Peroxisome-proliferator activator receptor-gamma activation decreases attachment of endometrial cells to peritoneal mesothelial cells in an in vitro model of the early endometriotic lesion

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ABSTRACT: The aim of this study was to investigate whether peroxisome proliferator-activated receptor (PPAR)-γ activation has an effect on the attachment of endometrial cells to peritoneal mesothelial cells in a well-established in vitro model of the early endometriotic lesion. The endometrial epithelial cell line EM42 and mesothelial cell line LP9 were used for this study. EM42 cells, LP9 cells or both were treated with the PPAR-γ agonist ciglitazone (CTZ) at varying concentrations (10, 20 and 40 μM) × 48 h with subsequent co-culture of EM42 and LP9 cells. The rate of EM42 attachment and invasion through LP9 cells was then assessed and compared with control (EM42 and LP9 cells co-cultured without prior treatment with CTZ). Next, attachment of CTZ-treated and untreated EM42 cells to hyaluronic acid (HA), a cell adhesion molecule (CAM) on peritoneal mesothelial cells, were assessed. Although there was no difference in EM42 attachment when LP9 cells alone were treated with CTZ, treatment of EM42 cells with 40 μM CTZ decreased EM42 attachment to LP9 cells by 27% (P < 0.01). Treatment of both EM42 and LP9 cells with 40 μM CTZ decreased EM42 attachment to LP9 by 37% (P < 0.01). Treatment of EM42 cells with 40 μM CTZ decreased attachment to HA by 66% (P = 0.056). CTZ did not decrease invasion of EM42 cells through the LP9 monolayer. CTZ may inhibit EM42 cell proliferation. In conclusion, CTZ significantly decreased EM42 attachment to LP9 cells and HA in an in vitro model of the early endometriotic lesion.

Key words: attachment / endometriosis / peritoneum / PPAR-γ / thiazolidinedione

Introduction
Endometriosis, defined as endometrial glands and stroma outside the uterine cavity, is present in ~10% of reproductive-aged women even though retrograde menstruation (Sampson, 1927) is prevalent in 90% of women (Halme et al., 1984). It has been suggested that cell adhesion molecules (CAMs) (Witz, 2003), in conjunction with aberrant autoimmune and inflammatory modulators (Lebovic et al., 2001), may account for this discrepancy by facilitating the attachment and invasion of endometrial tissue to peritoneal mesothelium. The attachment of endometrial epithelial and stromal cells to peritoneal mesothelium has been demonstrated to occur within 1 h using an in vitro model of the early endometriotic lesion (Witz et al., 1999, 2001, 2002, 2003). Furthermore, it has been suggested that CAMs such as mesothelial cell-associated hyaluronic acid (HA) and endometrial cell-associated CD44, as well as others, are involved in this attachment process (Dechaud et al., 2001; Klemmt et al., 2007; Griffith et al., 2009 Epub ahead of print). Peroxisome-proliferator activator receptor-gamma (PPAR-γ) agonists, such as the thiazolidinedione ciglitazone (CTZ), have...
anti-inflammatory, anti-proliferative and anti-angiogenic properties in several cell types. Therefore, PPAR-γ activation may have a role in endometriosis treatment. In fact, PPAR-γ receptors have been demonstrated in endometrial epithelial (Wanichkul et al., 2003) and stromal (Pritts et al., 2002) cells. PPAR-γ ligands reduced monocyte migration in an in vitro study of peritoneal fluid of women with endometriosis (Hornung et al., 2001). CTZ has been shown to decrease the number of peritoneal macrophages in a mouse model (Hornung et al., 2003). In endometriosis animal models, PPAR-γ agonists decreased the size and mean weight of endometriotic explants, induced epithelial regression in the rat model (Lebovic et al., 2004; Demiturk et al., 2006; Aytan et al., 2007) and reduced lesion surface area in the baboon model (Lebovic et al., 2007).

Current mainstays of medical therapy for endometriotic pain preclude pregnancy due to ovarian suppression. If PPAR-γ agonists are found to be clinically effective in the treatment of endometriosis, then they may show promise as a means for concurrently treating pain and facilitating fertility. PPAR-γ ligands have decreased the expression of CAMs in various cell types (Wang et al., 2002; Imamoto et al., 2004; Arnold et al., 2007). Accordingly, the purpose of this study was to evaluate the effect of PPAR-γ activation by CTZ on the attachment of endometrial cells to peritoneal mesothelial cells (PMS) in a novel, well-established in vitro model of the early endometriotic lesion (Fig. 1).

Materials and Methods

Approval for this study was obtained from the Institutional Review Boards of the University of Michigan at Ann Arbor and the University of Texas Health Science Center at San Antonio.

