RESEARCH COMMUNICATION

Inhibition of Breast Cancer Metastasis Via PITPNM3 by Pachymic Acid

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Abstract

Breast cancer metastasis is the most common cause of cancer-related death in women. Thus, seeking targets of breast tumor cells is an attractive goal towards improving clinical treatment. The present study showed that CCL18 from tumor-associated macrophages could promote breast cancer metastasis via PITPNM3. In addition, we found that pachymic acid (PA) could dose-dependently inhibit migration and invasion of MDA-MB-231cells ,with or without rCCL18 stimulation. Furthermore, evidence was obtained that PA could suppress the phosphorylation of PITPNM3 and the combination of CCL18 and PITPNM3. Therefore, we speculate that PA could inhibit breast cancer metastasis via PITPNM3.

Keywords: Breast cancer - metastasis - MDA-MB-231 - PITPNM3 - pachymic acid

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Introduction

Breast cancer is a leading cause of cancer death in women worldwide, which always accompany with metastasis. Even after removal of the primary tumor, micrometastases persist in various tissues of cancer patients (Mundy, 2002). The survival of these cancer cells mainly depend on the support provided by the microenvironment and the ability of these cells adapt to the microenvironment (Clines & Guise, 2005).

Macrophages are heterogeneous cells that respond differently to various microenvironmental signals and, thus, display distinct function (Martinez et al., 2009). Chemokine (C-C motif) ligand (CCL18) is a chemokine predominantly produced by monocyte-derived cells with M2 phenotype. Overexpression of CCL18 was observed in chronic inflammations, fibrotic diseases and various cancers.CCL18 from TAM can promote breast cancer metastasis via PITPNM3 which is the functional receptor of CCL18.

Pachymic acid (PA) is a lanostrane-type triterpenoid from P.cocos., which is also called Fuling in Chinese medicine. Previous studies have shown that PA could induce apoptosis in prostate cancer cells (Gapter et al., 2005). Lots of evidences have revealed that certain triterpenoids have anti-cancer activity, especially lanostane-type triterpenoids (Kaminaga et al., 1996; Ukiya et al., 2002). Another study has shown that PA could stimulate glucose uptake through enhance GLUT4 expression and translocation (Huang et al., 2010). In clinical, Fuling is an important component for treatment of breast cancer metastasis in traditional Chinese medicine. However, there hasn't been any research on effect of PA on breast cancer metastasis. Therefore, we pay attention to whether PA could suppress breast cancer metastasis, and how it works.

Materials and Methods

Cell culture

The human breast carcinoma cell line MDA-MB-231 were obtained from Cell Bank of Chinese Academic of Science and maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Industries, Inc.) at 37 °C in a humidified atmosphere of 95% and 5% CO_2.

Reagents

Pachymic acid (PA), whose purity was above 98%, was obtained from national institutes for food and drug control. Its structure was shown in Figure 1. It was dissolved in DMSO at the desired concentrations and diluted into media at a maximal concentration below 0.1%, which was also present in the corresponding controls.

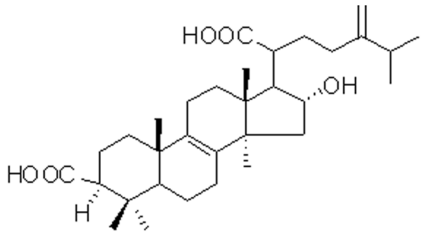

Figure 1. Structure of Pachymic Acid

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RNA interference assay

Small interfering RNA (siRNA) was transfected into MDA-MB-231 cells with LipofectamineTM 2000 (Invitrogen Corp.) according to the manufacturer's protocol (Cell Signaling Technology). The PITPNM3 siRNA used was obtained by R&D Systems.

Wound healing assay

For this assay, MDA-MB-231BO cells with rCCL18 stimulation (0, 5, 15 μg/mL), and cells interfered with siRNA were cultured in 24-well cell culture plates. A plastic pipette tip was drawn across the center of the plate to produce a 1mm-wide wound, followed by the incubation with PA (0, 10 , 20, 40 μg/mL). After 12 h, the movement of the cells into the wound was examined. Migration rate = (migration distances of PA treated cells/ migration distances of untreated cells) × 100%.

Transwell assay

Invasiveness into the reconstituted basement membrane Matrigel (Becton Dickinson Labware) was assayed. MDA-MB-231BO cells with rCCL18 stimulation (0, 5, 15 μg/mL), and cells interfered with siRNA were incubated with PA (0, 10, 20, 40 μg/mL) for 12 h, and then trypsinized to form single-cell suspension in DMEM (serum-free), which was added into the upper compartment of a Transwell cell culture chamber. Culture medium containing 10% (v/v) BS was served as chemo-attractant in the lower chamber. After 8 h of incubation, cells that still remained on the upper surface of the membrane were removed by a cotton swab, while cells having invaded through the Matrigel-precoated membrane filter (Becton Dickinson Labware) were fixed, stained and then counted with a microscope.

