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An Intracellular Form of Cathepsin B Contributes to Invasiveness in Cancer

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ABSTRACT

Cathepsin B is a lysosomal cysteine proteinase whose expression and trafficking are frequently altered in cancer, and plasma membrane and secreted forms are thought to contribute to the invasive and metastatic properties of malignant tumors. We have manipulated the expression of cathepsin B in several tumor cell lines and measured their capacity to invade through a reconstituted extracellular (Matrigel) matrix. Transient expression of human cathepsin B in a poorly metastatic B16F1 murine melanoma variant produced a 3–5-fold increase in cathepsin B activity and a comparable increase in invasiveness. Stable antisense cathepsin B-expressing clones of the highly metastatic human melanoma A375M and prostate carcinoma PC3M cell lines produced 40–50% less cathepsin B than control cells and were proportionately less invasive. In contrast, manipulating cathepsin B levels had no effect on cell migration across an uncoated membrane. The anionic cathepsin B inhibitor (L-3-*trans*-propylcarbamoyloxirane-2-carbony)-L-isoleucyl-L-proline (CA-074), at a concentration of 1 μM, caused a nearly quantitative inhibition of extracellular cathepsin B but had no effect on Matrigel invasion. In contrast, the equally potent but less selective inhibitor, *trans*-epoxysuccinyl-L-leucylamino(4-guanidino)butane (E-64) inhibited invasion by 75%. Surprisingly, at a concentration of 10 μM, CA-074 slowly permeated the cells, causing an 80–95% inhibition of intracellular cathepsin B after 12 h, the duration of the invasion assay. The membrane-permeant cathepsin B inhibitor, CA-074 methyl ester, and the higher concentration of CA-074 that inhibited intracellular cathepsin B both significantly reduced Matrigel invasion. Collectively, these results identify an intracellular role for cathepsin B in matrix degradation. They also indicate that caution should be exercised in assuming that CA-074 is unable to enter cells when it is used to inhibit biological processes of long duration.

INTRODUCTION

The most important characteristic of malignant tumors is their ability to invade surrounding tissues and metastasize. Proteolytic enzymes facilitate the dissemination of tumors by degrading the ECM[2] proteins that form the structural barriers that cells must cross to reach the vasculature (1–3). Cathepsin B is among the candidate proteinases believed to participate in invasion and metastasis. Cathepsin B mRNA or protein is often present in higher amounts in malignant tumors than in normal tissues or benign tumors (4–12). The intracellular trafficking of cathepsin B is frequently altered in malignant tumors, resulting in the increased secretion of precursor (13–15) and active forms of the enzyme (16), its redistribution from perinuclear lysosomes to peripheral vesicles (17), and its association with the plasma membrane (12, 18–23). Numerous clinical studies have confirmed a correlation between cathepsin B expression, disease progression, and clinical outcome for patients with diverse types of tumors (10, 24–29).

It is widely held that invasion is facilitated by a membrane or secreted form of cathepsin B that acts outside the cell to degrade ECM components at or adjacent to the surface of the invading cell. Cathepsin B has a broad pH optimum (16, 30) and is capable of degrading constituents of the ECM and basement membrane, such as type IV collagen, fibronectin, and laminin (31–33). Cathepsin B can also activate both the soluble and tumor cell receptor-bound forms of urokinase plaminogen activator (uPA) and other latent proteinases (34–36). In this study, we selectively manipulated the expression of cathepsin B in three different tumor cell lines. These cells were tested in an *in vitro* Matrigel invasion assay with membrane-permeant and impermeant inhibitors of cathepsin B. The results, which provided direct proof for the participation of cathepsin B in matrix penetration, also yielded the unexpected finding that an intracellular form of cathepsin B was required for Matrigel invasion.

MATERIALS AND METHODS

Construction of Expression Vectors. A cDNA encoding the full human preprocathepsin B was cloned into the multiple cloning site of expression plasmid pcDNA3. PCR was used to introduce *Bam*H I and *Xba* I restriction sites at the opposite ends of the cathepsin B coding region using plasmid phCB79-2 as the template (37). The 5′ primer (5′-GGATCCGCGGCAACCGCTCCGGCA) corresponded to the beginning of exon 1 and contained a flanking *Bam*H I site. The 3′ primer (5′-TCTAGATTAGATCTTTTCCCAGTA) corresponded to the end of exon 11 and contained the stop codon and a flanking *Xba*I site. To insert cathepsin B cDNA into pcDNA3 in the antisense orientation, the *Bam*H I and *Xba*I sites were reversed. All PCR reactions were carried out in a final volume of 100 μl and contained 10 μl of a 10× PCR buffer [200 mM Tris-HCl, 500 mM KCl (pH 8.4)], 1.5 mM $MgCl_2$, a deoxynucleotide triphosphate mixture (0.2 mM each), primers (50 pmol each), 2.5 units of Taq DNA polymerase (Life Technologies, Inc., St. Louis, MO), and 100 ng of the DNA template in distilled H_2O. The mixture was incubated for 3 min at 94°C and subjected to 35–40 cycles of amplification (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min) in a programmable minicycler (MJ Research, Watertown, MA).

Detection of Antisense Cathepsin B mRNA by the RT-linked PCR. Total cellular RNA was isolated using TRI reagent (Sigma, St. Louis, MO) according to the instructions of the supplier and reverse transcribed using Omniscript reverse transcriptase (Qiagen, Valencia, CA) and an oligo(dT) primer (Amersham Pharmacia, Piscataway, NJ). One μg of total RNA in 13 μl of RNase-free water was incubated at 65°C for 10 min and cooled on ice for 5 min. A master mixture that contained 2 μl of 10 × RT buffer (Qiagen), 2 μl of a deoxynucleotide triphosphate mixture (5 mM each), 1 μl of the oligo(dT) primer (10 μM), 1 μl of RNase inhibitor (10 units/μl), and 1 μl of reverse transcriptase was prepared fresh on ice. The RNA template was added to the mix, vortexed for 5 s, and centrifuged briefly. After incubation at 37°C for 60 min, 1 μl of the reaction mixture was removed for amplification by PCR (see above) in a final volume of 50 μl. The 5′ primer (5′-AGCCGTGGTCGCACCACTGCACACCA) corresponded to the minus strand of the cathepsin B near the beginning of exon 2. The 3′ primer (5′-TAGGAAAGGACAGTGGGAGTGGCACC) was complementary to a transcribed sequence in the pcDNA3 vector. These primers generated a 258-bp PCR product specific for the antisense cathepsin B mRNA encoded by the pcDNA3 expression plasmid.

