Inhibition of IDO1 suppresses cyclooxygenase-2 and matrix metalloproteinase-9 expression and decreases proliferation, adhesion and invasion of endometrial stromal cells

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ABSTRACT: Indoleamine 2,3-dioxygenase-1 (IDO1) is an intracellular enzyme that catalyses essential amino acid tryptophan along the kynurenine pathway. The aim of this study was to determine the impact of IDO1 expression on the biological characteristic of the endometrial stromal cells (ESCs). IDO1, cyclooxygenase-2 (COX-2) and matrix metalloproteinases (MMPs) in endometriotic ectopic stromal cells, endometriosis-derived eutopic stromal cells and normal ESCs (control) were detected by the in-cell Western analysis. After being treated with lipopolysaccharide, levo-1-methyl-tryptophan (L-1-MT) alone or a combination, a comparative analysis of the above protein expression was evaluated. The effects of IDO1 on ESCs proliferation, adhesion and invasion were detected through ELISA, adhesion assay and Matrigel invasion assay, respectively. The results showed that, contrary to healthy ESCs from control women, the expression of IDO1 was significantly higher in eutopic and ectopic ESCs obtained from women with endometriosis. Inhibition of IDO1 by L-1-MT suppressed the expression of COX-2 and MMP-9 in ESCs. It could also decrease the ESCs proliferation, adhesion and invasion, while stimulating ESCs decidualization. Thus, IDO1 is possibly involved in endometriosis pathogenesis via promoting COX-2 and MMP-9 expression and regulation of ESCs biological characteristics. The information may be useful for developing a new therapeutic strategy for endometriosis.

Key words: endometriosis / endometrial stromal cell / indoleamine 2,3-dioxygenase-1 / levo-1-methyl-tryptophan

Introduction

Endometriosis, a common gynecologic disorder, is defined as the presence of ectopic endometrial glands and stroma outside the uterine cavity. Despite extensive research, the etiology of endometriosis remains elusive. According to the implantation theory of Sampson (1927), retrograde menstruation, peritoneal adhesion of shed endometrial tissue, degradation of the extracellular matrix (ECM) and outgrowth of these endometrial cells are essential steps for endometriosis (Spuijbroek et al., 1992). Given this theory, a puzzle emerges that only a fraction of women develop endometriosis, whereas retrograde menstruation is observed in most women. Some research suggests that the intrinsic defect of the endometrium, at least partially, contributes to the pathogenesis of this disorder. To date, various aberrant factors in eutopic endometrium and ectopic endometriotic tissue from women with endometriosis, such as cytokine, growth factors, angiogenic factors, cancer-related molecules as well as immunosuppression factors are inferred to be involved in the occurrence and maintenance of endometriosis (Giudice and Kao, 2004).

Indoleamine 2,3-dioxygenase (IDO) is an intracellular heme enzyme that catalyses the initial and rate-limiting step in the metabolism of the essential amino acid tryptophan along the kynurenine pathway. It is expressed in a constitutive or inducible manner in lung, small and large intestine, colon, spleen, liver, kidney, stomach and brain. Regulation of IDO involves costimulatory signals during antigen presentation (ligands of CD80/CD86-CTLA4) as well as an infectious or inflammatory mechanism, through lipopolysaccharide (LPS) and interferon family members, respectively (King and Thomas, 2007). Traditionally,
IDO is thought to be an immune modulator through tryptophan depletion and via the generation of proapoptotic metabolites. Moreover, it plays a crucial role in various infectious diseases, fetal rejection, organ transplantation, neuropathology, autoimmune disorder and cancer by promoting immune tolerance (Entrican et al., 2009; Palafox et al., 2010; Soliman et al., 2010). In addition to the classic IDO (dubbed IDO1), a closely related variant called IDO2 has been reported recently (Murray, 2007). The corresponding genes have a similar genomic structure and are situated adjacent to each other on human chromosome 8. They are likely to have arisen by gene duplication before the origin of the tetrapods. Different enzymatic activities, and diverse expression pattern within tissues, suggest a distinct role for each protein. Besides, the expression of the protein in response to stimuli differs. For example, IDO1 is induced systemically in endothelial cells during infection, whereas IDO2 mRNA expression is unchanged or down-regulated (Ball et al., 2007). The indole-containing compound 1-methyl-tryptophan (1-MT) was identified as a competitive inhibitor of IDO (Sedlmayr et al., 2002). In a previous study, while the levo-1-methyl tryptophan (L-1-MT) was a more potent inhibitor of IDO1, the devo-1-methyl tryptophan (D-1-MT) was shown to have superior anti-tumor activity and to be more effective in inhibiting IDO2-expressing tolerogenic dendritic cells in preclinical models (Munn, 2006). However, recent human studies indicate that, whereas the IDO2 gene seems to be functional in murine models, it was not found to be functional in humans (Lob et al., 2008).

