Original Paper

Osteopontin Promotes Invasion, Migration and Epithelial-Mesenchymal Transition of Human Endometrial Carcinoma Cell HEC-1A Through AKT and ERK1/2 Signaling

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Key Words

Osteopontin • Endometrial cancer • Migration • Invasion • EMT

Abstract

Background/Aims: Osteopontin (OPN) is an Extracellular Matrix (ECM) molecule and is involved in many physiologic and pathologic processes, including cell adhesion, angiogenesis and tumor metastasis. OPN is a well-known multifunctional factor involved in various aspects of cancer progression, including endometrial cancer. In this study, we examined the significance of OPN in endometrial cancer. **Methods:** The proliferation, migration and invasion ability of HEC-1A cells were detected by Cell Counting Kit-8 (CCK-8), Wound scratch assay and transwell. Western blots were employed to detect the expression of Matrix metalloproteinase-2 (MMP-2) and epithelial-mesenchymal transition (EMT)-related factors in HEC-1A cells treated with rhOPN. **Results:** rhOPN promotes cell proliferation, migration and invasion in HEC-1A cells. rhOPN influenced EMT-related factors and MMP-2 expression in HEC-1A cells. rhOPN promoted HEC-1A cells migration, invasion and EMT through protein kinase B (PKB/AKT) and Extracellular regulated protein kinases (ERK1/2) signaling pathway. **Conclusions:** These results may open up a novel therapeutic strategy for endometrial cancer: namely, rhOPN have important roles in controlling growth of endometrial of cancer cells and suggest a novel target pathway for treatment of this cancer.

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Introduction

Endometrial carcinoma (EC) is the most common malignancy of the female genital tract and is increasing in frequency [1]. EC often causes abnormal postmenopausal bleeding as an initial symptom and is commonly detected as an early stage malignancy, with more than 75% of women with this disease diagnosed at stage I/II and the majority of them surgically curable [2, 3]. Thus, the overall prognosis for EC is excellent, with a 5-year survival rate approximating 80% [4, 5].

Tumor growth and metastasis requires a coordinated succession of events within the tumor microenvironment that involves proliferation, loss of cellular adhesion, degradation of the extracellular matrix (ECM), invasion into host stroma, cell migration, and angiogenesis [6, 7]. Growth factors and cytokines, serine proteases, matrix metalloproteinases (MMPs), cellular adhesion molecules, tumor cell stromal interactions, and phospholipids are actively involved in the regulation of these processes [8-10]. Thus, understanding the tumor microenvironment and its regulatory mechanism is critical for the development of effective therapies to treat metastatic endometrial carcinoma. Epithelial-to-mesenchymal transition (EMT) is important to tumor aggression and metastasis [11]. At a molecular level, EMT occurs as a result of the activity of some transcriptional factors, such as ZEB1/2, Twist and Snail, which suppress expression of the epithelial marker E-cadherin and induce the mesenchymal genes N-cadherin and Vimentin [12]. However, the mechanisms and pathways that initiate EMT programs are not comprehensively understood.

Osteopontin (OPN) is a SIBLING (small integrin-binding ligand, N-linked glycoprotein) that was first identified in 1986 in osteoblasts. OPN is an ECM molecule and is involved in many physiologic and pathologic processes, including cell adhesion [13], angiogenesis [14] and tumor metastasis [15, 16]. OPN is a well-known multifunctional factor involved in various aspects of cancer progression, including endometrial cancer. OPN is postulated to bind to integrin receptors at the cell surface of human endometrium to mediate cellular adhesion and migration during embryo implantation [17]. There are conflicting reports about the expression of OPN in endometrial cancer [18, 19]. Additionally, the molecular mechanisms that define the role of OPN in tumor progression and metastasis in endometrial cancer are incompletely understood.

In this paper, we were aimed to study the relationship between OPN and EC.