Cell Culture. B16F1, A375M, and PC3M were obtained from I. J. Fidler (M.D. Anderson Cancer Center, Houston, TX) and cultured as described previously (4, 37). The cells were detached from culture flask with a solution of 0.25% trypsin-1 mM EDTA (Life Technologies, Inc., Grand Island, NY) after reaching about 80% confluence. The cells were neutralized with an equal volume of fresh medium supplemented with 10% fetal bovine serum, collected by centrifugation at 4000 × *g*, resuspended in 10 ml of fresh medium, and

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[2] The abbreviations used are: ECM, extracellular matrix; uPA, urokinase-type plasminogen activator; E-64, *trans*-epoxysuccinyl-L-leucylamino(4-guanidino)butane; CA-074, (L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; CA-074Me, (L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline methyl ester; RT, reverse transcription; PET, polyethylene terephthalate.

passaged at a ratio of 1:10. Cells were subcultured approximately once a week for a maximum of three times. For invasion assays, the cells were detached from cell culture dishes with 1 mM EDTA in calcium and magnesium-free HBSS, rinsed with serum-free medium, collected by centrifugation, and resuspended at a concentration of 1.2×10^5 cells/ml in serum-free medium.

Transfection of Tumor Cells. B16F1 cells were seeded in 6-well plates at a density of 1.4×10^5 cells/well. After 24 h, each well was treated with a mixture containing 2 μg of plasmid DNA and 3 μl of Lipofectin reagent (Life Technologies, Inc.) according to the instructions of the supplier. The cells were harvested 36 h after transfection for use in the various assays described below. The stable transfection of PC3M and A375M cells was carried out with the Lipofectin reagent with the following modifications. Cells were seeded in 100-mm plates at a density of 4.0×10^5 cells/plate. After 24 h, each plate was treated with 10 μg of DNA and 20 μl of Lipofectin reagent. Twenty-four h after transfection, the cells were subcultured at a 1:3 ratio in complete MEM supplemented with 600 μg/ml G418. G418-resistant clones were collected after 14–21 days and maintained in culture with G418 as described above.

Cell Invasion and Motility Assays. Invasion assays were performed as described previously, with some modifications (38). Stock solutions (15 mg/ml) of Matrigel basement membrane matrix (Becton Dickinson Labware, Bedford, MA) were stored at 20°C in 100-μl aliquots. After thawing overnight on ice, the stock was diluted 1:50 with ice-cold serum-free culture medium and immediately applied in 100-μl aliquots to each of 6.4-mm PET membrane inserts (8 μm pores) that formed the upper chambers of 24-well multiwell invasion plates. The Matrigel was dehydrated overnight in open plates in a sterile tissue culture hood. The membranes were rehydrated for 2 h with 250 μl of serum-free medium, and the excess medium was removed by careful aspiration. NIH-3T3 cell-conditioned medium (750 μl) was added to the bottom of each well, which formed the lower chamber of the invasion plate. A suspension of 6×10^4 cells in 500 μl of serum-free medium was added to the surface of the Matrigel, and the plates were incubated for 12 h (A375M cells and PC3M cells) or for 24 h (B16F1 cells) at 37°C in a 95% air/5% CO_2 incubator. At the indicated times, the inserts were removed, the medium was discarded, and noninvading cells were removed from the upper surface with a cotton-tipped swab. The inserts were fixed in methanol and stained with Hemacolor (EM Diagnostic Systems, Gibbstown, NJ) according to the supplier's instructions. After drying in air, the membranes were detached from the inserts and fixed to a microscope slide, and the invading cells were counted at a magnification of ×40 under a microscope equipped with a ruled eye piece. The results were expressed as the number of cells present in the center of the field. Cell motility assays were performed in a similar manner except that the 8-μm pore PET membrane insert was not coated with Matrigel, and the duration of the incubation period was shortened. In some experiments, the protease inhibitor E-64, CA-074, or CA074-Me (Peptide Institute, Inc., Osaka, Japan) was added to the invasion chamber at the beginning of the incubation period.

To prepare the chemoattractant used in the lower well of the invasion chamber, NIH-3T3 cells were seeded in a 175-cm^2 flask in MEM supplemented with 10% fetal bovine serum. When cells became approximately 90% confluent, the medium was removed, and cells were washed once with serum-free MEM supplemented with 50 μg/ml ascorbic acid. The cells were then incubated for 24 h with 30 ml of serum-free MEM containing 50 μg/ml ascorbic acid. The conditioned medium was sterile filtered, divided into 5-ml aliquots, and frozen at −70°C. The conditioned medium was thawed and prewarmed to 37°C before use.

Measurement of Cathepsin B Activity and Protein. Media and cells were harvested and processed as described previously (4, 15). Cathepsin B was measured with the fluorogenic substrate N^{α}-CBZ-L-Arg-L-Arg-7-amido-4-methylcoumarin hydrochloride (Sigma) after a 30-min pretreatment with DTT and EDTA (4). The predominant form of cathepsin B secreted by tumor cells is the inactive proenzyme, and conditioned medium that was freed of cells contained no detectable, active cathepsin B (15). The latent cathepsin B precursor in the medium was measured after activation by limited proteolysis with pepsin as described previously (4, 15). In some experiments, the protease inhibitor E-64, CA-074, or CA074-Me was added to cell cultures in serum-free medium for a period equal to the duration of the invasion assay to determine its effect on intracellular active and latent secreted cathepsin B. Protein concentrations in cell lysates were determined by the Bradford method using the Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA) according to the instructions of the supplier.

Table 1 *Cathepsin B activity in B16F1 cells after transient transfection with the pcDNA3-CB expression plasmid*

Cathepsin B activity is the mean ± SE for $n = 3$ in B16F1 cell lysates 36 h after transfection with a human cathepsin B cDNA (B16F1-CB) or with the expression plasmid alone (B16F1-pCDNA3). Values are normalized for the recovery of cell protein. Experiments 1–3 correspond to experiments 1–3 in Fig. 2, and experiment 4 corresponds to motility in Fig. 2.

