Original Article

MAPK/ERK signal pathway involved expression of COX-2 and VEGF by IL-1\(\beta\) induced in human endometriosis stromal cells in vitro

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Received June 23, 2013; Accepted August 1, 2013; Epub September 15, 2013; Published October 1, 2013

Abstract: Objective: Now there are more and more evidences that Cyclooxygenase-2 (COX-2) plays an important role in angiogenesis of endometriosis (EMs). Vascular endothelial growth factor (VEGF) has a potent angiogenic activity. However, it is worth studying about the regulating mechanism of COX-2/COX-1 and VEGF in the development of human endometriosis in vitro. The current study was designed to investigate the effect of 4 cytokines on COX-2/COX-1 expression and the effect of IL-1\(\beta\) on VEGF release in human endometriosis stromal cells (ESC), and to explore the related signaling pathways involved in vitro. Methods: Isolation, culture and identification of ESC. Cells were treated with 4 cytokines, and the inhibitor mitogen-activated protein-Erk (MEK) and the inhibitor p38 mitogen-activated protein kinase (MAPK) prior to adding cytokine IL-1\(\beta\). COX-2 protein expression was measured by western blot and VEGF secretion was determined by ELISA. Results: Among four kinds of cytokines, IL-1\(\beta\) treatment increased COX-2 protein expression and VEGF release in three ESC, and TNF-\(\alpha\) had the same effect on COX-2 protein level as IL-1\(\beta\) only in ectopic and eutopic ESC, and MCSF had only slight effect on ectopic ESC. In contrast, cytokines had no effect on COX-1 expression. We also demonstrated that MAPK reduced the synthesis of COX-2 by IL-1\(\beta\) induced. COX-2 inhibitor reduced VEGF release by IL-1\(\beta\) induced. Conclusions: i) In human ESC in vitro, IL-1\(\beta\) up-regulated the COX-2 expression through the activation of p38 MAPK pathway, and not to COX-1. ii) Up-regulation of VEGF level by IL-1\(\beta\) treatment was found in human endometriosis stromal cell and COX-2 inhibitor was involved in this process.

Keywords: Endometriosis stromal cells (ESC), cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), cytokines, signal pathways, in vitro

Introduction

Cyclooxygenase (COX) is the rate-limiting enzyme in the metabolic conversion of arachidonic acid to prostaglandins (PGs). Two isosforms of COX have been described: COX-1 is constitutively expressed in many tissues, while COX-2 is inducible by mitogen [1], growing factors [2] and cytokines [3, 4]. Angiogenesis is a critical step in the process of endometriosis progressing [5]. Vascular endothelial growth factor (VEGF), which can be induced by COX-2, is one of the most potent angiogenic cytokines, has been shown to be highly up-regulated in various tumors, including ovary cancer [6], endometrial cancer [7], and other benign diseases, such as endometriosis [5, 8] and spontaneous abortion [9]. Several evidences [10-12] have demonstrated that IL-1\(\beta\) induced expression of the potent angiogenic factors VEGF and COX-2 in many malignant. And we have known that Cyclooxygenase-2 (COX-2) and VEGF play an important role in angiogenesis of endometriosis (EMs). Therefore, It is worth studying about the regulation mechanism of COX-2/COX-1 and VEGF in the development of human endometriosis, and involved the related signaling pathways in vitro. The aim of this current study was designed to investigate the effect of four cytokines on expression of COX-2/COX-1, and the VEGF release by IL-1\(\beta\) induced, and the related signaling pathways involved, to explore the new treatments in human endometriosis.

Materials and methods

Sample collection

In total, 57 women undergoing gynecological surgery were recruited to the study. 40 women
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(aged 33.2 ± 7.3 years; mean ± SD) had endometriosis and 17 women (aged 31.6 ± 8.1 years) did not have endometriosis but had parovarian cyst (n=5) and mature cystic teratoma of the ovary (n=12). Endometrial tissues were collected by curettage at the same time of surgery. In women with ovarian endometrioma, endometriotic tissues were collected from the walls of cysts. Formalin fixed tissues were for pathological diagnosis and fresh tissues were for cell culture. All women with or without endometriosis had regular menstrual cycles, and they had no hormonal treatment for at least 6 months before surgery. All samples were collected with informed consent from each woman and approval from the local ethics committee of the Second Xiangya Hospital of Central South University in Changsha in China.