EM42 cells

The endometrial epithelioid cell line EM42 was used as a surrogate for patient derived endometrial epithelial cells (EEC). This cell line has been characterized (Gill et al., 2001; Rong et al., 2002; Wanichkul et al., 2003) and has a similar rate of PMC attachment to patient derived EECs (Lucidi RS, Witz CA, unpublished). The EM42 cells (gift from Dr N. Desai, University MacDonald Women’s Hospital, Case Western Reserve University, Cleveland, OH, USA) were grown as monolayers in MEM-α modification (JRH Biosciences, Lenexa, KS, USA) containing antibiotic/antimycotics, 10 μg/ml insulin, α-glucose 0.3 μg/ml and 10% fetal calf serum (FCS). This cell line was used in this study because of its stability and in order to avoid patient-to-patient variability found in primary cultures. A stromal cell line was not available at the time the experiments were conducted.

Peritoneal model

Previous studies have shown similar rates of peritoneal cell binding to commercially available LP9 PMCs (NIH Aging Cell Repository, Cornell Institute for Medical Research, Camden, NJ, USA) to PMCs derived from parietal peritoneum and ovarian surface epithelium (Lucidi et al., 2005). This would suggest that LP9 PMCs are an appropriate experimental surrogate for patient derived PMCs. LP9 cells were grown in MCDB-131 Medium 199 (1:1) (Sigma, St Louis, MO, USA) supplemented with epidermal growth factor (20 ng/ml), l-glutamine (2 mM), hydrocortisone (400 ng/ml), 1% antibiotic/antimycotic, HEPES buffer and 15% FCS (supplemental elements used as per Cornell Institute for Medical Research, supplier of the LP9 cell line). The attachment and invasion assays were performed in stripped media (Sigma, St Louis, MO, USA) (10% charcoal/dextran treated Fetal Bovine Serum, Hyclone #SHI30 068.03, heat inactivated prior to adding to media) in order to evaluate the effect of CTZ in the absence of potential confounding effects of endogenous steroids.

Control and treatment groups for attachment experiments

Four groups consisted of: (i) Control without CTZ treatment, (ii) CTZ treatment of EM42 cells, (iii) CTZ treatment of LP9 cells and (iv) CTZ treatment of both EM42 and LP9 cells (Table I). CTZ concentration treatments were 10, 20 and 40 μM. The duration of CTZ treatment was 48 h. Co-culture of EM42 and LP9 cells were then performed for each experiment. EM42 cells were allowed to attach for 1 h and an attachment assay was performed as previously reported (Lucidi et al., 2005). In addition, the extent of attachment of CTZ-treated (n = 4 for each treatment group) and untreated (n = 4) EM42 cells to HA coated 96-well plates was assessed. (CTZ concentrations utilized: 10, 20 and 40 μM x 48 h).

Attachment assay

A previously described attachment assay (Lucidi et al., 2005) was used. EM42 cells were collected using non-enzymatic cell dissociation media (Sigma, St Louis, MO, USA), washed with stromal cell complete medium, and subsequently labeled with 5 μM calcein-AM (Molecular Probes, Inc., Eugene, OR, USA) for 20 min at 37°C. After resuspension in culture medium, EM42 cells were plated over confluent LP9 cells in 96-well plates at 20 000 cells per well in a total volume of 100 μl.

The 96-well plates were then cultured at 37°C for 1 h in 5% CO₂ in air. After incubating, the plates were assayed for Total Relative Fluorescence.

Table I Co-culture combinations depicted as the group name followed by whether or not the cell type in co-culture (EM42, LP9) was treated with CTZ or vehicle control for 48 h

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Figure 1 Graphic illustration of the attachment assay used as an in vitro model of the early endometriotic lesion. EM42 = endometrial epithelioid cell line; LP9 = peritoneal mesothelial cell line.

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on a Fluoroskan Ascent plate reader with Ascent Software (Thermo-Fisher Labsystems). Thereafter, plates were inverted, submerged in a bath of phosphate buffered saline (PBS) containing calcium and magnesium (Invitrogen; Carlsbad, CA, USA). The bath was placed on an orbital mixer (Barnstead/Thermolyne, Dubuque, IA, USA) set at 20 r.p.m. and incubated at 37°C in 5% CO₂ in air for 15 min. During this time, non-adherent endometrial cells precipitated under gravity from the 96-well plate to the bottom of the PBS bath. The washed plate was assayed for Attached Relative Fluorescence on the plate reader and the percentage of EM42 cells bound to LP9 cells was determined (Attached Relative Fluorescence after washing/Total Relative Fluorescence before washing) x 100) (Fig. 2). Each experiment was performed eight times, the mean of which was calculated to determine each data point. Experiments to evaluate the attachment of EM42 cells to HA were determined in a similar fashion with the exception of using HA-lined wells instead of LP9 cells grown in wells.

