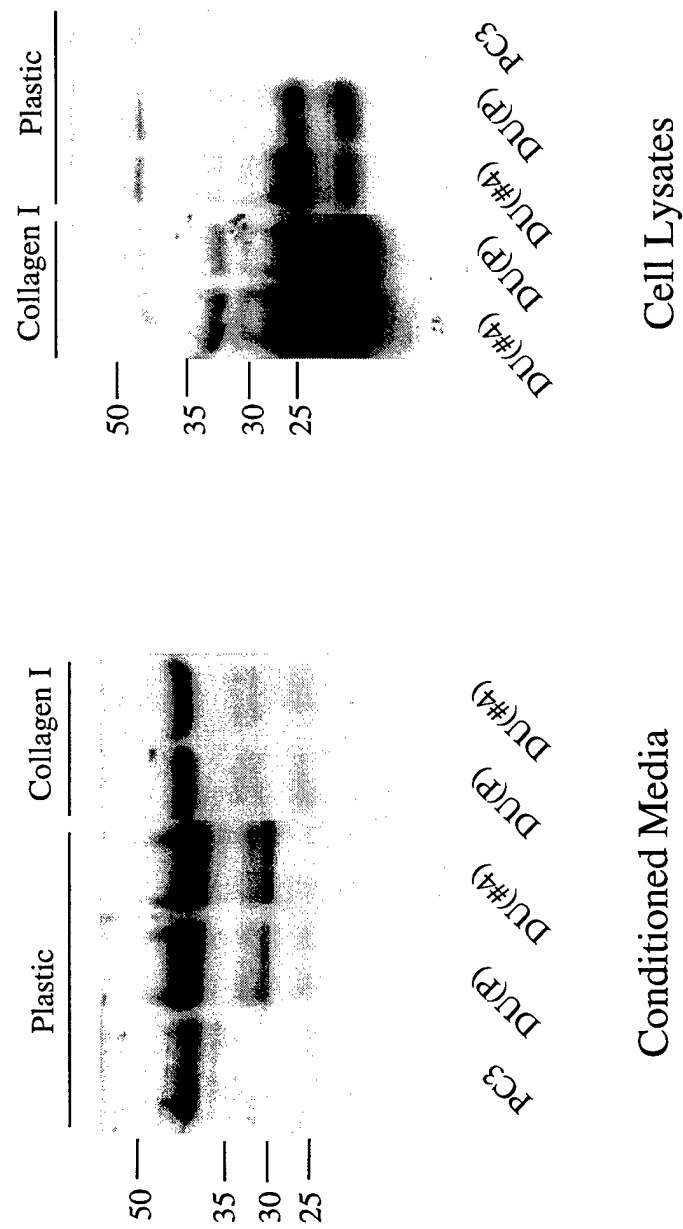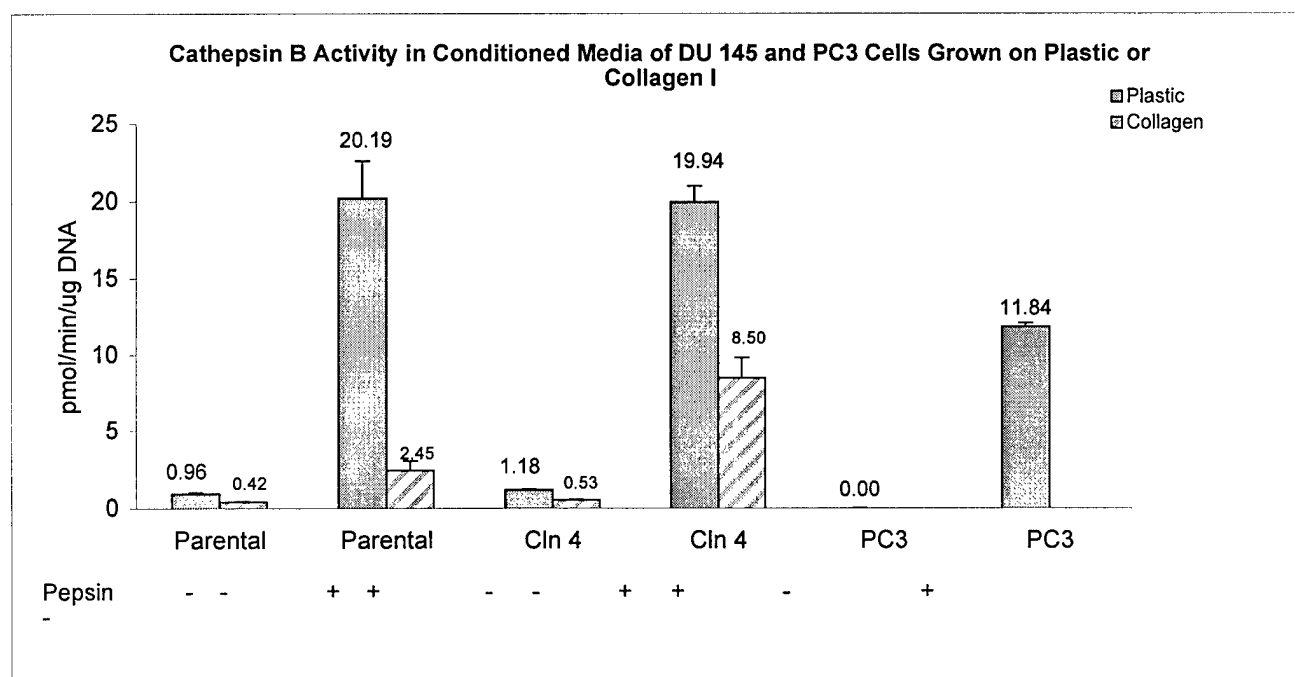
Figure 10

