Macrophage Migration Inhibitory Factor Is Involved in a Positive Feedback Loop Increasing Aromatase Expression in Endometriosis

Véronique Veillat,* Valérie Sengers,* Christine N. Metz,† Thierry Roger,‡ Mathieu Leboeuf,§ Jacques Mailloux,§ and Ali Akoum*§

From the Laboratory of Reproductive Endocrinology,* Research Center, Hospital of Saint-François of Assisi CHUQ, Québec, Canada; the Feinstein Institute for Medical Research,† Manhasset, New York; the Department of Infectious Diseases,‡ University Hospital Center of Vaudois and University of Lausanne, Lausanne, Switzerland; and the Department of Obstetrics and Gynecology,§ Faculty of Medicine, University of Laval, Québec, Canada

Immune–endocrine interplay may play a major role in the pathogenesis of endometriosis. In the present study, we have investigated the interaction between macrophage migration inhibitory factor (MIF), a major pro-inflammatory and growth-promoting factor markedly expressed in active endometriotic lesions, and estradiol (E2) in ectopic endometrial cells. Our data showed a significant increase of MIF protein secretion and mRNA expression in endometriotic cells in response to E2. MIF production was blocked by Fulvestrant, an estrogen receptor (ER) antagonist, and induced by ERα and ERβ selective agonists propylpyrazole-triol (PPT) and diarylpropionrile (DPN), respectively, thus demonstrating a specific receptor-mediated effect. Cell transfection with MIF promoter construct showed that E2 significantly stimulates MIF promoter activity. Interestingly, our data further revealed that MIF reciprocally stimulates aromatase protein and mRNA expression via a posttranscriptional mRNA stabilization mechanism, that E2 itself can upregulate aromatase expression, and that inhibition of endogenous MIF, using MIF specific siRNA, significantly inhibits E2-induced aromatase. Thus, the present study revealed the existence of a local positive feedback loop by which estrogen acts directly on ectopic endometrial cells to upregulate the expression of MIF, which, in turn, displays the capability of inducing the expression of aromatase, the key and rate-limiting enzyme for estrogen synthesis. Such interplay may have a considerable impact on the development of endometriosis. (Am J Pathol 2012, 181:917–927; http://dx.doi.org/10.1016/j.ajpath.2012.05.018)

Endometriosis is a common gynecological disorder that affects 5% to 15% of women of reproductive age and frequently causes abdominal pain, dysmenorrhea, dyspareunia, and infertility. Endometriosis is defined by the presence and growth of endometrial glands and stroma within the pelvic peritoneum and other extraterine sites. Its etiology is not well elucidated, but the transplantation, implantation, and growth of exfoliated menstrual debris on the peritoneal and ovarian surfaces are the most widely accepted explanation for its development. Because retrograde menstruation is observed in up to 90% of women, most investigators agree that additional factors including genetic predisposition, environmental toxins, hormonal factors, and immune dysfunctions are essential for the aberrant ectopic growth of endometrial tissue. Our previous studies identified macrophage migration inhibitory factor (MIF) as a potent mitogenic factor for endothelial cells released by ectopic endometrial cells. Originally described as a product of activated T lymphocytes that inhibits the random migration of cultured macrophages, MIF is now known as a multifunctional cytokine with potent proinflammatory, immuno-modulatory, and growth-promoting effects. MIF has been shown to be implicated in tumorigenesis as well as in the pathogenesis of many inflammatory and autoimmune diseases. Our subsequent studies further revealed a close relationship between MIF and the pathophysiology of endometriosis. MIF levels were markedly elevated in the peripheral blood, the peritoneal fluid as well as in the eutopic...
Endometriosis is known for being estrogen dependent. The disease arises during the reproductive phase of life, but can be sporadically found in postmenopausal women with relatively high concentrations of estrogens. The biologically active estrogen is 17β-estradiol (E2), and its effects are mediated through the two subtypes of the estrogen receptor (ER), usually referred to as ERα and ERβ. Endometriotic lesions express sex steroid receptors and can undergo cyclical changes that, however, do not proceed as clearly or as uniformly as in the intrauterine endometrium. It is well documented that ovarian steroid hormones play a critical role in the development of endometriosis. Current medical therapy of endometriosis is generally based on the suppression of ovarian function and the induction of hypoestrogenism, which reduce symptomatology and endometriotic lesion volume. Estradiol appeared even to be locally synthesized in endometriotic lesions.