Materials and Methods

Reagents

Human uterine epithelial cell line HEC-1A cells were acquired from the American Type Culture Collection (ATCC). Recombinant OPN protein (rhOPN) was purchased from R&D Systems. Rabbit anti-AKT and phosphor-AKT (Ser473) antibodies were purchased from Bioworld Technology. Rabbit anti-ERK, phospho-ERK 1/2, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies and U0126 (a specific inhibitor of MEK/ERK; 10-5M) and LY294002 (a specific inhibitor of p38; 10-6M) were purchased from Beyotime Institute of Biotechnology. MMP-2, E-cadherin, N-cadherin and Vimentin were purchased by Proteintech. Cell Counting Kit-8 was purchased by Dojindo. Enhanced chemiluminescence (ECL) assay kit was purchased from Amersham. Horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody, anti-mouse secondary antibody, biotinylated secondary antibody, streptavidin-horseradish peroxidase and diaminobenzidine (DAB)-peroxidase substrate were purchased from ZSGB-BIO. TRITC-conjugated goat anti-rabbit secondary antibody IgG and FITC-conjugated goat anti-mouse secondary antibody were purchased from Thermo.

Cell lines and culture conditions

HEC-1A cells were grown in McCoy's 5A supplement with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at 37°C under 5% CO₂ in humidified air.

Cell Proliferation Assay

Cell proliferation was detected by a Cell Counting Kit-8 assay. HEC-1A cells were suspended in McCoy's 5A medium supplemented with 15% heat-inactivated fetal bovine serum and subsequently seeded in 96-well plates and incubated for 24h. After that, plates were divided into four groups treated with different concentration of rhOPN (0 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml and 400 ng/ml). Then, plates were continued incubating 1 Days, 2 Days, 3 Days and 4 Days. After being incubated, the cultures were added 10 μ l CCK-8 solution to each well and incubated at 37°C for another 2 h. Optical density (OD) value of absorbance at 450 nm was measured by Thermo Scientific Fluoroskan Ascent FL. The results were plotted as means $\pm$ SEM of three independent experiments having three determinations per sample for each experiment.

Wound scratch assay

Cells were seeded into six-well plates and then cultured to 90% confluence. The confluent cell monolayer was wounded using a sterile 10 μ l pipette tip. The suspended cells were washed using normal growth medium. The scratch wound was captured after 48 h using an OLYMPUS IX81 microscope in three fields of view at 100 $\times$ magnification. The area of the open wound was quantified using Photoshop (Adobe).

Matrigel cell invasion assay

For matrigel cell invasion assay, transwell inserts (6.5 mm, Costar) containing polycarbonate filters with 8 mm pores were precoated with 50 μ l of 1 mg/ml matrigel matrix (Becton Dickinson) at 37°C for 4 h for gelling. 1.0×10^5 of HEC-1A cells in serum-free medium were plated in the upper chamber, whereas medium with 10% FBS was added to the lower chamber. After incubating for 24 h, the cells on the matrigel side of the inserts were removed by cotton swab. The inserts were fixed in methanol and stained with crystal violet. The number of invaded or migrated cells attached to the other side of the insert was counted under a light microscope (Olympus) in five random fields. Three independent experiments were performed. Numbers of invasive or migrated cells under different treatments were normalized to the control and expressed as a means of invasion or migration percentage (%) $\pm$ SEM.