IDO1 has been found to be present in the human female genital tract including the vagina, cervix, uterus, oviducts and maternal–fetal interface (Sedlmayr et al., 2002; King and Thomas, 2007), and evidence has demonstrated that high IDO1 expression in cancer cells was a reliable indicator for disease progression and prognosis in endometrial cancer (Ino et al., 2006). Since endometriosis and cancer are similar in several aspects such as unrestrained growth and cell invasion (Ueda et al., 2002), we speculate that IDO1 might be a possible candidate that which facilitates the survival and implantation of endometrial cells lodged outside the uterine cavity, and may also have interactions with other known abnormal factors in endometriosis. However, there have been few studies on IDO1 expression in the development of human endometriosis.

To elucidate the possible role of IDO1 in endometriosis, we examined the effects of IDO1 on the expression of cyclooxygenase-2 (COX-2) and matrix metalloproteinases (MMPs), proteins already implicated to be key players in endometriosis. We also studied whether IDO1 is involved in endometrial stromal cells (ESCs) proliferation, decidualization, adhesion and invasion, in the presence or absence of LPS stimulation or L-1-MT blockage.

### Materials and Methods

#### Patients and specimens

Patients treated by the laparoscopic surgical service for endometriosis, tubal ligation and dermoid cysts in our department were enrolled in this study. Inclusion criteria were: reproductive age (age 23–40 years), in the secretory phase of menstrual cycle, the absence of systemic pathologies, BMI >19 and <28 kg/m², no smoking, no drug therapy in the last 3 months.

The presence or absence of endometriosis was confirmed visually by laparoscopy and histological analysis. According to the revised America Fertility Society classification of endometriosis, all of the women with endometriosis were classified as stages III/IV (American Fertility Society, 1985). Endometriotic cyst wall tissue (ectopic endometrium), as well as eutopic endometrial samples (eutopic endometrium) were obtained from endometriosis patients (n = 18) during surgeries. Healthy endometrial samples (normal endometrium) were collected in fertile women (n = 18) who had undergone tubal ligation as controls. The median age was 29.8 ± 6.7 years for the group of women with endometriosis and 30.4 ± 8.1 years for the control group. Additional normal ovarian cortex tissues were obtained from 10 women (age range: 31.5 ± 7.2 years) undergoing laparoscopic ovarian cystectomy for dermoid cysts, and fixed in 4% formalin. According to established criteria of Noyes et al. (1950), all endometrial samples were confirmed histologically to be in the secretory phase of the menstrual cycle. Informed written consent was obtained from all participants, and the present protocol was approved by the Research Ethics Committee in Obstetrics and Gynecology Hospital, Shanghai Medical School, Fudan University.

All samples of at least 200 mg were collected under sterile conditions and divided into two parts. The first part was immediately fixed in 4% formalin for immunohistochemical studies, and the second part was transported to the laboratory on ice in DMEM (Dulbecco’s modified Eagle’s medium)/F-12 (Gibco, USA).

#### Isolation and culture of ESCs

We purified ESCs as described previously (Banu et al., 2008) with slight modification. Tissues were minced into 2 to 3-mm pieces and digested with collagenase type IV (0.1%; Sigma, USA) and deoxyribonuclease type I (DNase I; 3000 U; Sigma) with constant agitation for 70 min at 37°C. The resulting suspension was filtered through sterile 100- and 70-μm nylon strainers (Becton Dickinson) in turn to remove undigested tissue and epithelial cells. The filtrate was then centrifuged at 800g for 15 min to further remove leukocytes and erythrocytes, and washed with phosphate-buffered saline (PBS). After being centrifuged at 600g for 8 min, the ESCs were resuspended in DMEM/F-12 containing 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), and plated into culture flasks. The adherent stromal cells were cultured as monolayer in flasks and incubated in 5% CO₂ at 37°C, and the culture medium was replaced every 3 days. Cell viability was assessed by Trypan Blue exclusion assay (~89.7%). The purity of ESCs was ≥95%, as judged by diffuse and strong cytoplasmic immunostaining for vimentin and negative cellular staining for cytokeratin-7 (CK7) in immunocytochemistry.

