Combination of estrogen and dioxin is involved in the pathogenesis of endometriosis by promoting chemokine secretion and invasion of endometrial stromal cells

Jing Yu¹, Yun Wang¹, Wen-Hui Zhou¹, Ling Wang¹, Yin-Yan He¹ and Da-Jin Li¹,²,³

¹Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, People’s Republic of China; ²Department of Obstetrics and Gynecology, Affiliated Hospital, Hainan Medical College, Haikou 570102, People’s Republic of China
³Correspondence address. Laboratory for Reproductive Immunology, Hospital and Institute of Obstetrics and Gynecology, Fudan University Shanghai Medical College, Shanghai 200011, People’s Republic of China. Tel/Fax: +86-21-63457331; E-mail: djli@shmu.edu.cn

BACKGROUND: The CC chemokines, regulated on activation, normal T-cell expressed and secreted (RANTES) and macrophage-inflammatory protein-1alpha (MIP-1α), have been identified as potential contributors to the pathogenesis and the progression of endometriosis. Dioxin, an air pollutant, and estrogen also appear to be involved in endometriosis. The aim of this study was to probe into the effect of dioxin and estrogen on expression of the chemokines in endometriosis-associated cells, and to explore the pathogenesis of endometriosis. METHODS: Co-culture models were established to evaluate the secretion of human RANTES and MIP-1α. The effects of a dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD) and estrogen on the invasion of endometrial stromal cells (ESC) were also examined by using an invasion assay, and the translation and proteolytic activity of matrix metalloproteinase (MMP)-9 and MMP-2 in ESC were determined by western blot and zymography, respectively. RESULTS: Our results showed that the combination of 17β-estradiol and TCDD increased the secretion of RANTES and MIP-1α, promoted the invasiveness of ESC and increased the expression of MMP-2 and MMP-9 in ESC. Anti-RANTES, anti-MIP-1α neutralizing antibody or antibody against their receptors could effectively inhibit the invasiveness of ESC and the expression of MMP-2 and MMP-9. CONCLUSIONS: The combination of 17β-estradiol with TCDD may facilitate the onset of endometriosis and contribute to its development by increasing the invasion of ESC mediated by CC-motif chemokines.

Keywords: endometriosis; air pollutant; estrogen; chemokines

Introduction

Endometriosis, a chronic inflammatory disease, is a very frequent gynecological disorder in the fertile phase. Sampson’s theory of implantation of endometrial cells and fragments refluxed during the menstrual period is generally accepted in the pathogenesis. The retrograde menstruation occurs in almost all cycles, suggesting that the onset of endometriosis involves inefficient clearance of the menstrual efflux from the pelvis, a defect that is associated with the serous immune system (Dmowski et al., 1981; Halme et al., 1983, 1988). Indeed, endometriosis is often associated with inflammatory changes observed in the peritoneal fluid, which bathes the organs in the pelvic cavity, as well as in endometriotic implants (Witz and Schenken, 1997; Mulayim and Arici, 1999). One of the hallmarks of such a local inflammatory reaction is an increased number of activated peritoneal leukocytes, particularly macrophages (Haney et al., 1981; Halme et al., 1983; Olive et al., 1985; Hill et al., 1988). Paradoxically, instead of eliminating the misplaced endometrial tissue, activated macrophages appear to be involved in the ectopic adhesion, implantation and growth of the endometrial tissue. In fact, the peritoneal macrophages isolated from patients with endometriosis were found to have phenotypic and functional alterations leading to poor phagocytic capacity, which is highly associated with severity of endometriosis (Dmowski et al., 1990; Raiter-Tenenbaum et al., 1998), although the underlying mechanism remains uncharacterized. Therefore, the peritoneal macrophages may contribute to the development of endometriosis.

The potential contribution of macrophages to endometriosis motivated researchers to identify factors that may induce the recruitment of monocytes from the circulation into the ectopic endometrial tissues and peritoneal cavity. A series of research has shown that chemokines produced by the endometriotic tissue may contribute to a feed-forward cascade of events, which accentuates the recruitment of leukocytes into
the peritoneal cavity of patients with endometriosis (Akoum et al., 1995, 2001; Tseng et al., 1996; Hornung et al., 1997; Akoum, 2000). The CC subfamily of chemokines is characterized as major mediators of monocyte and T-cell migration into inflammatory loci (Wang et al., 1998; Murdoch and Finn, 2000). RANTES and macrophage-inflammatory protein-1α, which are major ligands of the receptors, CCR1 and CCR5, are members of the C–C chemokine family. RANTES is a chemoattractant for both monocytes and activated T-cells (Schall et al., 1990). RANTES and MIP-1α are known to be elevated in peritoneal fluid of women with endometriosis, and are commensurate with the stage of endometriosis. Most of the monocyte-chemotactic activity in peritoneal fluid from women with endometriosis appears to be mediated by RANTES (Hornung et al., 2001), whereas MIP-1α mediates neutrophil migration into immune inflammation milieu. These results indicate that the high incidence and the elevated expression levels of RANTES and MIP-1α are directly associated with a more advanced disease, suggesting that these chemokines might be involved in endometriosis progression.

The initial phase of endometriosis is an invasive event which requires extracellular matrix (ECM) breakdown (Spuijbroek et al., 1992; Bruner et al., 1997). Matrix metalloproteinases (MMPs) are essential in the remodeling of the ECM in normal development, growth and repair of tissues, and are implicated in cancer as well as in inflammatory and degenerative diseases. Several studies have shown an increase in the expression of MMP-1 (Kokorine et al., 1997), MMP-2 (Wenzl and Heinzl, 1998), MMP-3 (Cox et al., 2001), MMP-7 (Rodgers et al., 1993) and MMP-9 (Chung et al., 2001) in endometriotic tissue. Alteration of MMP-9 and MMP-2 are important factors in development of endometriosis (Chung et al., 2002; Collette et al., 2006).