Experiment	pcDNA3 (nmol/min/mg protein)	pcDNA3-CB (nmol/min/mg protein)	% CB activity relative to control[a]
1	2.1 ± 0.5	7.0 ± 0.5	350
2	2.6 ± 0.2	13.0 ± 0.9	500
3	1.9 ± 0.3	8.0 ± 0.4	400
4	2.3 ± 0.2	9.5 ± 0.7	400

[a] CB, cathepsin B.

RESULTS

Invasiveness of B16F1 Cells That Overexpress Cathepsin B. B16F1 murine melanoma cells of low metastatic potential in an i.v. experimental metastasis assay (4) were transiently transfected with either a pcDNA3-cathepsin B expression plasmid (pcDNA3-CB) or with the empty vector as described in "Materials and Methods." Based on the expression of a β-galactosidase reporter gene, at least 80% of the B16F1 cells took up the exogenous DNA. In four independent experiments (Table 1), cathepsin B specific activity at 36 h after transfection was 3.5–5-fold greater in lysates from cells treated with pcDNA3-CB than in those treated with the empty vector control. Fig. 1 shows a representative experiment performed in triplicate that compares the ability of these cells to invade through the reconstituted Matrigel ECM. The results from three such experiments, summarized in Fig. 2, show a highly significant increase in the invasiveness of cells in proportion to the elevation in level of cathepsin B (experiments 1–3 in Fig. 2 refer to experiments 1–3 in Table 1). In a separate measurement, cathepsin B was found to have no effect on the motility of B16F1 cells when they were seeded on uncoated PET filters (Fig. 2). Consequently, we conclude that cathepsin B is likely to have acted by increasing the ability of B16F1 cells to degrade and penetrate the Matrigel ECM.

Invasiveness of Human Tumor Cell Lines That Express an Antisense Cathepsin B RNA. The human melanoma and prostate carcinoma cell lines A375M and PC3M, which are highly metastatic in an i.v. lung colonization assay (39, 40), were stably transfected with the cathepsin B antisense expression plasmid pcDNA3-aCB or with the empty plasmid as a control. After selection with G418, resistant clones were collected and expanded as described in "Materials and Methods." All clones were assayed for intracellular and secreted cathepsin B in cell lysates and media, respectively. RT-PCR was used to measure the expression of antisense cathepsin B RNA. To distinguish between a possible endogenous antisense RNA (41) and the authentic product of the transgene, the primer set recognized the end of human antisense cathepsin B cDNA (109 bp) and adjacent transcribed sequences from the vector (149 bp). Eight of 15 PC3M clones transfected with pcDNA3-aCB showed some reduction in cathepsin B activity (Table 2) and yielded the expected 258-bp PCR product (Fig. 3). In four of these clones, the reduction in enzymatic activity was statistically significant, ranging from 20–50%. Cell lines that expressed the highest amounts of the antisense message showed the largest decrease in cathepsin B activity (Table 2 and Fig. 3). These cells were stable when passaged in the presence of G418. In contrast, only 2 of the 10 A375M clones resistant to G418, A375M-aCB1 and A375M-aCB5, showed significant reductions in cathepsin B levels of about 25% and 40%, respectively. In addition, A375M-aCB1 soon lost the antisense cathepsin B transgene and reverted to control levels of cathepsin B expression. Such a loss of a transgene in cells grown in the presence of G418 has been described previously (42). Repeated measurements of cathepsin B activity in A375M-aCB5 revealed a

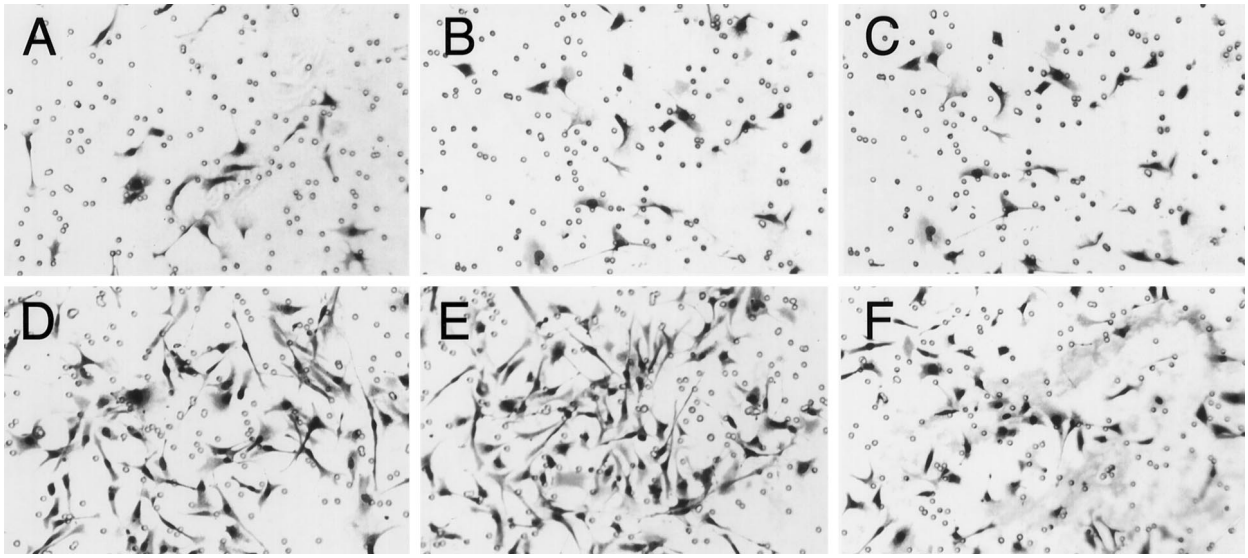

Fig. 1. Invasion of Matrigel by B16F1 cells transiently transfected with cathepsin B expression plasmid, pcDNA3-CB, or with the empty vector, pcDNA3. Cells were collected 36 h after transfection, dispersed in calcium and magnesium-free HBSS, and applied to the upper surface of a Matrigel-coated PET filter (30 μg/filter) in a modified Boyden invasion chamber as described in "Materials and Methods." After 24 h at 37°C in an atmosphere of 5% CO_2/95% air, the upper surface of the filter was scrubbed free of cells and Matrigel, the filter was fixed and stained, and the lower surface was photographed at a magnification of ×200. *A–C* show cells transfected with the empty vector control. *D–F* were obtained with cells transfected with pcDNA3-CB.

stable decrease in the intracellular and secreted forms of cathepsin B compared to the control (Fig. 4*A*) and a persistent expression of the antisense cathepsin B (Fig. 4*B*). Interestingly, qualitative RT-PCR detected no difference in cathepsin B mRNA between control clones containing the empty vector and clones expressing the antisense RNA in A375M and PC3M cells (data not shown). This suggests that the antisense cathepsin B RNA acted at a posttranscriptional step to decrease cathepsin B expression.

