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Original Article

Acidic extracellular microenvironment promotes the invasion and cathepsin B secretion of PC-3 cells

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Abstract: Background: This study aimed to investigate the effect of acidic microenvironment on the invasion of prostatic carcinoma PC-3 cells and to explore the potential mechanism. Material and methods: PC-3 cells were maintained in medium at different pHs (pH 7.4, pH 7.0 and pH 6.6). Invasion and metastasis of PC-3 cells were investigated *in vitro*. Acridine orange staining was performed, followed by laser confocal scanning microscopy for the localization of lysosomes. Western blot assay and ELISA were employed to evaluate the effect of acidic microenvironment on the cathepsin B secretion. Results: Acidic microenvironment remarkably promote the invasion and migration of PC-3 cells ($P<0.01$). Moreover, at acidic extracellular pH (pHe), an obvious shift of lysosomes from the perinuclear region to the periphery was observed. Western blot assay and ELISA revealed that acidic microenvironment promoted the cathepsin B secretion in PC- cells. Conclusion: Acidic microenvironment may significantly promote the invasion of PC-3 cells and increase the secretion of cathepsin B. This suggests that the acidic microenvironment induced invasion of PC- cells is related to the elevated cathepsin B secretion.

Keywords: Acidic extracellular microenvironment, human prostatic cancer, invasion, lysosome, cathepsin B

Introduction

When compared with developed countries, China has a low prevalence of prostate cancer (PC), but its incidence is increasing stably in recent years. The metastasis of PC is a main cause of death in PC patients. For patients with metastatic PC, surgical intervention and radiotherapy usually achieve a poor efficacy. Thus, the metastasis of PC determines the therapeutic efficacy and prognosis of PC patients.

In 1889, Peget proposed the 'seed and soil' hypothesis in which the metastasis of tumor cells is ascribed to the specific affinity of these cells (which he equated to the 'seed') to the milieu of certain organs (which he equated to the 'soil') [1]. In past more than 100 years, findings revealed that the metastasis of tumor cells is dependent on not only the characteristics of tumor cells (such as the activation and/or inactivation of tumor related genes), but the interaction between tumor cells and tumor microenvironment (such as the interaction of tumor cells with macrophages [2], vascular endothelial cells and fibroblasts in the interstitium of tumors [3] as well as acidic microenvironment [4]).

In the present study, prostate cancer PC-3 cells were maintained at different pH (pH 7.4, pH 7.0 and pH 6.6), aiming to investigate the effect of acidic microenvironment on the metastasis of PC-3 cells.

Materials and methods

Materials

Prostate cancer PC-3 cells were purchased from the Shanghai Cell Bank of Chinese Academy of Science. Fetal bovine serum (FBS; GEMINI, Cat No. 900-208), DMEM (Gibco, Cat No. 12430-047), acridine orange solution (Sigma, Cat No. A8097), cathepsin B ELISA kit (R&D; Cat No. DY2176), CathepsinB (Santa

Cruz; Cat No. sc-13985), β-actin (Santa Cruz; Cat No. sc-47778), ECL luminescent substrate kit (Pierce), Transwell chamber (inner diameter: 6.5 mm; pore size: 8.0 μm; BD) and Matrigel™ (BD) were used in the present study.

Preparation of medium for cell culture

The pH value of DMEM was adjusted to 7.4, 7.0 and 6.6 with HCl or NaOH. Following filtration, FBS was added to DMEM at a final ratio (V/V) of 10%.

Transwell invasion assay

Transwell chamber (inner diameter: 6.5 mm; pore size: 8.0 μm) was placed in 24-well plates. The upper chamber was pre-coated with 50 μg of Matrigel in DMEM and DMEM containing 10% FBS (800 μl) was added to lower chamber. Cells in logarithmic growth phase were harvested after digestion with trypsin and centrifugation. Then, cells were re-suspended in serum free DMEM, and added to upper chambers (2×10^5 cells/chamber), followed by incubation at pH 7.4, pH 7.0 or pH 6.6 for 48 h. The medium in the upper chamber was removed, and the upper chambers were taken out. Swabs were used to remove the cells in the upper chamber, and these cells were fixed in 4% paraformaldehyde, followed by crystal violet staining. Following washing, cells were air-dried, and observed under a light microscope and representative photographs were captured. Then, cells were incubated with 10% glacial acetic acid for 30 min. When the crystal violet was resolved, 100 μl of medium was collected from each group and added to 96-well plates, followed by measurement of absorbance at 560 nm with a microplate reader (infinite M 200 PRO). Experiment was done three times.