Western blot analysis

Proteins were prepared with the Mammalian Cell Extraction Kit (Bio Vision). Each sample (50μg) was subjected to SDS-polyacrylamide (10%) gel electrophoresis and electro-transferred onto a PVDF membrane. After blocked, the membranes were incubated with appropriate primary antibodies in blocking buffer overnight at 4 °C. Then the membranes were incubated with secondary antibody. At last, the membranes were visualized with ECL Plus reagent (GE Healthcare) and developed onto X-ray film (Kodak).

Flow cytometry analysis

MDA-MB-231 cells with rCCL18 stimulation (0, 5, 15 μg/mL) were incubated PA (0, 10, 20, 40 μg/mL). After 12h, cells were fixed, and then stained with an FITC -labled anti-CCL18 antibody. Then MDA-MB-231 cells were detected by flow cytometry (Beckman Coulter).

Statistics

Each experimental value was expressed as means ± standard deviation (SD). Statistical analysis was performed using the Origin7.5 software to evaluate the significance of differences between groups considered as *p < 0.05; **p < 0.01 and ***p < 0.001. All data points represented the mean of triplicates.

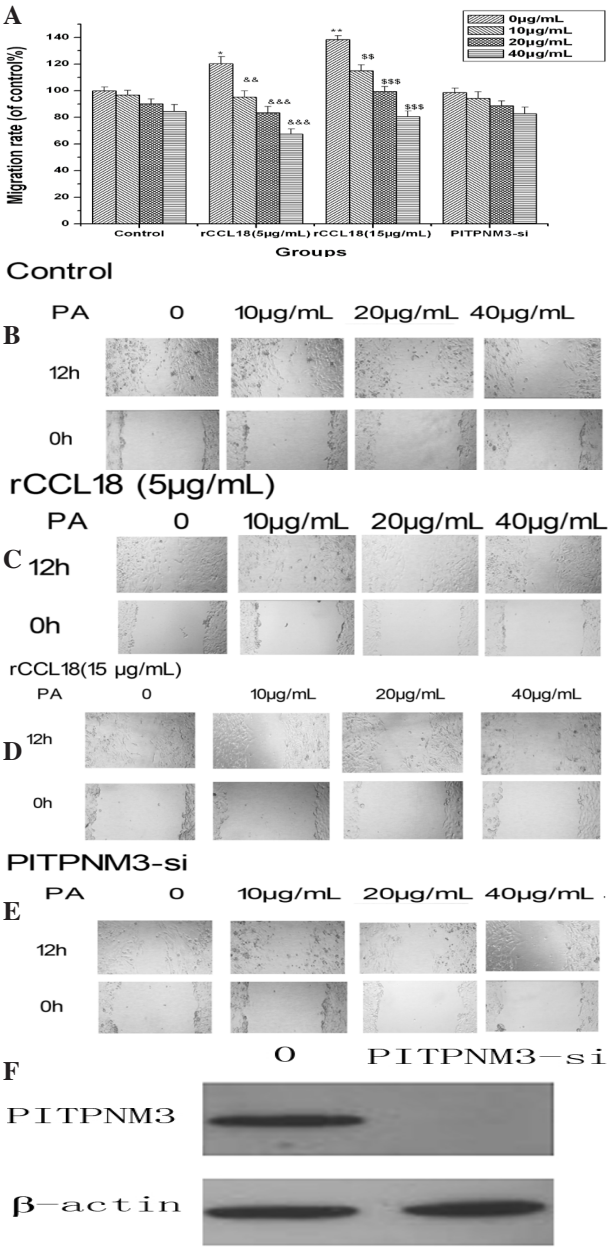

Figure 2. (A) Effect of PA on migration of MDA-MB-231 cells. Migration rate = (migration distances of drug treated cells/ migration distances of untreated cells) × 100%. Data were shown as the mean ±S.D, all experiments were conducted in triplicate. Significant differences from control were indicated by *p < 0.05, **p < 0.01, ***p < 0.001, compared to absence of PA in control group; $^{\&}$p < 0.05, $^{\&\&}$p < 0.01, $^{\&\&\&}$p < 0.001, compared to absence of PA in rCCL18 (5 μg/mL) group; $^{\$}$p < 0.05, $^{\$\$}$p < 0.01, $^{\$\$\$}$p < 0.001, compared to absence of PA in rCCL18 (15 μg/mL) group, and $^{\#}$p < 0.05, $^{\#\#}$p < 0.01, $^{\#\#\#}$p < 0.001, compared to absence of PA in PITPNM3-si group. (B) Effect of PA on migration of MDA-MB-231 cells in control group. (C) Effect of PA on migration of MDA-MB-231 cells in rCCL18 (5μg/mL) group. (D) Effect of PA on migration of MDA-MB-231 cells in rCCL18 (15μg/mL) group. (E) Effect of PA on migration of MDA-MB-231 cells in PITPNM3-si group. (F) Expression of PITPNM3 in MDA-MB-231 cells and ones interfered by PITPNM3-siRNA

