NEOPLASIA
www.neoplasia.com

Volume 10 Number 5 May 2008 pp. 481–488 481

Cathepsin B Mediates the pH-Dependent Proinvasive Activity of Tumor-Shed Microvesicles[1]

Ilaria Giusti*[,2], Sandra D'Ascenzo*[,2], Danilo Millimaggi*, Giulia Taraboletti[†], Gaspare Carta[‡], Nicola Franceschini[§], Antonio Pavan[¶] and Vincenza Dolo*

*Department of Experimental Medicine, University of L'Aquila, L'Aquila, Italy; [†]Department of Oncology, Mario Negri Institute for Pharmacological Research, Bergamo, Italy; [‡]Department of Surgical Sciences, University of L'Aquila, L'Aquila, Italy; [§]Department of Science and Biomedical Technologies, University of L'Aquila, L'Aquila, Italy; [¶]Department of Experimental Medicine, University of Rome "La Sapienza," Rome, Italy

Abstract

Vesicles shed by cancer cells are known to mediate several tumor-host interactions. Tumor microenvironment may, in turn, influence the release and the activity of tumor-shed microvesicles. In this study, we investigated the molecular mediators of the pH-dependent proinvasive activity of tumor-shed vesicles. Gelatinase zymography showed increased microvesicle activity of matrix metalloproteinases 9 and 2 as a result of acid exposure (pH 5.6) compared to pH 7.4. Thus, we reasoned that the cysteine protease cathepsin B might play a role in mediating the pH-dependent activation of gelatinases. Cathepsin B expression in tumor-shed microvesicles was confirmed by Western blot analysis and zymography. The activity of vesicle-associated cathepsin B measured using Z-Arg-Arg-pNA as substrate was significantly increased at acidic pH values. Inhibition of protease activity by the cysteine protease inhibitor, E-64, and treatment of ovarian cancer cells with small interfering RNA against cathepsin B suppressed the ability of tumor-shed microvesicles to stimulate both gelatinase activation and the invasiveness of endothelial cells observed at low pH values. We conclude that microvesicle shedding is a major secretory pathway for cathepsin B release from tumor cells. Hence, the acidic microenvironment found in most solid tumors may contribute to cathepsin B–mediated proinvasive capabilities of tumor-shed vesicles.

Neoplasia (2008) 10, 481–488

Introduction

Mounting evidence suggests that cancer is a complex biologic system affected by several factors that dampen or enhance the effects of genetic alterations [1]. Hence, it has become increasingly apparent that cancer is not a single-cell disease, and its growth, invasion, and metastasis are constantly modulated by the host [2]; its microenvironment, indeed, could exert a profound influence on the fate of potentially neoplastic cells [3]. In general, tumor-environment interactions are mediated by secreted growth factors, chemokines, cytokines, and cell-to-cell adhesion contacts. However, there has been a growing interest in a particular form of cell-to-cell communication that involves shed membrane microvesicles.

Shedding of membrane-derived microvesicles is a physiological phenomenon that accompanies cell activation and growth [4]. Intriguingly, tumor cells constitutively release microvesicles, transporting a broad array of biologically active molecules, including cell surface receptors, matrix metalloproteases, and adhesion molecules

Address all correspondence to: Prof. Vincenza Dolo, Dipartimento di Medicina Sperimentale, Università di L'Aquila, Via Vetoio-Coppito 2, I-67100, L'Aquila, Italy. E-mail: dolo@univaq.it

[1]This work was supported by grants from the Italian Ministry of University and Scientific and Technological Research to A. P. and V. D., from the European Union FP6, LSHC-CT-2003-503297 to G. T., and from Johnson & Johnson Medical S.p.A.

[2]The first two authors contributed equally to this work.

Received 18 January 2008; Revised 14 February 2008; Accepted 15 February 2008

Copyright © 2008 Neoplasia Press, Inc. All rights reserved 1522-8002/08/$25.00
DOI 10.1593/neo.08178

[5,6]. Tumor-shed vesicles have been implicated in a range of different biologic processes including regulation of tumor invasiveness and metastasis [5,7–11], drug resistance [12], and modulation of the host immune response [13–15]. In addition, numerous reports have shown that shed tumor vesicles may promote endothelial cell migration, invasion, and neovascularization [16–20].

The tumor-promoting activities of membrane-shed vesicles are modulated by the extracellular environment. In this regard, we have recently shown that the bioavailability of angiogenic factors released by tumor-shed vesicles depends on vesicle rupture induced by acidic pH in the microenvironment [19]. It remains unclear, however, whether the vesicle-mediated promotion of endothelial cells invasiveness could occur in a pH-dependent fashion.

Another mechanism whereby tumor-shed vesicles may exert their proinvasive abilities can involve the activity of vesicle-associated proteases [7,9], which in turn might be influenced by the pH of the tumor microenvironment.

Cathepsins are cysteine proteinases that primarily function as endopeptidases within endolysosomal compartments in normal cells; they are involved in physiological processes such as protein turnover, bone remodeling, reproduction, keratinocyte differentiation, and apoptosis [21,22]. Multiple mechanisms increase cathepsins' expression in tumors and in tumor-associated cells, including genetic amplification or alternative splicing [21]. In tumors, cysteine proteinases can be secreted, bound to cell membrane, or released by shedding vesicles [23].

Cathepsin B is highly upregulated in several malignant cells at the mRNA, protein, and activity levels [21,24]. Notably, cathepsin B activity is the result of several levels of regulation, including transcription, posttranscription processing, translation, and glycosylation [25,26]. Additionally, cathepsin B has been shown to facilitate direct degradation of extracellular matrix (ECM) proteins [21] and activate other proteases capable of degrading ECM [27,28]. Although both extracellular and intracellular forms of cathepsin B in tumor cells are thought to play a major role in the degradation of ECM [29,30], the secretory pathways for cathepsin B release from tumor cells remain poorly understood; therefore, the mechanisms by which cathepsin B could activate the other nonacid-dependent gelatinases is not only poorly described but also controversial.

This study was designed to investigate the molecular mediators of the pH-dependent proinvasive activity of tumor-shed vesicles. Specifically, we investigated whether the cysteine protease cathepsin B may play a role in mediating the pH-dependent activation of gelatinases.

Materials and Methods

Cell Culture

The CABA I cell line was established from the ascitic fluid of a patient with ovarian carcinoma before any drug treatment [31]. Cells were grown as monolayers in RPMI 1640 (Euroclone, Devon, UK) with 5% fetal calf serum, 2 mM glutamine, penicillin and streptomycin. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cord veins and grown on 1% gelatin-coated flasks in DMEM supplemented with 10% fetal calf serum, 10% newborn calf serum, 20 mM HEPES, 6 U/ml heparin, 2 mM glutamine, 50 μg/ml endothelial cell growth factor (crude extract from bovine brain), penicillin, and streptomycin. Cells from the third to fifth passages of culture were used.