#### Immunohistochemistry

Immunohistochemistry was performed on specimens of human ovary tissue, normal, eutopic and ectopic endometrium from patients with or without endometriosis. Paraffin sections were cut at a thickness of 5 μm. Slides were dewaxed, hydrated and quenched in 3% hydrogen peroxide. They were then soaked in 0.01 M citric acid (pH 6.0) for 20 min at 95°C for antigen retrieval. After cooling, slides were blocked for 60 min using 1% bovine serum albumin (BSA; Hyclone) in tris-buffered saline. The samples were then incubated with mouse anti-human monoclonal IDO1 antibody (1:167, Abcam, USA) at 4°C for 12 h in a humidity chamber. As a negative control, the primary antibody was replaced with isotype-matched immunoglobulin G (IgG) (Sino-America Co. Ltd). Two washings with PBS were performed before the slides were incubated with the secondary antibody (biotinylated anti-mouse, Vector Laboratories, Burlingame, USA). The slides were developed in peroxidase substrate solution (Vector Laboratories) for 3.5 min, counterstained with hematoxylin for 10 min and then washed in running tap water for 30 min. Finally, the slides were evaluated under Olympus BX51 + DP70 microscope (Olympus Optical, Tokyo, Japan).
Immunocytochemistry

Eutopic ESCs were cultured on culture dish and allowed to grow to ~70–80% confluence. The isolated ESCs were identified by immunocytochemistry. The cells were fixed in 4% formalin for 20 min at room temperature, washed twice with PBS and then permeabilized for 10 min with 1% Triton-100 (Sigma) in PBS. Non-specific binding of antibodies was blocked by incubation with a solution of 1% BSA in PBS for 30 min. Then, the mouse anti-human vimentin monoclonal antibody (1:100, Zymed Laboratories, USA), CK7 antibodies (1:100, Zymed Laboratories, USA), IDO1 antibody (1:167, Abcam) and isotype-matched IgG (Sino-America Co. Ltd) were added overnight at 4°C. Cells were stained by Vectastain Elite ABC kit (Vector Laboratories) as described in immunohistochemistry. All dishes were counterstained with hematoxylin and evaluated under Olympus BX51 + DP70 microscope.

In vitro treatment of ESCs

To evaluate the dose and time effect of LPS and L-1-MT on ESCs, the cells were incubated with LPS (at 1, 10 and 100 ng/ml; Sigma), L-1-MT (at 0.01, 0.05 and 0.25 mM; Sigma) and with vehicle (1% dimethylsulfoxide, DMSO; Sigma) as a negative control for 24, 48 or 72 h, respectively, in cell 3-bromo-2′-deoxyuridine (BrdU) proliferation assay. The dose and time chosen in the following studies were based on our findings in the proliferation assay. The cultured ESCs were stimulated with LPS (10 ng/ml), L-1-MT (0.05 mM) alone or in combination for 24 h, with vehicle (1% DMSO) as control. After incubation, the cells were processed to determine the expression of molecules (IDO1, COX-2, MMP-2 and MMP-9) at the protein level and the decidualization, adhesion and invasion ability.