RT-PCR of Cathepsin K in DU145 cells

Figure 11

Western Analysis with anti-Cathepsin B

Conditioned Media

Cell Lysates

Figure 12

Cathepsin B Activity in DU 145 and PC3 Cells Grown on Plastic or Collagen I

	Parental	Parental	Cln 4	Cln 4	PC3	PC3
CA074	– –	+ +	– –	+ +	–	+	–

Cathepsin B Activity in Conditioned Media of DU 145 and PC3 Cells Grown on Plastic or Collagen I

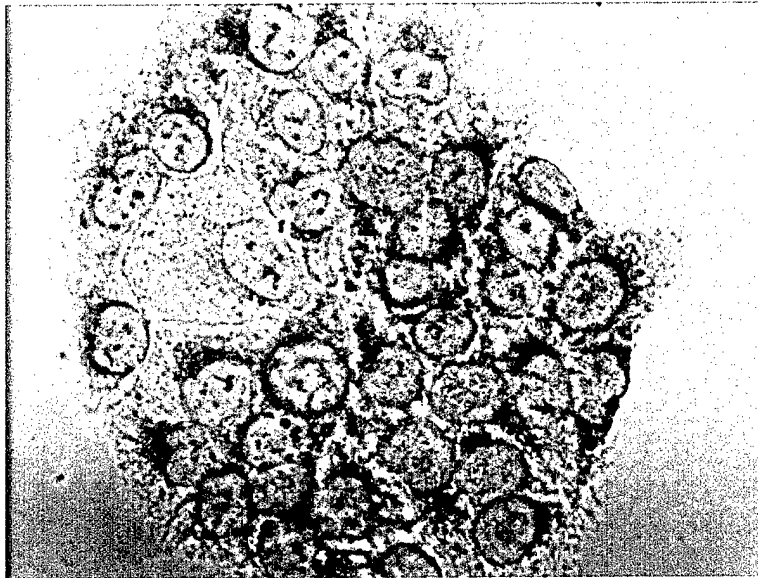|	Parental	Parental	Cln 4	Cln 4	PC3	PC3
Pepsin	– –	+ +	– –	+ +	–	+
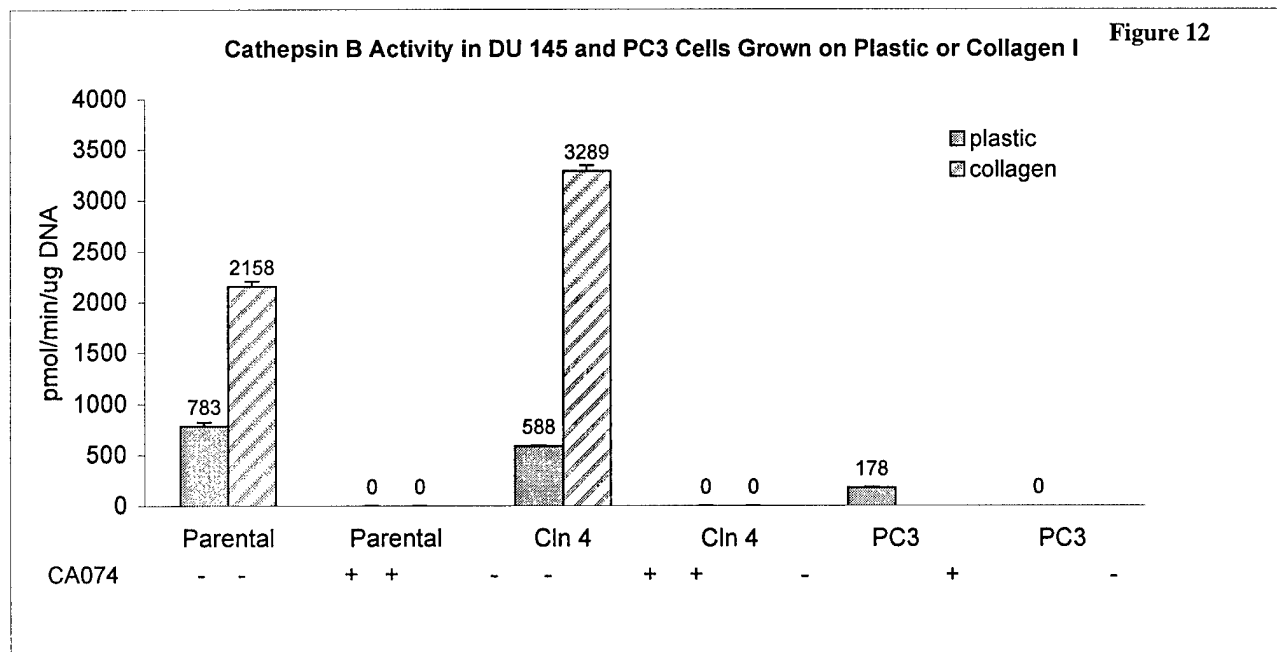
Figure 13
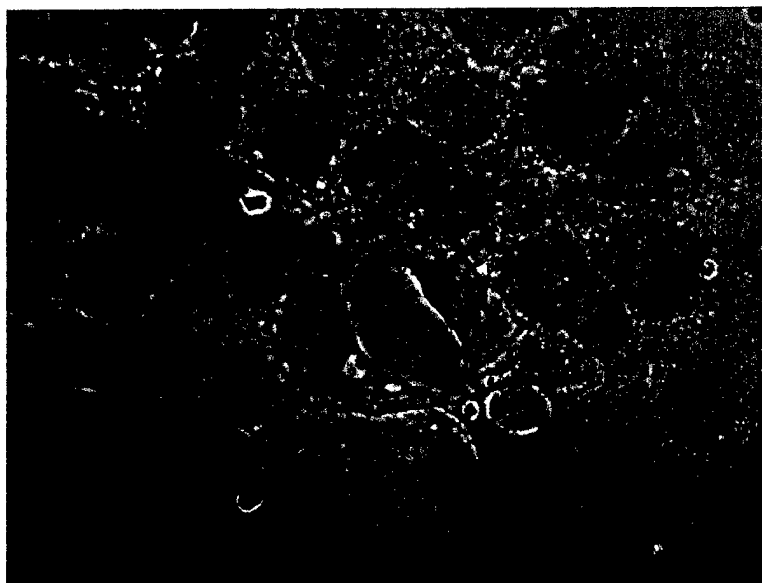
DU145 (EGFP Clone #4)