Isolation of Membrane Vesicles from Cell-Conditioned Medium

Vesicles were prepared as previously described [4]. Conditioned medium obtained as above was centrifuged at 600*g* for 15 minutes and then at 1500*g* for 15 minutes to remove cells and large debris. Supernatants were centrifuged at 100,000*g* for 1 hour at 4°C. Vesicles were quantified based on measurements of vesicle-associated protein levels using the method of Bradford (Bio-Rad, Milan, Italy), with bovine serum albumin (Sigma, St. Louis, MO) as standard.

Pelleted microvesicles were resuspended in citrate-phosphate buffer at three different pH values (5.6, 6.0, and 7.4) and were incubated for 30 minutes; after incubation, the acidic pH was made to neutral pH value diluting vesicles in a convenient volume of RPMI 1640; measurements of pH was controlled using pH indicator sticks. Each experiment was conducted with or without cathepsin B inhibitors.

Motility and Invasion Assay

Endothelial cell invasion was assayed using modified Boyden chambers with polycarbonate polyvinyl pyrrolidone–free Nucleopore filters (pore size, 8 μm) [32]. Vesicles, added at the indicated concentrations to the upper compartment of the chamber, were used as external stimuli. Buffer alone or conditioned medium from NIH3T3 cells were used as negative and positive reference controls, respectively. For motility (chemotaxis), filters were coated with 0.1% gelatin. For invasion, filters were coated with a thick layer of the reconstituted basement membrane Matrigel (Beckton Dickinson, Bedford, MA; 0.5 mg/ml) which cells must degrade to migrate through the filter. Human umbilical vein endothelial cells were detached, washed in DMEM–0.1% bovine serum albumin, resuspended in the same medium at a concentration of 5×10^5/ml, and added to the upper compartment of the chamber. After 4 hours (motility) or 6 hours (invasion), filters were stained with Diff-Quik (Baxter, Dudingen, Switzerland), and the migrated cells in five high-power fields were counted.

Gelatin Zymography

Gelatin zymography was performed using sodium dodecyl sulfate–polyacrylamide gels (SDS-PAGE, 7.5%) copolymerized with 1 mg/ml gelatin type B (Sigma). After resuspension and incubation in the specific buffer, vesicles (2–16 μg) were diluted in SDS-PAGE sample buffer in nonreducing conditions without heating. After electrophoresis, gels were washed twice for 30 minutes in 2.5% Triton X-100 at room temperature and were incubated overnight in collagenase buffer (50 mM Tris–HCl, pH 7.4, containing 5 mM $CaCl_2$ and 120 mM NaCl) at 37°C. Gels were stained with Coomassie Blue R 250 (Bio-Rad) using a mixture of methanol–acetic acid–water (4:1:5) for 1 hour and were destained in the same solution without dye. Gelatinases' activities were visualized as distinct bands, indicating proteolysis of the substrate. The supernatant of HT1080 fibrosarcoma cells was used as a reference standard for matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9). The broad-spectrum cysteine protease inhibitor E-64 (0–50 μM; Bachem, Bubendorf, Switzerland) was used to analyze the effect of cathepsin B inhibition on the activation of gelatinases.

Western Blot Analysis

Vesicles (20 μg) from CABA I cells were resolved by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions and were transferred to nitrocellulose

membranes (Schleicher & Schuell, Dassel, Germany). Nonspecific binding sites were blocked by overnight incubation with 10% nonfat dry milk in TBS-T containing 0.5% Tween-20. Blots were incubated with antibodies against human cathepsin B (1:2500 dilution of anti–cathepsin B; Biomol, Plymouth Meeting, PA) for 1 hour, followed by peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), in blocking buffer. After washing, reactive bands were visualized using a chemiluminescence detection kit (ECL; Amersham-Pharmacia, Uppsala, Sweden).

Cysteine Protease Zymography

Cysteine protease activity (cathepsins B and L) was detected by SDS-PAGE using gels copolymerized with gelatine (0.3 mg/ml). Samples were mixed with nonreducing SDS sample buffer and were subjected to SDS-PAGE (12.5% acrylamide). After electrophoresis and washing (50 mM sodium acetate pH 5.5, 100 mM sodium chloride, 10 mM cysteine, 2.5% Triton X-100), gels were incubated in acetate buffer 50 mM (pH 4.8) containing 20 mM cysteine and 1 mM EDTA for 20 hours at 37°C.

The nature of the gelatinolytic activity was defined by using the broad-spectrum cysteine protease inhibitor E-64 (50 μM in acetate buffer). To distinguish the gelatinolytic activity due to cathepsin B activity, the cathepsin L inhibitor Z-FY-CHO (Calbiochem, San Diego, CA) was used.

Cathepsin B Activity Assay

Cathepsin B activity in vesicles or cell extracts was determined using a spectrophotometric assay. The specific cathepsin B substrate, Z-Arg-Arg-pNA (Biomol), was used. Substrate hydrolysis was monitored by following the absorbance variation at 38°C, using a lambda 19 spectrophotometer (Perkin-Elmer, Rahway, NJ) equipped with thermostatically controlled cells. The reaction was spectrophotometrically recorded at 405 nm. Z-Arg-Arg-pNA was monitored at 405 nm using two different buffer systems: 1) 50 mM sodium acetate pH 5.5 containing 2.5 mM DTT and 2.5 mM EDTA; and 2) 0.1% Triton X-100 or PBS pH 7.4 containing the same additives. A change in the extinction coefficient of +8800/M per centimeter was used to compute units of enzyme activity. A total of 500 μl of reaction buffer containing 5 to 20 μM substrate was preincubated for 5 minutes before the addition of either cathepsin B purified from human liver (Biomol) for reference, tumor-shed vesicles, or of protein extracts from the CABA I cell lines. The reaction was monitored for 15 minutes. All measurements were done at least in triplicate in different vesicle or protein extract preparations.

For the inhibition experiments, we used the selective cell-impermeable cathepsin B inhibitor CA-074 (20 μM) [33], the cell-permeable cathepsin B inhibitor CA-074Me (20 μM) [34], and the broad-spectrum cysteine protease inhibitor E-64 (20 μM). All inhibitors were obtained from Bachem.