Recently, environmental contaminants have been also suggested to play a role in the pathogenesis of endometriosis. Previous work on primates has shown that exposure to the dioxin 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is associated with an increased prevalence and severity of endometriosis (Rier and Foster, 2002). TCDD, one of the family of chlorinated aromatic hydrocarbons, is a highly toxic environmental contaminant derived from sources of 2,4,5-trichlorophenol (Landers and Bunce, 1991; Birnbaum, 1994). The myriad of biological effects of TCDD is believed to be mediated essentially via the aryl hydrocarbon receptor (AhR; Whitlock, 1993), which must form a complex with the AhR nuclear translocator (ARNT) to activate TCDD responses (Hoffman et al., 1991; Landers and Bunce, 1991; Reys et al., 1992; Greenlee et al., 1994). TCDD exposure has been shown to induce both CYP1A1 (Charles and Shiverick, 1997; Kress and Greenlee, 1997) and CYP1B1 (Hakkola et al., 1997) in various tissues. Both CYP1A1 and CYP1B1 are 17β-estradiol hydroxylases (Spink et al., 1992; Hayes et al., 1996). The heteromeric complex acts as a signal transducer and transcription factor for target genes, including cytochromes P450 1A1, 1A2 and 1B1 (CYP1A1, CYP1A2 and CYP1B1), and growth regulatory genes involved in cell proliferation, differentiation and inflammation (Zhao et al., 2002). Since endometriosis is an estrogen-dependent disease (Dizerega et al., 1980; Sensky and Liu, 1980; Cramer et al., 1986; Kiesel and Rennebaum, 1990), and the inflammatory milieu in the peritoneal cavity of women with endometriosis has been extensively characterized, altered metabolism of estradiol by TCDD or other dioxin-like halogenated aromatic hydrocarbons, and pro-inflammatory effects of TCDD may be involved in the pathogenesis of endometriosis. Our previous work showed that the combination of 17β-estradiol and TCDD up-regulated CXCR1 expression in endometrial stromal cells (ESC), and promoted secretion of IL-8, a ligand of CXCR1, in co-culture of ESC–HPCMC cells (Shi et al., 2006).

In the present study, we first observed the effects of 17β-estradiol and TCDD on RANTES and MIP-1α secretion of the endometriosis-associated cells in the co-culture unit, and then studied the expression of MMP-9 and MMP-2 and invasiveness of the ESC. To better understand the role of RANTES and MIP-1α in the progression of endometriosis, we investigated the regulation of RANTES and MIP-1α expression and the underlying mechanisms of their potential pro-invasion activity.

Materials and Methods

Tissue collection and cell culture

All the eutopic endometrial tissues were obtained from 15 patients with endometriosis (mean age 40.2 years; range 31–44) at the time of laparoscopy or by uterine curettage, in the Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College. The endometriotic tissues included peritoneal implants (n = 3) and ovarian endometriomas (n = 12). The patients were classified according to the revised American Fertility Society (AFS) classification: 4 in Stage 1 and 11 in Stage 2. Patients had not received any GnRH analog or other hormonal drug in the 6 months prior to the surgical procedure. Before surgery, informed consent was obtained from each patient using protocols approved by the Human Investigation Committee in Hospital of Obstetrics and Gynecology, Fudan University Shanghai Medical College. All the samples were obtained in the proliferative phase of the cycle, which was confirmed histologically according to established criteria. The eutopic tissues were collected under sterile conditions and transported to the laboratory on ice in 1:1 formula of DMEM (Dulbecco’s modified Eagle’s medium)/F-12 (Gibco, USA) with 10% fetal calf serum (FCS; HyClone, Logan, UT, USA). The minced eutopic endometrium was digested with collagenase type A (0.1%; Sigma, USA) for 30 min at 37°C with constant agitation. The tissue pieces were filtrated through a 200 μm wire sieve to remove debris. Following gentle centrifugation, the supernatant was discarded, and the cells were resuspended in DMEM/F-12. The ESCs were separated from epithelial cells by passing them over a 400 μm wire sieve. The filtrated suspension was layered over Ficoll, and centrifuged at 1200 g for 20 min to further remove leukocytes and erythrocytes, and the middle layer was collected and then washed with D-Hanks. The ESCs were placed in a culture flask, and allowed to adhere for 20 min. The adherent stromal cells were cultured as monolayer in flasks with DMEM/F-12 containing 1% FCS, 20 mmol/l HEPES, 100 IU/ml penicillin and 100 μg/ml streptomycin and incubated in 5% CO2 at 37°C. This method supplied a 95% purity of ESC.

HMrSV5 (HPCMC, a human peritoneal mesothelial cell line provided by Prof. Jian Yao, the First People’s Hospital, Shanghai, China) and human monocyte U937 cell line (purchased from Bank of Cell, Chinese Academy of Sciences, Shanghai, China) were...
maintained in DMEM (Gibco, USA) with 10% FCS and Roswell Park Memorial Institute (RPMI)1640 medium (Life Technologies) with 10% bovine calf serum, respectively, and containing 20 mmol/l HEPES, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified, CO₂-controlled (5%) incubator. The medium was changed every other day.

**Cell co-culture unit**

**Contact co-culture units of two sorts of cells**

The ESC or HPMC were cultured in 24-well plates at a concentration of 1 × 10⁵ cells/well until adhering to the plastic. The media was removed, and then ESC or U937 cells were applied, respectively, over HPMC or ESC at the same concentration. The total number of the cells in co-culture unit was 2 × 10⁶ cells/well. The cells were cultured in a final volume of 1 ml fresh DMEM with 2.5% FCS for 48 h. HPMC, ESC and U937 cells of 1 × 10⁵ cells/well cultured alone in the same media were used as controls. Each experiment was carried out in triplicate and repeated three times.