Western Blot

Cells were washed in PBS before incubation with Lysis Buffer (1% Triton X-100, 150 mM NaCl, 10mM Tris, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM Na_3VO_4 , 0.2 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40) on ice for 10 min. The cell lysates were clarified by centrifugation at 9,000 g for 15 min and the supernatants were collected. Protein concentration was determined with the Coomassie Protein Assay reagent using bovine serum albumin (BSA) as a standard. Equal amounts of protein extracts (30 μ g) were separated by 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose filter (NC) membranes. The membranes were blocked in 5% non-fatmilk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 2 hours at room temperature and probed with primary antibodies overnight at 4°C (MMP-2, 1:400; N-cadherin, E-cadherin and Vimentin, 1:1000; AKT, p-AKT, ERK1/2 and p-ERK1/2, 1:500; GAPDH, 1:5000). The membranes were washed with TBST three times. Then the membranes were incubated with horseradish peroxidase-conjugated antibody for 1 hour at room temperature. After washed with TBST four times, the membranes were detected using ECL and visualized using Bio-Rad Laboratories. Western blots shown are representative of at least three independent experiments. Densitometry of each band for the target protein was quantified by densitometry analysis with Labworks 4.6. The protein band intensity was quantified by the mean $\pm$ SEM of three experiments for each group as determined from densitometry relative to GAPDH or AKT or ERK1/2.

Gelatin zymography assay

Cell culture media were standardized according to the protein contents and subjected to 10% SDS-PAGE containing 1 mg/ml gelatin. After electrophoresis, the gel was washed at room temperature for 1 h in 2.5% triton X-100 (v:v), 50 mM TrisHCl (pH7.5) to remove SDS, and then incubated at 37°C overnight in a buffer containing 150 mM NaCl, 5 mM CaCl_2 and 50 mM Tris-HCl (pH7.6). The gel was subsequently stained with 0.1% (w/v) Coomassie Brilliant Blue R- 250, and destained in 10% (v/v) methanol and 5% (v/v) glacial acetic acid to reveal zones with gelatinase activity. The results were analyzed using Image Jversion 1.47 software (NIH). All zymography experiments were repeated in triplicate.

Indirect Immunofluorescence staining

After washing with PBS, cells grown on coverslips were fixed in 4% paraformaldehyde following 20 min and washed in PBS three times. Nonspecific binding sites were blocked by incubation with 1% goat serum in PBS at room temperature for 1 h. Slides were incubated with N-cadherin, E-cadherin and Vimentin antibody (all 1:100) overnight at 4°C. After washing three times with PBS, slides were incubated with TRITC-conjugated goat anti-rabbit antibody (1:100) for 1 h at 37°C. After washed three times, slides were incubated with DAPI for 5 min at room temperature and washed three times again. Specimens were mounted in PBS containing 90% glycerol and 1.0% p-phenylenediamine and subsequently monitored under an Olympus BX51 immunofluorescence microscope.

Data analysis

All data were presented as the mean $\pm$ standard error mean (SEM). Statistical analysis was performed using one-way ANOVA or t-test (for data normally distributed). * $P < 0.05$, ** $P < 0.01$.

Results

rhOPN promotes cell proliferation, migration and invasion in HEC-1A cells

We used different concentration of rhOPN (0 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml and 400 ng/ml) treated with HEC-1A cells firstly. The CCK-8 results showed that rhOPN could significantly increase the proliferation ability of HEC-1A cells. The proliferation ability of HEC-1A reached the peak at the point of 300 ng/ml (Fig. 1A). Then we used wound scratch assay and transwell to detect the migration and invasion ability of HEC-1A. We found that rhOPN could promote HEC-1A migration (Fig. 1B and D) and invasion (Fig. 1C and E). And the peak was both at the point of 300 ng/ml.

rhOPN induced MMP-2 expression in HEC-1A cells

To determine whether OPN induces MMP-2 expression, HEC-1A cells were treated without or with rhOPN (300 ng/ml) for 4, 8, 12, 24, 48 h and the expression of MMP-2 was detected by western blot. The results indicated that rhOPN induced MMP-2 expression in a time-dependent manner in HEC-1A cells, and the protein expression of MMP-2 reached the peak at the point of 24 h (Fig. 3A and B). We also studied the dose-dependent response of rhOPN (24 h) for 0, 100, 200, 300 and 400 ng/ml. It showed that the protein expression of MMP-2 increased in a dose-dependent manner and that 300 ng/ml rhOPN induced the highest level of MMP-2 expression compared with other doses (Fig. 3C and D). We also used the Gelatin zymography assay to detect the expression of MMP-2, a key factor of cell migration and invasion. These results showed that the secretion of MMP-2 was obviously increased after treated with 300 ng/ml rhOPN than untreated group (Fig. 2E and F).