Main reagents

DMEM/F12 medium was from GIBCO (USA), NS398 (COX-2 inhibitor), IV-type collagenase and progesterone were from Sigma (USA), trypsin was from Amresco Inc (USA), anti-human monoclonal antibodies against vimentin, keratin, prolactin mouse and fetal bovine serum were from Wuhan Boster (China), interleukin-1beta (IL-1β), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) were from Pierce Biotechnology, Inc (Rockford, IL, USA). Macrophage colony stimulating factor (MCSF) and vascular endothelial growth factor (VEGF) development ELISA Kit were from PeproTech, Inc (Rocky Hill, NJ, USA), protein assay kit was from Bio-RAD (Hercules, CA, USA), antibody against COX-2 was from Cayman Chemicals (Ann arbor, MI, USA), antibody against COX-1 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the second antibody, a horseradish peroxidase-conjugated anti-mouse antibody and ECL plus western blotting detection system were obtained from Amersham (Piscataway, NJ, USA). PD98058 (MEK inhibitor) and SB203580 (p38 MAPK inhibitor) were obtained from Calbiochem (La Jolla, CA, USA). Unless otherwise stated above, other chemicals were from Wuhan Boster (China).

Isolation, culture, and identification of endometriotic stromal cells (ESC)

Surgical operation procedure was carried out in our hospital. Primary endometriotic stromal cells were cultured by the following steps described by Morimoto [13] and Liu [14]: Endometriotic tissues (ectopic and eutopic) and normal endometrial tissues were dissected after rinsing, then digested for 2-3 h until the tissue disappeared by adding 0.1% IV collagenase solution and 0.25% trypsin digestion at pH 7.4 at 37°C, and then isolated cells with 100 μm and 38 μm nylon cell strainers, centrifuged at 800 rpm for 5 min, removed the supernatant, added DMEM/F12 medium (containing 10% newborn bovine serum), finally cell growth and morphology changes were observed under inverted microscope, cell counting and cell growth curve can be made. 10⁴/mL cell were seeded into 25 cm² cell culture plate at 37°C with 5% CO₂ incubator, semi-amount replacement of medium every 2-3 d, till cell fusion ≥80%, the culture of primary ESC was completed. We identified the cultured ESC using vimentin, cytokeratin, and prolactin (PRL), due to PRL was produced only by endometrial stromal cell in non-pregnant but not gland and fibroblast, so we used PRL to identify ESC. Cells were identified directly in 6-well culture plates, passaged adhesive cell, and replaced the medium, added 10⁻⁸ mol/L progesterone stimulating 6 d. The ESC were identified according to instructions by immunocytochemical ABC kit, positive staining cell was vimentin and PRL, and negative staining cell was cytokeratin. Three wells were repeated, and took the average value.

The third generation Cells were seeded at 1x10⁵ cells/ml in 100 mm tissue culture plates (10 ml/plate) for western blotting and incubated with 10% serum-containing medium for 72 h until they were confluent. and then the confluent cells were treated with serum-free fresh DMEM/F12 medium of either vehicle or cytokines for 24 h. For inhibitor studies, 10 uM SB203580 or 10 uM PD98059 was added to the culture medium 1 h prior to adding IL-1β. Three wells were repeated, and took the average value.