Cell migration assay

Transwell chamber (inner diameter: 6.5 mm; pore size: 8.0 μm) was used here for both invasion assay and migration assay, except for the upper chamber being not pre-coated with Matrigel in migration assay. The detection of cell migration was similar to the detection of cell invasion, except for the upper chamber being not pre-coated with Matrigel. Cells in logarithmic growth phase were digested with trypsin, centrifuged and re-suspended in serum free DMEM. Then, cells were added to upper chambers (2×10^5 cells/chamber) and maintained in DMEM at different pH values (pH 7.4, pH 7.0 and pH 6.6) for 24 h. The medium was removed and upper chambers were taken out of plates. Swabs were used to remove the cells in upper chambers, and these cells were then fixed in 4% paraformaldehyde, followed by crystal violet staining. Cells were observed under a light microscope and representative photographs were captured, followed by cell counting [5]. There were 4 samples in each group, and five fields were randomly selected at 100×, followed by cell counting. Experiment was done three times.

Detection of cathepsin B

PC-3 cells were maintained in DMEM for 24 h and then in serum free DMEM at different pH values (pH 7.4, pH 7.0 and pH 6.6) for 48 h. Then, the supernatant was harvested and proteins were extracted from cells, followed by Western blot assay. In brief, proteins were subjected to SDS-PAGE (12% separating gel + 6% stacking gel) and then transferred onto NC membrane which was then blocked in 5% non-fat mild for 1 h. The membrane was incubated with mouse Rac1 primary antibody (1:500) and mouse β-actin primary antibody (1:500) at 4°C over-night, followed by washing in TBST. Then, the membrane was treated with HRP conjugated goat anti-mouse IgG (1:1000) for 2 h, followed by washing in TBST. Visualization was done with ECL kit. The cathepsin B protein in the supernatant was detected by ELISA according to manufacturer's instructions.

Lysosome staining

PC-13 cells were maintained in DMEM at different pH values (pH 7.4, pH 7.0 and pH 6.6) for 48 h, and then incubated with acridine orange at 5 μg/ml for 10 min at 37°C in an environment with 5% CO_2. Subsequently, cells were washed in PBS thrice and then observed under a laser scanning confocal microscope at excitation wavelength of 488 nm and emission wavelength of 610 nm.

Statistical analysis

Statistical analysis was performed with SPSS version 13.0. Comparisons between two groups were done with t test. Data are expressed as mean ± standard deviation (SD). Data were expressed as mean ± standard deviation (SD). A value of $P<0.05$ was considered statistically significant.

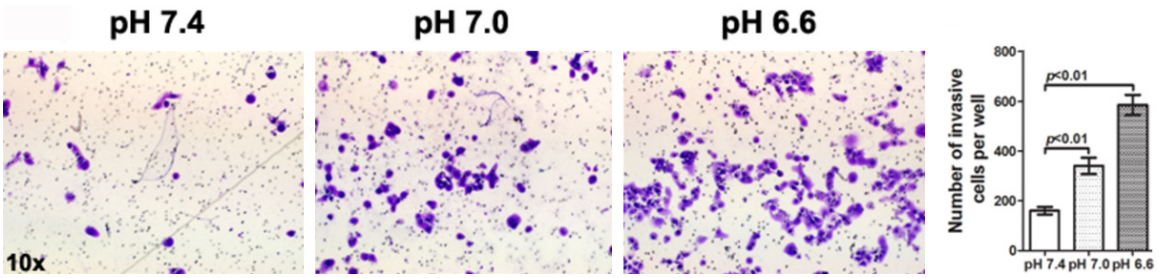

Figure 1. Acidic extracellular microenvironment promotes PC-3 cell invasion *in vitro*. The number of invasive cells significantly increased after culture at pH 6.6 as compared to pH 7.0 and pH 7.4 groups (n=3, *P*<0.01). Magnification: ×100.