rhOPN influenced EMT-related factors expression in HEC-1A cells

We treated HEC-1A cells with rhOPN for 24 h at the concentration of 300 ng/ml, then used western blot and Indirect Immunofluorescence staining to detect the expression of N-cadherin, E-cadherin and Vimentin. The western blot results showed that the expression of N-cadherin and Vimentin were significantly increased than untreated cells, and the expression of E-cadherin was decreased after treated with rhOPN (Fig. 3A, B, C and D). The immunofluorescence showed the same results (Fig. 3E).

rhOPN promoted HEC-1A cells migration, invasion and EMT through AKT and ERK1/2 signal pathway

To determine whether rhOPN promoted HEC-1A cells migration, invasion and EMT through p-AKT and p-ERK1/2 signal pathway, HEC-1A cells were treated with rhOPN at 0 ng/ml, 100 ng/ml, 200 ng/ml, 300 ng/ml and 400 ng/ml for 24 h firstly. The expression of p-AKT and p-ERK1/2 were detected by western blot. Results indicated that rhOPN could

Fig. 1. rhOPN promotes HEC-1A cells proliferation, migration and invasion. (A) Cells were seeded in 96-well plates and detected the ability of HEC-1A cells proliferation by CCK-8; (B and D) Cells were seeded in 6-well plates and detected the ability of HEC-1A cells migration by Wound scratch assay (magnification, $\times 40$); (C and E) Cells were suspended in McCoy's 5A medium supplemented with 15% heat-inactivated fetal bovine serum and subsequently seeded in 96-well plates and detected the ability of HEC-1A cells invasion by transwell (magnification, $\times 10$).

regulate AKT and ERK signal pathway (Fig. 4). Then we co-treated HEC-1A cells with rhOPN and LY294002/U0126. The results (Fig. 5 and 6) showed that LY294002 and U0126 can significantly decrease the expression of MMP-2, Vimentin and N-cadherin, while increased the expression of E-cadherin. Compared to treated with rhOPN group, co-treated with rhOPN and LY294002/U0126 group was obviously decreased the expression of MMP-2, Vimentin and N-cadherin, while increased the expression of E-cadherin.

Fig. 2. Recombinant human OPN (rhOPN) induces MMP-2 expression and secretion (A and B). HEC-1A cells were treated with various concentrations of rhOPN for 24 h; (C and D) HEC-1A cells were treated for various periods with rhOPN at a concentration of 300 ng/ml. (E and F) HEC-1A cells were treated with rhOPN at 300ng/ml. * $P < 0.05$.

Fig. 3. rhOPN influences E-cadherin, Vimentin and N-cadherin expression in HEC-1A cells. (A-D) HEC-1A cells were treated with rhOPN at 300ng/ml for 24h. Use western blot to detect the expression of E-cadherin, Vimentin and N-cadherin; (E) HEC-1A cells were treated with rhOPN at 300ng/ml for 24h. Use immunofluorescence staining to detect the expression and location of E-cadherin, Vimentin and N-cadherin (bar = 50μm).

Fig. 4. Recombinant human OPN (rhOPN) stimulates ERK1/2 and AKT signaling pathway expression and secretion. HEC-1A cells were treated with various concentrations of rhOPN for 24 h (A) The expression of p-AKT and AKT were detected by western blot; (B) The expression of p-ERK1/2 and ERK1/2 were detected by western blot. * $P < 0.05$, ** $P < 0.01$.