Western blot

Cells were lysed in ice-cold RIPA protein lysis buffer with protease inhibitors (1x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 ug/ml aprotime, 1 mM sodium orthovanadate, 1 ug/ml phenyl-methylsulfonyl fluoride). The protein concentration
was determined by using BIO-RAD protein assay with bovine serum albumin as the standard. An equal amount of total cell lysate (40 ug) were solubilized in the sample buffer by boiling for 5 minutes and subjected to electrophoresis on a 10% polyacrylamide gel containing sodium dodecylsulfate (SDS), and then transferred onto a nitrocellulose membrane. The membranes were incubated in a blocking buffer (1x Tris-buffer-saline (TBS) containing 5% non-fat, dried milk and 0.1% Tween 20) for 1 hour at room temperature or overnight at 4°C, and then incubated with the monoclonal mouse COX-2 or COX-1 primary antibody at 1:1000 dilution for 1 hour at room temperature. Following the primary antibody incubation, the membranes were then washed three times in TBS containing 0.1% Tween 20 and then incubated for 30 minutes with the second antibodies labeled with horseradish peroxidase. Protein bands were visualized using enhanced chemiluminescence (ECL kit).

VEGF ELISA

Cells (1x10⁵/ml) were seeded in 6-well plates (2 ml/well) in 10% serum-containing medium, after the cells reached or over 80% confluence, they were stimulated with IL-1β (10 ng/ml) with or without 100 uM of NS398 (a specific COX-2 inhibitor) in serum-free medium for 24 h. Cells incubated in serum-free medium without IL-1β were used as controls. The experimental media was centrifuged at 1100 g for 10 minutes at 4°C, removed debris and stored at -70°C for test.

Statistics

Data were expressed as means ± standard error of the mean (SEM). Significance was tested by Two-tailed Student’s t-test or Two-way ANOVA with Dunnett’s post-hoc analysis for comparison of each treated group to the non-treated control when appropriate. P values less than 0.05 were considered statistically significant.

Results

Expression of COX-2 by 4 cytokines in human endometriosis ESC by Western Blotting in vitro

Confluent cells (≥80%) were treated for 24 h with: 10 ng/ml interleukine-1β (IL-1β, lane 2); 15 ng/ml tumor necrosis factor-α (TNF-α, lane 3); 10 ng/ml interferon-γ (IF-γ, lane 4) and 10 ng/ml microphage colony stimulating factor (M-CSF, lane 5), respectively. Lane 1 was the control group. After 24 h, proteins were extracted and 40 ug of each sample was analyzed by western blotting using mouse anti-human COX-2 antibody. Results were shown in Figure 1: i) Expression of COX-2 in human ectopic endometriosis stromal cell was higher than the
eutopic or normal ESC (P<0.05), but the eutopic and normal had no difference (P>0.05) without CK treatment; ii) Ectopic or eutopic ESC expressed significance higher levels of COX-2 protein with IL-1β or TNF-α treatment group compared to the other two groups of CK treatments or the control, there was statistics difference (P<0.05), especially, the IL-1β had the strongest COX-2 expression (P<0.01). iii) The M-CSF group increased COX-2 expression in ectopic (P=0.04) and in eutopic (P=0.55). The IFN-r had no effect on three ESC (P>0.05).

Expression of COX-1 by 4 cytokines in human endometriosis ESC by western blotting in vitro

Confluent cells (≥80%) were treated for 24 h with: 10 ng/ml IL-1β (lane 2); 15 ng/ml TNF-α (lane 3); 10 ng/ml IF-γ (lane 4) and 10 ng/ml M-CSF (lane 5), respectively. Lane 1 showed the results of the control experiment. After 24 h, proteins were extracted and 40 μg of each sample was analyzed by western blotting using mouse anti-human COX-1 antibody. Results were shown in Figure 2: Expression of COX-1 was expressed in normal, eutopic and ectopic ESC, the normal was higher than the eutopic or ectopic, there was statistical difference (P<0.05), the eutopic and ectopic had no statistical difference (P>0.05). And the expression of COX-1 had no significance differences after 4 kinds CK (IL-1β, TNF-α, IFN-γ and M-CSF) was added (P>0.05).