**Contact co-culture units of U937–ESC–HPMC**

The three sorts of the cells were plated in the proportion of 1:1:1. The HPMC were cultured in 24-well plates at a concentration of 1 × 10⁵ cells/well until adhering to the plastic bottom. The media was removed, and then ESC were applied over HPMC at the same concentration. After ESC adhered to the plastic and HPMC, the media was removed again. U937 cells were applied over ESC and HPMC at the same concentration. The total number of the three sorts of the cells of co-culture unit was 3 × 10⁶ cells/well. The cells were cultured in a final volume of 1 ml fresh DMEM with 2.5% FCS for 48 h. Each experiment was carried out in triplicate, and repeated three times.

**Transwell co-culture units of the three cells**

The three sorts of the cells were also plated in the proportion of 1:1:1. The ESC, HPMC and U937 cells at 1 × 10⁵ cells per well were plated, respectively, in the lower or upper compartment of Costar transwell cell culture chamber inserts (0.4 μm, 12 mm diameter). There were three models with different combinations of these cells. First, U937/HPMC–ESC (U/H–E), U937 cells were plated in the upper compartment, and the HPMC and ESC in contact were in the lower compartment. Second, HPMC/ESC–U937 (H/U–E), HPMC were in the upper compartment, and the ESC and U937 in contact were in the lower compartment. Third, ESC/HPMC–U937 (E/H–U), ESCs were in the upper compartment, and the HPMC and U937 in contact were in the lower compartment. The total number of the three sorts of the cells in co-culture unit was 3 × 10⁶ cells/well. Each experiment was carried out in triplicate, and repeated three times.

After co-culture, the ESC, HPMC and HPMC–ESC in co-culture were stained using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA). An immunoperoxidase staining was performed by using anti-human vimentin and anti-human cytokeratin monoclonal antibody (Maixin Co. Ltd, Fuzhou, China). An isotypic control was used at the same concentration.

**Treatment in vitro with estrogen or TCDD**

After serum starvation for 12 h, ESC, HPMC, U937 cells and every co-culture unit were treated, respectively, with 17β-estradiol at 10⁻⁸ mol/l (Sigma, USA), TCDD at 1 nmol/l (Sigma, USA) or the combination of 17β-estradiol and TCDD for 48 h, respectively, with vehicle (DMSO) as controls. Each experiment was carried out in triplicate, and repeated three times.

**Enzyme-linked immunosorbent assay for determination of rantes and mip-1α**

The culture supernatants were harvested, centrifuged to remove cellular debris, and stored at −80°C until being assayed by enzyme-linked immunosorbent assay (ELISA). The RANTES and MIP-1α concentrations in the culture supernatant were quantified by ELISA kits (R&D Systems, Wiesbaden, Germany) according to the manufacturer’s instructions. The limit of detection for MIP-1α was <10 pg/ml. The limit of detection for RANTES was <2 pg/ml. Each experiment was carried out in triplicate, and repeated three times.

**Matrigel invasion assay**

The invasion of ESC across Matrigel was evaluated objectively in invasion chambers with 6.4 mm diameters and 8 μm pore sizes (Corning, Corning, NY, USA). Invasion chambers coated with 6 μl Matrigel were placed in a 24-well plate. The purified ESCs (2 × 10⁵ in 200 μl DMEM with 1% FCS) were plated in the upper chamber. There were two groups in terms of different cells at the lower compartments. In the first group, there were no cells in the lower compartments; 17β-estradiol at 10⁻⁸ mol/l, TCDD at 1 nmol/l, the combination of 17β-estradiol and TCDD, hrRANTES (R&D Systems, Abingdon, UK) at 10 ng/ml, hrRANTES combined with anti-RANTES neutralizing antibody (R&D Systems) at 2.5 μg/ml, hrMIP-1α at 10 ng/ml (R&D Systems) or hrMIP-1α combined with anti-MIP-1α neutralizing antibody at 2.5 μg/ml (R&D Systems) were added to both upper compartments and lower compartments, respectively. The lower chamber was filled with 800 μl DMEM with 10% FBS. The second group was the co-culture of HPMC and U937 cells in the lower compartment and the ESC were plated in the upper compartment. Anti-RANTES, anti-MIP-1α or anti-CCR1 neutralizing antibody (MBL, Japan) at 10 μg/ml or anti-CCR5 neutralizing antibody (BD) at 5 μg/ml was added to both upper compartments and lower compartments, respectively. The mouse IgG acted as an isotypic control. Then, 17β-estradiol and TCDD combined with every different neutralizing antibody was added to the compartments. The cells were then incubated at 37°C for 48 h.

The inserts were removed, washed in phosphate-buffered saline (PBS) and non-invading cells 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 methanol for 10 min at room temperature and then stained with hematoxylin. The result was observed under Olympus BX51+DP70 fluorescence microscope (Olympus, Tokyo, Japan). The cells that migrated to the lower surface were counted in five predetermined fields at a magnification of ×400. Each experiment was carried out in triplicate, and repeated three times.

**Gelatin zymography**

The culture medium in the upper chamber of the invasion assay was harvested, and the proteolytic activity of both MMP-9 and MMP-2 was measured by the gelatin zymography described by Yoshizaki, with light modification (Yoshizaki et al., 1997). Briefly, the culture supernatants, collected from upper chamber of the invasion assay, containing 10 μg of total protein were mixed with sodium dodecyl sulfate (SDS) loading buffer, applied in each lane and electrophoresed on 10% SDS–polyacrylamide gels copolymerized with 0.2% gelatin. After electrophoresis, the gel was rinsed in 2.5% Triton-X 100 for 1 h to remove SDS. The gel was incubated for 12 h at 37°C in 50 mM Tris–HCl (pH 7.5), 200 mM NaCl and 10 mM CaCl₂, and stained with 2.5% Coomassie Blue R250 (Sigma Chemical Co., St Louis, MO, USA) dissolved in 40% (v/v) methanol and 10% acetic acid. The gels were then rinsed in three different decolorant solutions.
(A: 30% methanol, 10% acetic acid; B: 20% methanol, 10% acetic acid and C: 10% methanol, 5% acetic acid), respectively. The gel was photographed and assayed by the Odyssey Infrared Imaging System. The gelatinolytic activity was visualized as a clear white band against a dark background of stained gelatin. The proteolytic activity of MMP was equal to the intensity of MMP-9 or MMP-2 of the treatment group over that of the control. The experiments were carried out in triplicate, and were repeated three times.