Fig. 5. rhOPN promotes EMT-related expression and MMP-2 secretion through AKT signaling pathway in HEC-1A cells. Cells were treated with LY294002 (a specific inhibitor of PI3K/AKT signaling pathway, 10^{-6} M) and/or rhOPN. The expression of MMP-2 (on the top of Fig. 5A), E-cadherin, Vimentin and N-cadherin were detected by western blot. The secretion of MMP-2 (at the bottom of Fig. 5A) was detected by Gelatin zymography assay. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Discussion

Endometrial carcinoma is one of the most common malignant diseases of female reproductive tract affecting 2–3 % of women. A major problem in the therapeutic

Fig. 6. rhOPN promotes EMT-related expression and MMP-2 secretion through ERK1/2 signaling pathway in HEC-1A cells. Cells were treated with U0126 (a specific inhibitor of ERK1/2 signaling pathway, 10^{-5} M) and/or rhOPN. The expression of MMP-2 (on the top of Fig. 6A), E-cadherin, Vimentin and N-cadherin were detected by western blot. The secretion of MMP-2 (at the bottom of Fig. 6A) was detected by Gelatin zymography assay. * $P < 0.05$, ** $P < 0.01$.

management of endometrial cancer is the growth and metastases in distant organs, but the genes orchestrating the process are yet to be identified for the rational design of new treatment.

OPN is a member of the extracellular matrix protein. It is a non-collagenous, sialic acid-rich, and glycosylated phosphoprotein [20, 21]. It has an N-terminal signal sequence, a highly acidic region consisting of nine consecutive aspartic acid residues, and a GRGDS cell adhesion sequence predicted to be flanked by the β -sheet structure [22]. This protein has a functional thrombin cleavage site and is a substrate for tissue transglutaminase [21]. OPN binds with type I collagen [23], fibronectin [24], and osteocalcin [25]. Several highly metastatic transformed cells synthesize higher level of OPN than the nontumorigenic cells [26]. OPN causes cell adhesion, cell migration, ECM invasion, and cell proliferation by interacting with its receptor $\alpha v \beta 3$ integrin in various cell types [27]. OPN induces pro-MMP-2 activation by binding to its receptor $\alpha v \beta 3$ integrin [28, 29].

MMPs are ECM degrading enzymes that play critical role in embryogenesis, tissue remodeling, inflammation, and angiogenesis [30]. MMP-2 (also called type IV collagenase or gelatinase A) degrades several ECM proteins such as fibronectin, laminin, type I collagen, and proteoglycans [31]. Earlier reports have indicated that the increased levels of MMP-2 correlate with the invasive properties of several tumor cells [31, 32].

The EMT is a complex process involved in embryonic development, and was first recognized as a hallmark of embryogenesis in the early 1980s [33]. EMT is involved in tumor migration, invasion process and considered to play an important role in increasing cell invasion and metastatic potential of cancer cells [34]. During this process, epithelial cells lose their defining characteristics and acquire mesenchymal properties, including loss of cell-cell adhesion, increased motility and invasiveness, resistance to apoptosis and changes in cellular morphology [35]. The EMT has been identified as a driver of metastasis

and tumor invasion, allowing cells to detach from their niche and migrate through blood and lymphatic vessels to invade different organs. That is to say that EMT makes tumor cells more invasion properties [36, 37].

In this paper, we found that rhOPN could promote HEC-1A cell migration, invasion and proliferation (Fig. 1) and increase MMP-2 expression and secretion (Fig. 2). It has been study that OPN can activate PI3K/AKT and ERK1/2 signaling. So we detected that rhOPN influenced these progressions through AKT and ERK1/2 signaling pathway (Fig. 4, 5, 6) [38, 39].

Since OPN can mediate cell adhesion, cell-cell interaction, invasion and colony formation in tumor cells in experimental systems, this molecule is a key candidate for a tumor prognostic marker and a potential target for therapeutic intervention in control of metastasis.

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Disclosure Statement

None of the authors listed in this manuscript have a conflict of interest.

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