In-cell Western

According to the description by Egorina et al. (2006), we used a newly set-up assay called in-cell Western to determine the in-cell protein level of interest. Normal, eutopic and ectopic ESCs in 96-well plates were cultured with or without LPS (10 ng/ml), L-1-MT (0.05 mM) for 24 h, respectively. After being fixed with 4% formaldehyde 10 min at room temperature and washed with 0.1% Triton in PBS for five times, the cells were blocked by 150 μl of Li-COR Odyssey Blocking Buffer (Li-COR Biosciences, Lincoln, Nebraska, USA) for 90 min at room temperature. Then, the cells were incubated with mouse anti-human IDO1 (1:30, Abcam), mouse anti-human monoclonal MMP-2 (clone 36006, 20 μg/ml, R&D Systems, Abingdon, UK). The polyclonal antibody of housekeeping protein actin, rabbit anti-human actin (1:50, Santa Cruz Biotechnology, USA) was meanwhile added to each well as an internal control. However, for rabbit anti-human COX-2 (1:30, Cell Signaling Technology, USA), rabbit anti-human MMP-9 (20 μg/ml, R&D Systems) detection group, homologue mouse anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:50, Santa Cruz Biotechnology) served as an internal control. After overnight treatment at 4°C, the wells were then incubated with corresponding IRDye™ 700DX (red fluorescence)-conjugated goat anti-mouse fluorescence secondary antibody (1:50, Rockland Inc., USA) and IRDye® 800DX (green fluorescence)-conjugated goat anti-rabbit fluorescence secondary antibody (1:50, Rockland Inc.). This procedure must be carried out in the dark to prevent fluorescence quenching. Images of target proteins were obtained by using the Odyssey Infrared Imaging System (Li-COR Biosciences German version of Ltd). The expression level of the correspondent molecules was calculated as the ratio of the intensity of target proteins to actin or GAPDH. The well edges were excluded when fluorescence was quantified because there is non-specific fluorescence emission at the edge.

The BrdU proliferation assay

Normal and eutopic ESCs were seeded at a density of 1 × 10⁴ cells/well in 96-well flat-bottom microwells, and starved with DMEM/F-12 containing 1% FBS for 12 h. Then they were cultured in the presence or absence of LPS (at 1, 10, 100 ng/ml) or L-1-MT (at 0.01, 0.05, 0.25 mM) for 24, 48 or 72 h. The ability of ESC proliferation was detected by BrdU proliferation ELISA system (Millipore, USA) according to the manufacturer’s instructions. The absorbance values (at 450 nm) read in the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria) represent the rate of DNA synthesis and correspond to the number of proliferating cells. The values were normalized to those measured in control cells absorbance, which was set to 1.

In vitro decidualization of ESCs

ESCs decidualization was induced by the addition of 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) and measured by prolactin (PRL) level in the culture supernatants as described previously (Klemmt et al., 2006). Normal and eutopic ESCs were seeded into 24-well plates (2 × 10⁵ cells/well) and treated with phenol red-free DMEM/F-12 medium containing 2% dextran-coated charcoal-treated FBS with or without LPS (10 ng/ml) and L-1-MT (0.05 mM) in the presence of 8-Br-cAMP (0.5 mM; Sigma, UK). The medium was changed every 3 days. Culture supernatants (9 days) were collected and centrifuged to remove cellular debris, and stored at ~80°C for PRL measurements. PRL level was detected with the PRL ELISA Kits (Alpha biology; USA) according to the manufacturer’s protocol. The absorbance value was measured by a DigiScan Microplate Reader. Cultured cells were homogenized, and the total protein in the homogenized cells was measured by a biocinchoninic acid protein assay kit (Pierce; USA). Data were standardized by total protein of cell lysates.

Cell adhesion assay

Cell adhesion assays were performed with the Cell Adhesion Assay Kit (Cell Biolabs; San Diego, CA, USA) according to the manufacturer’s protocol. After being treated with or without LPS (10 ng/ml), L-1-MT (0.05 mM) for 24 h, normal or eutopic ESCs (1 × 10⁵/well) were seeded in the 48-well plates coated with various growth substrates including fibronectin, collagen type I, collagen type IV, laminin type I and fibrinogen. Following incubation at 37°C for 60 min and the removal of unattached cells by PBS washes, cell stain solution and extraction solution were added to the wells. Finally, the extracted samples were transferred to a 96-well plate and detected the optical density 560 nm in DigiScan Microplate Reader.