Cathepsin B Gene Silencing

We silenced cathepsin B by using cathepsin B small interfering RNA (siRNA) (h) sc-29238, with scrambled oligos sc-37007 as control and sc-29528 siRNA transfection reagents (Santa Cruz Biotechnology) according to the manufacturer's protocol. Briefly, the cells were incubated overnight at 37°C in medium without antibiotics, treated according to the transfection protocol, and then incubated for 6 hours at 37°C. At the end of incubation, the medium containing twice the amount of normal serum and antibiotics was added. After an incubation period of 72 hours, reverse transcription–polymerase chain reaction (RT-PCR), cathepsin B zymography, and Western blot analysis were performed to confirm that the CABA I cell transfection was successful. The same results were obtained with nonsilenced CABA I cells transfected with scrambled oligos and CABA I wild-type.

Real-Time PCR for Cathepsin B

Total RNA was isolated using SV Total RNA Isolation System (Promega, Madison, WI), and cDNA was synthesized from 5 μg RNA using the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's protocol. Each real-time PCR reaction was prepared in triplicate and contained 2.0 μl of cDNA. Polymerase chain reaction was carried out using SYBR-green detection of PCR products in real-time (Roche Diagnostics, Indianapolis, IN). The sequences of the primers used for PCR were as follows: cathepsin B forward, 5′-CCAGGGAGCAAGACAGAGAC-3′, reverse 5′-GAGACTGGCGTTCTCCAAAG-3′ (163 bp); GADPH forward 5′-GGCCTCCAAGGAGTAAGACC-3′, GADPH reverse 5′-AGGGGTCTACATGGCAACTG-3′ (147 bp). A comparative ΔC_t method was used to determine gene expression. Expression levels were normalized to the expression levels of the housekeeping gene *GADPH*. To determine the relative expression level of cathepsin B, the expression in CABA I cells was arbitrarily set at 1.

Statistical Analysis

All data shown are from at least three independent experiments and are expressed as mean ± SD. Statistical significance was determined by using the Mann-Whitney *U* test. Calculations were performed using GraphPad Prism 4 software (GraphPad, San Diego, CA). Statistical significance was set at $P < .05$.

Results

Endothelial Cell Invasiveness Is Increased By Tumor-Shed Vesicles Exposed to Acidic pH

We have previously shown that acidic pH increases the chemotactic and proinvasive activities of tumor-shed membrane vesicles [19]. The chemotactic activity was mainly due to the vascular endothelial growth factor (VEGF) released from microvesicles; it was previously demonstrated that transient exposure to low extracellular pH induces an increased steady-state level of VEGF mRNA expression, which correlates with prolonged VEGF mRNA stability and rise in VEGF secretion [35].

However, little is known about the molecular mediators involved in the increased proinvasive activity. To determine whether the ability of tumor-shed vesicles to stimulate endothelial cell invasiveness may occur in a pH-dependent fashion, in this study, we used the CABA I cell model. These cells have the capacity to release membrane vesicles that stimulate endothelial cell motility and invasiveness [19]. Differently from other cell models [19], the exposure of CABA I–shed vesicles to acidic pH (6.0 and 5.6) increased their proinvasive activity (Figure 1*A*), with no effect on their chemotactic activity (Figure 1*B*). We therefore selected CABA I cells as a means to investigate the effect of pH on the proinvasive activity of vesicles, independently from the effect on pure motility.

Differently from pure motility, invasiveness requires enzymatic degradation of ECM. We thus investigated whether acidic pH could

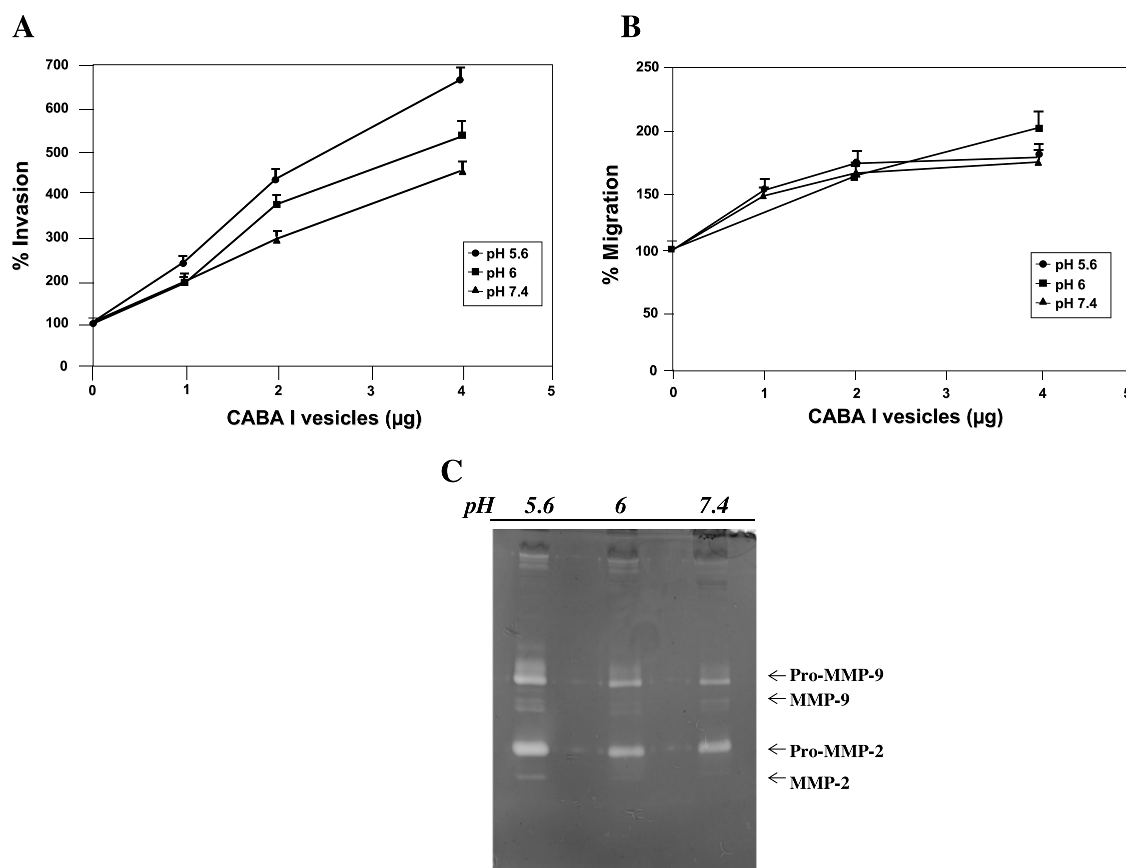

Figure 1. Effect of tumor-shed vesicles on endothelial cell motility and invasiveness. Human umbilical vein endothelial cell invasiveness (A) and motility (B) were tested in the Boyden chamber. Vesicles shed by human ovarian carcinoma cells CABA I were used as external stimuli. Data (mean ± SD of three independent experiments) represent the number of cells that had migrated in five high-power fields expressed in percentage, counting untreated cells as 100%, $P < .05$. (C) Effect of acidic pH on vesicle-associated gelatinases.