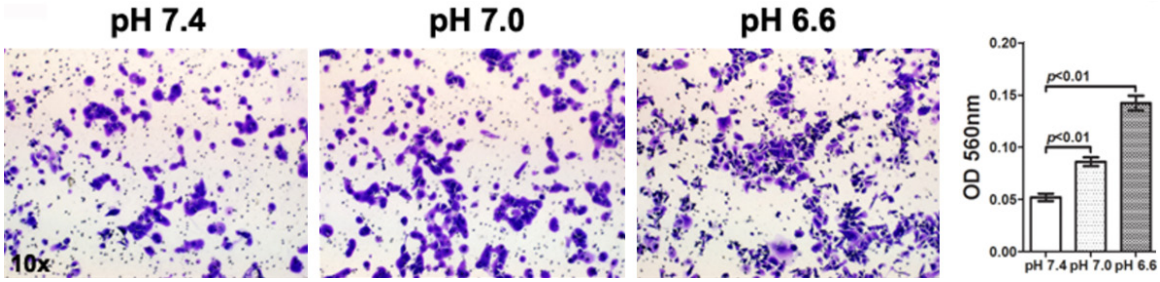

Figure 2. Acidic extracellular microenvironment promotes PC-3 cell migration *in vitro*. The number of migrated cells significantly increased after culture at pH 6.6 as compared to pH 7.0 and pH 7.4 groups (n=3, *P*<0.01). Magnification: ×100.

Results

Acidic microenvironment promotes the invasion of PC-3 cells

In Transwell chamber assay, there were 3 samples in each group (pH 7.4 group, pH 7.0 group and pH 6.6 group), and experiment was done thrice. Cells were incubated at different pH values for 48 h, and the upper chambers were taken out. Swabs were used to remove cells in the upper chambers. These cells were then fixed in 4% paraformaldehyde, followed by crystal violet staining. Representative photographs were captured under a light microscope [4], followed by cell counting. Results showed the number of invasive cells was 160.33±27.15, 340.33±56.75 and 585.33±69.37 in pH7.4 group, pH7.0 group and pH6.6 group, respectively, showing marked difference (P<0.01; **Figure 1**).

Acidic microenvironment promotes the migration of PC-3 cells

In the detection of PC-3 cell migration, there were 3 samples in each group (pH 7.4 group, pH 7.0 group and pH 6.6 group), and experiment was done thrice. Cells were incubated at different pH values for 24 h, and the upper chambers were taken out. Swabs were used to remove cells in the upper chambers. These cells were then fixed in 4% paraformaldehyde, followed by crystal violet staining. Representative photographs were captured under a light microscope [6]. Then, 10% glacial acetic acid (2 ml) was added to resolve crystal violet, and 100 μl of solution was transferred into a 96-well plate. Absorbance was measured at 560 nm with a microplate reader (infinite M200 PRO). The optical density was 0.0520±0.0063, 0.0861±0.0074 and 0.1423±0.0127 in pH 7.4 group, pH 7.0 group and pH 6.6 group, respectively, showing significant difference (P<0.01; **Figure 2**).

Acidic microenvironment promotes cathepsin B secretion of PC-3 cells

PC-3 cells were maintained for 24 h and the grown in serum free medium at pH 7.4, pH 7.0 or pH 6.6 for 48 h. Proteins were extracted from cells for Western blot assay, and the supernatant was harvested for further ELISA.