COX-2 was up-regulated by IL-1β induced in ESC, the effect could be inhibited by SB203580

Confluent cells (≥80%) were treated for 24 h with various concentrations of IL-1β; 10 ng/ml IL-1β + PD98059 10 uM and 10 ng/ml IL-1β + SB203580 10 uM, respectively. (PD98059 is inhibitor of MEK1/2, MEK: mitogen-activated protein-Erk kinase, Erk: extracellular signal-regulated kinase; SB203580 is an inhibitor of p38 mitogen-activated protein kinase, MAPK inhibitor). Cells were pretreated with different kinase inhibitors (PD98059 and SB203580) 1 h before treatment with IL-1β 10 ng/ml. After 24 h, proteins were extracted and 40 μg of each sample was analyzed by western blotting. Lane 1 showed the result of the control experiment. Results were shown in Figure 3: i) The level of COX-2 protein had statistical increased after ectopic ESC incubated with IL-1β 0.1 ng/ml (P<0.05), with IL-1β concentration increasing, the level of COX-2 protein reached and maintained the highest in ectopic ESC at IL-1β 1 ng/ml, and there was statistical difference (P<0.01); ii) In eutopic ESC, the increasing of COX-2 protein was in a dose-dependent manner with IL-1β concentration increasing (P<0.05); iii) While in normal ESC, COX-2 had slightly increased at IL-1β 0.1 ng/ml (p=0.06), and no additional increased at IL-1β higher concentration (P>0.05). iv) Inhibitor of P38 MAPK SB203580 blocked expression of COX-2 by IL-1β induced in three cells, and there was marked statistical difference in eutopic or ectopic ESC (P<0.01); while inhibitor of MEK1/2 PD98059 was found to have synergetic or no role with IL-1β on COX-2 expression in three ESC (P>0.05).

VEGF release increased after IL-1β treatment in three ESC, the effect could be inhibited by NS398

Three ESC were incubated with IL-1β 10 ng/ml in serum-free medium for 24 hours, with or without 100 uM NS398 (a specific COX-2 inhibitor). The control was cell in serum-free medium without IL-1β. VEGF was detected by ELISA. Results were shown in Figure 4: VEGF release increased obviously after IL-1β treatment in three ESC in vitro, there was statistical difference (P<0.05), the effect was the strongest on ectopic (P<0.01). The increased VEGF level by IL-1β induced could be inhibited by NS398, a specific COX-2 inhibitor, and there was statistical difference (P<0.05), especially in the ectopic (P<0.01).

Discussion

Endometriosis is a common gynecological disease in women of childbearing age. Its pathogenesis is unclear, recurrent rate is high, treatment is thorny, these make endometriosis become difficult and hot research. At present, a lot of evidences suggest that pelvic inflammation and other immunological changes are a consequence of endometriosis. The development of non-invasive diagnostic tools based on cytokines and autoantibodies could be of great benefit in the clinical management of endometriosis. The elimination of the inflammatory reaction associated with endometriosis might bring about new treatment options.
Because of ethical issues, it is difficult to control study endometriosis in the human body, especially impossible to do traumatic examine and early drug test. Therefore, Endometriosis models in vitro will help to research its pathogenesis and treatment [8, 15]. We had successfully isolated, cultured, and identified the endometriosis stromal cells in vitro. Although the pathogenesis of endometriosis vary, the ectopic endometrium to grow successfully must have new blood vessels to provide blood. Therefore, angiogenesis plays very important role in the pathogenesis in endometriosis [16]. For instance, high concentration VEGF has been detected in peritoneal fluid from women with moderate to severe endometriosis [17-20], and also secreted in endometriotic lesions [8], which may be as a downstream consequence of pro-inflammatory cytokine IL-1β being activated [21, 22]. Therefore, whether VEGF level will change with IL-1β treatment in ESC in vitro, and how about its adjustment mechanism, which are worthy further study.