Plastic
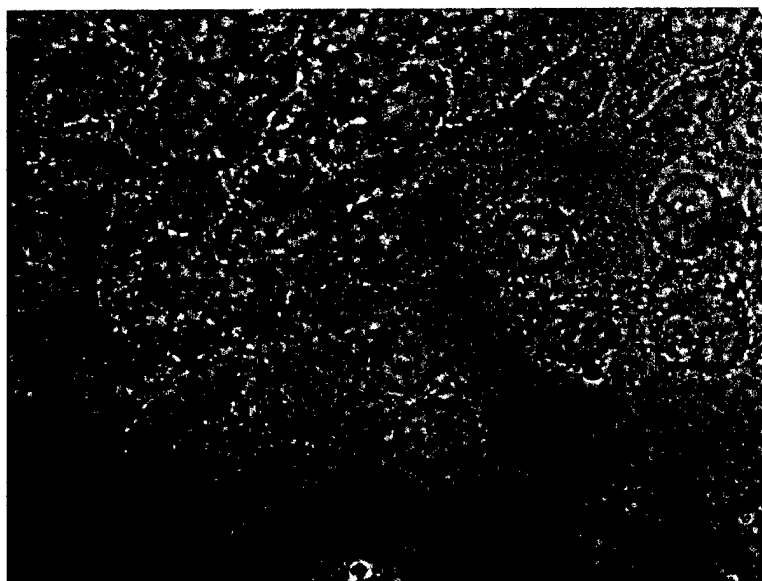
Collagen I

Anti-Cathepsin B

Figure 14