Matrigel invasion assays demonstrated that down-regulation of cathepsin B reduces the capacity of the human prostate and melanoma cell lines to invade across the reconstituted Matrigel basement membrane. The results for clones PC3M-aCB9 and A375M-aCB5 are shown in Fig. 5 and clearly demonstrate that cathepsin B contributes to the invasive potential of these cells. A similar inhibition of invasion was observed for clone PC3M-aCB13 when compared to the empty vector control (data not shown). Decreasing cathepsin B had no effect on the ability of PC3M and A375M cells to traverse an uncoated PET filter, indicating that cathepsin B levels do not influence motility in these cells.

Table 2 *Cathepsin B activity in cell lysates and media from PC3M clones transfected with a human antisense cathepsin B cDNA*

Cathepsin B activity was measured in cell lysates and serum-free media. The activity of secreted procathepsin B was measured after activation with pepsin (4, 15). The values are normalized for cellular protein and are expressed as the mean ± SE of three determinations. Statistical significance was determined by the Student-Newman-Keuls multiple comparison test.

	Cell lysates		Media	
Clone	nmol/min/mg protein	% Control	nmol/min/mg protein	% Control
PC3M-pcDNA3	14.0 ± 0.2	100	1.00 ± 0.02	100
PC3M-aCB1	12.2 ± 0.2[a]	87	0.80 ± 0.01[a]	80
PC3M-aCB8	13.0 ± 0.2	93	0.90 ± 0.05	90
PC3M-aCB9	7.8 ± 0.1[a]	56	0.60 ± 0.02[a]	60
PC3M-aCB7	13.4 ± 0.2	96	0.90 ± 0.06	90
PC3M-aCB5	13.0 ± 0.2	93	0.90 ± 0.05	90
PC3M-aCB10	9.0 ± 0.3[a]	64	0.70 ± 0.03[b]	70
PC3M-aCB6	13.0 ± 0.2	93	0.90 ± 0.04	90
PC3M-aCB13	7.1 ± 0.1[a]	51	0.50 ± 0.02[a]	50

[a] $P < 0.001$.
[b] $P < 0.01$.

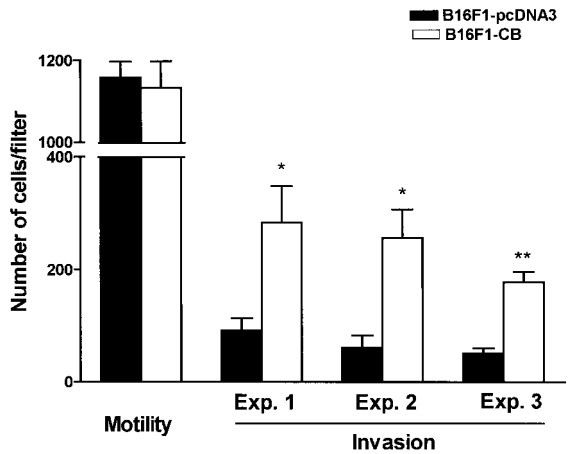

Fig. 2. Effect of increased cathepsin B expression on the motility and invasiveness of B16F1 murine melanoma cells. Motility was measured with uncoated 8-μm-pore PET filter inserts, and B16F1 cells were transfected with pcDNA3 or pcDNA3-CB (experiment 4, Table 1). After 6 h, the cells on the underside of the filter were counted. Invasion was measured with Matrigel-coated filter inserts as described in the Fig. 1 legend. Experiments 1–3 correspond to experiments 1–3 in Table 1. Each *bar* represents the mean ± SE of three independent measurements. *, statistical significance at $P < 0.05$; **, statistical significance at $P < 0.005$.

Effects of Specific Inhibitors on Cathepsin B Activity and Invasion. The molecular genetic manipulations in this study altered the levels of both the intracellular and secreted forms of cathepsin B. We therefore sought to use the cathepsin B-specific inhibitors, CA-074 and its membrane permeant analogue, CA-074Me, to distinguish between extracellular and intracellular forms of cathepsin B (43). However, because invasion assays are often performed over periods of 8–24 h, we thought it necessary to confirm the selectivity of the two inhibitors at longer incubation times. PC3M and A375M cells were incubated with 0, 1, or 10 μM CA-074 or CA-074Me in serum-free medium for 12 h at 37°C in an atmosphere of 5% CO_2/95% air. The cell monolayer was thoroughly washed, and the cells were collected and resuspended in extraction buffer. Measurements of cathepsin B activity in cell lysates revealed a concentration-dependent inhibition by CA-074, a presumptive nonpermeant cathepsin B inhibitor. With 10 μM CA-074, inhibitions ranged from about 80% in B16F1 cells to >90% in PC3M and A375M cells (Table 3). At a concentration of 1

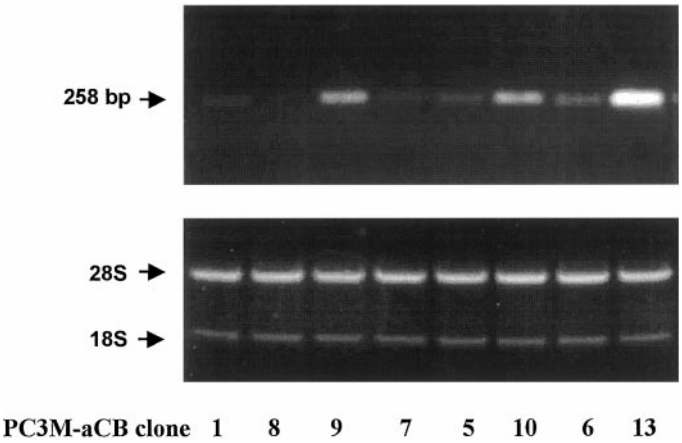

Fig. 3. Levels of antisense cathepsin B mRNA in stable clones of PC3M cells. One μg of total RNA from G418-resistant clones transfected with pcDNA3-aCB was reverse transcribed and amplified as described in "Materials and Methods." The *top panel* shows the expected 258-bp PCR product derived from the cathepsin B antisense RNA that was detected in all the clones. The product was not detected after amplification of RNA from wild-type PC3M cells or from PC3M cells transfected with the control plasmid (data not shown). The *bottom panel* shows the electrophoresis of total RNA used in the RT-PCR reaction and demonstrates that there were no significant differences in the amounts and integrity of the RNA. The *top* and *bottom* bands correspond to 28S and 18S rRNA, respectively.

