Matrigel influences morphology and cathepsin B distribution of prostate cancer PC3 cells

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Abstract. Increases in expression and activity of matrix-degrading enzymes such as the cysteine proteinases cathepsins B and L, and abnormal levels of their inhibitors, the cystatins, are associated with tumor cell invasion and metastasis. Environmental conditions have been shown to be causative factors in the development of a metastatic/invasive phenotype. We hypothesized that cell-matrix interactions affect the expression and activity of cathepsins B and L and their inhibitors in the prostate cancer cell lines, PC3 and DU145. To test this possibility, PC3 and DU145 were plated on uncoated surfaces or on surfaces coated with the reconstituted basement membrane, Matrigel. The cells were analyzed for cathepsins B and L immunolocalization, protein expression and activity 48 h after plating. Our data demonstrated that cathepsins B and L displayed a distinct punctate distribution with little colocalization; individual cells displayed a predominant staining for one or the other enzyme. Cathepsin B had a perinuclear distribution in PC3 grown on uncoated surfaces but a more peripheral staining in PC3 plated on Matrigel. Localization of cathepsin L remained predominantly perinuclear regardless of the plating surface. In addition to the translocation of cathepsin B from a perinuclear distribution to the cell periphery, growth of PC3 on Matrigel shifted cathepsin B activity from the cell extract to the media. There were no significant changes in cathepsins B and L immunolocalization or activity in DU145 with regard to plating surfaces. Likewise, the activity of endogenous cysteine proteinase inhibitors (CPIs) and protein expression of cystatin C remained unchanged in both cell lines. In conclusion, the interaction of PC3 prostate cancer cells with extracellular matrix components affects the distribution of cathepsin B protein and activity.

Introduction

A primary cause of death from cancer is invasion and metastasis of cells from the primary tumor to distant sites. The ability of a cancer cell to detach from adjacent cells and its supporting extracellular matrix is partly dependent on the activities of a number of proteinases including metalloproteinases, plasminogen activators, and the cathepsins (1-4). These proteinases facilitate the breakdown of cell adhesion molecules, basement membranes, vascular channels, and possibly other cell regulatory molecules; their activity may also be responsible for the release of regulatory factors from the surrounding matrix. It is no surprise that the activities and expressions of many proteinases are increased in a number of cancers and cancer cell lines. This study focuses on the cysteine proteinases, cathepsins B and L. Cathepsins B and L are lysosomal proteinases; they acquire mannose-6-phosphate (M6P) residues during their synthesis that target them to lysosomal vesicles via the M6P receptor. In many cancer cells, however, cathepsins B and L are either secreted or associate with the plasma membrane and with caveolae (5-8). Both proteinases can degrade components of the extracellular matrix in vitro (9-14) and can activate other matrix-degrading enzymes (15,16) suggesting that they contribute in a significant way to the metastatic ability of cancer cells. Lysosomal degradation of matrix components by the cathepsins has also been demonstrated in cancer cells (17,18).

The extracellular matrix is an important regulator of cellular differentiation and behavior; it also plays an active role in the development of the metastatic phenotype. A hallmark of metastasizing cells is their increased ability to migrate and invade adjacent tissue in most cases by altered expression of cell-cell and cell-matrix adhesion molecules. Adhesion molecules, in turn, participate in cell signaling cascades that relay information from the environment to the cell nucleus with resulting changes in gene expression patterns and cell behavior (19-21). Migration and invasion also involve cytoskeletal rearrangements necessary for cell locomotion; cytoskeletal components are responsible for the arrangement and location of cell organelles including lysosomes. We propose that the changes in cathepsin expression, activity and location result from the interaction of cancer cells with tumor-altered components of the extracellular matrix. Matrigel is a reconstituted basement membrane composed predominantly of laminin but also contains collagen IV, heparin sulfate proteoglycans,

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entactin, and nidogen (BD Biosciences, SPC354234). Matrigel is used in many studies of cell-matrix interactions including prostate cell lines (22). Matrigel enhanced the growth of human prostate carcinoma cell lines injected into nude mice (23) and increased their invasive and tumorigenic properties (24). Since cathepsins B and L are implicated in prostate tumor growth and invasion (3,4,25-29) we analyzed the effect of cell-Matrigel interactions on the expression and activity of cathepsins B and L in the prostate cancer cell lines, PC3 and DU145. This study demonstrates that the location of active cathepsin B is modulated by the interaction of PC3 cells with Matrigel.