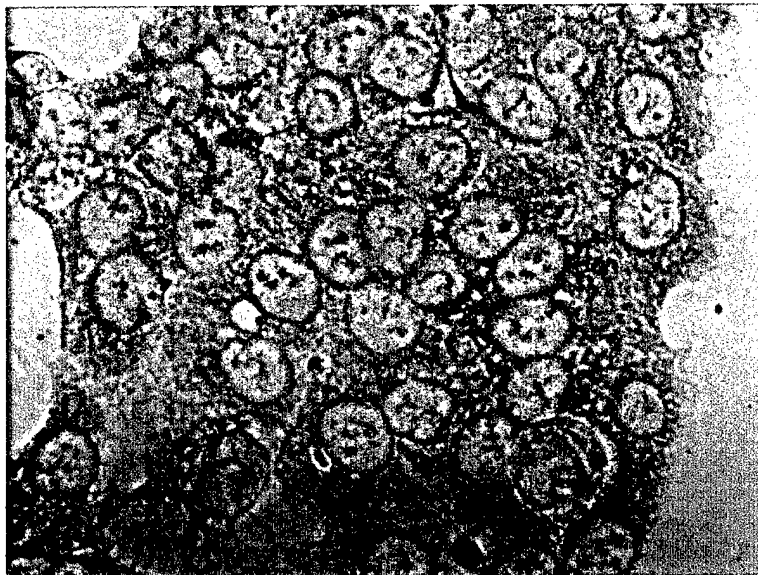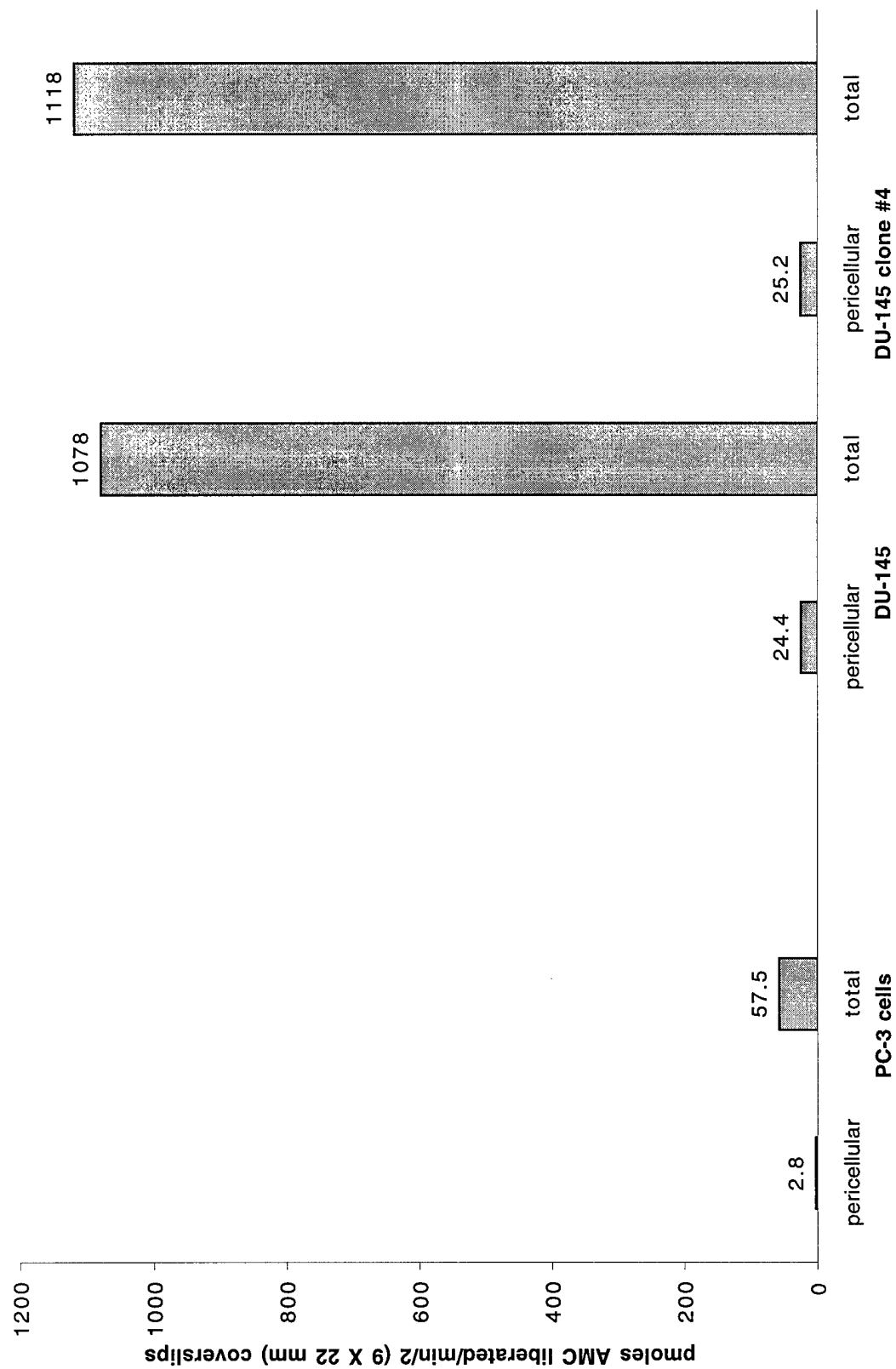## DU145 (EGFP Clone#4)