A

Cells	Intracellular CB (*nmol/min/mg protein*)	% Control	Secreted CB (*nmol/min/mg protein*)	% Control
A375M-pcDNA3	24 ± 0.3	100	2.0 ± 0.1	100
A375M-aCB5	16 ± 0.4[a]	66	1.2 ± 0.1[b]	60

B

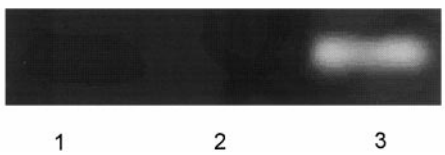

Fig. 4. Levels of intracellular cathepsin B, secreted latent cathepsin B, and antisense cathepsin B RNA in clone A375M-aCB5. A375M cells were transfected with expression plasmid pcDNA3 containing an antisense cathepsin B cDNA (A375M-aCB5) or with the empty vector (A375M-pcDNA3). *A*, cathepsin B activity in cell lysates and serum-free media is expressed as the mean ± SE of three separate determinations. Secreted cathepsin B was measured after pepsin treatment to activate the proenzyme. Statistically different from control at $P < 0.001$ ([a]) and $P < 0.005$ ([b]). *B*, RT-PCR was used to measure cathepsin B antisense RNA in clone A375M-aCB5. One μg of total cellular RNA was reverse transcribed and amplified as described in "Materials and Methods." The expected 258-bp PCR product was detected with clone A375M-aCB5 (*Lane 3*), but not with the control clone after stable transfection with the empty plasmid (*Lane 1*) or from wild-type cells (*Lane 2*).

μM, CA-074 reduced intracellular cathepsin B activity to a lesser extent (Table 3). Both concentrations of CA-074 essentially produced a near quantitative inhibition of the secreted, latent, pepsin-activatable form of cathepsin B in the culture medium (Table 3).

To further examine CA-074 effects on whole cells, we determined the time course for inhibition of intracellular cathepsin B in PC3M cells with 10 μM CA-074 (Fig. 6). At the indicated times, the medium was removed, the cell monolayer was washed repeatedly to remove excess inhibitor, and cathepsin B was measured in the cell lysates. Fig. 6 shows a significant reduction in the activity of intracellular cathepsin B over time in PC3M cells with 10 μM CA-074. In the absence of inhibitor, no change in cathepsin B activity was observed during the same period. Taken together, the results in Table 3 and Fig. 6 indicate that CA-074, although negatively charged at physiological pH, is slowly internalized by cells in a concentration-dependent manner. In contrast to CA-074, the general cysteine proteinase inhibitor, E-64, had no effect on intracellular cathepsin B activity in all three cell lines studies, even after a 12-h exposure to the inhibitor (data not shown).

Various concentrations of CA-074, CA-074Me, or E-64 were added together with tumor cells to the upper well of the invasion chamber to determine the nature and location of proteases that may be involved in ECM invasion. In some experiments, the cells had been genetically manipulated to alter cathepsin B expression. The results are summarized in Table 4. CA-074Me, at a concentration of 10 μM, produced a near quantitative inhibition of intracellular cathepsin B and inhibited Matrigel invasion by 50–75%, depending on the cell line. CA-074 produced divergent effects on invasion, depending on the concentration. At 1 μM, a concentration that produced a >95% inhibition of extracellular cathepsin B but only partially affected the intracellular enzyme (Table 3), CA-074 actually increased the number of invading cells. This increase, which ranged from 30–90%, depending on the cell line, was highly significant. At a concentration of 10 μM, CA-074 significantly inhibited invasion to a similar extent as CA-074Me. This effect of 10 μM CA-074 was presumably due to its inhibition of intracellular cathepsin B. E-64, a general inhibitor of extracellular cysteine proteinases, strongly inhibited (about 75%) the ability of PC3M cells to penetrate the reconstituted ECM when present at concentrations of 1 and 5 μM. Because E-64 and CA-074 are equally effective inhibitors of cathepsin B (Refs. 43 and 44; Table 3), the observation that 1 μM CA-074 and E-64 have opposite effects implies the participation of a second, non-cathepsin B-like proteinase acting outside the cell to promote Matrigel invasion. CA-074, CA-074Me, and E-64 had no effect on cell migration across uncoated PET filters