Materials and methods

Materials. The prostate cancer cell lines, DU145 and PC3, were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Matrigel was obtained from Becton-Dickinson (Bedford, MA). All other culture reagents were obtained from Gibco/Invitrogen (Grand Island, NY). The cathepsin substrates, Z-phe-arg-aminoethylcoumarin (Z-phe-arg-NMec), Z-arg-arg-aminomethylcoumarin (Z-arg-arg-AMC), and the standard 7-aminomethylcoumarin (AMC) were purchased from Bachem (Torrance, CA). Centricon YM10 columns were from Millipore (Bedford, MA). Reagent sources for the immunolocalization studies were as follows: 4',6-diamidino-2-phenylindole (DAPI) for DNA staining (Molecular Probes, Eugene, OR), polyclonal antibody to cathepsin L (Calbiochem, San Diego, CA), and polyclonal antibody to cathepsin B (R&D Systems, Minneapolis, MN). Secondary antibodies were donkey anti-rabbit conjugated to Rhodamine Red-X for cathepsin L and donkey anti-goat conjugated to Cy2 for cathepsin B; slides were incubated with secondary antibodies for 1 h at room temperature following reaction with the primary antibody and extensive washing with PBS-Tween. Coverslips were mounted on glass slides with Mowiol (Calbiochem, La Jolla, CA) and examined with an Olympus confocal microscope.

Western blot analysis. Cells were collected by scraping in PBS and centrifuged at 5,000 g for 5 min. The cell pellet was resuspended in lysis buffer (1% nonidet P-40, 0.5% deoxycholate, 1% SDS, 1 mM sodium orthovanadate, 0.5 mM PMSF, 10 μg/ml aprotinin, 20 μg/ml leupeptin), lysed by sonication and placed on ice for 10 min. The homogenate was centrifuged at 13,100 rpm for 10 min at 4°C and the supernatant saved. Protein (50 μg) from each sample was mixed (1:2, v/v) with sample buffer (125 mM Tris-Cl, pH 6.5, 20% (v/v) glycerol, 4% SDS, 0.025% bromophenol blue, 0.1 M DTT) and boiled for 5 min. Samples were loaded on 12% (for cathepsins B and L) or 16% (for cathepsin C) precast SDS-PAGE gels and run at constant voltage in a Tris-glycine running buffer (25 mM Trizma base, 0.192 M glycine, 0.1% SDS).

Preparation of samples for Western blot analysis of cathepsins B and L and cystatin C in the media was as follows: 10 μl of the appropriate antibody was added to media (amount equivalent to 100 μg protein in media and previously concentrated 100-fold with a Centricon-YM10 column) and incubated overnight at 4°C with gentle rocking. The immunocomplex was captured with 20 μl of protein G beads (ATTO, Work In Progress, Germany) and eluted with 40 μl of a solution of 2 M urea, 20 mM DTT, 20 mM EDTA, 1% Triton X-100, and 0.5% octyl glucoside. The eluate was loaded onto SDS-PAGE gels and run at constant voltage in a Tris-glycine running buffer (25 mM Trizma base, 0.192 M glycine, 0.1% SDS).

Following electrophoresis, proteins were transferred to nitrocellulose membranes using the Invitrogen Trans-blot apparatus according to the manufacturer's specifications. The membranes were immersed in blocking buffer (5% NFDM in TBS-Tween) for 1 h at room temperature followed by incubation in primary antibody (diluted 1:1000 for cathepsins B and L, and cystatin C) (diluted 1:2000) for 1 h at room temperature. Cathepsins B and L and cystatin C protein bands were detected by chemiluminescence.
using the ECL kit and procedure specified by Amersham Corp. (Piscataway, NJ).

**Assays.** Cells were fed with serum-free media 12-18 h prior to harvesting for enzyme and inhibitor assays. On the day of harvesting, the serum-free medium was collected for proteinase and inhibitor assays. The cells were washed with PBS, pH 7.2, collected in PBS, centrifuged at 3,000 g for 5 min, and the pellet resuspended in PBS. The cells were lysed using a Tissue Tearor (Biospec Products Inc., Bartlesville, OK) at high speed for 5 sec, and immediately placed on ice. The cell pellet and homogenate were checked microscopically at each step to ensure that the cells were completely lysed. The homogenate was centrifuged at 15,000 g for 15 min to remove cell residue, and the supernatant saved.