Western blot analysis was performed by using anti-MMP-2 monoclonal antibody and anti-MMP-9 monoclonal antibody (Chemicon, USA). We established two culture models similar to that for the invasive assay. The ESC cultured alone received the same treatment. The ESC/HPMC-U937 were treated with 17β-estradiol at 10⁻³ mol/l, TCDD at 1 nmol/l, the combination of 17β-estradiol and TCDD, the combination of 17β-estradiol, TCDD and anti-RANTES neutralizing antibody, the combination of 17β-estradiol, TCDD and anti-MIP-1α neutralizing antibody, and the combination of 17β-estradiol, TCDD and anti-CCR5 neutralizing antibody. The cells were lysed in 1% NP-40, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 100 mg/ml PMSF, 1 mg/ml Aprotinin and 0.1% SDS for 10 min at 48°C. The nuclei were removed by centrifugation at 12,000 g at 4°C for 10 min, and the cell lysates were assayed for protein contents using the Bradford protein assay. The proteins (50 mg) were resuspended in sample buffer (2% SDS, 62.5 mM Tris, pH 6.8, 0.1% bromophenol blue and 2.5% 2-mercaptoethanol, 10% glycerol), separated on 10% SDS–polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane by electrotransfer for 1 h. After being soaked in blocking buffer [13 TBS with blocking reagent (5% milk)], the membrane was incubated with primary antibody overnight at 4°C. The blots were developed using the HRP-linked secondary antibody and a chemiluminescent detection system. The experiments were repeated three times.

Statistics
All values were shown in the mean ± SEM. One-way ANOVA was used to detect the difference of RANTES, MIP-1α secretion, invasion, MMP-9 or MMP-2 expression in human ESC. Differences were accepted as significant at P < 0.05.

Results
Effect of the co-culture on MIP-1α secretion of the endometriosis-associated cells
The respective culture of ESC and U937 cells for 48 h produced no secretion of RANTES, but the RANTES secretion by U937 cell lines was significantly higher than that of ESC and HPMC, respectively (P < 0.01). The RANTES secretion of the contact co-culture unit of HPMC and U937 (H–U) was 32.9-fold and 6.79-fold higher than that of the co-culture ESC–HPMC and ESC–U937 units, respectively (P < 0.01). The non-contact co-culture of three cells and the contact co-culture of three sorts of the cells showed significant effect on RANTES secretion. The contact co-culture unit of three cells secreted more RATNES, which was 8.8-fold higher than the total production of the respective culture of ESC, HPMC and U937 cells (P < 0.01). Among the three non-contact co-culture units, the RATNES production of the co-culture unit of ESC/HPMC–U937 was 1.66-fold and 1.34-fold higher than that of the non-contact co-culture units, U937/HPMC–ESC and HPMC/ESC–U937, respectively (P < 0.01). The RATNES production of the ESC/HPMC–U937 co-culture unit was 7.46-fold higher than the total production of the respective ESC, HPMC and U937 cells (P < 0.01). Meanwhile, the RANTES secretion of the non-contact co-culture of U/H–E, H/E–U or E/H–U was significantly higher than that of H–E, E–U and H–U, respectively (P < 0.01). The results above showed that the direct contact and non-contact of the corresponding cells could promote the secretion of RANTES, and the inflamed peritoneum might play an important role in pathogenesis of endometriosis (Fig. 1A). Cells morphous of the co-culture unit U937–HPMC–ESC, U937–HPMC and U937–ESC were observed by using an inverted microscope (Fig. 1A–C). Characterization of ESC–HPMC, HPMC and ESC showed that HPMC expressed both vimentin and cytokeratin. ESC expressed vimentin, but did not express cytokeratin. Therefore, in the HPMC–ESC co-culture unit, all the cells expressed vimentin (Fig. 2D–L).

Effect of estradiol and/or TCDD on RANTES production in respective culture and contact co-culture of ESC, HPMC and U937 cells
The 17β-estradiol alone showed no effect on the RANTES secretion of the endometriosis-associated cells. The combination of 17β-estradiol with TCDD had a synergistic effect on RANTES secretion of the ESC cultured alone (P < 0.05). The combination of 17β-estradiol with TCDD significantly decreased RANTES secretion of U937 cells (P < 0.05), but TCDD decreased RANTES secretion of U937 cell cultured alone (P < 0.05). Therefore, 17β-estradiol did not show a synergistic effect with TCDD on the RANTES secretion of...
Compared with the control, the 17\(\beta\)-estradiol had no obvious effect on RANTES production in the contact co-culture unit of ESC, HPMC and U937 cells. TCDD decreased RANTES secretion in this culture unit (\(P < 0.05\)). Therefore, although the combination of TCDD with 17\(\beta\)-estradiol significantly decreased RANTES secretion of the contact co-culture unit (\(P < 0.05\)), they showed no synergistic effect (Fig. 1C).

**Effect of estradiol and/or TCDD on rantes secretion in the non-contact co-culture of ESC, HPMC and U937 cells**

In the co-culture unit of U937/HPMC–ESC, 17\(\beta\)-estradiol and/or TCDD showed no effect on RANTES secretion. In the HPMC/ESC–U937 unit, estradiol had no effect on RANTES secretion, but TCDD promoted significantly RANTES secretion (\(P < 0.05\)). Although the 17\(\beta\)-estradiol combined with TCDD increased RANTES secretion (\(P < 0.05\)), they showed no synergistic effect (Fig. 1C). In the co-culture unit of ESC/HPMC–U937, 17\(\beta\)-estradiol or TCDD alone did not promote RANTES secretion, but they significantly stimulated RANTES secretion when they were in combination (\(P < 0.05\), Fig. 1C).