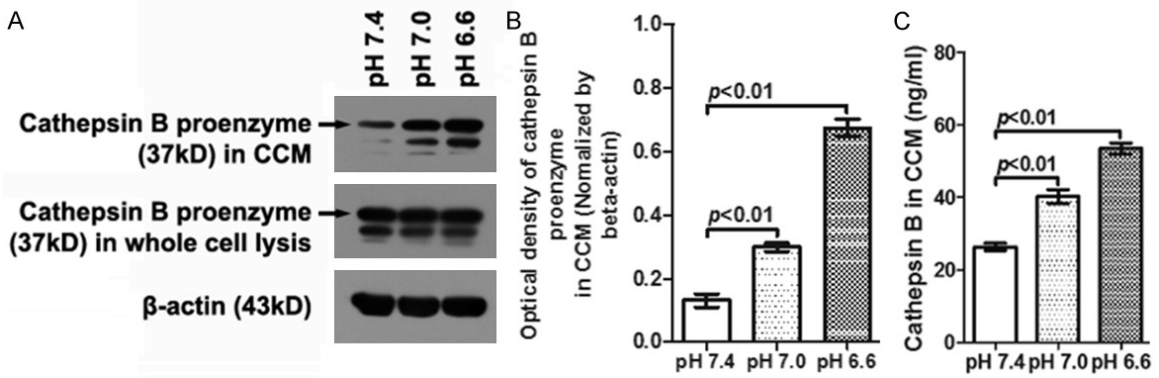

Figure 3. Acidic extracellular microenvironment enhances cathepsin B secretion in PC-3 cells. Cathepsin B in the supernatant of pH 6.6 group was higher than that in pH 7.4, but there was no significant difference between pH 6.6 group and pH 7.4 group (A). When compared with pH 7.4 group, acidic extracellular microenvironment significantly enhanced the cathepsin B secretion in PC-3 cells. OD was detected and OD of cathepsin B was normalized to that of β-actin. The cathepsin B proenzyme protein content was also detected in the supernatant (n=3) (B). ELISA assay obtained the same results (C).

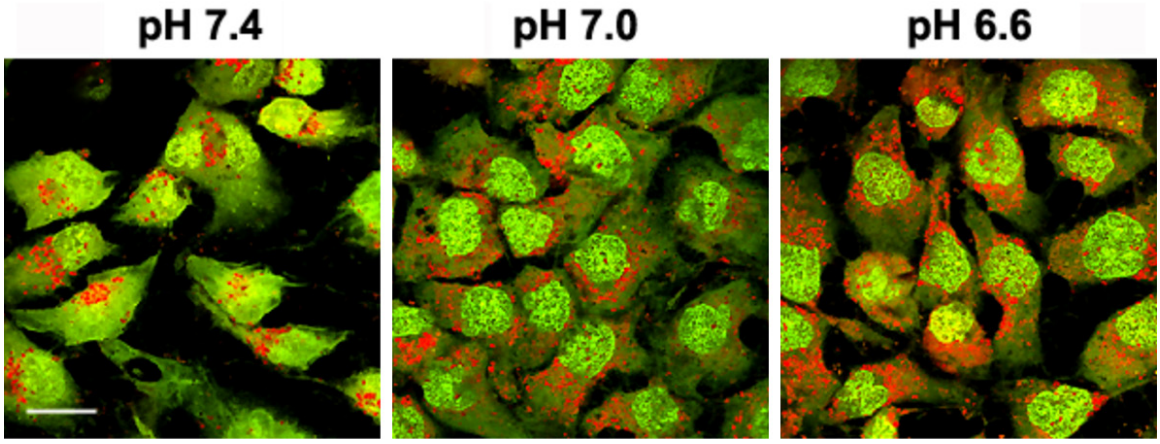

Figure 4. Acidic extracellular environment shifts the intracellular lysosomes from perinuclear region to cell periphery. Examples from three images are shown for each pH to demonstrate that, in control cells (pH 7.4), lysosomes mainly localized at perinuclear region, whereas in cells in acidic microenvironment (pH 7.0 and pH 6.6), lysosomes showed a shift toward cell periphery and a diffuse distribution (Bar=50 μm).

Results showed the cathepsin B in the supernatant of pH 6.6 group was higher than that in pH 7.4, but there was no significant difference between pH 6.6 group and pH 7.4 group (**Figure 3A**). The cathepsin B content of the supernatant normalized to β-actin was 0.1320±0.0356 in pH 7.4 group (n=3), 0.2995±0.0235 in pH 7.0 group (n=3) and 0.6756±0.0472 in pH 6.6 group (n=3), showing marked difference (P<0.01; **Figure 3B**). ELISA of the supernatant showed total cathepsin B in the supernatant in pH 6.6 group was significantly higher than that in pH 7.4 group. The total cathepsin B was 26.32±1.81 ng/ml in pH 7.4 group (n=3), 40.23±3.32 ng/ml in pH 7.0 group (n=3) and 53.42±2.68 ng/ml in pH 6.6 group (n=3), showing significant difference (P<0.01; **Figure 3C**).

Acidic microenvironment promotes shift of intracellular lysosomes from perinuclear region to periphery of PC-3 cells

Cells were maintained on coverslips at different pH values for 48 h, followed by acridine orange staining of intracellular lysosomes. Five cells were randomly selected in each group, and five lysosomes which farthest from the nucleus were selected to measure the maximal lysosome-to-nucleus distance in each cell [5]. Cells were observed under a laser scanning confocal

microscope. When compared with pH 7.4 group, the intracellular lysosomes shifted from perinuclear region to periphery and from cluster-like distribution to diffuse distribution in PC-3 cells of pH 7.0 group and pH 6.6 group (**Figure 4**). The maximal lysosome-to-nucleus distance (white arrow in **Figure 4**) was 8.92±3.27 μm in pH 7.4 group (n=25), 19.80±3.94 μm in pH 7.0 group (n=25) and 32.84±8.23 μm in pH 6.6 group (n=25), showing significant difference ($F=114.581$, $P<0.01$).