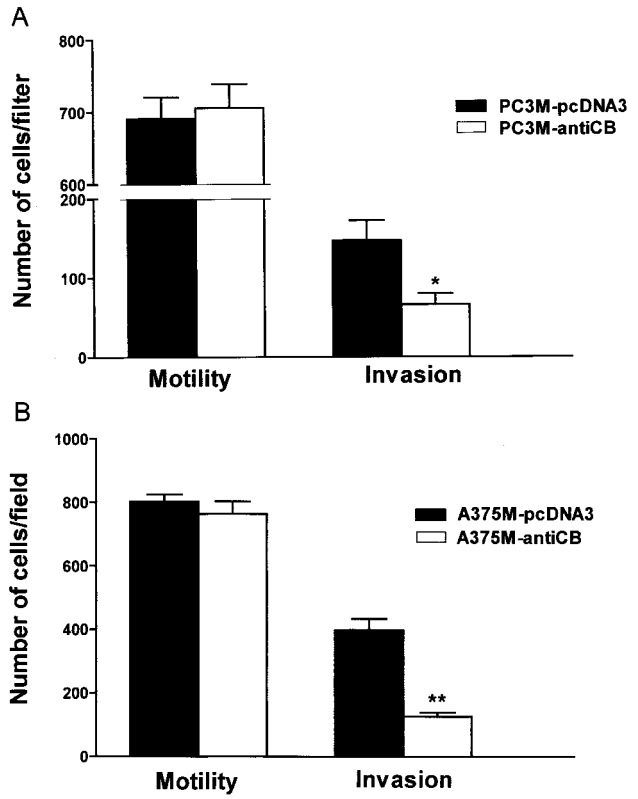

Fig. 5. The effect of decreased cathepsin B expression on motility and invasiveness of human tumor cells. Cells were stably transfected with pcDNA3 plasmid (control) or with pcDNA3 plasmid containing cathepsin B cDNA oriented in the antisense direction. Motility was measured after 6 h by counting the number of cells that traversed an uncoated PET filter. Invasion was measured after 12 h by counting the number of cells that traversed a PET filter coated with Matrigel (30 μg/filter). The *bars* represent the mean ± SE of nine separate determinations. *, statistical significance at $P < 0.005$, **, statistical significance at $P < 0.0001$.

at any of the concentrations studied (data not shown), indicating that their target proteases do not contribute to cell motility.

DISCUSSION

Previous evidence linking cathepsin B to tumor cell invasion and metastasis is substantial but mostly correlative and therefore indirect. Recently, two groups have used molecular genetic techniques to manipulate cathepsin B levels in established tumor cell lines. Stable transfection of human cathepsin B cDNA into a murine squamous cell carcinoma resulted in a nearly 3-fold increase in the level of secreted procathepsin B and a 22% increase in invasiveness across a Matrigel reconstituted basement membrane (45). Conversely, stable transfection of a human osteosarcoma with an antisense cathepsin B cDNA resulted in a 50–60% reduction in cathepsin B activity and a 25–40% inhibition of invasiveness in the Matrigel assay (46). Here we have extended those results by demonstrating that cathepsin B contributes to invasive potential in three additional, well-characterized tumor lines. Transient transfection of a poorly metastatic B16F1 melanoma (4) with human cathepsin B cDNA caused a 3.5–5-fold increase in cathepsin B activity and a 3–4-fold increase in invasiveness when both were measured 36 h later. Similarly, stable clones derived from the metastatic human prostate PC3M and melanoma A375M cell lines (39, 40) transfected with an antisense human cathepsin B cDNA displayed a 35–45% decrease in cathepsin B activity and a 55–65% decrease in Matrigel invasion. Because invasiveness through Matrigel is often correlated with metastatic potential *in vivo* (47–50), these data are further confirmation of a causal relationship between increased levels of cathepsin B and malignancy. Our inability to affect cell migration across an uncoated filter indicates that cathepsin B and other cysteine proteinases act by promoting matrix degradation and have little effect on cell motility *per se* in this system.

The cells in these studies were chosen because their potential to colonize the lung and other organs in an experimental assay of metastasis can be correlated with their level of expression of cathepsin B. In a previous comparison of three B16 melanoma variants, we observed large differences in cathepsin B mRNA and activity that were proportional to metastatic potential. B16F1 produced the least cathepsin B and the smallest number of lung colonies in an experimental assay of metastasis (4). Similarly, the metastatic human tumor cell lines, A375M and PC3M, express more (2–4-fold) cathepsin B mRNA than the parental cell lines from which they were derived (37 and unpublished observations). Therefore, we reasoned that these cells could be particularly sensitive to manipulation of cathepsin B expression. In agreement, we observed larger changes in invasiveness than were seen in the previous cathepsin B overexpression study (45).

Conflicting results are reported for the contributions of cysteine proteinases to cell motility. Exposure of human transitional carcinoma cells to E-64 resulted in a dose-dependent reduction in motility (51). E-64 at a concentration of 100 μM inhibited the motility of A2058 melanoma cells by 50% and the motility of W256 carcinosarcoma

Table 3 *Inhibition of cathepsin B by cysteine protease inhibitors*

Cells were incubated in serum-free medium for 12 h with or without the indicated concentration of inhibitor. After the cell monolayer was thoroughly washed, cell lysates and media samples were collected, processed, and analyzed for cathepsin B activity as described in "Materials and Methods." Values are normalized for cell protein and are the mean ± SE of three determinations.

	Cell lysate		Medium	
Treatment	nmol/min/mg	% Control	nmol/min/mg	% Control
B16F1 cells				
None	2.60 ± 0.10	100	0.082 ± 0.007	100
CA-074, 1 μM	1.90 ± 0.08	73	0.0013 ± 0.0003	2
CA-074, 10 μM	0.46 ± 0.03	18	0.0005 ± 0.0001	<0.01
CA-074Me, 1 μM	0.36 ± 0.04	14		
CA-074Me, 10 μM	0.08 ± 0.01	3		
None	3.00 ± 0.05	100		
E-64, 1 μM			0.0063 ± 0.0009	8
E-64, 5 μM	2.90 ± 0.07	97		
E-64, 10 μM			0.0057 ± 0.0012	7
PC3M cells				
None	2.14 ± 0.20	100		
CA-074, 1 μM	1.13 ± 0.20	53		
CA-074, 10 μM	0.15 ± 0.03	7		
CA-074Me, 1 μM	ND[a]	0		
CA-074Me, 10 μM	ND	0		
None	4.20 ± 0.07	100		
E-64, 5 μM	4.40 ± 0.10	105		
A375M cells				
None	7.00 ± 0.03	100		
CA-074, 1 μM	4.50 ± 0.30	64		
CA-074, 10 μM	0.40 ± 0.04	6		
CA-074Me, 1 μM	0.020 ± 0.003	<0.01		
CA-074Me, 10 μM	ND	0		
None	10.00 ± 0.10	100		
E-64, 5 μM	9.80 ± 0.10	98		

[a] ND, not detectable.