Results

Effect of PA on the migration of MDA-MB-231 cells

To evaluate the inhibition effect of PA on the migration of MDA-MB-231 cells, wound healing assay was used. As shown in Figure 2, cellular motility was obviously

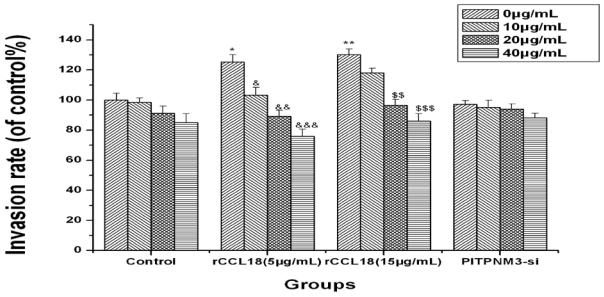

Figure 3. Effect of PA on Invasion of MDA-MB-231 Cells. Invasion rate = (the number of invading cells in drug treated team/the number of invading cells in untreated team) × 100%. Data were shown as the mean ±S.D., all experiments were conducted in triplicate. Significant differences from control were indicated by $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to absence of PA in control group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$, compared to absence of PA in rCCL18 (5 μg/mL) group; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$, compared to absence of PA in rCCL18 (15 μg/mL) group, and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, compared to absence of PA in PITPNM3-si group

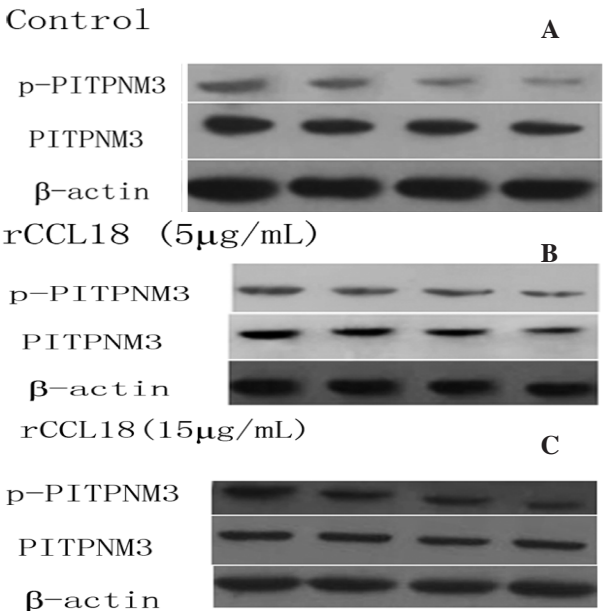

Figure 4. Western Blot Analysis. (A) Effect of PA on the expression of PITPNM3 and p-PITPNM3 in MDA-MB-231 cells of the control group. (B) Effect of PA on the expression of PITPNM3 and p-PITPNM3 in MDA-MB-231 cells of rCCL18-5μg/mL group.(C) Effect of PA on the expression of PITPNM3 and p-PITPNM3 in MDA-MB-231 cells of rCCL18-15μg/mL group. After 12 h incubation with PA (0, 10, 20, 40 μg/mL), the protein of MDA-MB-231 cells were prepared. β-actin was used as a loading control. Western blots were representative of three independent experiments. Experiment was repeated three times with similar results

inhibited in a dose-dependent manner by PA all the groups expect the one interfered with PITPNM3 siRNA, and the changes of cellular in the present of CCL18 (5, 15 μg/mL) were greater than changes at the absence of rCCL18. The results above indicated that PA could inhibit the migration of MDA-MB-231 cells via the CCL18- -PITPNM3 combination.

Effect of PA on the invasion of MDA-MB-231 cells

To investigate the inhibition effort of PA on the invasion of MDA-MB-231 cells, the Transwell assay was