influence the activity of MMP-2 and MMP-9 in CABA I–shed vesicles. To achieve this goal, vesicles isolated from CABA I–conditioned medium were exposed at three different pH values (5.6, 6.0, and 7.4) and were subsequently analyzed by gelatin zymography. The pattern of the lytic bands is shown in Figure 1*C*. Bands corresponding to the activated forms of MMP-2 and MMP-9 were more evident at acidic pH (5.6) compared to pH 7.4. Unexpectedly, bands of lysis corresponding to the proenzyme forms of MMP-2 and MMP-9 were both more marked at lower pH values. These findings indicate that acidic pH is involved in the activation of vesicle-associated gelatinases which, in turn, may promote endothelial cell invasiveness.

Presence of Cathepsin B in Shed Vesicles

In the light of these results, we reasoned that the cysteine protease cathepsin B, whose activity is acidic pH-dependent [36], might play a role in mediating activation of vesicle-associated gelatinases. Firstly, we asked whether vesicles from CABA I cells contained cathepsin B. In keeping with our hypothesis, Western blot analysis demonstrated the presence of cathepsin B in shed membrane vesicles. Notably, the vesicle-associated forms of cathepsin B were different from those found in cell extracts (Figure 2*A*). Accordingly, cell extracts showed bands with molecular masses of 15 to 30 kDa corresponding to those of the active enzyme, whereas shed vesicles showed a band with a molecular mass of 41 kDa corresponding to the zymogen (proform) of cathepsin B.

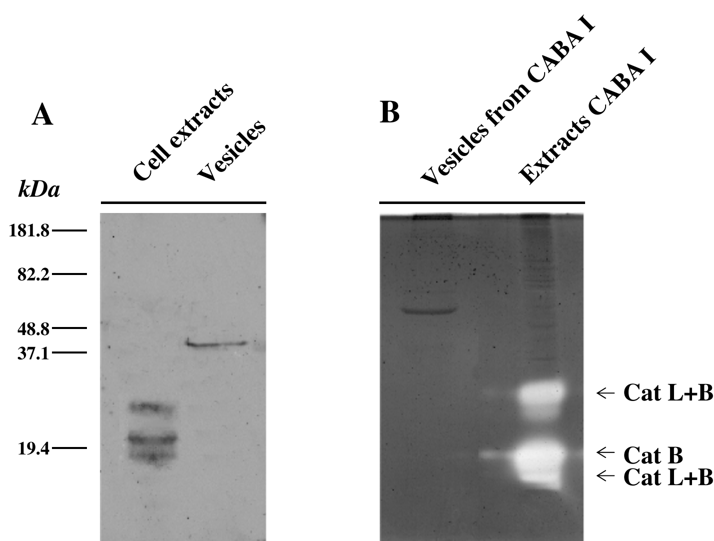

Figure 2. Presence of cathepsin B in CABA I–shed vesicles. (A) Western blot analysis of cell extracts and vesicle-associated cathepsin B. (B) Cysteine zymography of extracts and vesicle-associated cathepsins B and L. The identification of cathepsin B was confirmed through the inhibition of cathepsin L (not shown).

Table 1. Vesicles- and Cell Extracts–Associated Enzymatic Activity.

	Enzymatic Activity (mol/min × mg protein)	
	pH 7.4	pH 5.6
Cell extracts	$5.7 \times 10^{-10} \pm 2.8 \times 10^{-11*}$	$1.9 \times 10^{-8} \pm 9.5 \times 10^{-10*}$
Vesicles	$6.1 \times 10^{-10} \pm 3 \times 10^{-11*}$	$7.9 \times 10^{-7} \pm 3.9 \times 10^{-8*}$

Data are the mean ± SD of three independent experiments.
$^*P < .05$.

Cathepsin B activity in CABA I vesicles and extracts was detected by means of cathepsin B gelatin zymography; because both cathepsins (B and L) are detected by means of this technique, we confirmed the identity of the bands as cathepsin B by using a selective cathepsin L inhibitor (data not shown). In keeping with the results of Western blot analysis, the active forms of cathepsin B were detected in CABA I extracts as bands of gelatinolysis with molecular masses of 15 to 30 kDa. In contrast, no gelatinolytic activity was detected in CABA I–shed vesicles (Figure 2*B*). This result was not surprising given the absence of procathepsin B activity in this assay. In addition, gelatinase activity in cells' extracts was completely ablated by the general cysteine proteinase inhibitor, E-64, thereby confirming identification of cathepsins (data not shown).

We then investigated whether acidic pH could influence the activity of cathepsin B in CABA I–shed vesicles and cell extracts. To achieve this goal, cathepsin B activity was measured in cell extracts and vesicles exposed at two different pH values (5.6 and 7.4). Specifically, enzymatic activity was measured using Z-Arg-Arg-pNA as substrate under different pH conditions (Table 1). Both the activity and the stability of cathepsin B were assessed. The results showed that vesicle-associated cathepsin B activity was increased 1300-fold at acidic pH values compared to physiological pH 7.4. Similarly, cathepsin B activity in cells' extract was increased 33-fold at pH 5.6 *versus* pH 7.4. Cleavage was completely inhibited by E-64. A moderate inhibition was seen with the use of cathepsin B inhibitors CA-074 and CA-074Me (data not shown).

Role of Cathepsin B in the pH-Dependent Proinvasive Activity of Vesicles

To investigate the role of cathepsin B in mediating vesicle-induced endothelial invasiveness observed at low pH values, we followed two distinct experimental approaches.

Vesicles collected from CABA I cells were incubated in pH 5.6 and pH 7.4 buffer, either with or without E-64 (50 μM), and were tested for their ability to induce endothelial cells' invasiveness (Figure 3*A*). Vesicles incubated at pH 5.6 showed an increased proinvasive activity compared to vesicles incubated at pH 7.4. The pH-dependent increment in the proinvasive activity of tumor-shed vesicles was completely abrogated by the typical cysteine protease inhibitor E-64. After exposure to E-64, endothelial cells' invasiveness induced by vesicles was similar to that observed at physiological pH. These findings indicate that cysteine cathepsins play a critical role in the biologic activity of tumor-shed vesicles at acidic pH. Conversely, cathepsins are not critically involved in the biologic activity of tumor-shed vesicles at physiological pH. Accordingly, E-64 had only a marginal effect on vesicle-induced endothelial cell invasiveness at pH 7.4.