Figure 3. COX-2 was up-regulated by IL-1β in ESC, the effect was inhibited by p38 MAPK signaling pathway. i) The level of COX-2 protein had statistical increased after ectopic ESC incubated with IL-1β 0.1 ng/ml (P<0.05), with IL-1β concentration increasing, the level of COX-2 protein reached and maintained the highest at IL-1β 1 ng/ml, and there was statistical difference (P<0.01); ii) In eutopic ESC, the increasing of COX-2 protein was in a dose-dependent manner with IL-1β concentration increasing (P<0.05); iii) While in normal ESC, COX-2 had slightly increased at IL-1β 0.1 ng/ml (p=0.06), and no additional increased at IL-1β higher concentration (P>0.05). iv) Inhibitor of P38 MAPK SB203580 blocked expression of COX-2 by IL-1β induced in three cells, and there was marked statistical difference in eutopic or ectopic ESC (P<0.01); while inhibitor of MEK1/2 PD98059 was found to have synergistic or no role with IL-1β on COX-2 expression in three ESC (P>0.05).

Figure 4. VEGF release increased by IL-1β induced, and the effect was inhibited by NS398 in three ESC. VEGF release increased obviously after IL-1β treatment in three ESC in vitro, there was statistical difference (P<0.05), the effect was the strongest on ectopic (P<0.01). The increased VEGF level by IL-1β induced could be inhibited by NS398, a specific COX-2 inhibitor, and there was statistical difference (P<0.05), especially in the ectopic (P<0.01).

VEGF is involved in the development of blood vessels, which is the key in the growth and implant of ectopic endometrium, VEGF is related to COX-2. Numerous studies have confirmed the association between COX-2 overexpression and tumor progression, and increased angiogenesis (VEGF expression) has been involved in several malignancies including gastric [21], colon [22], prostate [23], breast [24], and pancreatic cancers [25, 26]. In addition, numerous studies in vivo have demonstrated that COX-2 mediated VEGF expression in numerous cell lines [27]; however, this may be parts dependent of tumor, since COX-2 inhibitors do not produce this effect in all tumors. In addition, another clinical trial with anti-angiogenic factors have been less effective than predicted from mouse models [28], suggesting angiogenesis may be orchestrated by a more complex set of cytokines, growth factors, cell types [29], and by difference singling pathway [30].

Like tumor metastases, endometriotic implants require neovascularization to proliferate and invade into ectopic sites of the host. Whether COX-2 and VEGF produce this effect on endometriosis, by what signal pathway, and which is worth studying about regulation mechanism of COX-2/COX-1 and VEGF in the development of human endometriosis. Therefore, the current study was designed to investigate the effect of 4 cytokines on COX-2/COX-1 expression and IL-1β on the effect of VEGF release in human endometriosis stromal cells (ESC) in vitro, and explore the related signaling pathways.
MAPK/ERK signal pathway involved expression of COX-2 by IL-1β induced in endometriosis ESC