Of note, early-stage and active endometriotic implants correlate with E2 levels in peritoneal fluid. However, despite the crucial role that estrogen plays in the maintenance and progression of endometriosis, the mechanisms underlying its excessive local production and capacity to promote ectopic endometrial cell growth and influence the immuno-inflammatory process occurring in endometriosis women have not been yet clearly elucidated. The present study was designed to assess whether there is any relationship between MIF expression and local estrogen production in endometriotic cells. Herein, we reveal the existence of a positive feedback loop between estrogen and MIF, operating locally in ectopic endometrial implants. Estrogen appeared to act directly on ectopic endometrial cells to upregulate the expression of MIF, which, in turn, displayed the capability of inducing the expression of aromatase, the key rate-limiting enzyme for estrogen synthesis. Such an immuno–endocrine interplay may have a considerable impact on the development of endometriosis, taking into account the critical role of estrogens in the pathogenesis of the disease and the well-documented role of MIF as a key mediator of inflammation, angiogenesis, tissue remodeling, and cell growth.

Materials and Methods

Subjects and Tissue Handling

Women who participated in this study had provided a written informed consent for a research protocol approved by the Saint-François d’Assise Hospital Ethics Committee on Human Research. Endometriotic tissue specimens were obtained from women with endometriosis (n = 14, mean age = 31.5 ± 2.6 years). These women consulted for infertility and/or pelvic pain and were found to have endometriosis during laparoscopy or laparotomy (Table 1). All subjects had no endometrial hyperplasia or

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*Precise location of cultured lesion unknown.

ELISA, enzyme-linked immunosorbent assay; ICF, immunocytofluorescence; P, proliferative; qRT-PCR, quantitative real-time polymerase chain reaction; S, secretory; UK, unknown.
neoplasia and had not received any anti-inflammatory or hormonal medication for at least 3 months before surgery.

Biopsy samples were immediately placed at 4°C in sterile Hanks’ balanced salt solution (HBSS) containing 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin and directly used for cell culture. A subsample from each biopsy sample was snap-frozen in liquid nitrogen with Tissue-Tek OCT compound (Miles, Elkhart, IN) and stored at −80°C for historical analyses. All endometriotic biopsy samples were characterized histologically by the presence of endometrial-like glands. The stated last menstrual period was used to classify tissues as proliferative or secretory, and histological assessment of endometrial biopsy samples according to the criteria of Noyes was used to ascertain the menstrual cycle phase.25

Cell Culture and Stimulation

As previously described,26 endometriotic tissue was minced into small pieces and dissociated with collagenase. Cells were pelleted by centrifugation (200 × g for 10 minutes). Stromal fibroblast-like cells were separated and plated in DMEM-F12 (HyClone, Logan, UT) containing 10 μg/mL of insulin, 5 μg/mL of transferrin, 10% fetal bovine serum (FBS), and 1% antibiotics (Invitrogen, Burlington, ON, Canada) at 37°C, 5% CO2. Stromal cells were characterized morphologically in culture by light microscopy and immunocytochemically with specific monoclonal antibodies to cytokeratins, vimentin, and CD10, as described below (immunocytofluorescence). The culture medium was changed every 48 hours, and cultures were passaged at confluence. For stimulation, the complete medium was discarded from confluent cultures and replaced overnight with phenol red-free DMEM-F12 medium without serum. The following day, cells were exposed to E2 (1, 3, 5, 10-estratrien-3, 17β-diol 3 benzoate) (10−8 M and 10−10 M) (Sigma-Aldrich, St. Louis, MO) or to MIF (0 to 50 ng/mL) for different periods of time in fresh, phenol red–free medium without serum. These concentrations take into account E2 and MIF levels according to the criteria of Noyes.27

