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Involvement of NADPH oxidase and NF-κB activation in CXCL1 induction by vascular endothelial growth factor in human endometrial epithelial cells of patients with adenomyosis

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ABSTRACT

Chemokines were known to participate in inflammation and angiogenesis but have been recently recognized to be involved in embryonic implantation and endometrium-related pathologies. Among these chemokines, the CXC chemokines, such as CXCL1, have potential roles to work as biomarkers to identify patients with uterine adenomyosis. In this study, human endometrial epithelial cells (HEECs) were derived from patients' endometrium with adenomyosis. The inductive effects of CXCL1 production by various mediators/growth factors were investigated in the HEECs. Of the tested mediators, VEGF was found to be the most effective. The immunohistochemistry and RT-PCR analysis revealed a positive staining for VEGF and CXCL1 at the epithelium and the presence of CXCL1 in the human endometrium specimens, respectively. The CXCL1 induction by VEGF could be reduced by the antagonist for VEGF receptor (VEGFR), and by the inhibitors for NADPH oxidase and NF-κB signaling pathway. However, it was not affected by sex hormones and the inhibitors for MAPKs, PI-3K, protein kinase A and C. In parallel, VEGF induced p47 phox NADPH oxidase activation, IκBα phosphorylation, NF-κB translocation and NF-κB-DNA complex formation in the HEECs. Moreover, the CXCL1 released by the HEECs with VEGF stimulation attracted vascular endothelial cell migration. Taken together, we show that VEGF and CXCL1 are expressed in epithelium of the endometrium with adenomyosis and demonstrate here for the first time that VEGF is capable of inducing CXCL1 expression in HEECs through VEGFR, p47 phox NADPH oxidase and NF-κB signaling pathway, which is functionally required for attracting vascular endothelial cell migration.

1. Introduction

Chemokines are a group of small heparin-binding proteins that can be divided into four subfamilies. Among these, CXC chemokines are defined by the arrangement of the first two of four invariant cysteine residues found in most chemokines and are further subgrouped according to the presence or absence of a three amino acid 'ELR' (Glu-Leu-Arg) motif preceding the CXC sequence. The CXCL1 belongs to CXC chemokines, which can bind to CXCR2 chemokine receptor and induce chemotaxis of circulating leukocytes to the sites of inflammation (Charo and Ransohoff, 2006). The CXCL1 was implicated in blood vessel formation and related to oncogenic activity (Baggiolini et al., 1994; Loukinova et al., 2001) and has been demonstrated recently to be as an angiogenic growth factor to enhance angiogenesis (Caunt et al., 2006). It is not detectable in the epithelium and microvascular endothelium in nonpathological endometrium but is expressed in chronic endometritis (Kitaya and Yasuo, 2010). The CXCL1 and other chemokines have a potential to work as biomarkers to identify patients with endometriosis (Borrelli et al., 2013) and the ELR$^+$ CXC group of chemokines (including CXCL1, −8, and −5) are angiogenic and can promote epithelial ovarian cancer (EOC) (Rainczuk et al., 2012). For example, both CXCL1 (Bolitho et al., 2010) and CXCL8 (Schutyser et al., 2002) are up-regulated in ascites fluid from advanced EOC patients.

Vascular endothelial growth factor (VEGF) and endometrial angiogenesis play a critical role in successful embryonic implantation and normal maternal production of VEGF is likewise

Abbreviations: ERK, extracellular matrix-regulated kinase; HEEC, human endometrial epithelial cell; HUVEC, human umbilical vein endothelial cell; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NADPH oxidase, nicotinamide adenine dinucleotide phosphate oxidase; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; PI-3K, phosphoinositide-3 kinase.

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necessary for decidual vascularization (Burton et al., 2009). However, abnormal angiogenesis may contribute to several different endometrium-related pathologies including endometrial cancer, endometriosis, adenomyosis, menorrhagia, and breakthrough bleeding (Fraser and Lunn, 2000; Gargett and Rogers, 2001). Among these pathologies, a causal relation between adenomyosis and infertility has been repeatedly suggested (Sunkara and Khan 2012; Tomassetti et al., 2013). Adenomyosis appears to impact negatively on *in vitro* fertilization/intra-cytoplasmic sperm injection outcome owing to reduced likelihood of clinical pregnancy and implantation (Vercellini et al., 2014).

Uterine adenomyosis is a pathological condition characterized by the presence of endometrial glands and stroma within the myometrium (Maheshwari et al., 2012). The neovascularization can be considered as a major pathological feature of adenomyosis/endometriosis. In addition, the angiogenesis is required for the invasion of ectopic endometrial implants into pelvic sites and their subsequent proliferation (Taylor et al., 2007; Goteri et al., 2009; Kang et al., 2009; Huang et al., 2014). These suggest a certain role of neovascularization/angiogenesis in the pathogenesis of adenomyosis.