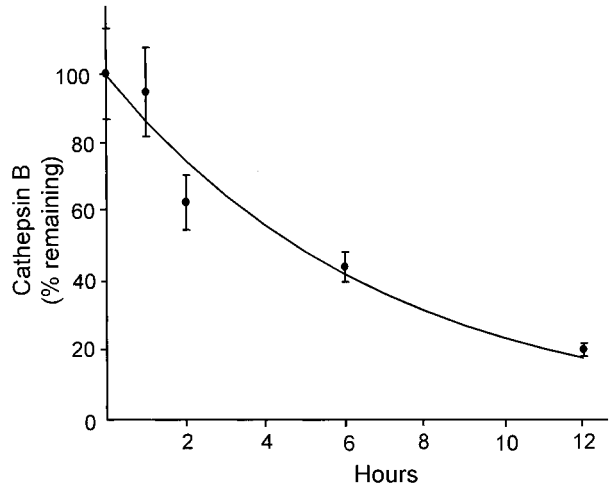

Fig. 6. Time-dependent inhibition of intracellular cathepsin B by 10 μM CA-074 in PC3M cells. The cells were incubated in serum-free media with or without 10 μM CA-074. At the indicated times, the medium was removed, the cell monolayer was washed repeatedly to remove excess inhibitor, and the cells were lysed and assayed for cathepsin B activity as described in "Materials and Methods." The *ordinate* is the relative cathepsin B activity expressed as percentage of the control. The curve was calculated for a first-order decay with a half-life of 4.8 h.

Table 4 *Effect of cysteine protease inhibitors on the invasion of Matrigel by tumor cells*

Matrigel invasion was determined in the absence (control) or presence of the indicated inhibitor as described in "Materials and Methods." The values are the mean ± SE of the indicated number of replicate determinations. Statistical significance was determined by the Student-Newman-Keuls multiple comparison test.

Cells	Inhibitor	Invasion (% of control)
B16F1-pCDNA3	1 μM CA-074	169.4 ± 8.6[a] (n = 3)
	10 μM CA-074Me	42.2 ± 5.7[a]
B16F1-CB[b]	1 μM CA-074	152.0 ± 14.8[c] (n = 6)
	10 μM CA-074Me	44.3 ± 4.0[c]
PC3M	1 μM CA-074	131.7 ± 4.5[c] (n = 9)
	10 μM CA-074	71.5 ± 4.0[c]
	10 μM CA-074Me	51.6 ± 3.5[c]
	1 μM E-64	24.5 ± 2.5[c]
	5 μM E-64[d]	27.8 ± 2.7[c]; 23.2 ± 2.8[c]
A375M-pCDNA3	1 μM CA-074	158.9 ± 13.4[e] (n = 6)
	10 μM CA-074Me	22.6 ± 4.3[c]
A375M-aCB5	1 μM CA-074	198.5 ± 37.9[c] (n = 6)
	10 μM CA-074Me	27.3 ± 6.2[a]

[a] $P < 0.05$.
[b] In this experiment, cells transfected with human cathepsin B (B16F1-CB) were 2.7-fold more invasive than the empty vector control (B16F1-pcDNA3).
[c] $P < 0.001$.
[d] Two independent experiments, each with n = 9 replicates.
[e] $P < 0.01$.

cells by 70% (52). Krueger *et al.* (46) observed a 25–50% inhibition of motility by human osteosarcoma cells that expressed an antisense cathepsin B. In contrast, and similar to our results, cathepsin B and cysteine proteinase inhibitors did not alter the motility of murine SCC-VII squamous carcinoma cells through uncoated filters but did affect their invasiveness through Matrigel (45). Similarly, high concentrations of E-64 inhibited the invasiveness but not the motility of human malignant melanoma (LOX) cells (53).

It is generally assumed that cathepsin B exerts its effects by acting outside the invading cell to degrade ECM components adjacent to the cell surface. This conclusion is based on the following observations. The intracellular trafficking of cathepsin B is frequently altered in transformed cells, which can result in the secretion of precursor and active forms of the enzyme (14–16, 54) and their association with the plasma membrane (21, 23). Inhibitors of cathepsin B that are thought to be unable to permeate cells are found to suppress experimental metastasis *in vivo* (55–57) and invasion *in vitro* (35, 45, 51, 53, 58). In addition, cathepsin B is capable of degrading specific ECM components (31–33, 59) and activating extracellular proteinases, such as soluble and receptor-bound pro-uPA and pro-stromelysin (34–36). However, most of these observations either lack the force of proof or are subject to alternate interpretations. For example, invasion studies using the presumptive extracellular cathepsin B-specific inhibitor, CA-074, do not always report measuring intracellular cathepsin B to confirm a lack of effect. Indeed, there are many reports of inhibition of intracellular cathepsin B by the related inhibitor, E-64 (for example, Ref. 56).

CA-074Me is a membrane-permeant pro-inhibitor, which, after internalization and conversion to CA-074, inactivates intracellular cathepsin B (43). At a concentration of 10 μM, CA-074Me completely abolished intracellular cathepsin B activity and produced a 45–75% inhibition of Matrigel invasion under similar conditions, suggesting an important intracellular function for cathepsin B in matrix degradation. In contrast, CA-074 produced divergent effects depending on concentration. At 1 μM, a concentration previously shown to produce a modest increase in intracellular cathepsin B in fibroblasts (43), CA-074 consistently and substantially increased the invasiveness of the three tumor cell lines in this study. At 10 μM, CA-074 had the opposite effect of decreasing invasiveness. This result was surprising because 1 and 10 μM CA-074 were equally effective in neutralizing the extracellular cathepsin B activity generated by activating the latent cathepsin B precursor (Ref. 4; Table 3). However, activity measurements revealed that CA-074 also reduced intracellular cathepsin B activity in the three cell lines in a time- and concentration-dependent manner. With 10 μM CA-074, the half-life for inhibition of intracellular cathepsin B in PC3M cells was about 5 h, which is less than the duration of the Matrigel invasion assay (12–24 h). However, at a concentration of 1 μM, CA-074 predominantly inhibited extracellular cathepsin B (Table 3). In contrast to CA-074, E-64, which has a broader range of inhibitable targets, reduced invasion but had no effect on intracellular cathepsin B activity at the two concentrations studied (Tables 3 and 4). E-64 also had no effect on cell motility. Thus, inhibition of invasion but not motility by E-64 indicates that one or more cysteine proteinases other than cathepsin B can act outside the cell to promote matrix degradation.