To verify whether cathepsins may be responsible for the pH-dependent modulation of vesicle gelatinases, vesicles shed by CABA I cells were resuspended in buffer at pH 5.6 in the presence of E-64 (0–50 μM). We subsequently analyzed the lytic bands by means of gelatin zymography (Figure 3*B*). In the presence of the cysteine inhibitor E-64, the bands corresponding to activated MMP-2 and MMP-9 were not detectable. Moreover, E-64 appeared to modulate the activity of the proenzymatic forms of both gelatinases. Accordingly, fainter bands were discernible in the presence of E-64.

To evaluate the role of one specific cysteine protease, cathepsin B, CABA I cells were transfected with a specific siRNA against cathepsin B to silence its expression. Silencing was confirmed by the use of real-time PCR, Western blot analysis, and by zymography studies. RT-PCR analysis showed a ~10-fold reduction in target mRNA compared to control cells (Figure 4*A*). Western blot analysis showed three different forms (15, 20, and 30 kDa) in extracts from control cells

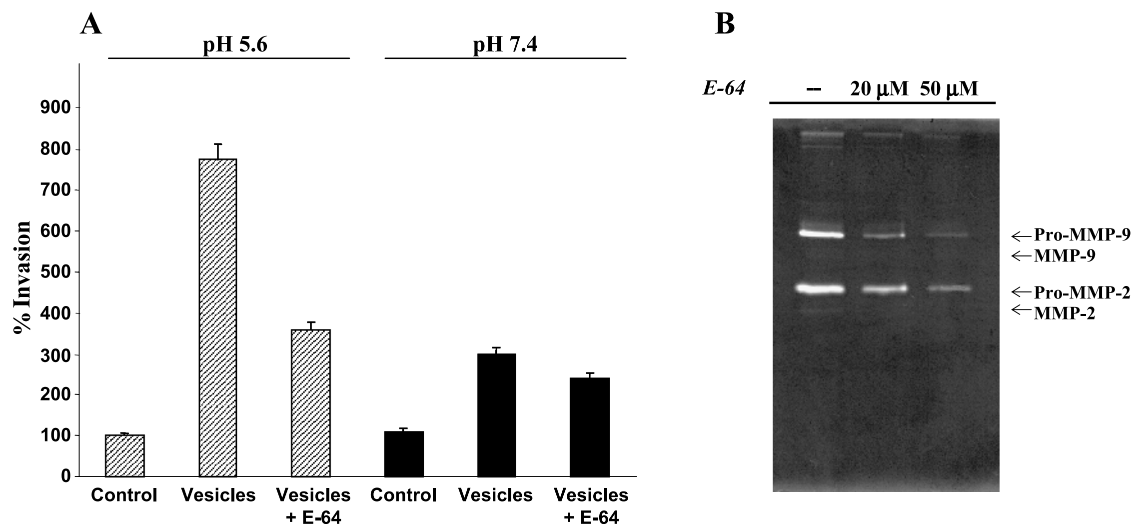

Figure 3. Effect of cathepsin B inhibition. (A) Vesicles shed from CABA I were isolated and tested for their ability to stimulate HUVEC invasiveness at pH 5.6 and 7.4, either with or without the cysteine proteinase inhibitor E-64 (50 μM). Data (mean ± SD of three independent experiments) represent the number of cells that had migrated in five high-power fields expressed in percentage, counting untreated cells as 100%, $P < .05$. (B) Zymographic analysis of vesicle-associated gelatinases incubated with inhibitor E-64 0 to 50 μM.

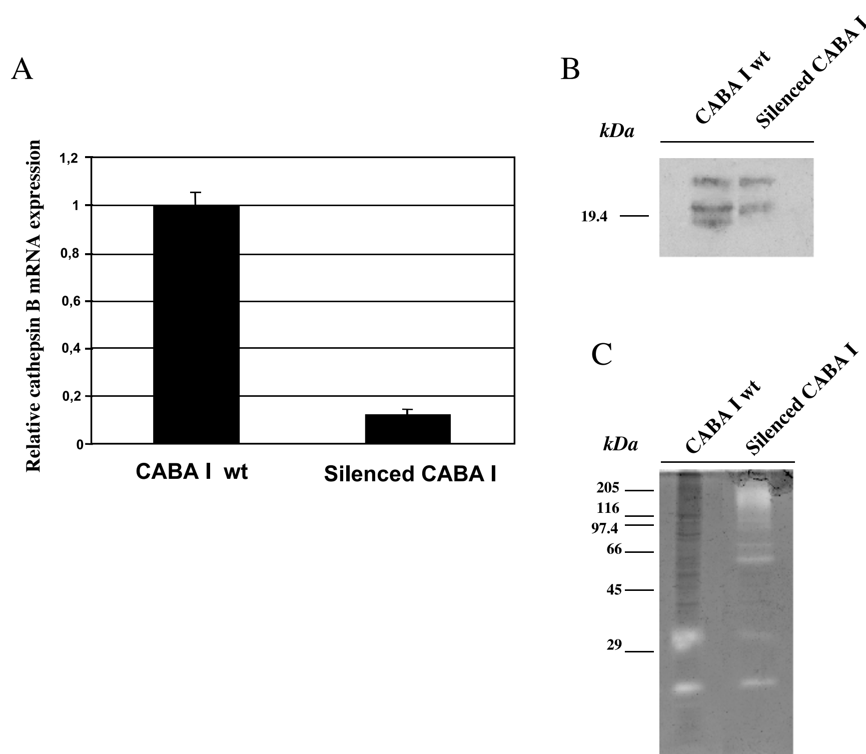

Figure 4. Effectiveness of cathepsin B siRNA silencing in CABA I cells. (A) RT-PCR analysis of parental control and silenced CABA I cells. (B) Western blot analysis of cathepsin B in cellular extracts. (C) Cysteine zymography of cathepsins B and L in cellular extracts.

compared to two different forms (20 and 30 kDa) in extracts from silenced cells. These two bands appeared less intense compared to control bands (Figure 4*B*). Similarly, gelatin zymography showed three different forms (15, 20, and 30 kDa) in extracts from parental CABA I compared to two different forms (20 and 30 kDa) in extracts from silenced cells. These two bands appeared less intense compared to control bands (Figure 4*C*).