As described previously (Lucidi et al., 2005), confocal laser scanning microscopy with an Olympus IX70 inverted microscope using a X40 1.35 N.A. oil immersion objective and the Olympus Fluoview System—Nagano, Japan) documented the attachment of EM42 cells to LP9 cells.

**Invasion assay**

For this study, LP9 PMCs were grown to confluence on Matrigel™ coated 24-well invasion chambers containing membranes with 8 μM pores (BD Bioscience, San Jose, CA, USA). EM42 cells were grown to near confluence, with two study groups, Control (n = 8 replicates) and CTZ 40 μM (n = 8 replicates), labeled with CellTracker Green® (Molecular Probes, Inc., Eugene, OR, USA), and placed over the LP9 covered membranes (50 000 cells/well). Preliminary experiments demonstrated that 25 000 endometrial cells per well produced a uniform distribution of endometrial cells without crowding or stacking of cells; however, due to the less invasive nature of EM42 cells, 50 000 cells per well have been used for invasion experiments. The membranes were incubated at 37°C in 5% CO₂ in air and cultures were interrupted at 24 h. Cells not invaded, located on the upper surface of the membranes, were mechanically removed with a cotton tip applicator, and the membranes were fixed in cold formaldehyde. Each invasion assay was run in triplicate.

The membranes were then treated with Hoescht 33342 (Invitrogen, Grand Island, NY, USA), a fluorescent nuclear stain, to identify cell nuclei. The number of invaded cells on the bottom of the coated membranes was determined using a fluorescence microscope with a 20× objective. Images were obtained from eight standardized, non-overlapping fields on the central portion of each membrane, representing ~40% of the total surface area. The number of invaded endometrial cells were counted by identifying a Hoescht labeled nucleus surrounded by CellTracker™ Green labeled cytoplasm.

**Proliferation assay**

Cell proliferation was assessed using the Cell Titre-Glo luminescence assay (Promega Inc., Madison, WI, USA). Briefly, EM42 cells were plated onto 96-well plates and grown to subconfluence. The EM42 cells were then treated with depleted media for 24 h and subsequently either received CTZ treatment (n = 12 replicates at various doses) or no treatment (n = 12 replicates) for 48 additional hours. Proliferation was measured by Cell Titre-Glo assay on the Thermo Fisher Fluoroskan Fluorometer.

**Statistics**

Comparisons of attachment among groups as well as proliferation data were made by ANOVA using SPSS (SPSS, Inc., Chicago, IL, USA) and post hoc analyses were performed by the stringent Bonferroni method. Comparisons of invasion between control and CTZ 40 μM groups were made by Student t-test. P < 0.05 was considered statistically significant.

**Results**

There was no difference in the rate of EM42 attachment when LP9 cells alone were treated with CTZ at the 10, 20 and 40 μM doses prior to co-culture with EM42 cells. When EM42 cells were treated with 40 μM CTZ, there was a 27% decrease in binding to LP9 cells (46.3 versus 62.7%; P < 0.01) (Fig. 3). When both EM42 and LP9 cells were treated with 40 μM CTZ, there was a 37% decrease in EM42 attachment to LP9 cells (39.7 versus 62.7%; P < 0.01) (Fig. 3).

CTZ treatment of EM42 cells (40 μM) led to a 66% decreased rate of attachment to HA-coated wells in comparison to control (P = 0.056) (Fig. 4). CTZ treatment did not decrease the invasion of EM42 cells through the monolayer of LP9 cells in any of the treatment groups (data not shown: comparisons tested for statistical significance by ANOVA).

CTZ treatment of EM42 cells appeared to have an inhibitory effect on cell proliferation. The average relative luminescence for the CTZ 40 μM group was decreased by 37.8% that of the control group (P < 0.01) (Fig. 5). The decreased proliferation effect may be due to interference with EM42 attachment to plastic after cell division. Cytotoxicity is unlikely since propidium iodide staining of EM42 cells and experiments measuring the apoptotic marker Annexin V (S.K. Kavoussi and D.I. Lebovic, unpublished data) showed no difference between control and CTZ treated endometrial and endometriotic cell lines.
The aim of this study was to determine the potential effects of PPAR-γ activation on the attachment of endometrial cells to peritoneal cells in vitro. CTZ treatment significantly decreased EM42 binding to LP9 cells in an in vitro model of the early endometriotic lesion. These results suggest that CTZ could significantly decrease binding of EECs to mesothelial cells and, therefore, may inhibit the initial steps in the pathogenesis of the development of the early endometriotic lesion. A further extension of these experiments showed that CTZ treatment decreased EM42 binding to HA, the PMC CAM which has been shown to be involved in attachment via the endometrial cell CAM CD44 (Dechau et al., 2001). Although the mechanism of action remains to be elucidated, these results appear to support the hypothesis that CAM down-regulation plays a role in the decreased attachment of endometrial cells to peritoneal mesothelium after exposure to a PPAR-γ agonist. Further study is necessary to differentiate the extent of decreased attachment that is due to decreased proliferation of endometrial cells in comparison to that attributable to potential down-regulation of CAM.