Axys

Chemicon

Anti-Cathepsin K

Figure 15

Pericellular and Total Cathepsin B in PC-3 and DU-145 cells

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration that interaction of prostate carcinoma cells with collagen I, the organic matrix of bone, increases expression of transcripts for both cathepsin B and cathepsin K.

- Demonstration that interaction of prostate carcinoma cells with collagen I, the organic matrix of bone, increases the expression of cathepsin B protein.

- Demonstration that interaction of prostate carcinoma cells with collagen I, the organic matrix of bone, increases cathepsin B activity.

- Demonstration that interaction of prostate carcinoma cells with collagen I, the organic matrix of bone, reduces secretion of cathepsin B.

REPORTABLE OUTCOMES

- Development of cell lines:

- Stable EGFP-expressing clones of DU145 human prostate carcinoma cells.

CONCLUSIONS

We have made interesting initial observations which indicate that expression in human prostate cancer cells of the two cysteine proteases cathepsins B and K can be induced by collagen I and thus presumably by interaction of the prostate cancer cells with bone during their metastasis. We have made similar observations in breast fibroblasts and in that case shown that ligation of collagen binding integrins is responsible for the induction. If further studies confirm our initial observations, we will need to perform comparable mechanistic studies with the prostate cancer cell lines. We are already analyzing the molecular mechanisms underlying increased expression of cathepsin B in human cancer and thus have genomic clones of the cathepsin B promoter. Combining the present analyses with analyses of the cathepsin B promoter may well be of future interest. The results to date indicate that therapeutic interventions to reduce bone metastasis by human prostate cancers may need to differ from those used against the primary tumors or metastases to other sites.

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APPENDICES

Biosketch for Dr. Radhika Bakhshi, Postdoctoral Fellow.

BIOGRAPHICAL SKETCHES

Provide the following information for the key personnel listed on the budget page for the initial budget period			
NAME Radhika Bakhshi, Ph.D.		POSITION TITLE Post-Doctoral Fellow	
EDUCATION/TRAINING (Begin with baccalaureate or other initial professional education, such as nursing, and include post-doctoral training.)			
INSTITUTION AND LOCATION	DEGREE (IF APPLICABLE)	YEAR(S)	FIELD OF STUDY
University of Delhi, New Delhi, India	B.Sc.	1989-92	Biochemistry
University of Delhi, New Delhi, India	M.Sc	1992-94	Biochemistry
All India Institute of Medical Science, New Delhi, India	Ph.D.	1994-00	Molecular Biology
Wayne State University, Detroit, MI	Postdoc	2000-	

Professional Experience in Prostate Cancer Research: Dr. Bakhshi has not previously worked in prostate cancer research. Her doctoral studies were on the cloning and characterization of human Cathepsin L promoter, a lysosomal cysteine protease, and included studies in glioma cells. Thus, she has the appropriate training to carry out the proposed studies on lysosomal cysteine proteases in metastasis of prostate cancer to bone.

Employment / Experience

2000-present **Post-Doctoral Fellow,** Department of Pharmacology, Wayne State University School of Medicine

Publications

Bakhshi R, Goel A, Chauhan S.S, Cloning and Characterization of human cathepsin L promoter. *Submitted.*

Goel A, **Bakhshi R,** Chauhan S.S., Tissue specific stabilization of human cathepsin L mRNA splice variants by protein interaction with their 5' untranslated region. *In preparation.*