Cyclooxygenase (COX) regulates the rate-limiting conversion of arachidonic acid to prostaglandin H2 (PGH2) in the prostaglandin synthesis pathway. Two isoforms of COX have been described: COX-1 is constitutively expressed in many tissues, while COX-2 is inducible by mitogens, growing factors and cytokines [1-4]. It’s reported [31] that high levels of cyclooxygenase-2 (COX-2) have expressed in peritoneal macrophages and endometriotic tissue in women with endometriosis, which might be one of causes in endometriosis. Our study found the COX-1/COX-2 expressed in human three ESC by Western Blotting, COX-2 was mainly in ectopic ESC, while COX-1 was mainly in normal ESC. Our result was consistent with the above mentioned: COX-2 caused EMs, while COX-1 is constitutive gene. In order to further prove the COX-2 regulation mechanism on endometriosis ESC, we intervened with four cytokines: interleukine-1 (IL-1β), tumor necrosis factor-α (TNF-α), interferon-r (IFN-r) and microphage colony stimulating factor (M-CSF). The results revealed: the IL-1β could induce the highest level COX-2 expression (P<0.01), TNF-α had the same as effect on the ectopic and eutopic ESC, but the effect of other two cytokines (IFN-r and M-CSF) is not obvious. What is more, the effect was found only in COX-2, not COX-1. This further pointed out COX-1 is constitutively expressed, while COX-2 was induced by CK. The result was consistent with the above reported. At the same time, this study also found the level of COX-2 protein had statistical increased in ectopic or eutopic ESC at IL-1β ≥1 ng/ml than the control (P<0.01); while there was no additional increase at IL-1β higher concentration in normal cell (P>0.05). These suggested that pro-inflammatory factor IL-1β and COX-2 played an important role in the formation of endometriosis. Meanwhile, which further demonstrated the occurrence of endometriosis was associated with abnormal eutopic endometrium, this supported eutopic endometrium decision theory [32]. In addition, inhibitor of P38 MAPK SB203580 blocked induction of COX-2 by IL-1β in ectopic and eutopic cells (P<0.01), while inhibitor of MEK1/2 PD98059 was found to have synergetic or no role on COX-2 expression with IL-1β, which further suggested that IL-1β regulated the COX-2 expression through the activation of p38 MAP kinase pathway in human endometriosis stromal cells, not via MEK signal pathway. From what has been discussed above: inhibitor P38 MAPK (SB203580) may become one of the new treatments in endometriosis.

VEGF release was inhibited by COX-2 inhibitor (NS938) in human ESC in vitro

In the above study, we investigated the effect of IL-1β on COX-2 protein expression; COX-2 played an important role in the formation of endometriosis. Now, we investigated VEGF secretion in the human endometriosis ESC, change of VEGF secretion with IL-1β treatment and the possible COX-2 signaling pathways involved. To test this, normal, eutopic and ectopic ESC were stimulated with IL-1β with or without 100 uM NS398 (a specific Cox-2 inhibitor) in serum-free medium for 24 h. VEGF was detected by ELISA. Our study found: three ESC could secreted VEGF, there was no difference among them (P>0.05), IL-1β treatment increased obviously VEGF release in three ESC (P<0.05), the effect was the strongest in ectopic (P<0.01). The increased VEGF release by IL-1β could be inhibited by NS398, a specific Cox-2 inhibitor in the three ESCs, there was statistical difference (P<0.01). The results indicated that IL-1β could up-regulate VEGF level in human endometriosis stromal cell in vitro, and IL-1β induced VEGF expression via COX-2 signaling pathway. That is to say that VEGF took part in the pathogenesis of endometriosis, and COX-2 signaling pathway is involved in this process. It’s well known that nonsteroidal anti-inflammatory drugs can suppress the occurrence and progression of malignancies such as colorectal cancers. However, the precise mechanism of these actions remains unknown. Yoshida [12] evaluated the role of an inducible COX-2 in tumor-associated angiogenesis and tumor growth, and identified the downstream molecules involved using a mouse model of sponge angiogenesis. In this model, they found that VEGF expression was down-regulated by selective COX-2 inhibition NS-938. Our results were consistent with their reported. Simultaneously, Yoshida also found that the inhibition of the COX-2/VEGF-dependent pathway was effective in tumor-associated angiogenesis, tumor growth, and tumor metastasis [12].
Our study demonstrated: COX-2 expressed in ectopic ESC in vitro, not COX-1; VEGF expressed in three ESC; and IL-1β mainly up-regulated the expression of COX-2 and VEGF in ectopic and eutopic ESC, especially in ectopic ESC. Which suggested COX-2 and VEGF played an important role in the formation of endometriosis. IL-1β up-regulated the COX-2 expression, not the COX-1, through the activation of p38 MAPK pathway. Up-regulation of VEGF level was found in three ESC by IL-1β, and COX-2 inhibitor was involved in this process. These provided new theoretical and experimental basis for endometriosis new treatment.

Acknowledgements

The project was supported by Hunan Province Health Department Fund Project (2010-016) and Hunan Province Natural Science Fund Project (2012jj3100).

Disclosure of conflict of interest

None.

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