Previous studies have explored the potential of the effects of PPAR-γ agonists on inhibiting CAM expression in the setting of inflammatory disease processes. Imamoto showed that pioglitazone influenced monocyte to endothelial cell binding by inhibiting vascular cell adhesion molecule-1 (VCAM-1) expression on activated endothelial cells (Imamoto et al., 2004). In the same study, neutrophil to endothelial cell binding was influenced by the inhibition of CD11b/CD18 up-regulation on activated neutrophils. In addition, Wang et al. (2002) demonstrated that PPAR-γ agonist suppressed vascular adhesion molecule expression in endothelial cells as well as the ensuing leukocyte recruitment. Another group of investigators showed that PPAR-γ agonists down-regulated the expression of intercellular cell adhesion molecule-1 (ICAM-1) on human lung epithelial cells, resulting in a reduced β2-integrin mediated adhesion of monocyte effector cells to monolayers of these respiratory syncytial virus-infected human lung cells (Arnold et al., 2007).

Studies with a focus on the effects of PPAR-γ agonists in animal models of endometriosis have shown promise. Lebovic showed that the administration of a PPAR-γ agonist diminished the burden of endometriosis in the rat model (Lebovic et al., 2004). Demirturk found that PPAR-γ agonists may have a preventative application as evidenced by a diminution of the induction of endometriosis in the rat model (Demirturk et al., 2006). And, finally, in the baboon model of endometriosis (Lebovic et al., 2007) it has been shown that thiazolidinediones can effectively reduce the burden of disease.

Prevention of endometrial cell-mesothelial cell attachment and lesion formation could potentially benefit those who suffer from endometriosis-associated pelvic pain and/or infertility. One potential use for PPAR-γ agonist therapy could include the secondary prevention of endometriotic lesion formation in those who have had surgery for endometriosis. For example, post-operative administration of a PPAR-γ ligand could be limited to times of menstrual flow. Furthermore, such therapy could theoretically be administered between the time of surgery and assisted reproduction cycles in attempts to optimize pregnancy rates. In addition, patients with heavy menstrual flow and duration, for whom pelvic pain, not fertility, is the primary concern, may benefit from a combination of exogenous hormone and PPAR-γ agonist therapy.

The safety of PPAR-γ agonists in early pregnancy has been suggested by recent studies. Studies in mice have shown that rosiglitazone does not have an adverse impact on embryo or neonatal development (Klinker et al., 2001). Although the mechanism of action remains to be elucidated, these results appear to support the hypothesis that CAM down-regulation plays a role in the decreased attachment of endometrial cells to peritoneal mesothelium after exposure to a PPAR-γ agonist. Further study is necessary to differentiate the extent of decreased attachment that is due to decreased proliferation of endometrial cells in comparison to that attributable to potential down-regulation of CAM.

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At the time that this study was performed, CTZ was chosen for these experiments due to its commercial availability and wide use in the literature to study the effects of PPAR-γ activation. Although CTZ is not in clinical use, pharmacokinetic calculations show that the 40 μM dose is the equivalent of pioglitazone 4.3 μM which closely approximates a pioglitazone 45 mg pill. It should be mentioned that the 2007 meta-analysis suggesting that the PPAR-γ agonist rosiglitazone increases the risk of cardiac ischemia among type 2 diabetics (Nissen and Wolski, 2007) shifted the potential extension of the results of our study to the clinical use of pioglitazone, a PPAR-γ agonist that has been shown to have a favorable safety profile in type 2 diabetics, in terms of cardiovascular health (Lincoff et al., 2007).

Combining the encouraging findings from in vitro studies and animal models using PPAR-γ agonists, it is conceivable that this class of drug could be utilized for both preventative and therapeutic interventions. Further studies are currently underway in efforts to determine the clinical efficacy of PPAR-γ agonists as a potential treatment option for endometriosis.

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**References**