As expected, microvesicles shed by CABA I cells with silenced cathepsin B showed no enzymatic activity as determined by the Z-Arg-Arg-pNA substrate (data not shown). Microvesicles isolated from

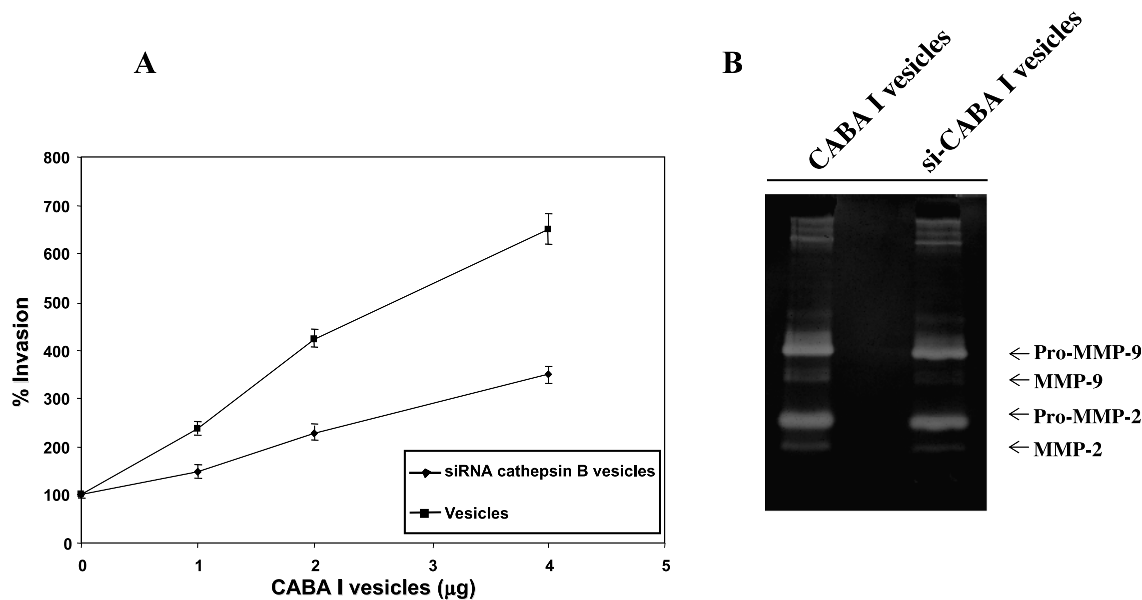

Figure 5. Effect of cathepsin B gene silencing. (A) Vesicles shed from CABA I cells, either parental control and silenced, were isolated and tested for their ability to stimulate HUVEC invasiveness at pH 5.6. Data (mean ± SD of three independent experiments) represent the number of cells that had migrated in five high-power fields expressed in percentage, counting untreated cells as 100%, $P < .05$. (B) Zymographic analysis of vesicle-associated gelatinases.

CABA I cells with silenced cathepsin B expression were less effective inducers of proinvasive activity in HUVECs compared to parental cell–derived microvesicles at pH 5.6 (Figure 5*A*).

We then investigated whether cathepsin B silencing may affect gelatinase activity in CABA I–shed microvesicles at pH 5.6. The pattern of the lytic bands detected after zymography is presented in Figure 5*B*. Bands for both the proenzymatic forms of MMP-2 and MMP-9 were less intense in vesicles isolated from CABA I cells with silenced cathepsin B. Additionally, bands corresponding to the active forms of gelatinases were not detectable. Altogether, these findings confirmed a crucial role of cathepsin B in mediating gelatinases' activation in a pH-dependent fashion.

Discussion

In recent years, growing interest has been focused on tumor-released microvesicles in the light of their ability to transport a wide array of biologically active molecules involved in malignant transformation and/or progression [10,12,13,37–39]. It has been thus hypothesized that tumor-shed vesicles may deliver detrimental signals to the tumor microenvironment independently from direct cell-to-cell contact [9]. Accordingly, tumor-shed microvesicles may bear cell surface receptors, adhesion molecules, and metalloproteinases, thereby modulating the tumor-invasive capabilities and promoting tumor angiogenesis [4,16–18].

We have recently demonstrated that tumor-shed vesicles transport VEGF and that the bioavailability of angiogenic factor depends on vesicle rupture induced by acidic pH in the microenvironment [19]. Of interest, the extracellular pH in solid tumors is generally acidic because of elevated anaerobic glycolysis and impaired clearance of acidic metabolic wastes [40]. Acidification of extracellular environment is advantageous to the tumor because it promotes angiogenesis through acid-induced release of VEGF and because, indirectly, promotes ECM degradation by inducing adjacent normal cell (such as fibroblasts and macrophages) to release proteolytic enzymes such as cathepsin B [41]. Therefore, the release of vesicle-associated VEGF triggered by low pH values suggests that the activity of tumor-shed vesicles may be regulated by the tumor microenvironment in a pH-dependent fashion.

Our current findings indicate that low pH may promote the invasive capacities of tumor-shed vesicles. Although acidic pH did not increase the chemotactic activity of CABA I–shed vesicles, we have demonstrated that the exposure of shed vesicles to acidic medium (pH 6.0 and 5.6) increased the ability of tumor vesicles to promote endothelial cell invasiveness. Because MMP-2 and MMP-9 activities were increased in tumor-shed vesicle after exposure to acidic medium, we reasoned that the cysteine protease cathepsin B might play a role in mediating the pH-dependent activation of gelatinases.

The lysosomal cysteine protease cathepsin B is implicated in the degradation of ECM, a critical step in tumor dissemination and angiogenesis. Cathepsin B has a pH optimum of 4.5 to 5.5 and has been shown to lose its activity at neutral pH [42]. Of interest, it has been previously demonstrated that cathepsin B appears to undergo autodegradation at neutral pH [43]. Vesicles shed by CABA I cells appear to contain the inactive proenzyme of cathepsin B, as demonstrated by Western blot analysis. However, this technique has a low sensitivity for detecting the presence of small amounts of active mature cathepsin B, whose presence is demonstrated by a more sensitive enzymatic activity assay. Additionally, acidic conditions were shown to increase the activity of cathepsin B in tumor-shed microvesicles, thereby suggesting an ability of this enzyme to increase invasiveness in a pH-dependent manner. Unexpectedly, we found a higher cathepsin B activity in tumor-shed vesicles compared to total cell extracts. This finding suggests that microvesicles hijack cathepsin B as a means to promote malignant progression.

There are at least two lines of evidence supporting a crucial role for vesicle-associated cathepsin B in mediating pH-dependent tumor invasiveness. Firstly, the synthetic cysteine protease inhibitor E-64 was shown to diminish the activity of vesicle-associated gelatinases and to reduce the ability of tumor-shed microvesicles to induce endothelial cells' invasiveness at neutral pH. Moreover, vesicles shed by ovarian carcinoma cell line CABA I with silenced cathepsin B expression had reduced proinvasive ability and diminished activity of gelatinases compared to parental cell–derived microvesicles.