Recently, a study showed that estrogen can induce VEGF expression in endometrial epithelial cells, cause pro-angiogenic activity, and promote adenomyosis (Huang et al., 2014). Meanwhile, we found a previous report showing that VEGF induces CXCL1 and −8 expression in endothelial cells (Hao et al., 2009). We therefore hypothesized that VEGF and CXCL1 may play a role in adenomyosis. Based on the hypothesis, the aim of this study was to investigate the CXCL1 expression and regulation in the specimens and human endometrial epithelial cells (HEECs) from endometrium with adenomyosis. Which mediators/growth factors could induce CXCL1 release in the HEECs and whether CXCL1 was present in the endometrium specimens were investigated in this study. We observed that a marked stimulatory effect by VEGF on the HEECs and a positive staining for VEGF/CXCL1 expression in the human endometrium specimens. Following these observations, the mechanism of action of VEGF on CXCL1 release in HEECs was further explored throughout this study.

2. Materials and methods

2.1. Materials

Thrombin, bradykinin (BK), PD98059, SB202190, SP600125, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). SU5416 (SU) and SB225002 were purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). Human recombinant VEGF was purchased from Prospect Biotech (Rehovot, Israel). Human EGF, IGF, and bFGF were from Invitrogen Life Technologies (Carlsbad, CA, USA). The antibody (Ab) raised against p-ERK1/2 was from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The Abs raised against p-PI-3K and p-JNK were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Human IP-10, SDF-1, and TNF-α were from R&D systems, Inc. (MN, USA). ATP and ADP were purchased from Affymetrix USB Products (Santa Clara, CA, USA). U46619 was from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). The Ab for α-tubulin was purchased from EMD Millipore (Billerica, MA, USA).

2.2. Preparation of human endometrial epithelial cells (HEECs) and human umbilical vein endothelial cells (HUVECs)

This study has been approved by the Ethics Committee of the Cathay General Hospital, Taipei City, Taiwan and conducted with the written informed consent to the patients. The endometrium samples were obtained via hysterectomy from the patients with adenomyosis and the HEECs were isolated from the endometrium of the uterus. The procedures of isolation and culture of the human glandular epithelial cells has been described previously (Bongso et al., 1988; Bilotas et al., 2007). The HEECs were cultured in Dulbecco's modified Eagle's medium F12 containing 100 U/ml of penicillin, 100 μg/ml of streptomycin and 250 ng/ml of fungizone (Thermo Fisher Scientific, NY, USA) with 10% fetal bovine serum (Thermo Fisher Scientific). The cells were characterized by indirect immunofluorescence staining using mouse monoclonal anti-cytokeratin 56 kDa (Serotec Ltd, Oxford, UK) followed by anti-immunoglobulin-fluorescein isothiocyanate (Dako Ltd, Cambridge, UK) as a secondary Ab. HUVECs were prepared and characterized by a method described previously (Lo et al., 2014). Isolated HUVECs were maintained in M199 containing 20% FBS (fetal bovine serum), 30 μg/ml endothelial cell growth supplement (ECGS), 4 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and fungizone (250 ng/ml) (Thermo Fisher Scientific, NY, USA).

2.3. Measurement of secreted CXCL1 in culture medium by ELISA

The secretion of CXCL1 in culture medium was determined using the human CXCL1 ELISA Development kit (R&D Systems, Inc., MN, USA), according to the protocol provided by the manufacturer. Briefly, the HEECs were treated with vehicle, VEGF or other reagents. The culture medium was collected and centrifuged, and the secreted CXCL1 in the culture medium was measured spectrophotometrically in an ELISA reader at 450 nm. The absolute concentration of CXCL1 in the culture medium was calculated from a standard curve.

2.4. Immunohistochemistry (IHC) of CXCL1 and VEGF expression patterns in endometrium

IHC was performed to identify CXCL1 and VEGF expression in the endometrium specimens. Briefly, tissue sections were deparaffinized, and the slides were hydrated through graded ethanol before use. The sections were then washed in TBS (Tris-buffered saline containing 1% $CaCl_2$), immersed in sodium citrate buffer (pH 6.0), and heated on a water bath for 20 min. After blocking with buffer containing 10% FBS, the slides were incubated at 4 °C overnight with primary Ab specific for CXCL1 or VEGF (LifeSpan BioSciences, Inc., Seattle, WA, USA). The secondary antibodies were used at a dilution of 1:250. After additional washing, the slides were stained with one-step 3-amino-9-ethylcarbazole (AEC; Biogenex) for 5–30 min. Sections were counterstained in hematoxylin for 20–40 s, washed with tap water, and mounted with 100% glycerol.

2.5. Cell viability/proliferation assay

Cell viability/proliferation was assayed as previously described (Shieh et al., 2010). Briefly, the cells were incubated with 0.5 mg/ml MTT for 2 h at 37 °C. Formazan crystals resulting from MTT reduction were dissolved by adding DMSO. The absorbance of the supernatant was then measured spectrophotometrically in an ELISA reader at 550 nm.

2.6. Cell lysate preparation and western blot analysis

The cell lysates were prepared as previously described (Lai et al., 2015). The total proteins were separated by electrophoresis through sodium dodecyl sulfate polyacrylamide gels, electroblotted onto polyvinylidene fluoride membranes, and then probed using a primary Ab. The immunoblots were developed using Immobilon Western Chemiluminescent HRP Substrate (EMD