Cysteine cathepsin assays were done according to the procedure of Barrett and Kirschke (32) as described by Colella et al. (25). Briefly, cathepsin L activity of the cell supernatant was measured using 12.5 μM Z-phe-arg-NMec as substrate in 250 mM acetate buffer, pH 5.4, containing 2.5 mM EDTA, and 1 μM dithiothreitol (DTT). The Z-phe-arg-NMec substrate is hydrolyzed by cathepsin L and to a small extent by cathepsin B; therefore, the activity is referred to as cathepsin L+B activity (32). Cathepsin B was measured with 12.5 μM Z-arg-arg-NMec in 250 mM phosphate buffer, pH 6.0, containing 2.5 mM EDTA and 1 μM DTT. The reactions were stopped by the addition of 100 mM monochloroacetic acid in 100 mM acetate buffer, pH 4.3. Fluorescence was measured in a Perkin-Elmer spectrofluorometer at an excitation wavelength of 370 nm and an emission wavelength of 460 nm. Activity is expressed as μUnit/μg protein where 1 Unit is defined as 1 μmol substrate released per minute. Protein concentration was determined by the Lowry method using bovine serum albumin as standard (33).

Statistics were calculated using the Student's t-test where P≤0.05 was considered significant. All values are the mean ± standard error of at least three separate experiments.

**Results**

**Morphology of cells plated on Matrigel.** The interactions between epithelial cells and components of their supporting matrix orchestrate the development of specific cell phenotypes and subsequently tissue and organ characteristics. Both cellular and stromal characteristics are altered during progression of cancer to the metastatic state; this includes the release of proteolytic enzymes that can also modify the external milieu (37). To determine if attachment to Matrigel affected cathepsin B and L expression in prostate cancer cell lines, PC3 and DU145 were initially plated on uncoated surfaces or surfaces coated with a thin gel of Matrigel (0.5-0.6 mg per cm²). There are no apparent differences in morphology between cells growing on uncoated surfaces (A and D) or surfaces coated with a thin layer of Matrigel (B and E). Cells attached to a Matrigel gel clump together and form linear arrays (C and F). Size bar indicated in Fig. 1E.

There are no direct assays for measuring cathepsin activity, total cysteine proteinase inhibitor (CPI) activity was indirectly measured in cell extract and conditioned media as described (25). Briefly, samples (cell extracts and conditioned media) were boiled for 5 min, centrifuged for 10 min at 15,000 g and the supernatant incubated with the cysteine proteinase, papain using Z-phe-arg-NMec as substrate as described above for cathepsin L+B. Inhibitory activity is expressed as μU inhibitory activity per μg protein where 1 μU of inhibitory activity represents the amount of papain activity inhibited per minute.

Since there are no direct assays for measuring cystatin activity, total cysteine proteinase inhibitor (CPI) activity was indirectly measured in cell extract and conditioned media as described (25). Briefly, samples (cell extracts and conditioned media) were boiled for 5 min, centrifuged for 10 min at 15,000 g and the supernatant incubated with the cysteine proteinase, papain using Z-phe-arg-NMec as substrate as described above for cathepsin L+B. Inhibitory activity is expressed as μU inhibitory activity per μg protein where 1 μU of inhibitory activity represents the amount of papain activity inhibited per minute.

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The aggregates were seen as early as 4-5 h following plating. The goal of this study was to determine the effects of extracellular matrix components on cathepsins L and B distribution and activity. Therefore, to eliminate the possibility that changes in cell shape also affect cathepsins L and B expression, cells were plated on a thin layer of Matrigel (70-80 μg Matrigel per cm²). The difference between a thin layer of Matrigel versus a thin gel is that the thin layer provides a complex protein layer for cell attachment without forming a ‘basement membrane’ type gel as the thin gel provides (Tech sheet, BD Bioscience SPC354234). There was no difference in the morphology of PC3 and DU145 plated on uncoated surfaces (Fig. 1A and D) compared to cells plated on a thin layer of Matrigel (Fig. 1B and E). We continued our studies using a thin layer of Matrigel to maintain consistency in cell shape between cells on uncoated and coated surfaces.