Cell invasion assay

The invasion of ESCs across Matrigel was evaluated in invasion chambers following our previous procedure (Li et al., 2011). The cells inserts (8 μm pore size, 6.5 mm diameter, Corning, USA) coated with 15–25 μl Matrigel were placed in a 24-well plate. The normal ESCs or ectopic ESCs at a density of 2 × 10⁵ cells/well were plated in the upper chamber, while the lower chamber were added with or without 800 μl LPS (10 ng/ml) and L-1-MT (0.05 mM) for 24 h. The medium used here was DMEM/F-12 containing 1% charcoal stripped FCS. After washing in PBS, the non-invaded cells in the inserts, together with the Matrigel, were removed from the upper surface of the filter by wiping with a cotton bud. The inserts were then fixed in 4% formalin for 10 min at room temperature and stained with hematoxylin. The result was observed under an Olympus BX51 + DP70 microscope. The cells that migrated to the lower surfaces were counted at a magnification of ×200 in five predetermined fields.
Statistical analysis

One-way analysis of variance with the post hoc test was used in multiple comparisons of our study. Differences were considered as statistically significant at $P < 0.05$.

Results

Expression of IDO1 in endometrium and isolated ESCs

As shown in Fig. 1A, IDO1 was mainly expressed in glandular and surface epithelial cells of endometrium or endometriotic tissue, while it was conversely rare in the ovary cortex. The staining intensity of IDO1 in the endometrial stroma differed from strong to absent.

Experiments were then designed to determine whether positive staining of IDO1 in endometrial stroma were attributed to ESCs, since leukocytes in the stroma might also be the IDO1-positive cells. The results, indeed, showed that almost all of the isolated eutopic ESCs were stained for IDO1, and meanwhile positive for vimentin rather than CK7. No staining was seen when isotype-matched IgG was used as a primary antibody (Fig. 1B). Thus the presence of IDO1 in ESCs was demonstrated (Fig. 1B).

Comparative study of IDO1, COX-2, MMP-2 and MMP-9 expression profiles between normal, eutopic and ectopic ESCs

In the present study, differences between normal, eutopic and ectopic ESCs in protein expression profiles were detected by means of in-cell Western. We found that, both eutopic and ectopic ESCs expressed a higher level of COX-2 and MMP-9, as well as IDO1 than normal ESCs ($P < 0.05$; Fig. 1C, D and F). But no variation of IDO1, COX-2 and MMP-9 expression was found between eutopic and ectopic ESCs ($P > 0.05$; Fig. 1C, D and F). And there were no significant differences of MMP-2 expression in the three cell types ($P > 0.05$; Fig. 1E).

In vitro effects of L-1-MT on IDO1, COX-2, MMP-2 and MMP-9 expression in LPS-stimulated ESCs

In BrdU proliferation assay, neither LPS (at 1, 100 ng/ml) nor L-1-MT (at 0.01, 0.25 mM) had any effect on the proliferation of normal and eutopic ESCs after 24 h incubation, and the control group (1% DMSO) led to significant decrease of cell viability after 48 and 72 h compared with 24 h ($P > 0.05$; data not shown). On the basis of these discoveries, in further studies, we used LPS and L-1-MT in a dose of 10 ng/ml and 0.05 mM, respectively, and the experiments were carried out up to 24 h only.

As shown in Fig. 2A, IDO1 expression was ~1.7- and 1.5-fold of the control in normal and eutopic ESCs with LPS stimulation (LPS versus control; $P < 0.05$), and the up-regulation was abolished by L-1-MT (LPS plus L-1-MT versus LPS; $P < 0.05$). Moreover, in the presence of L-1-MT alone, the concentration of IDO1 was also significantly reduced (L-1-MT versus control; $P < 0.05$).

Similar to the observation for IDO1 expression, COX-2 and MMP-9 expressions in both normal and eutopic ESC were up-regulated by LPS after 24 h treatment (LPS versus control; $P < 0.05$, Fig. 2B and D), and the effects of LPS could be reversed by L-1-MT administration (LPS plus L-1-MT versus LPS; $P < 0.05$; Fig. 2B and D). However, no difference was found with the sole administration of L-1-MT as far as the expression of COX-2 and MMP-9 was concerned (L-1-MT versus control; $P > 0.05$; Fig. 2B and D). Conversely, MMP-2 expression was not influenced by LPS, L-1-MT alone or LPS plus L-1-MT ($P > 0.05$ versus control; Fig. 2C). These results provided evidence that IDO1 had its ability to promote the expression of COX-2 and MMP-9 in ESCs.