Real-Time PCR

Reverse transcription and quantitative real-time PCR (qRT-PCR) were performed according to our previously described procedures.30 Briefly, total RNA was extracted using the Trizol reagent (Invitrogen) and reverse transcribed into cDNA in the presence of random primers. The cDNA was then used as a template for quantitative real-time PCR with gene specific primers. Real-time PCR reactions were carried out in an ABI 7000 Thermal Cycler (Applied Biosystems, Foster City, CA) using 2 μL of RT product, 0.5 μL of each primer, and 12.5 μL of Platinum SYBR Green qPCR SuperMix-UDG with ROX (Invitrogen). Amplification conditions were as follows: hold for 2 minutes at 95°C, followed by two-step PCR for 40 cycles of 95°C for 15 seconds, followed by 55°C (MIF), 60°C (aromatase), or 60°C (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) for 40 seconds. MIF primers were 5’-CGCAAGACCGCTCTACAG-3’ (forward) and 5’-GAGTTGTCTCCAGCCCAT-3’ (reverse). Aromatase primers were 5’-CTCAGAATGTGGCAGAATCT-3’ (forward) and 5’-ACACGTCCACATAGCGGATT-3’ (reverse). GAPDH primers were 5’-CAGGCCCGCTTCTCTCTGA-3’ (forward) and 5’-TGGGGAGCCTAGTTGAGA-3’ (reverse). The amplification of MIF, aromatase, and GAPDH cDNA gave rise to products of 125, 224, and 102 bp, respectively. Quantification of MIF or aromatase mRNA was performed by using a relative quantification method. For each experimental sample, MIF or aromatase mRNA levels were normalized to GAPDH mRNA levels. After each run, melting curve analysis (55 to 95°C) was performed to verify the specificity of the PCR reaction. All samples were tested in triplicate, and each run included no-template and no-reverse transcription controls.

ELISA

MIF concentrations were measured using an ELISA procedure developed in the laboratory as previously described.31 To summarize, a mouse monoclonal anti-human MIF antibody (5 μg/mL in PBS, R&D Systems, Minneapolis, MN) was incubated in ELISA plates overnight at 4°C. Afterward, nonspecific sites were blocked with Superblock Blocking Buffer (Pierce Biotechnology, Rockford, IL) and incubated for 1 hour with agitation at 37°C. Following appropriate washes, the supernatant was added in its respective well and incubated for 1 hour with agitation at 37°C. A rabbit polyclonal anti-human-MIF IgG antibody (1/1000 in PBS/BSA 3%) (Santa Cruz Biotechnology, Santa Cruz, CA) was then added for 1 hour at 37°C, followed by biotin-SP–conjugated affiniPure goat anti-rabbit IgG (1/5000 in PBS/BSA 3%) (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour with agitation at 37°C, peroxidase-conjugated streptavidin (1/1000 in PBS/BSA 3%) (Jackson ImmunoResearch Laboratories) for 45 minutes with agitation at 37°C and finally 3, 3, 5, 5’-tetramethylbenzidine (TMB) (Bio-Rad Laboratories, Hercules, CA) for revelation. The optical density was measured at 450 nm, and MIF concentrations were extrapolated from a standard curve using recombinant human MIF (R&D Systems). The sensitivity limit of the assay was 100 pg/mL, with intra- and interassay coefficients of variation <4%.
Plasmid Preparation, Transfections, and Luciferase Assays

A 3-kb fragment from the human MIF gene was amplified by PCR and cloned into the pGL3-basic vector (Promega, Madison, WI), as previously described.33 A 935-bp fragment of promoter II regulatory region of the P450arom gene was amplified by PCR and cloned into the pGL3-basic vector (Promega, Madison, WI) using the following primers: 5′-GCATTAAATGATGACTCCTCT-3′ (forward) and 5′-GATCCTACTCTGGTTTATAATTGAT-3′ (reverse). Transfections were performed in triplicate in 24-well plates using Plus Reagent and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Endometriotic stromal cell transient transfection was performed with 0.2 μg of human MIF (hMIF) promoter-pGL3 luciferase reporter vector, P450arom promoter-pGL3 luciferase reporter vector, or pGL3 control vector as previously reported.34 The pBind plasmid encoding Renilla luciferase (Promega) was used to normalize transfection efficiency. After 3 hours of incubation, cells were washed with sterile PBS and incubated for 24 hours in phenol red-free medium without serum before being stimulated with 10−9 M and 10−19 M E2 for 48 hours. Cell extracts were assayed for firefly and Renilla luciferase activities using the dual luciferase reporter assay system (Promega, Madison, WI), as instructed by the manufacturer. Endometriotic stromal cells were also transiently transfected with 0.4 μg of pCMX-hERα, pCMX-hERβ, and pCMX (control) expression vectors (gift from Dr Vincent Giguère, McGill University Health Centre, Montreal, QC, Canada).35

RNA Interference

Cells were transfected with 50 nmol/L siRNA oligonucleotides using siPORT Amine Transfection Agent (Applied Biosystems/Ambion, Austin, TX) following the manufacturer’s protocol. The targeted base sequence for human MIF was 5′-CAACTCCATGGCTCAA-3′ (corresponding to the 3′ end of the open reading frame) (Dharmacon, Lafayette, CO).36 As negative control, a commercially available siRNA referred to as Silencer Negative Control #1 siRNA (AM4611; Applied Biosystems/Ambion) was used. Cells were incubated at 37°C for 48 hours and subjected to stimulation with 10−9 M E2 for 48 hours.