**Effect of estradiol and/or TCDD on MIP-1\(\alpha\) secretion in the respective culture and contact co-culture of ESC, HPMC and U937 cells**

The 17\(\beta\)-estradiol and TCDD alone had no effect on the MIP-1\(\alpha\) secretion of ESC, but in combination, they promoted...
IgG (IgG (I) /C2 stained by anti-cytokeratin mAb (J)

In the non-contact co-culture unit of HPMC–U937 cells

ESC expressed vimentin and did not express cytokeratin. The ESC–HPMC–U937 invasion model was significantly higher than that of ESC cultured alone, which shows that the co-culture improves significantly the invasiveness of ESC cultured alone and in the ESC/HPMC–U937 co-culture system.

The invasive index of cells under different conditions was normalized to the control. The invasion of ESC through the matrigel-coated membranes was taken by microscopic morphology at ×200 magnification. The ESC was incubated in the presence of vehicle (A), 10−8 mol/l 17β-estradiol (B), 1 nmol/l TCDD (C), 17β-estradiol and TCDD in combination (D), co-culture in the presence of vehicle (E), co-culture treated with 10−8 mol/l 17β-estradiol (F), co-culture treated with 1 nmol/l TCDD (G) co-culture treated with the combination of 17β-estradiol and TCDD (H) (×200). The results in invasive index showed that the combination of 17β-estradiol with TCDD improved invasion of ESC cultured alone and in the ESC/HPMC–U937 co-culture; the invasion of ESC was increased and significantly increased after stimulation by the combination of 17β-estradiol with TCDD (I). Data are expressed as mean ± SEM. *P < 0.05 compared with the ESC cultured alone control; **P < 0.01 compared with the co-culture control.

ESC secretion of MIP-1α (P < 0.05). Compared with the control, both 17β-estradiol and TCDD significantly increased MIP-1α secretion in the contact co-culture unit of ESC–HPMC–U937 (P < 0.05), but 17β-estradiol combined with TCDD showed no effect on the MIP-1α secretion (Fig. 1D).

**Effect of estradiol and/or TCDD on MIP-1α secretion in the non-contact co-culture of ESC, HPMC and U937 cells**

In the non-contact co-culture unit of HPMC/ESC–U937 and U937/HPMC–ESC, estradiol and/or TCDD had no effect on MIP-1α secretion. In the co-culture unit of ESC/HPMC–U937, neither 17β-estradiol nor TCDD alone changed MIP-1α secretion, but in combination, they significantly stimulated MIP-1α secretion (P < 0.05, Fig. 1D).

The combination of estradiol with TCDD and co-culture of ESC, HPMC and U937 cells promoted invasion in vitro of ESC through increasing secretion of rantes and MIP-1α

To testify the effects of estradiol and TCDD on the invasion of ESC, a matrigel-based transwell assay was carried out. The freshly isolated ESCs were added to the upper chamber, and then treated, respectively, with estradiol, TCDD or the combination of estradiol with TCDD. The number of cells migrating to the lower surface was counted in 48 h of incubation. As shown in Fig. 3A–D, 17β-estradiol combined with TCDD significantly increased invasion of human ESC. The invasive index of ESC was significantly higher compared with the vehicle controls (P < 0.05, Fig. 3I). Moreover, the invasion of ESC in the ESC/HPMC–U937 invasion model was significantly higher than that of ESC cultured alone, which shows that the co-culture improves significantly the invasiveness of ESC (P < 0.05, Fig. 3E and I). Furthermore, the invasiveness of ESC in the E/H–U model treated with the combination of 17β-estradiol with TCDD was significantly higher than that of the co-culture control without treatment (P < 0.05, Fig. 3H and I).

Our research showed that the RANTES and MIP-1α secretions in the ESC culture alone, and the non-contact co-culture of the three sorts of the cells, ESC/HPMC–U937, were significantly higher after treated with the combination.

**Figure 2:** Cells morphous of co-culture units and characterization of ESC–HPMC, HPMC and ESC.

Cells morphous of co-culture unit U937–HPMC–ESC (A), U937–HPMC (B) and U937–ESC (C) were observed by using inverted microscope (×100). HPMC expressed both vimentin and cytokeratin. ESC expressed vimentin and did not express cytokeratin. The ESC–HPMC stained by anti-vimentin mAb (D). The ESC–HPMC stained by anti-cytokeratin mAb (J). The ESC–HPMC stained by anti-vimentin mAb (G). The HPMC stained by anti-cytokeratin mAb (H). The HPMC stained by isotypical IgG (I). The ESC stained by anti-vimentin mAb (J). The ESC stained by anti-cytokeratin mAb (K). The ESC stained by isotypical IgG (I) (×200).

**Figure 3:** The combination of 17β-estradiol with TCDD enhanced invasiveness of ESC cultured alone and the co-culture-improved invasiveness of ESC in the ESC/HPMC–U937 co-culture system.

The invasive index of cells under different conditions was normalized to the control. The invasion of ESC through the matrigel-coated membranes was taken by microscopic morphology at ×200 magnification. The ESC was incubated in the presence of vehicle (A), 10−8 mol/l 17β-estradiol (B), 1 nmol/l TCDD (C), 17β-estradiol and TCDD in combination (D), co-culture in the presence of vehicle (E), co-culture treated with 10−8 mol/l 17β-estradiol (F), co-culture treated with 1 nmol/l TCDD (G) co-culture treated with the combination of 17β-estradiol and TCDD (H) (×200). The results in invasive index showed that the combination of 17β-estradiol with TCDD improved invasion of ESC cultured alone and in the ESC/HPMC–U937 co-culture; the invasion of ESC was increased and significantly increased after stimulation by the combination of 17β-estradiol with TCDD (I). Data are expressed as mean ± SEM. *P < 0.05 compared with the ESC cultured alone control; **P < 0.01 compared with the co-culture control.
neutralizing antibody or anti-CCR5 neutralizing antibody, anti-RANTES neutralizing antibody, anti-MIP-1α.