Immunolocalization of cathepsins B and L. Co-immunolocalization of cathepsins B and L was performed on prostate cancer cell lines plated on glass coverslips or coverslips coated with 70-80 μg Matrigel per cm². Cathepsins L and B appear as punctate material typical of a lysosomal/endosomal distribution in PC3 cells (Fig. 2). Individual cells show a predominant staining for either cathepsin B (green) or L (red). There is little co-localization of cathepsins B and L (as indicated by yellow-orange staining). PC3 cells on Matrigel demonstrate more cathepsin B staining toward the cell periphery (arrowhead, B) when compared to cells plated on uncoated surfaces. The distribution of cathepsin L is similar in cells plated on coated or uncoated surfaces. DU145 cells demonstrated similar distribution of cathepsins B and L as PC3 plated on uncoated surfaces; however, B and L localization did not change when DU145 was plated on Matrigel (data not shown).

Cathepsin L immunolocalization did not appear to be affected by the presence of Matrigel; cathepsin L displayed a perinuclear distribution in all groups of cells examined.

Cathepsin L+B, cathepsin B and cysteine proteinase inhibitor (CPI) activities. Cathepsin L+B, cathepsin B, and total CPI activities were measured in extracts and media of PC3 and DU145 cells grown on either uncoated surfaces or surfaces coated with a thin layer of Matrigel. DU145 exhibited higher cathepsin L+B (Fig. 3A and B) and cathepsin B (Fig. 3D and E) activity both in the cell extract and medium compared to PC3 consistent with our previous results (25) and with those of Freidrich et al (3). Cathepsin L+B activity was detected only in media that had been previously incubated with formic acid; the absence of proforms of cathepsin L on Western blots (Fig. 5) suggests that incubation of media at low pH destroys strong cathepsin L-cystatin complexes (34) rather than the presence of pro-forms of cathepsin L. Furthermore, incubation of media with formic acid results in the loss of measurable cathepsin B activity suggesting that cathepsin B is also secreted in its active form (Fig. 3E).

Matrigel did not significantly affect either cell-associated or secreted cathepsin L+B activity of PC3 and DU145 cells (Fig. 3); however, the percent of total cathepsin L+B activity secreted by PC3 was significantly higher when grown on Matrigel compared to plastic (Fig. 3C).

The distribution of cathepsin B activity between the cell extracts and media was affected by growth on Matrigel in the two cell lines (Fig. 3D-F). A decrease in intracellular activity of cathepsin B and an increase in secreted cathepsin B was observed in PC3 cells plated on Matrigel compared to cells plated on non-coated controls. In contrast, DU145 cells on Matrigel exhibited increase intracellular B activity but no significant difference in secreted activity (Fig. 3D and E). The
percent of total cathepsin B secreted confirms an increased secretion by PC3 but not DU145 (Fig. 3F).

Total cysteine proteinase inhibitory (CPI) activity was determined by the ability of cell extracts and media samples to inhibit the cysteine proteinase, papain. PC3 and DU145 had similar amounts of CPI activity in extracts (Fig. 4A) and media (Fig. 4B) as shown previously (25). Matrigel did not significantly affect cell-associated or secreted CPI activity in either cell line (Fig. 4).

Western blot analysis. Western analysis of cathepsins B and L and cystatin C was performed on cell extracts and media to determine if growth on Matrigel affected the protein levels of the cysteine proteinases and their endogenous CPI, cystatin C. Fig. 5 is a representative of three separate blots performed per protein. Cathepsins B and L appear as bands corresponding to the 31 kDa active forms of both proteinases. The inactive proforms of cathepsins B or L were not detected in the cell extracts or media. Western analysis indicates an increase in secreted cathepsin B protein in PC3 plated on Matrigel consistent with our activity data (Figs. 5 and 3B, respectively). An increase in the amount of secreted cathepsin B protein from DU145 grown on Matrigel is also evident in Western blots; however, the increase in secreted cathepsin B activity was not significantly different from cells grown on uncoated surfaces (Figs. 5 and 3E, respectively).
In agreement with cathepsin L activity data, Matrigel did not induce any changes in cell-associated or secreted cathepsin L protein in DU145 or PC3 cells.

A 14 kDa band corresponding to the cysteine proteinase inhibitor, cystatin C was present in the extract and media from both cell lines (Fig. 5); the amounts of cystatin C protein were equivalent in cells grown on Matrigel to those grown on plastic.

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Discussion

This study demonstrates that the location of the lysosomal proteinase, cathepsin B, is affected by a cell's interaction with basement membrane components. This phenomenon is specific for cathepsin B as cathepsin L is not affected. Furthermore, we found that cathepsins B and L do not co-localize suggesting lysosomal sorting pathways specific for each proteinase. Others also have shown that cathepsins B and L do not co-localize to the same vesicle (16,41) suggesting differential localization of cathepsins B and L in some cells.