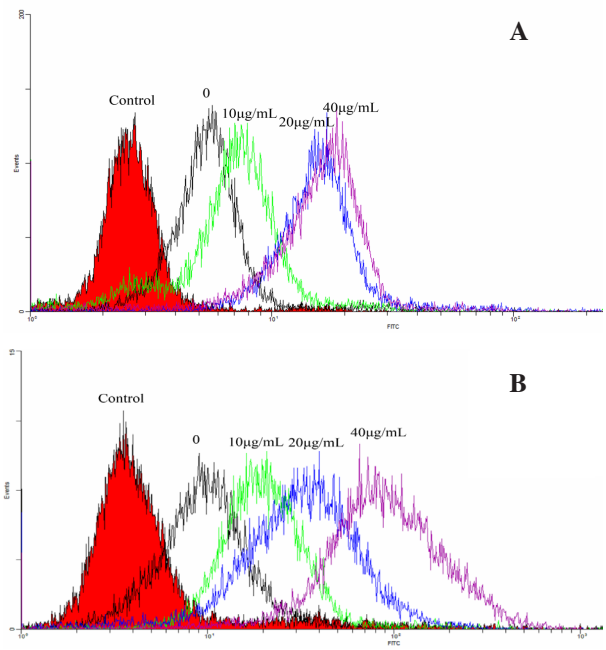

Figure 5. (A) Effect of PA (0, 10, 20, 40 μg/mL) on the CCL18 expression of MDA-MB-231 cells in rCCL18-5μg/mL group. (B) Effect of PA (0, 10, 20, 40 μg/mL) on the CCL18 expression of MDA-MB-231 cells in rCCL18-15 μg/mL group. The histograms demonstrated the fluorescence intensity of CCL18, while control values were shown as gray areas. All experiments were conducted in triplicate

conducted. As shown in Figure 3, the invasion ability of cells were inhibited in a dose-dependent manner by PA all the group expect the one interfered with siRNA, and the similar changes with the wound healing assay were observed between these groups in this assay. From these results obtained, it was revealed that PA was capable to inhibit the invasion of MDA-MB-231 cell via CCL18-PITPNM3.

Effect of PA on PITPNM3 expressions in protein

To illuminate whether the present of PA could affect PITPNM3 expression in protein level, western blot was used. As shown in Fig.4, the expressions of PIPNM3 in the groups stimulated with CCL18 (0, 5, 15 μg/mL) had been almost unchanged. However, PA could dose-dependently suppress the phosphorylation of PITPNM3.

Effect of PA on combination of CCL18 and PITPNM

To observe whether PA could inhibit CCL18 locating on the cell surface with PITPNM3, flow cytometry was used. From the results of Figure 4, we could find that there is no obvious change of PITPNM3 expressions in each group. If the expression of CCL18 changed, we would speculate that PA can affect combination of CCL18 and PITPNM3. From Figure 5, we could find that PA could dose-dependently suppress fluorescence intensity of CCL18 in present of CCL18 (5, 15 μg/mL) that just indicated PA could inhibit relative expressions of CCL18, which meant PA could inhibit the combination of CCL18 and PITPNM3. Thus, the inhibition on the combination of CCL18 and PITPNM3 may be a main path to restrain metastasis.

Discussion

Breast cancer metastasis is always an obstacle to clinical treatment. Breast cancer survival rate falls from about 90% to 20% for metastatic diseases (Fougere et al., 2010; Wang et al., 2011). In the process of metastasis, it is vital for tumor cell to arrest in mediums, regulated by series of stimulus or inhibitors in the microenvironment. CCL18 triggers integrin clustering and ECM adherence of tumor cells, which eventually contribute to enhanced invasion and metastasis (Zohny & Fayed, 2010; Narita et al., 2011). Essentially, CCL18 could specifically bind to PITPNM3, stimulate calcium signaling and induce directional migration. As the functional receptor of CCL18, PITPNM3 could be phosghorylated and then trigger a series of actions, such as activation of praline-rich tyrosine kinase (Pyk2), a key component of focal adhesion complex (FAC) (Lev et al., 1999; Zohny & Fayed, 2010). In our study, we chose MDA-MB-231 cells, a breast cancer line with metastasis potential. The expression of PITPNM3 was high, but the expression of CCL18 could not be detected in MDA-MB-231 cells (Chen et al., 2011). Then, we established a model, as MDA-MB-231 cells were co-cultured with rCCL18, which predominantly produced by monocyte-derived cells with M2 phenotype, in order to imitate the microenvironment in vivo. Pachymic acid (PA), a lanostrane-type triterpenoid from P.cocos, has widely used for treatment of cancers in traditional Chinese medicine. Previous study has shown that PA had some effect on apoptosis of prostate cancer cells, however, the inhibition was not so great. Therefore, we commit ourselves to finding out a main path to suppress cancers.

In this study, it has shown that PA could dose-dependently inhibit migration and invasion of MDA-MB-231cells with or without rCCL18 stimulation. Meanwhile, PA could suppress the migration and invasion greater in present of rCCL18, so we have a question whether PA has effect on the functional receptor of CCL18. Furthermore, the expressions of PITPNM3 have been almost unchanged, but we also have found that PA could obviously dose-dependently suppress phosphorylation of PITPNM3. However, PA has great inhibition to the combination of CCL18 and PITPNM3, which may be another main reason for PA to suppress the migration and invasion in microenvironment. Therefore, we speculate that PA could inhibit breast cancer metastasis via PITPNM3.

Acknowledgements

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