L-1-MT decreases cell survival and enhances decidualization in ESCs

In the cell proliferation assay, the number of normal and eutopic ESCs in the presence of LPS stimulation were 1.7- and 1.9-fold of the control, respectively (LPS versus control; $P < 0.05$; Fig. 3A), but a striking decrease of proliferation index could be observed in the presence of L-1-MT (L-1-MT versus control; $P < 0.05$; LPS plus L-1-MT versus LPS, $P < 0.05$; Fig. 3A). Moreover, in the proliferation ability of eutopic ESCs is significantly higher compared with the normal ESCs in the presence or absence of LPS or L-1-MT (eutopic versus normal, $P < 0.05$; Fig. 3A). Our data demonstrated that IDO1 enhances normal and eutopic ESCs proliferation.

As shown in Fig. 3B, L-1-MT demonstrated its efficacy in inducing ESCs decidualization. Eutopic ESCs showed a marked reduced capacity of decidualization (PRL secretion) in vitro ($P < 0.05$ versus control). L-1-MT treatment alone caused a significant increase in PRL levels in the culture supernatant of both normal and eutopic ESCs (L-1-MT versus control, $P < 0.05$). Moreover, the secretion of PRL by LPS-stimulated ESCs was strikingly increased after L-1-MT administration (LPS plus L-1-MT versus LPS, $P < 0.05$).

L-1-MT suppresses adhesion and invasion of ESCs

Based on the above findings, experiments were then designed to determine whether IDO1 plays a role in regulating ESCs adhesion and invasion. In the control group, eutopic ESCs were found to have a significantly higher adhesion ability to attach to a range of ECM components (fibronectin, type I collagen, type IV collagen, fibrinogen) than normal ESCs (eutopic versus normal, $P < 0.05$; Fig. 4). LPS alone could significantly enhance the adhesion ability of both normal and eutopic ESCs on fibronectin, type I collagen, type IV collagen and fibrinogen (LPS versus control, $P < 0.05$; Fig. 4A, B, C and E). Conversely, adding L-1-MT could reverse the effects of LPS mentioned above (LPS plus L-1-MT versus LPS, $P < 0.05$; Fig. 4A, B, C and E), and the inhibitory effect of L-1-MT was extremely limited on fibronectin, type I collagen, type IV collagen and fibrinogen. Neither LPS nor L-1-MT treatment could alter the adhesion of ESCs to type I laminin (LPS or L-1-MT versus control, $P > 0.05$; Fig. 4D). We thus obtain the conclusion that the enhancement of ESCs adhesion ability induced by LPS could be attenuated by L-1-MT, and IDO1 might acts as an accelerator in ESCs adhesion to certain ECM components.

We next investigated whether IDO1 could regulate the cellular invasion of ESCs. Our results showed that the invasiveness of eutopic ESCs was 1.8-fold of the normal group ($P < 0.05$; Fig. 4F). L-1-MT significantly diminished the LPS-triggered invasion ability of normal and eutopic ESCs (LPS plus L-1-MT versus LPS; $P < 0.05$; Fig. 4F). All of the above suggest that an increase of IDO1 expression is possibly...
The role of IDO1 in endometriosis

Figure 1 (A) Representative micrographs for IDO1 immunostaining in normal endometrium from women without endometriosis (i), eutopic endometrium of women with endometriosis (ii), normal ovary tissue from women without endometriosis (iii) and ectopic endometrium from the ovary endometriotic lesion (iv), respectively. Negative antibody isotype staining of the normal tissue was represented as control (v). Magnification, ×200. (B) The characterization of human ESCs by immunocytochemistry. Cultured eutopic endometriotic stromal cells were stained with anti-CK7 antibody (i), anti-vimentin antibody (ii), anti-IDO1 antibody (iii) or mouse IgG isotype-matched control (iv). Magnification, ×200. The expression of IDO1 (C), COX-2 (D), MMP-9 (F) were statistically significantly higher in eutopic (lanes 3, 4) and ectopic ESCs (lanes 5, 6) than in normal ESCs (lanes 1, 2), as analyzed by in-cell Western. There was no statistically significant difference concerning the expression of MMP-2 (E). Representative data were shown as well as the mean ± SD of 12 different experiments. Scale bar = 50 μm. *p < 0.05 versus control (normal ESCs).
Figure 2  IDO1 (A), COX-2 (B), MMP-2 (C) and MMP-9 (D) expression quantification obtained by in-cell Western in ESCs isolated from control women (normal) and from women affected by endometriosis (Eutopic) after treatment with vehicle (1% DMSO) as control (lane 1), LPS (10 ng/ml; lane 2) alone or with L-1-MT (0.05 mM; lane 3) alone, or coadministration of LPS and L-1-MT for 24 h (lane 4). Inhibiting the function of IDO1 (A) by L-1-MT was able to significantly attenuate expression of COX-2 (B) and MMP-9 (D) in eutopic ESCs, except MMP-2 (C). *P < 0.05, versus vehicle control; **P < 0.01, versus vehicle control; #P < 0.05, versus normal ESC; ##P < 0.01 versus normal ESC; D P < 0.05 versus LPS group; DDP < 0.01 versus LPS group. Representative data as well as the mean ± SD of nine independent experiments were shown.
associated with the unique biological characteristic of the eutopic ESCs, and it accelerates ESCs proliferation, adhesion and invasion ability while decreasing the decidualization of ESCs.