The combination of 17β-estradiol with TCDD and the co-culture of ESC, HPMC and U937 cells enhanced the proteolytic activity of MMP-9 and MMP-2 by ESC through increased secretion of RANTES and MIP-1α.

Both MMP-9 and MMP-2 exert their proteolytic activity through secretion into the ECM (Vu and Werb, 2000). Therefore, the supernatant from the upper chamber in the invasion assay were collected, and the gelatinolytic activity of MMP-9 and MMP-2 was analyzed by gelatin zymography. It was clearly shown in Fig. 6 that the activity of MMP-9 and MMP-2 of ESC to degrade gelatin was significantly increased after treatment with the combination of 17β-estradiol with TCDD. The hrRANTES and hrMIP-1α also increased the activity of MMP-9 and MMP-2, which could be inhibited by anti-RANTES or anti-MIP-1α neutralizing antibody. The activity of MMP-9 and MMP-2 in the co-culture unit of matrigel invasive assay was significantly higher than that of the ESC cultured alone (Figs 6 and 7). Interestingly, anti-RANTES, anti-MIP-1α, anti-CCR1 or anti-CCR5 neutralizing antibody inhibited the increased activity of MMP of ESC in the co-culture (Fig. 7A). The data above suggest that RANTES or MIP-1α may play an important role in promoting proteolytic activity in ESC in the development of endometriosis. After treatment by the combination of 17β-estradiol with TCDD, the activities of MMP-9 and MMP-2 were further increased, and anti-RANTES neutralizing antibody, anti-MIP-1α neutralizing antibody, anti-CCR1 neutralizing antibody or anti-CCR5 neutralizing antibody remarkably inhibited the enhanced activity of MMP-9 and MMP-2, but did not block the activity increase of MMP, induced by the combination of 17β-estradiol and TCDD completely (Fig. 7B). This suggests that the combination of 17β-estradiol with TCDD may promote proteolytic activity of MMP-9 and MMP-2 of ESC through stimulating RANTES and MIP-1α secretion and other factors. From the results above, it is concluded that the combination of 17β-estradiol with TCDD can up-regulate the expression and activity of MMP-9 and MMP-2 in the ESC, in which RANTES and MIP-1α may play a modulating role.

The combination of estradiol with TCDD and the co-culture of ESC, HPMC and U937 cells up-regulated the protein translation of MMP-9 and MMP-2 in the ESC.

It was shown in Fig. 8 that the protein translation of both MMP-9 and MMP-2 in the ESC was increased significantly after treated with the combination of 17β-estradiol with TCDD, and hrRANTES and hrMIP-1α also increased the protein translation of MMP-9 and MMP-2 of the ESC. Anti-RANTES neutralizing antibody and anti-MIP-1α neutralizing antibody could inhibit the increase. In the co-culture unit of ESC/HPMC–U937, the combination of 17β-estradiol and TCDD up-regulated the protein translation of MMP-9 and MMP-2 in the ESC. Anti-RANTES neutralizing antibody, anti-MIP-1α neutralizing antibody, anti-CCR1 neutralizing antibody or anti-CCR5 neutralizing antibody effectively inhibited the effect.

Yu et al.
abolished the enhanced protein expression induced by the combination of 17β-estradiol and TCDD (Fig. 9).

The ELISA data showed that 17β-estradiol combined with TCDD increased RANTES and MIP-1α secretion, whereas their neutralizing antibodies and the neutralizing antibodies of their receptors could inhibit the effect partially, which suggests that the combination of 17β-estradiol with TCDD may promote the invasiveness of ESC via promoting the CC-motif chemokines secretion. Since the neutralizing antibodies only decreased the MMP-2 and the MMP-9 proteins partially, the increased protein levels of MMP-9 and MMP-2 may be due to other factors induced by 17β-estradiol and TCDD. It is also possible that the transcriptional activation of the two genes or the up-regulation of protein expression is directly exerted by 17β-estradiol and TCDD. Further research is necessary to elucidate the exact mechanisms.
Discussion

The discriminating factor(s) in the development of active endometriosis probably involves a complex array of potentially interactive molecules including steroid exposure, immunological disturbances, genetic predisposition and, perhaps, environmental toxin exposure (Osteen et al., 2003). The adherence and invasion of the retrograded endometrial cells into the peritoneum is a key step for the early stage of endometriosis, and the retrograded ESC are responsible for this adherence and implantation of endometrium to peritoneum in the early stage of endometriosis (Witz et al., 2001). On the other hand, leukocytes, including macrophages and their numerous products, may be involved in the onset and development of endometriosis (Vinatier et al., 1996). Endometriosis is actually a chronic inflammation that recruits a series of immune cells. An increased number of active macrophages have been found in peritoneal fluid of patients with endometriosis (Wu et al., 2002). The increase in infiltrating macrophages in the peritoneal cavity not only accumulates in ectopic tissues but also harbors in peritoneum (Khan et al., 2004). So the interplay among the ESC, HPMC and mono-macrophages may be a major contributor to determination of the disease progression. In view of this, we constructed the co-culture unit of two cells including ESC and HPMC or ESC and U937 in the present study. We found that the co-culture of HPMC and U937 cells could induce RANTES and MIP-1α secretion significantly; however, co-culture of ESC-U937 and ESC-HPMC only increased secretion of RANTES and MIP-1α slightly. This suggests that ESCs in the shed endometrium represent a foreign entity, initiating the acute inflammatory response by recruiting monocytes, and the infiltrated monocytes exert the greater effect after harboring in peritoneum, and the cross-talk between the mesothelial cells and the macrophages increase the synthesis of RANTES and MIP-1α.