Discussion

Available studies reveal the solid cancers in humans and animals are acidic and their pH value may approach 6.0 [6-9]. Martinez-Zaguilan et al [10] employed membrane invasion culture system to investigate the effect of acidic microenvironment on cells. Their results showed C8161 cells and A375p cells became highly invasive in acidic microenvironment (pH 6.8) as compared to that at pH 7.4. In China, investigators also found that liver cancer cells were more invasive and more likely to metastasize in acidic microenvironment ($P<0.01$) and the activation of MMP-2 and MMP-2 increased significantly in this environment although MMP-2 expression remained unchanged [11]. In the present study, PC-3 cells were maintained at different pH values, and results showed acidic microenvironment could promote the invasion and migration of prostate cancer PC-3 cells.

In the metastasis of cancer cells, the degradation of extracellular matrix (ECM) is a prerequisite for the migration of cancer cells from the primary cancer and the subsequent metastasis. The degradation of ECM requires matrix metalloproteinases (MMPs). In addition, MMPs also mediate the slicing and release of receptors on cells, the activation and inactivation of cytokines and chemokines and the release of apoptosis related ligands, all of which are able to promote the proliferation, adhesion, separation, migration (degradation of E-cadherin by MMP-3 or MMP-7) [12], differentiation and angiogenesis of cancer cells and simultaneously inhibit cancer cell apoptosis and host defense, leading to the metastasis of cancer cells [5].

In recent years, studies on MMPs and its activators show acidic environment may promote the activation of MMPs such as cathepsin B. The activation, secretion and distribution of cathepsin B are closely related to pH value. The acidic microenvironment in a cancer may induce the peripheral distribution of cathepsin B in cells and also increase its secretion. At the cancer margin, cathepsin B is highly active [13-15]. Rozhin et al [16] found focal acidic microenvironment induced the peripheral distribution of cathepsin B and increased its secretion, and cathepsin B was highly active at the cancer margin [13]. Cathepsin B may not only degrade fibrinogen and type IX and XI cartilage collagen, but laminin [15], fibronectin [17] and type IV collagen [18] in the basement membrane. Antisense technique was employed to reduce cathepsin B expression in osteosarcoma MNNG/HOS cells [19, 20], and results showed the invasion and migration of these cells reduced significantly. In the present study, Western blot assay and ELISA confirmed that cathepsin B in the supernatant of pH 6.6 group was significantly higher than that in pH 7.4 group, but cathepsin B was comparable between pH 7.4 group and pH 6.6 group.

Cathepsin B is one of cathepsins closely related to cancers in the lysosomes. In the carcinogenesis, the location and permeability of lysosomes change, and a large amount of cathepsins are released into cytoplasm. Cathepsins in the cytoplasm, together with MMPs and blood plasminogen activation system, participates in the degradation of ECM, leading to the increased movement and invasion of cancer cells and the elevated angiogenesis for cancer growth. In these processes, Cathepsins, especially Cathepsins B, D and L, show a significant change in expression. Thus, the Cathepsin content may be employed as a reliable marker of malignant tumors and is helpful for the diagnosis of malignancies [21, 22].

In addition, Glunde et al [23] found, in acidic microenvironment (pH 6.8 and 6.4), the lysosomes shifted from perinuclear region to cell tentacles and periphery in breast cancer cells. In the present study, acridine orange staining of lysosomes was performed. Results showed, when compared with pH 7.4 group, the lysosomes in pH 7.0 group and pH 6.6 group showed a shift from perinuclear region to cell periphery and from cluster-like distribution to diffuse distribution. This indicates that acidic

microenvironment promotes the invasion of PC-3 cells and the secretion of cathepsin B, which might be ascribed to the elevated migration of lysosomes in PC-3 cells in acidic microenvironment.

Conclusions

Acidic microenvironment may significantly promote the invasion of PC-3 cells and increase the secretion of cathepsin B. This suggests that the acidic microenvironment induced invasion of PC- cells is related to the elevated cathepsin B secretion.

Acknowledgements

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Disclosure of conflict of interest

None.

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