European Journal of Obstetrics & Gynecology and Reproductive Biology 165 (2012) 307–312

Contents lists available at SciVerse ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

17β-Oestradiol activates proteolysis and increases invasion through phosphatidylinositol 3-kinase pathway in human cervical cancer cells

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ARTICLE INFO

Article history:
Received 15 January 2012
Received in revised form 24 May 2012
Accepted 25 July 2012

Keywords:
Oestrogen
Proteolysis
Invasion
Cervical neoplasm
Signal pathway

ABSTRACT

Objective: Despite evidence that oestrogen may play an important role in the carcinogenesis of cervical cancer, its action and mechanism in cervical cancer invasion are not well defined.
Study design: The invasion induced by 17β-oestradiol (E2) was measured by invasion assay. Real-time polymerase chain reaction (PCR), Western blot, enzyme-linked immunosorbent assay (ELISA) and gelatin zymography were used to study the role of E2 on metastasis-related proteases. The signal pathway was also investigated.
Results: E2 was found to significantly enhance the invasion of cervical cell lines including HeLa, CaSki and SiHa cells, but not C33A cells. Moreover, E2 10^{-8} M increased the expression and activation of matrix metalloproteinases (MMP-2 and MMP-9) in HeLa and CaSki cells, as shown by real-time PCR, Western blot, ELISA and gelatin zymography. The expression of tissue inhibitor of metalloproteinases (TIMP-1 and TIMP-2) was decreased significantly by E2. Pretreatment with GM6001 10 μM (total MMP inhibitor) or SB-3CT 20 μM (specific gelatinase inhibitor) blocked the pro-invasive effect of E2. E2 was found to induce invasion via the phosphatidylinositol 3-kinase (PI3K) signalling pathway.
Conclusion: E2 may contribute to cervical cancer metastasis through activation of proteolysis and increased invasion via the PI3K pathway.

1. Introduction

Cervical cancer is the third most commonly diagnosed cancer, and the fourth leading cause of cancer death in women, accounting for 9% (529,800) of all new cancer cases and 8% (275,100) of all cancer deaths among women in 2008 [1].

Human papillomaviruses (HPVs) are associated with more than 99% of cervical cancers, and are the major aetiological cause. Cervical cancers only develop in a minority of women with high-risk HPVs, however, suggesting that the development of cervical cancer is a multifactorial process [2]. As well as HPVs, environmental [3], genetic [4], biological and hormonal factors are also likely to be involved in the development of cervical cancer. An effect of 17β-oestradiol (E2) in human cervical cancer has been hypothesized based on two observations: (1) long-term use of oral contraceptives leads to a 2–4-fold increase in the risk of cervical cancer [5]; and (2) women are exposed to elevated levels of oestrogen during pregnancy which increases the risk of cervical cancer up to 3.8-fold [6]. As such, the role of oestrogen in the genesis of cervical cancer is evident, but the action and mechanism of oestrogen in the invasion of cervical cancer are not well characterized.

Matrix metalloproteinases (MMPs) and the urokinase-type plasminogen activator (uPA) system have been explored in cervical cancer and other cancers [7–9]. When MMPs or uPA are secreted from cells and activated through proteolytic cleavage, they can degrade the extracellular matrix to increase invasion, and their activity can be neutralized by their specific inhibitor [e.g. tissue inhibitor of metalloproteinase (TIMP) for MMP and plasminogen activator inhibitor (PAI)-1 for uPA [10]]. The MMP family has many members, of which MMP-2 and MMP-9 have been detected in human cervical high-grade lesions and cervical cancers [8,11]. Low expression of TIMP-2 or high expression of MMP-2 has been demonstrated in cancer of the uterine cervix [8]. PAI-1 concentration showed a positive correlation with advanced tumour stage, and increased levels are related to a poor prognosis in cervical cancer [9].

Oestrogen is known to interact with intracellular receptors that act as nuclear transcription factors, and there is abundant evidence that oestrogen can also act via its membrane receptor and cytoplasmic signalling cascades related to cell growth, preservation and differentiation [12].

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http://dx.doi.org/10.1016/j.ejogrb.2012.07.026

It was hypothesized that E2 may play a role in cervical cancer invasion through MMPs and the uPA system. The present study investigated: (1) the role of E2 in the invasion of cervical cancer cells; (2) the role of E2 in metastasis-related proteases; and (3) the involvement of signal pathways induced by E2.

2. Materials and methods

2.1. Materials

E2, PD98059 (a MEK1 inhibitor), LY294002 [phosphatidylinositol 3-kinase (PI3K) inhibitor] and SB203580 [p38 mitogen-activated protein kinase (p38 MAPK) inhibitor] were acquired from Sigma–Aldrich Corp (St. Louis, MO, USA). GM6001 (broad-spectrum MMP inhibitor) and SB-3CT (selective MMP-2/MMP-9 inhibitor) were obtained from Chemicon (Temecula, CA, USA). If the inhibitory effect of SB-3CT was found to be similar to that of GM6001, this could imply that MMP-2 and MMP-9 alone, and no other groups of MMPs, play a critical role in cervical cancer invasion induced by E2.

2.2. Antibodies

MMP-2, phospho-AKT (Ser473) (pAKT) and AKT antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). TIMP-2, uPA and PAI-1 antibodies were acquired from Santa Cruz Biotechnology Ltd. (Santa Cruz, CA, USA). MMP-9 and TIMP-1 antibodies were obtained from Epitomics (Burlingame, CA, USA), and GAPDH antibody was acquired from Kangcheng Biotechnology Ltd. (Shanghai, China).