Figure 6: The combination of 17β-estradiol with TCDD, hrRANTES and hrMIP-1α promoted the secretion of MMP-9 and MMP-2 by ESC cultured alone. The secretion of MMP-9 (92 kDa) and MMP-2 (72 kDa) of ESC was measured by gelatin zymography. Secretion was visualized as a clear white band against a dark background of stained gelatin. The results showed that the hrRANTES of 10 ng/ml and hrMIP-1α of 10 ng/ml improved secretion of MMP-2 and MMP-9, and their neutralizing antibody inhibited the increased secretion of MMP-2 and MMP-9 by ESC (A). Data are expressed as mean ± SEM. *P < 0.01, compared with the ESC cultured alone control.

Figure 7: The co-culture and combination of 17β-estradiol with TCDD promoted the secretion of MMP-9 and MMP-2 by ESC depending partially on RANTES, MIP-1α/CCR1, CCR5 interaction. The co-culture model is ESC/HPMC–U937 which is same as the model of Fig. 5. The secretion of MMP-9 (92 kDa) and MMP-2 (72 kDa) was measured by gelatin zymography. Secretion was visualized as a clear white band against a dark background of stained gelatin. Compared with the control (mouse IgG), the zymographic photography of the secretion of MMP-9 and MMP-2 produced by ESC in the co-culture system was inhibited by anti-CCR1 neutralizing antibody, anti-CCR5 neutralizing antibody, anti-RANTES neutralizing antibody or anti-MIP-1α neutralizing antibody (A). After treatment with 17β-estradiol and TCDD, activities of MMP-2 and MMP-9 of ESC in the E/H–U co-culture unit were increased significantly. Anti-CCR1 neutralizing antibody, anti-CCR5 neutralizing antibody, anti-RANTES neutralizing antibody or anti-MIP-1α neutralizing antibody could inhibit but not block the activity of MMP induced by the combination of 17β-estradiol and TCDD completely (B). Data are expressed as mean ± SEM. *P < 0.01, compared with the co-culture control. # P < 0.01, compared with the co-culture unit treated with the combination of 17β-estradiol and TCDD.
Estrogen and dioxin, chemokine secretion and endometriosis

After monocytes are recruited into the peritoneal cavity, the abdominal milieu became more complex. ESC, HPMC and mono-macrophages interact with each other. Therefore, the interaction of these cells may aggravate peritoneal inflammation. So we established the different co-culture unit of these cells, respectively, to mimic the peritoneal local inflammation. This model included four groups in terms of different combinations of the three sorts of the cells. Different models represented different situations of the ectopic tissues. We found that RANTES and MIP-1α secretion in the direct co-culture model of ESC–HPMC–U937 and the indirect co-culture model of ESC/HPMC–U937 (HPMC and U937 cells contacted with each other in this model), were significantly higher than in the other two models, in which HPMC did not contact directly with U937 cells, which suggests that the inflamed peritoneum plays an important role in the peritoneal cavity with endometriosis and secrets more RANTES and MIP-1α in a paracrine or/and autocrine manner. This prime event is followed by an increased migration of immune cells (T-cells and monocytes) into the loci, resulting thereafter in their ability to secrete cytokines that further stimulate the expression of RANTES. The endpoint of such a process is a high level of RANTES and MIP-1α in the peritoneal cavity, which may then mediate processes that contribute to endometriosis progression. Thus, mono-macrophages may create a positive loop to amplify the signal for the establishment and growth of endometriotic tissue in the ectopic sites. Some research showed that IL-1β produced by mono-macrophages promotes RANTES secretion from ESC, but other complex mechanisms may take part in chemokine secretion, and need further research. Therefore, mono-macrophages in the peritoneal cavity play a very important role in the pathogenesis and development of endometriosis.

It has been found in the present study that estradiol or TCDD alone did not promote ESC to secrete RANTES and MIP-1α, but interestingly, the secretion of RANTES and MIP-1α were up-regulated by estradiol and TCDD in combination. Moreover, in the presence of 17β-estradiol and TCDD, RANTES and MIP-1α secretion of the non-contact co-culture model of ESC/HPMC-U937 increased even further suggesting vicious cycles of endometrium attachment, but 17β-estradiol or TCDD alone exerted no effect. Surprisingly, in the direct co-culture model, the combination of 17β-estradiol with TCDD down-regulated secretion of RANTES, and had no effect on MIP-1α secretion, the underlying mechanism of which is still unclear. The interplay of these cells may activate different signaling pathways and gene transcription. Therefore, the cross-talk between the different kinds of cells may play a very important role in the pathogenesis of endometriosis.

Figure 8: The combination of 17β-estradiol with TCDD, hrRANTES and hrMIP-1α promoted the expression of MMP-9 and MMP-2 protein by ESC.

The secretion of MMP-9 (92 kDa) and MMP-2 (72 kDa) was measured by western blot. The protein expression of MMP 9 and MMP 2 produced by ESC (A). The results showed that the hrRANTES of 10 ng/ml and hrMIP-1α of 10 ng/ml improved expression of MMP-2 and MMP-9 and their neutralizing antibodies (anti-RANTES antibody of 2.5 µg/ml and anti-MIP-1α antibody of 2.5 µg/ml) inhibited their effect on expression of MMP-2 and MMP-9 by ESC. *P < 0.01, compared with the ESC cultured alone control.

Figure 9: The co-culture and combination of 17β-estradiol with TCDD promoted the expression of MMP-9 and MMP-2 by ESC depending partially on RANTES, MIP-1α/CCR1, CCR5 interaction. The secretion of MMP-9 and MMP-2 was measured by western blot. The expression of MMP-9 and MMP-2 produced by ESC in co-culture system treated with 17β-estradiol, TCDD or 17β-estradiol + TCDD and combined with each different neutralizing antibody. The anti-RANTES neutralizing antibody, anti-MIP-1α neutralizing antibody, anti-CCR1 neutralizing antibody and anti-CCR5 neutralizing antibody decreased the expression of MMPs but did not block expression of MMPs induced by the combination of 17β-estradiol and TCDD completely (A). *P < 0.01, compared with the co-culture unit control. #P < 0.01, compared with the co-culture unit treated with the combination of 17β-estradiol and TCDD.
high concentrations of estradiol in women with endometriosis in the inflammation milieu, and aggravated by TCDD, promote the development of endometriosis.