Cathepsin B may contribute to invasiveness in ovarian cancer by various direct and indirect mechanisms. The proteolytic activity of cathepsin B facilitates direct degradation of several ECM proteins, including fibronectin, laminin, tenascin C, and type IV collagen [44]. Moreover, cathepsin B can indirectly promote invasiveness by activating other matrix-degrading proteolytic systems such as MMPs and urokinase-type plasminogen activator [27] and by promoting degradation of tissue inhibitors of metalloproteinases [28]. Interestingly, hereto we have shown that cathepsin B may play a role in promoting the pH-dependent expression of the proenzymatic forms of MMP-2 and MMP-9. Future studies are needed to further clarify the mechanisms whereby this phenomenon occurs. In any case, the effect of vesicle-associated cathepsin B appears subjected to low pH values, as shown by the poor inhibitory activity on vesicle-induced invasiveness exerted by E-64 at physiological pH. This finding is in agreement with the acidic pH optimum for cathepsin B peptidase activity.

Altogether, our results indicate that cathepsin B may be carried within CABA I–shed vesicles. In addition, it is posited that an amount of vesicle-associated cathepsin B pool may be active in conditions of acidic pH typical of tumor settings. Alternatively, cathepsin B may be released from tumor-shed vesicles in an inactive form that can be activated by proteolytic processing [45], autocatalytic procathepsin B cleavage [36,46], or through interaction with cell surface glycosaminoglycans [21].

In conclusion, we have demonstrated that tumor-shed microvesicles may induce proangiogenic activities of endothelial cells by a cathepsin B–mediated mechanism. The tumor-promoting activities of vesicle-associated cathepsin B may be activated in conditions of acidic pH typical of tumor microenvironment.

Acknowledgments

We thank Enzo Emanuele for expert editorial assistance.

References

[1] Yan L, Shahied L, Tang Y, Kesavan P, and Nakada MT (2004). Tumor-host interactions. *Preclinica* **2**, 422–426.

[2] Zigrino P, Loffek S, and Mauch C (2005). Tumor-stroma interactions: their role in the control of tumor cell invasion. *Biochimie* **87**, 321–328.

[3] Laconi E (2007). The evolving concept of tumor microenvironments. *Bioessays* **29**, 738–744.

[4] Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, and Dolo V (2002). Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle–associated components by endothelial cells. *Am J Pathol* **160**, 673–680.

[5] Dolo V, Ginestra A, Ghersi G, Nagase H, and Vittorelli ML (1994). Human breast carcinoma cells cultured in the presence of serum shed membrane vesicles rich in gelatinolytic activities. *J Submicrosc Cytol Pathol* **26**, 173–810.

[6] Dolo V, Adobati E, Canevari S, Picone MA, and Vittorelli ML (1995). Membrane vesicles shed into the extracellular medium by human breast carcinoma cells carry tumor-associated surface antigens. *Clin Exp Metastasis* **13**, 277–286.

[7] Dolo V, Ginestra A, Cassara D, Violini S, Lucania G, Torrisi MR, Nagase H, Canevari S, Pavan A, and Vittorelli ML (1998). Selective localization of matrix metalloproteinase 9, beta1 integrins, and human lymphocyte antigen class I molecules on membrane vesicles shed by 8701-BC breast carcinoma cells. *Cancer Res* **58**, 4468–4474.

[8] Dolo V, D'Ascenzo S, Violini S, Pompucci L, Festuccia C, Ginestra A, Vittorelli ML, Canevari S, and Pavan A (1999). Matrix-degrading proteinases are shed in membrane vesicles by ovarian cancer cells *in vivo* and *in vitro*. *Clin Exp Metastasis* **7**, 131–140.

[9] Ginestra A, Monea S, Seghezzi G, Dolo V, Nagase H, Mignatti P, and Vittorelli ML (1997). Urokinase plasminogen activator and gelatinases are associated with membrane vesicles shed by human HT-1080 fibrosarcoma cells. *J Biol Chem* **272**, 17216–17221.

[10] Angelucci A, D'Ascenzo S, Festuccia C, Gravina GL, Bologna M, Dolo V, and Pavan A (2000). Vesicle-associated urokinase plasminogen activator promotes invasion in prostate cancer cell lines. *Clin Exp Metastasis* **18**, 163–170.

[11] Poste G and Nicolson GL (1980). Arrest and metastasis of blood-borne tumor cells are modified by fusion of plasma membrane vesicles from highly metastatic cells. *Proc Natl Acad Sci USA* **77**, 399–403.

[12] Shedden K, Xie XT, Chandaroy P, Chang YT, and Rosania GR (2003). Expulsion of small molecules in vesicles shed by cancer cells: association with gene expression and chemosensitivity profiles. *Cancer Res* **63**, 4331–4337.

[13] Dolo V, Pizzurro P, Ginestra A, and Vittorelli ML (1995). Inhibitory effects of vesicles shed by human breast carcinoma cells on lymphocyte ^{3}H thymidine incorporation, are neutralised by anti TGF-beta antibodies. *J Submicrosc Cytol Pathol* **27**, 535–541.

[14] Albanese J, Meterissian S, Kontogiannea M, Dubreuil C, Hand A, Sorba S, and Dainiak N (1998). Biologically active Fas antigen and its cognate ligand are expressed on plasma membrane–derived extracellular vesicles. *Blood* **91**, 3862–3874.

[15] Andre F, Schartz NE, Chaput N, Flament C, Raposo G, Amigorena S, Angevin E, and Zitvogel L (2002). Tumor-derived exosomes: a new source of tumor rejection antigens. *Vaccine* **20**, A28–A31.

[16] Taverna S, Ghersi G, Ginestra A, Rigogliuso S, Pecorella S, Alaimo G, Saladino F, Dolo V, Dell'Era P, Pavan A, et al. (2003). Shedding of membrane vesicles mediates fibroblast growth factor-2 release from cells. *J Biol Chem* **278**, 51911–51919.

[17] Kim CW, Lee HM, Lee TH, Kang C, Kleinman HK, and Gho YS (2002). Extracellular membrane vesicles from tumor cells promote angiogenesis via sphingomyelin. *Cancer Res* **62**, 6312–6317.

[18] Janowska-Wieczorek A, Wysoczynski M, Kijowski J, Marquez-Curtis L, Machalinski B, Ratajczak J, and Ratajczak MZ (2005). Microvesicles derived from activated platelets induce metastasis and angiogenesis in lung cancer. *Int J Cancer* **113**, 752–760.