Immunocytofluorescence

All immunostaining steps were carried out at room temperature. Cells were washed in PBS, fixed for 15 minutes in formaldehyde (3.8% in PBS), and permeabilized with Triton-X-100 (1% in PBS) for 15 minutes. Immunostaining of cytokeratins (monoclonal mouse anti-epithelial keratin-AE1, AE3 mix, 10 μg/mL in PBS/BSA 0.2%/TWEEN 0.01%, Santa Ana, CA) (negative immunostaining) and vimentin (monoclonal mouse anti-vimentin antibody, 0.1 μg/mL in PBS/BSA 0.2%/TWEEN 0.01%, a gift from Dr. Michel Vincent, Centre Hospitalier de l’Université Laval, Québec, QC, Canada) (positive immunostaining) was carried out as previously described.26 Cells were also immunostained using a monoclonal mouse antibody specific to CD10 (4 μg/mL in PBS/BSA 0.2%/TWEEN 0.01%) (Leica Microsystems, Concord, ON, Canada). Cells were then incubated for 1 hour with Alexa Fluor 568-goat anti-mouse IgG antibody (1:5000) (Invitrogen), rinsed with PBS/0.1% Tween-20, counterstained with DAPI (1:2000 dilution in PBS/0.1% Tween 20) (Sigma-Aldrich) and mounted with p-Phenylenediamine (PPD)Mowiol (Calbiochem, San Diego, CA). Cells incubated with equivalent concentrations of mouse IgGs were included as negative controls.

For ERα and ERβ, cells were either transfected with pCMX-hERα and pCMX-hERβ expression vectors (1600 ng/well) or left untreated, fixed, and permeabilized as described earlier and successively incubated with mouse monoclonal anti-ERα antibody (Novocastra Laboratories, Newcastle on Tyne, UK) or goat polyclonal anti-ERβ antibody (Santa Cruz Biotechnology) (1/100 in PBS/BSA 0.2%/TWEEN 0.01%) for 90 minutes, biotin-SP–conjugated affinityPure goat anti-mouse IgG or biotin-SP–conjugated affinityPure rabbit anti-goat IgG (Jackson Immunoresearch Laboratories) (1/500 in PBS/BSA 0.2%/TWEEN 0.01%) for 1 hour and streptavidin-alexa fluor 488 conjugate (Invitrogen) (1/100 in PBS/BSA 0.2%) for 1 hour. Mouse or goat IgGs, used at concentrations equivalent to that of the primary mouse monoclonal anti-ERα antibody or the goat polyclonal anti-ERβ antibody, were used as negative controls. For aromatase, cells were successively incubated with mouse monoclonal anti-human Cyt P450 aromatase (AbD Serotec, Raleigh, NC) (1:50 in PBS/BSA 0.2%/TWEEN 0.01%), biotin-SP–conjugated affinityPure goat anti-mouse IgG (Jackson Immunoresearch Laboratories) (1/500 in PBS/BSA 0.2%/TWEEN 0.01%) for 1 hour, and streptavidin-alexa fluor 488 conjugate (Invitrogen) (1/100 in PBS/BSA 0.2%) for 1 hour. Mouse IgGs, used at a concentration equivalent to that of the primary mouse monoclonal anti-human Cyt P450 aromatase, were included as negative controls. A human granulosa-like tumor cell line (KGN, BioResource Center, RIKEN Tsukuba Institute, Ibaraki, Japan) was used as a positive control for aromatase expression (data not shown). DAPI was used for counterstaining. Slides were then mounted in PPD/Mowiol and viewed using a microscope (Olympus Corporation model BX51, Tokyo, Japan). The intensity of ERα and ERβ immunostaining in transfected and nontransfected cells was determined in four different and randomly selected areas using Image-Pro Express 5.1 software (Media Cybernetics, Bethesda, MD).