Interestingly, in our matrigel invasion assay, the invasiveness of ESC treated with the combination of 17β-estradiol with TCDD was higher than when treated with either one. The hrRANTES or hrMIP-1α could also increase the invasiveness of ESC, whereas the anti-RANTES neutralizing antibody or the anti-MIP-1α neutralizing antibody could inhibit the invasiveness of ESC. The combination of 17β-estradiol with TCDD, or hrRANTES or hrMIP-1α enhanced the expression and activity of MMP-9 and MMP-2 by ESC. On the other hand, in the co-culture of HPMC and U937 in the lower compartment, the invasiveness of ESC was significantly higher than with ESC alone, and the combination of 17β-estradiol and TCDD improved the invasiveness of ESC further. The anti-RANTES neutralizing antibody, anti-MIP-1α neutralizing antibody, anti-CCR1 neutralizing antibody or anti-CCR5 neutralizing antibody could decrease but not block the invasiveness of ESC completely, which suggests that RANTES or MIP-1α may increase the invasiveness of ESC, and 17β-estradiol and TCDD may promote the invasiveness of ESC by increasing secretion of the two chemokines. However, other cytokines and chemokines induced by 17β-estradiol and TCDD may be involved in the progress. IL-8, another chemokine, increases the MMP activity and the invasiveness of ESC (Mulayin et al., 2004). IL-1β and TNF-α can stimulate MMP-1, MMP-2, and MMP-3 mRNA levels and promote MMP-1 and MMP-3 protein secretion, but not increase extracellular MMP inducer (EMMPRIN) (Braundmeier and Nowak, 2006). Therefore, the co-culture and the combination of 17β-estradiol with TCDD may improve the invasiveness of ESC by inducing other cytokines and mechanisms. The same occurs with the expression and activity of MMP-9 and MMP-2 in ESC.

Our results suggest that RANTES and MIP-1α may act in paracrine and indirect mechanisms, and also directly on the ESC, to support endometriosis progression. One mechanism by which RANTES and MIP-1α may affect endometriosis progression is their capacity to up-regulate the expression of MMPs in ESC. The RANTES and MIP-1α induce the infiltration of monocytes into the peritoneal cavity and the ectopic tissues followed by up-regulation of the expression of MMPs.

Our present study has demonstrated that blocking CCR1 or CCR5, the receptors for RANTES and MIP-1α, decreases the invasiveness of ESC and the expression and activity of MMP-9 and MMP-2 in ESC. The expression of RANTES and MIP-1α receptors by the ESC may enable RANTES derived from the ESC, mesothelial cells or from inflammatory cells to induce invasion-promoting properties in the ESC. It was shown that RANTES and MIP-1α promote expression of MMP-9 and MMP-2 in the ESC.

In conclusion, based on the results of our present study as well as the relevant studies on the potential contribution of RANTES and MIP-1α to endometriosis progression, a hypothetical model may be proposed to illustrate the complex set of interactions through which retrograde endometrium activates inflammation and promotes inflammation cells to migrate into PC. Interaction of the endometriosis-associated cells increases the secretion of RANTES and MIP-1α. High levels of estrogen of women with endometriosis and TCDD increase the chemokine secretion further. The two chemokines may act concomitantly to induce monocyte and T-cell migration into the ectopic site. Thereafter, the increased RANTES and MIP-1α expression gives rise to invasion-promoting activities of ESC by up-regulating secretion of MMPs or other growth factors or mediators of angiogenesis. This process may eventually result in the aggravation of endometriosis. Therefore, the altered regulation of endometrial MMP expression in response to steroids may represent a mechanism linking the invasive potential of refluxed endometrium to the establishment of this disease, as occurs in certain women (Osteen et al., 2003). Meanwhile, it is possible that estradiol and TCDD may promote the invasiveness of ESC by inducing other chemokines or cytokines. RANTES and MIP-1α in the ectopic tissues may induce MMP expression by acting on the monocytes as well as other cells. The ability of RANTES to promote vascularity may act in conjunction with the elevated expression of MMPs to facilitate invasion formation. Furthermore, the infiltrating monocytes, or other adjacent stroma cells may secret TNF-α, resulting in over-expression of MMPs by the ESC, and the elevated expression of MMPs and increased vascularity may be key events in the disease progression, facilitating tissue degradation, and invasive formation. Our results suggest that estradiol and TCDD can coordinate to evoke and aggravate the inflammatory response of the endometriotic focus-associated cells in vitro by stimulating pro-inflammatory cytokine secretion (e.g. RANTES and MIP-1α), leading to a persistent and serious inflammation. The RANTES and MIP-1α and the other cellular products could play a significant role in the endometriosis progression of endometriosis. The ability of RANTES and MIP-1α to facilitate invasive formations is in full agreement with high levels of RANTES and MIP-1α expression observed in advanced disease.

Acknowledgements
The human peritoneal mesothelial cell line HMRSV5 was generously provided by Prof. Jian Yao (The First People’s Hospital, Shanghai, China).

Funding
This work is supported by National Basic Research Program of China 2006CB944009 (to D.-J.L.), supported by Foundation for Key Medical Subject of Shanghai No. 05-III 016 (to D.-J.L.), Shanghai Leading Academic Discipline Project B117 (to D.-J.L.), and Program for Outstanding Medical Academic Leader of Shanghai (to D.-J.L.).

References
Estrogen and dioxin, chemokine secretion and endometriosis


Submitted on October 8, 2007; resubmitted on March 13, 2008; accepted on March 26, 2008.