[19] Taraboletti G, D'Ascenzo S, Giusti I, Marchetti D, Borsotti P, Millimaggi D, Giavazzi R, Pavan A, and Dolo V (2006). Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH. *Neoplasia* **8**, 96–103.

[20] Millimaggi D, Mari M, D'Ascenzo S, Carosa E, Jannini EA, Zucker S, Carta G, Pavan A, and Dolo V (2007). Tumor vesicle–associated CD147 modulates the angiogenic capability of endothelial cells. *Neoplasia* **9**, 349–357.

[21] Mohamed MM and Sloane BF (2006). Cysteine cathepsins: multifunctional enzymes in cancer. *Nat Rev Cancer* **6**, 764–765.

[22] Jedeszko C and Sloane BF (2004). Cysteine cathepsins in human cancer. *Biol Chem* **385**, 1017–1027.

[23] Cavanaugh PG, Sloane BF, Bajkowski AS, Gasic GJ, Gasic TB, and Honn KV (1983). Involvement of a cathepsin B–like cysteine proteinase in platelet aggregation induced by tumor cells and their shed membrane vesicles. *Clin Exp Metastasis* **1**, 297–307.

[24] Roshy S, Sloane BF, and Moin K (2003). Pericellular cathepsin B and malignant progression. *Cancer Metastasis Rev* **22**, 271–286.

[25] Sloane BF, Moin K, Sameni M, Tait LR, Rozhin J, and Ziegler G (1994). Membrane association of cathepsin B can be induced by transfection of human breast epithelial cells with c-Ha-*ras* oncogene. *J Cell Sci* **107**, 373–384.

[26] Sinha AA, Jamuar MP, Wilson MJ, Rozhin J, and Sloane BF (2001). Plasma membrane association of cathepsin B in human prostate cancer: biochemical and immunogold electron microscopic analysis. *Prostate* **49**, 172–184.

[27] Schmitt M, Jänicke F, and Graeff H (1997). Tumor-associated proteases. *Fibrinolysis* **6**, 3–26.

[28] Kostoulas G, Lang A, Nagase H, and Baici A (1999). Stimulation of angiogenesis through cathepsin B inactivation of the tissue inhibitors of matrix metalloproteinases. *FEBS Lett* **455**, 286–290.

[29] Sameni M, Moin K, and Sloane BF (2000). Imaging proteolysis by living human breast cancer cells. *Neoplasia* **2**, 496–504.

[30] Premzl A, Zavasnik-Bergant V, Turk V, and Kos J (2003). Intracellular and extracellular cathepsin B facilitate invasion of MCF-10A neoT cells through reconstituted extracellular matrix *in vitro*. *Exp Cell Res* **283**, 206–214.

[31] Dolo V, Ginestra A, Violini S, Miotti S, Festuccia C, Miceli D, Migliavacca M, Rinaudo C, Romano FM, Brisdelli F, et al. (1997). Ultrastructural and phenotypic characterization of CABA I, a new human ovarian cancer cell line. *Oncol Res* **9**, 129–138.

[32] Taraboletti G, Roberts D, Liotta LA, and Giavazzi R (1990). Platelet thrombospondin modulates endothelial cell adhesion, motility, and growth: a potential angiogenesis regulatory factor. *J Cell Biol* **111**, 765–772.

[33] Yamamoto A, Hara T, Tomoo K, Ishida T, Fujii T, Hata Y, Murata M, and Kitamura K (1997). Binding mode of CA-074, a specific irreversible inhibitor, to bovine cathepsin B as determined by X-ray crystal analysis of the complex. *J Biochem* **121**, 974–977.

[34] Montaser M, Lalmanach G, and Mach L (2002). CA-074, but not its methyl ester CA-074Me, is a selective inhibitor of cathepsin B within living cells. *Biol Chem* **383**, 1305–1308.

[35] Shi Q, Le X, Wang B, Abbruzzese JL, Xiong Q, He Y, and Xie K (2001). Regulation of vascular endothelial growth factor expression by acidosis in human cancer cells. *Oncogene* **20**, 3751–3756.

[36] Dickinson DP (2002). Cysteine peptidases of mammals: their biological roles and potential effects in the oral cavity and other tissues in health and disease. *Crit Rev Oral Biol Med* **13**, 238–275.

[37] Zucker S, Wieman JM, Lysik RM, Wilkie DP, Ramamurthy N, and Lane B (1987). Metastatic mouse melanoma cells release collagen-gelatin degrading metalloproteinases as components of shed membrane vesicles. *Biochim Biophys Acta* **924**, 225–237.

[38] Lerner MP, Lucid SW, Wen GJ, and Nordquist RE (1983). Selected area membrane shedding by tumor cells. *Cancer Lett* **20**, 125–130.

[39] Kim JW, Wieckowski E, Taylor DD, Reichert TE, Watkins S, and Whiteside TL (2005). Fas ligand–positive membranous vesicles isolated from sera of patients with oral cancer induce apoptosis of activated T lymphocytes. *Clin Cancer Res* **11**, 1010–1020.

[40] Gatenby RA and Gillies RJ (2004). Why do cancers have high aerobic glycolysis? *Nat Rev Cancer* **4**, 891–899.

[41] Gatenby RA, Gawlinski ET, Gmitro AF, Kaylor B, and Gillies RJ (2006). Acid-mediated tumor invasion: a multidisciplinary study. *Cancer Res* **66** (10), 5216–5223.

[42] Linebaugh BE, Sameni M, Day NA, Sloane BF, and Keppler D (1999). Exocytosis of active cathepsin B enzyme at pH 7.0, inhibition and molecular mass. *Eur J Biochem* **264**, 100–109.

[43] Buck MR, Karustis DG, Day NA, Honn KV, and Sloane BF (1992). Degradation of extracellular–matrix proteins by human cathepsin B from normal and tumor tissues. *Biochem J* **282**, 273–278.

[44] Mai J, Sameni M, Mikkelsen T, and Sloane BF (2002). Degradation of extracellular matrix protein tenascin-C by cathepsin B: an interaction involved in the progression of gliomas. *Biol Chem* **383**, 1407–1413.

[45] Yan S, Sameni M, and Sloane BF (1998). Cathepsin B and human tumor progression. *Biol Chem* **379**, 113–123.

[46] Rozman J, Stojan J, Kuhelj R, Turk V, and Turk B (1999). Autocatalytic processing of recombinant human procathepsin B is a bimolecular process. *FEBS Lett* **459**, 358–362.