Western Blotting

Briefly, total proteins were extracted from cell cultures using a lysis buffer containing 0.5% Triton X-100, 10 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 2 mmol/L ethyleneglycoltetaeractic acid, 2 mmol/L ethylenediaminetetaeractic acid, 0.02% NaN3, and a mix of antiproteases composed of 5 μmol/L of aprotinin, 63 μmol/L of leupeptin, and 3 mmol/L of phenylmethylsulfonyl fluoride. Cell homogenate was then incubated at 4°C for 45 minutes under gentle shaking, and centrifuged at 11,000 × g
for 30 minutes to recover the soluble extract, the total protein concentration for which was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. A mouse monoclonal anti-human aromatase (AbD Serotec) (1:5000 in PBS/BSA 0.2%/TWEEN 0.05%) was used for detection, followed by Fc-specific peroxidase-labeled goat anti-mouse antibody (1:10000 dilution in PBS/BSA/TWEEN 0.05%) (Jackson ImmunoResearch Laboratories) and enhanced chemiluminescence system (BM chemiluminescence blotting substrate [POD]) (Roche Diagnostics, Laval, QC, Canada), respectively. Membranes were exposed to Biomax film (Eastman Kodak, Rochester, NY). Positive controls included total proteins from human placental tissue. Membranes were rebotted with a rabbit anti-actin antibody (1/1000 dilution in PBS/BSA/TWEEN 20) (Sigma-Aldrich) to ensure equal protein loading.

Statistical Analysis

Data followed a parametric distribution and were therefore expressed as mean ± SD. An unpaired t-test was used for comparing the means of two groups, and one-way analysis of variance followed by the Bonferroni test was performed for multiple comparisons. Differences were considered as statistically significant whenever a P value less than 0.05 occurred. All experiments were performed in duplicate and repeated at least three times. All analyses were performed using GraphPad Software, Prism 3.0 (GraphPad Software, San Diego, CA).

Results

Endometriotic stromal cells, isolated and characterized according to our previously reported procedure, were vimentin positive and cytokeratin negative and expressed CD10, an immunohistochemical marker of endometrial stroma that has been used to confirm diagnosis of endometriosis. Analysis of MIF expression in endometriotic stromal cells in response to E2 treatment was first quantified by ELISA. Thus, endometriotic stromal cells were exposed to varying concentrations of E2 for different time periods. Our data showed a spontaneous release of MIF in the culture medium devoid of any stimulus and a significant induction of MIF secretion in cells exposed to $10^{-8}$ M and $10^{-8}$ M E2 ($P < 0.001$) (Figure 2A). The effects of E2 on MIF gene expression in endometriotic stromal cells was then assessed using qRT-PCR. MIF mRNA was detectable in endometriotic cells that were not exposed to exogenous stimuli. However, cell exposure to $10^{-9}$ and $10^{-8}$ M E2 for 48 hours significantly increased MIF mRNA levels ($P < 0.01$) (Figure 2B). As illustrated in Figure 2C, MIF secretion was time dependent and found to be significant in cells treated with $10^{-9}$ M E2 for 24 and 48 hours as compared with nontreated cells ($P < 0.01$) or cells treated with $10^{-9}$ M E2 for 6 hours ($P < 0.01$). Time-course analysis further showed a significant increase of MIF mRNA levels in cells treated with $10^{-9}$ M E2 for 24 and 48 hours compared with control cells (incubated for the same time with the culture medium alone without E2) ($P < 0.01$) (Figure 2D). However, no statistically significant differences were noted between E2-induced MIF mRNA levels after 6, 24, and 48 hours of treatment.

To investigate the specificity of E2-induced MIF expression, cells were preincubated for 1 hour with Fulvestrant ($10^{-8}$ M), a pure estrogen receptor antagonist, before E2 treatment for 48 hours. Our data showed that Fulvestrant significantly suppressed the ability of E2 to induce MIF protein secretion ($P < 0.01$) (Figure 2E) and mRNA synthesis ($P < 0.01$) (Figure 2F). Note that cells incubated only with Fulvestrant did not show any changes in MIF secretion and mRNA compared with con-
control. To further determine whether E2 can be involved in MIF gene transcription, the transcriptional activity of MIF promoter was evaluated by transfecting endometriotic stromal cells with hMIF promoter-pGL3 luciferase reporter vector or with the pGL3 control vector in the presence or absence of E2. The results showed a marked increase in MIF promoter activity with E2 treatment compared to control.**