**Discussion**

In the present study, we first demonstrated the higher expression of IDO1 in eutopic and ectopic ESCs derived from patients with endometriosis, and IDO1 dependent COX-2, MMP-9 expression in ESCs. L-1-MT, the inhibitor of IDO1, led to the greater level of ESCs decidualization, while offering inhibitory activity in ESCs proliferation, adhesion and invasion ability.

A current paradigm is that local inflammation in pelvic cavity plays an important role in the pathophysiology of endometriosis and various proinflammatory molecules are suggested to promote the progress of the disease (Osuga et al., 2002; Wu and Ho, 2003). The present study has shown that IDO1, which could be up-regulated by the inflammation factor LPS, stimulated the proliferation and invasion of normal and eutopic ESCs, while offering inhibitory activity in ESCs proliferation, adhesion and invasion ability.

The role of IDO1 in endometriosis

**Figure 3** ESCs isolated from control women (normal) and from women affected by endometriosis (eutopic) were treated with vehicle (1% DMSO) as control (lane 1), LPS (10 ng/ml; lane 2) alone, or with L-1-MT (0.05 mM; lane 3) alone, or co-administration of LPS and L-1-MT for 24 h (lane 4). Cell proliferation was determined by BrdU incorporation (A), and cells were allowed to decidualize in vitro in response to 0.5 mM of 8-Br-cAMP for 9 days, then PRL secretion by ESCs into supernatants was measured in triplicate by ELISA to comprehend the decidualization of ESCs (B). L-1-MT treatment of ESCs resulted in enhance of decidualization and reduction of proliferation. The columns represent the mean ± SD calculus of six different experiments. *P < 0.05 versus vehicle control; **P < 0.01 versus vehicle control; #P < 0.05 versus normal ESC; D P < 0.05 versus LPS group.
development of endometriosis through multiple mechanisms, including increased migration and invasiveness (Collette et al., 2006; Banu et al., 2008). Previous studies have suggested that COX-2 expression is increased in the eutopic endometrium and the ovarian endometriotic tissue of patients with endometriosis (Cho et al., 2009) and its derivative PGs, particularly PGE₂, can explain most of the symptoms of endometriosis (Chishima et al., 2002), especially the induction of pain. Conversely, selective inhibition of PGE₂ receptors suppresses

Figure 4  Cell adhesion properties and invasive ability of ESCs were analyzed. LPS-stimulated ESCs showed decreased adhesion (A–E) and invasiveness (F) after treated with L-1-MT. The significant inhibiting effects on migration and invasion by L-1-MT were also observed for ectopic ESCs (not indicated in figures). Results were highly reproducible in six independent experiments. Data were shown as the mean ± SD of six independent experiments. *p < 0.05, **p < 0.01 versus vehicle control; *p < 0.05 versus normal ESC; *p < 0.01 versus normal ESC; #p < 0.05 versus LPS group; ##p < 0.01 versus LPS group.
the expression and activity of MMPs, and decreases migration and invasion of human immortalized endometriotic epithelial and stromal cells into Matrigel (Lee et al., 2011). In the present study, we have validated that eutopic and ectopic ESCs expressed a higher level of COX-2 and MMP-9 than the normal group, which is consistent with the previous works. Besides, COX-2 triggered by LPS was reversed by the L-1-MT exposure, a specific inhibitor of IDO1, which highly suggested an intrinsic correlation between IDO1 and COX-2. Thus, the present finding that L-1-MT decreased COX-2 and MMP-9 expression in ESCs provides further evidence for involvement of IDO1 in endometriosis. Additionally, pain induced by PGE2 is the major symptom reported by women with endometriosis, and potential of the IDO1 inhibition in the treatment of inflammatory pain arouses our interest.