Cathepsins B, L and other lysosomal cathepsins can be sorted following synthesis of their pro-forms to either lysosomes, regulated secretory lysosomes/vesicles, or constitutive secretory vesicles; evidence for targeting to each of these vesicles has been reported (42,43). Pro-cathepsins B and L are processed to mature forms in the acidic milieu of the lysosome; the mature forms include a single chain of ~30-31 kDa that is further processed to the double chain species of ~25 and 5 kDa. Pro-cathepsin B, its mature forms or both are reportedly secreted from cancer cells depending on cell type and environmental factors. The pro-form of cathepsin B is secreted when normal and tumor breast fibroblasts are plated on collagen I gels (44) whereas collagen I induces the secretion of pro- and mature forms of cathepsin B from invasive melanoma cells (45). An active cathepsin B species of ~31 kDa is secreted pericellularly from a number of human cell lines (46). DU145 cells secrete mature, active forms of cathepsin B following their interaction with either type I collagen or bone fragments (47). In the present study, the secretion of active cathepsin B was up-regulated by Matrigel in PC3 but not in DU145 cells. This discrepancy may be due to the different types of matrices used in the two studies: Matrigel is composed predominantly of type IV collagen whereas bone is composed on Matrigel. An additional 25 kDa band was detected in the media of both cell lines; this band may represent cystatin C dimerization (38,39) or different glycoforms of the inhibitor. Various glycoforms of cystatin C similar in size to our 25 kDa band were also detected in media conditioned by hippocampal stem cells (40).

Figure 5. Western blot analysis of cathepsins L and B and cystatin C in cell extracts and conditioned media from PC3 (lanes 1 and 2) and DU145 (lanes 3 and 4) grown on uncoated (lanes 1 and 3) or Matrigel-coated (gray bars) surfaces. The cell extracts and media were boiled for 5 min, centrifuged to remove debris, and incubated with purified papain to measure total CPI activity. CPI activity in the cell extracts (A) or media (B) of both cell lines was not significantly different when comparing cells grown on Matrigel to those grown on plastic.

Figure 4. Cysteine proteinase inhibitor (CPI) activity in cells grown on uncoated (black bars) or Matrigel-coated (gray bars) surfaces. The cell extracts and media were boiled for 5 min, centrifuged to remove debris, and incubated with purified papain to measure total CPI activity. CPI activity in the cell extracts (A) or media (B) of both cell lines was not significantly different when comparing cells grown on Matrigel to those grown on plastic.
of type I collagen. Cell binding to extracellular matrices occurs through surface integrins and specific integrin subunits have been implicated to play a role in cathepsin B secretion (44,45). PC3 expresses different integrin subunits compared to DU145 and these integrin subunits correlate with an increased invasive ability of PC3 (48). The differences in integrin subunit expression between PC3 and DU145 could also be responsible for the significant increase in secreted cathepsin B activity by PC3 when grown on Matrigel.

Recent evidence demonstrated that a number of matrix-degrading proteolytic enzymes associate with caveoleae (49), which are membrane lipid rafts that function in signal transduction, endocytosis, and lipid transport (50). Caveoleae may function as organizing centers for proteolytic events leading to matrix degradation and invasion. Caveolin-1 is a major structural component of caveoleae; caveolin-1 may play a crucial role in prostate cancer growth and is secreted by prostate cancer cells (51,52). Cathepsin B localizes to caveoleae of colon cancer cells and caveolin-1 is implicated in the secretion of cathepsin B from colorectal carcinoma cells (8,53). It is possible that the selectivity of cathepsin B secretion from PC3 cells and not cathepsin L is due to the association of cathepsin B with caveolin-1. Although there is strong evidence that caveolin-1 functions as a tumor promoter in prostate cancer, its role in modulating cathepsin B secretion in prostate cancer cells is not known and needs to be assessed.

In this study, cells plated on uncoated surfaces or on a thin layer of Matrigel exhibited similar morphologies, but differed remarkably from cells plated on a gel-like matrix. PC3 and DU145 formed typical monolayers on uncoated surfaces and thin Matrigel gels; whereas on gels, the cells clumped together and formed web-like structures connecting aggregates of cells. Similar patterns of cell organization were seen in human airway smooth muscle (54), primary rat mammary tumor cell lines. Int J Oncol 19: 1333-1339, 2001

References