The difference in the ability of CA-074 and E-64 to inhibit intracellular cathepsin B might seem surprising because both compounds are ionic at pH 7. However, structural differences between these inhibitors can account for their behaviors. CA-074 is a monocarboxylic acid, and at pH 7, the ionic form of CA-074 is in equilibrium with a small but appreciable amount of the neutral carboxylic acid form (about 0.1% of the total), which should be similar to CA-074Me in its ability to permeate the cell (Eq. A).

$$CA\text{-}074^{-1}_{out} + H^{+}_{out} \rightleftarrows Ca\text{-}074^{0}_{out} \rightleftarrows CA\text{-}074^{0}_{in} \rightleftarrows Ca\text{-}074^{-1}_{in} + H^{+}_{in} \quad (A)$$

Therefore, the initial difference in the concentration of CA-074 across the plasma membrane could drive a slow uptake of inhibitor through the neutral intermediate. In contrast, E-64 contains in addition a guanidinium group, pKa > 12, so that the concentration of the neutral, membrane-soluble form of E-64 is vanishingly small (less than 10^{-6}% of the total).

Our conclusion that some cultured cells can slowly take up CA-074 may be in disagreement with a recent report by Bogyo *et al.* (60) that indicates that the anionic CA-074 derivatives, CA-074b or MB-074, at concentrations as high as 100 μM, have no effect on cathepsin B in cultured mouse dendritic cells. Similarly, these authors report that periodic i.p. injections of CA-074b in mice over a period of 2 days did not decrease cathepsin B in isolated spleen cells from treated animals. However, in the cell culture experiment, the duration of exposure of the cells to the inhibitors was not reported. In addition, the *in vivo* experiment did not eliminate the possibility of differences in the sensitivity of various tissues to CA-074. Thus, Maekawa *et al.* (61) reported that periodic peritoneal injection of CA-074 decreased cathepsin B activity in peritoneal macrophages and liver by about 60–80% but only decreased cathepsin B in a peripheral lymph node by 20–30%. In any case, we believe the observed time- and concentration-dependent inhibition of cathepsin B in cultured cells by CA-074 is best explained by a slow uptake of the inhibitor.

Our observation that 10 μM CA-074 inhibits the migration of tumor cells across a Matrigel barrier is similar to the result of Coulibaly *et al.* (45), except that we attribute this inhibition to the inactivation of intracellular cathepsin B by 10 μM CA-074. We base this conclusion on the failure of 1 μM CA-074 to inhibit invasion, although it is effective at neutralizing any extracellular cathepsin B activity that can be generated from the pool of latent cathepsin B precursor present in conditioned medium (Table 3). It might be argued that the inability of 1 μM CA-074 to inhibit invasion is due to a kinetic effect. At the lower inhibitor concentration, the rate of the irreversible inactivation of extracellular cathepsin B is too slow to prevent some of the newly exposed enzyme from degrading matrix component(s) in contact with the invading cell. However, the observation that 1 μM E-64 is strongly inhibitory argues for the participation of other extracellular cysteine proteinase(s) in Matrigel invasion. Thus, E-64 and CA-074 are similarly reactive against cathepsin B, with second-order inhibition rate constants of 8.94×10^4 M^{-1}s^{-1} and 11.2×10^4 M^{-1}s^{-1}, respectively (43, 44). If 1 μM E-64 had targeted an extracellular form of cathepsin B in invasion, then 1 μM CA-074 should have also been inhibitory. Furthermore, the kinetic argument does not explain the stimulation of invasion that occurs with 1 μM CA-074 (see below).

Our findings on the effects of cysteine proteinase inhibitors on tumor cell invasion resemble those of Hill *et al.* (62) and others (63, 64) in studies of bone resorption by osteoclasts. Specifically, bone resorption was inhibited by CA-074Me and E-64, but not by CA-074. Thus, an intracellular form of cathepsin B, together with an unspecified extracellular cysteine proteinase, appears to be important to the degradation of the organic matrix of bone and one or more of the basement membrane constituents that make up Matrigel. Some of the proteinases implicated in cancer, like the matrix metalloproteinases and uPA, may be part of a hierarchical network or a cascade of interacting enzymes (36, 65). The observation that an intracellular form of cathepsin B is required for invasion through Matrigel may argue for a network of proteinases with cathepsin B acting in an early step within the cell (62). *In vitro*, cathepsin B can activate precursors of serine proteinases, such as pro-uPA (34, 58), and metalloproteinases, such as pro-stromelysin (36). The missorting of cathepsin B to the secretory pathway in transformed cells (15, 17, 54, 66–68) in-

creases the likelihood that it could activate a secreted enzyme required for matrix degradation. Alternatively, efficient matrix degradation might require cooperation between proteinases acting outside the cell and lysosomal enzymes acting in endocytic vesicles in the peripheral cytoplasm (60, 69, 70). Recently, Ahram *et al.* (71) reported that the activated GTP-binding protein, Rac1, increased cell invasion *in vitro* by a mechanism that included increased endocytosis and the accumulation of degraded matrix proteins in cathepsin B-containing, perinuclear lysosomes. The accumulation of the degraded substrate in cathepsin B-containing vesicles was inhibited by CA-074Me and by E-64d, a membrane-permeant form of E-64.

Also noteworthy is our observation that 1 μM CA-074, which predominantly inhibited extracellular cathepsin B, significantly increased the invasiveness tumor cells. One explanation is that the secreted form of cathepsin B interferes with invasion by degrading ECM components or cell surface receptors necessary for cell to matrix attachment, migration through the matrix, or matrix to cell signaling. Tsuboi and Rifkin (72) observed that Bowes melanoma and HT1080 cells, which were characterized as high secretors of tissue-type plasminogen activator and uPA, respectively, were poorly invasive in an amnion invasion assay. However, invasion could be increased by either inhibitors of plasmin, the plasminogen activators, or matrix metalloproteinases. In contrast, Osmond cells, which produce low levels of uPA, were highly invasive in the amnion assay, and their ability to invade could be inhibited by agents that blocked plasmin or plasminogen activation. It was proposed that high levels of plasmin, generated by uPA or tissue-type plasminogen activator, could cause uncontrolled matrix degradation that disrupted a critical interaction between cells and matrix at an early stage of invasion. Extracellular cathepsin B might act in a similar manner to block invasion by cells that are high cathepsin B producers. A possible interpretation of our inhibitor results is that cathepsin B acts inside the cell to promote the degradation of the ECM and outside the cell to inhibit migration through the ECM, with the former effect predominating in the tumor cell lines we have chosen to study.

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