Although endometriotic lesions are benign, they share certain characteristics with malignancies and can be defined as an invasive disease. Invasion is a multiphase process constituted by different coordinated interdependent steps, controlled by cross-talk mechanisms between cells and extracellular microenvironment (Alessandro and Kohn, 2002). In the present study, we demonstrated that eutopic ESCs were found to have a significantly higher primary adhesion ability than that of normal ESC in a range of ECM components (fibronectin, type I collagen, type IV collagen and fibrinogen), as well as an elevated invasiveness and survival ability. Our findings were in line with previous findings (Witz et al., 2001). Furthermore, individual L-1-MT treatment had no effect on the initial attachment of both ESCs derived from the normal control and ESCs derived from the eutopic group. Moreover, L-1-MT can abolish the increased adhesion capability of ESCs induced by LPS in fibronectin, type I collagen, type IV collagen and fibrinogen in a significant manner. Based on these findings and data from cancer research, it is tempting to speculate that inducible IDO1 expression is an important determinant of the way in which ESCs interact with their extracellular environment in vitro, but the intrinsic IDO1 is inadequate to impact the adhesion of ESCs. In a comparative study of invasive assay, a marked difference was observed in basal invasion or in the invasion response to L-1-MT used alone or in combination with LPS. Thus, IDO1 assumes a direct role in controlling interactions with endometrial cells and the surrounding extracellular environment.

We also examined that cells of the eutopic endometrium have an inherent higher capacity to proliferate induced by IDO1. Additionally, PRL levels were low in the culture supernatant of endometriotic stromal cells compared with the normal ESCs, which suggests a reduced capacity of endometriotic stromal cells for decidualization at ectopic sites, and our observations are in agreement with the findings of Minici et al. (2008). L-1-MT treatment significantly induced decidualization of endometriotic stromal cells in vitro evidenced by increased PRL. It could be speculated that, the milieu surrounding the uterine cavity may be involved in impaired eutopic ESCs decidualization, partially due to increased level of IDO1. Since decidualization is a critical process for the successful establishment and maintenance of pregnancy, a role for IDO1 in endometriosis-related infertility could also been suggested here. Furthermore, Kudo et al. (2004) showed that in a cell culture model, the level of IDO1 was suppressed specifically by progesterone-induced decidualization of isolated ESCs. Since endometriosis is defined as a chronic inflammatory condition with impaired progesterone responsiveness, the elevated expression of IDO1 in the endometriotic lesion could be well explained. To our knowledge, there is no report available in the literature that demonstrates the effect of IDO1 on PRL levels.

In conclusion, our finding provides evidence that elevated IDO1 expression in the eutopic and ectopic ESCs may promote the expression of COX-2 and MMP-9, induce abnormal increase of ESCs growth, and initiate the invasion and implantation of the shed endometrium to peritoneum. It finally leads to the formation of endometriotic lesions and the abnormal decidualization of endometrium. This information will be helpful for further investigation of the pathogenesis and therapeutics of endometriosis. Taking into the consideration the potential of L-1-MT for cancer therapy and the already running clinical trials (Opitz et al., 2011), the L-1-MT releasing intrauterine device system might be applied to both contraception and prevention of endometriosis, and further work needs to be done to identify IDO1-based therapies with no disruption of hormonal balance which could be of clinical relevance.

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Authors’ roles

J.M. performed the cell isolate, in-cell Western, proliferation assay, invasion assay and took the lead in the writing of the manuscript. D.D. was responsible for immunohistochemistry, immunocytochemistry and adhesion assay. M.-Q.L. participated in cell culture, decidualization assay and data analysis. D.-J.L. reviewed the study arrangement and paper. L.-P.J. and X.-Y.Z. were in charge of the study design, overseeing the completion of the study, editing and finalizing of the manuscript.

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Conflict of interest

None declared.

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