2.3. Cell culture and treatment

The cervical cancer cell lines HeLa, C33A, SiHa and CaSki, were purchased from the American Type Culture Collection (Manassas, VA, USA), and cultured in MEM or RPMI 1640 culture solution without phenol red (GIBCO BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum (FBS) at 37 °C (95% humidity and 5% CO_2 atmosphere).

Following incubation with different E2 concentrations for 48 h, the invasion of cervical cancer cells was examined by the invasion assay. To investigate the regulation of MMP-2, MMP-9, TIMP-1, TIMP-2, uPA and PAI-1, the cells were plated and then treated for an additional 24 h (mRNA) or 48 h (protein analysis with E2 10^{-8} M). Following treatment with E2 10^{-8} M for 48 h, conditioned medium was collected for zymography or enzyme-linked immunosorbent assay (ELISA). In order to evaluate the effect of the inhibitors, including GM6001, SB-3CT, PD98059, LY294002 and SB203580, the cells were pretreated with the inhibitor for 30 min, and E2 10^{-8} M was added for 48 h to analyse the invasion assay. In order to investigate the signal pathways, the cells were plated and then treated for up to 120 min with E2 10^{-8} M. Following 30 min of pretreatment with LY294002, the cells were treated with E2 10^{-8} M for 30 min (pAKT) or 48 h (MMP2 and MMP9) for analysis by Western blot.

2.4. Invasion assay

Polycarbonate filters (8 μm) were coated with Matrigel and placed in a modified Boyden chamber. Trypsinized cells (1.0×10^5) were resuspended in medium containing 0.5% FBS and various concentrations of E2, and added to the top chamber in the presence or absence of pretreatment with GM6001 10 μM (total MMP inhibitor), SB-3CT 20 μM (specific gelatinase inhibitor), PD98059 10 μM (MEK1 inhibitor), LY294002 10 μM (PI3K inhibitor) or SB203580 20 μM (p38 MAPK inhibitor) for 30 min. Culture medium containing 1% FBS was added to the bottom chamber and the cells were incubated for 48 h. Next, the filters were fixed and stained while non-invading cells were removed. The invading cells were counted using a microscope, and a minimum of 15 fields per filter were counted.

2.5. Real-time polymerase chain reaction

Total RNA was prepared by RNAprep pure Cell Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The primers used for SYBR Green real-time polymerase chain reaction (PCR) were as follows: MMP-2, 5′-CCGCAGTGACGGAAAGATGT-3′ and 5′-CACTTGCGGTCGTCATCGTA-3′; MMP-9, 5′-GGACGATGCCTGCAACGT-3′ and 5′-CAAATACAGCTGGTTCCCAATCT-3′; GAPDH, 5′-ATGGAAATCCCATCACCATCTT-3′ and 5′-CGCCCCACTTGATTTTGG-3′; uPA, 5′-CGCTTTCTTGCTGGTTGTCA-3′ and 5′-CCCAGTCTCTTCTTACAGCTGATG-3′; PAI-1, 5′-CCGCCGCCTCTTCCA-3′ and 5′-GCCATCATGGGCACAGAGA-3′; TIMP-1, 5′-ACCATGGCCCCCTTTGA-3′ and 5′-CAGCCACAGCAACAACAGGAT-3′; and TIMP-2, 5′-AGCATTTGACCCAGAGTGGAA-3′ and 5′-CCAAAGGAAAGACCTGAAGGA-3′ [10]. These primers were obtained from Takara (Dalian, China). Real-time PCR was performed using the Eppendorf System with a 96-well plate. The reactions were set up with 12.5 μl One Step SYBR RT-PCR Buffer III, 0.5 μl Takara Ex Taq HS (5 U/μl), 0.5 μl PrimeScript RT Enzyme Mix II, 0.5 μl PCR Forward Primer (10 μM), 0.5 μl PCR reverse primer (10 μM, 2 μl total RNA 100 ng) and 8.5 μL RNase Free dH_2O. Real-time PCR conditions were as follows: 42 °C for 5 min followed by 95 °C for 10 s, and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative expression of mRNA was calculated using the comparative threshold cycle (CT) method.

2.6. Immunoblotting assay

Cells were lysed and total protein (30 μg) was separated on 8–12% SDS-polyacrylamide gels to determine the expression of MMP-2, MMP-9, TIMP-1, TIMP-2, uPA, PAI-1, GAPDH, pAKT and AKT. The membrane was immunoblotted using specific primary antibodies at 4 °C overnight after electrotransferring the proteins to a nitrocellulose membrane (Amersham Pharmacia Biotech). The signals were discerned with secondary antibody for 30 min and visualized through an enhanced chemiluminescence system (Amersham Pharmacia Biotech) Uppsala, SE.

2.7. Zymography

The conditioned medium was separated on an 8% SDS-polyacrylamide gel containing 0.1% gelatin. After electrophoresis, the gel was washed twice in 2.5% Triton X-100 and then incubated overnight at 37 °C in buffer [0.1 mM glycine, 10 mM $CaCl_2$, 1 μM $ZnCl_2$ (pH 8.3)]. The gel was stained with Coomassie blue G-250 to visualize activity.

2.8. ELISA

The concentrations of MMP-2 and MMP-9 in conditioned medium were measured with an ELISA kit (Amersham Pharmacia Biotech). The absorbance of the samples was examined at 450 nm with a microplate spectrophotometer. A standard curve was used to calculate the concentrations of MMP-2 and MMP-9.

2.9. Data analysis

Data are means ± standard errors of three individual experiments performed in triplicate. Data were analysed by one-way analysis of variance followed by Dunnett's test, and $P < 0.05$ was considered to