**Figure 2.** E2 induces MIF secretion, mRNA expression, and promoter activity in endometriotic stromal cells. Cell cultures were stimulated with E2 as described in Materials and Methods. MIF protein concentration in the culture supernatant was measured by ELISA and expressed as percentage of control (culture medium devoid of any stimulus). Total RNA was extracted and reverse transcribed, then MIF and GAPDH (internal control) cDNAs were amplified and quantified by real-time PCR. MIF mRNA levels were normalized to those of corresponding GAPDH mRNA levels and expressed as percentage of control. ‡‡ means /H11006

**Figure 3.** ERα and ERβ are involved in E2-mediated MIF expression in endometriotic stromal cells. Cells were transiently transfected with pCMX-hERα, pCMX-hERβ or pCMX (control vector) as described in Materials and Methods. A: Immunocytofluorescence analysis of ERα and ERβ before and after cell transfection. Note the increase in the intensity of staining in cells transfected with pCMX-hERα (upper, middle) and pCMX-hERβ (lower, middle) compared with nontransfected cells (left). B: MIF protein secretion and mRNA steady-state levels, respectively, after cell transfection and stimulation with E2 (10^-9 M) for 48 hours. Data are mean ± SD from three different endometriotic lesions (patients 1, 2, and 6; Table 1). C: MIF protein secretion in cells transfected with the empty vector pCMX (right). B: The intensity of immunostaining in transfected cells was determined and expressed as percentage of control [immunostaining intensity in non-transfected (NT) cells]. **P < 0.01 compared with NT; *P < 0.05 and ††P < 0.01 compared with cells transfected with pCMX (P = 0.01 and P < 0.05, respectively). Data are from patient 1 and representative of endometriotic cell cultures from three different subjects (patients 1, 2, and 6, Table 1). Scale bar = 50 μm. Original magnification, ×20. C and D: MIF protein secretion and mRNA steady-state levels, respectively, after cell transfection and stimulation with E2 (10^-9 M) for 48 hours. Data are mean ± SD from different endometriotic lesions (patients 3, 4, and 7). E2-treated cells for 4 hours, †P < 0.01 and ††P < 0.01 compared with control (no E2) for the same time period); ‡‡P < 0.01 and †‡‡P < 0.001 compared with control (cells transfected with the empty vector pCMX) for a further 48 hours. Data are mean ± SD from three different endometriotic lesions (patients 1, 2, and 6, Table 1).
increase of luciferase activity in cells transfected with hMIF promoter construct and exposed E2 \((P < 0.001)\) as compared with cells incubated with the control medium alone (control), whereas a very faint luciferase activity was detected in cells transfected with the empty pGL3 control vector and treated with E2 (Figure 2G).

The biological effects of E2 are mediated via binding and activation of two receptors ER\(\alpha\) and ER\(\beta\). Endometriotic stromal cells were transfected with pCMX-hER\(\alpha\) or pCMX-hER\(\beta\) expression vectors, and the effect of E2 on MIF synthesis and secretion was examined. Immunocytofluorescence analysis showed that transfected cells did express higher levels of ER\(\alpha\) or ER\(\beta\) than nontransfected cells (Figure 3A). Quantitative analysis showed that the intensity of ER\(\alpha\) and ER\(\beta\) immunostaining was significantly higher in cells transfected with pCMX-hER\(\alpha\) and pCMX-hER\(\beta\), respectively, compared with nontransfected cells (\(P < 0.01\)) or cells transfected with the control empty vector pCMX (\(P < 0.01\) and \(P < 0.05\), respectively) (Figure 3B). Our data further showed that, in cells transfected with the control empty expression vector (pCMX), E2 significantly increased MIF protein secretion (\(P < 0.05\)) and mRNA synthesis (\(P < 0.01\)) as compared with cells incubated with the control medium alone (Figure 3, C and D). Such an effect of E2 was even more significant in cells transfected with pCMX-hER\(\alpha\) or pCMX-hER\(\beta\) expression vector, either for MIF protein secretion or mRNA synthesis (\(P < 0.01\) and \(P < 0.001\), respectively). However, as compared with cells transfected with the control empty expression vector and exposed to E2, the increase in the E2-induced MIF secretion was statistically significant in cells transfected with pCMX-hER\(\alpha\) (\(P < 0.05\)), but did not reach statistical significance in cells transfected with pCMX-hER\(\beta\) (\(P = 0.09\)). A similar pattern of response was obtained for MIF mRNA as only cells overexpressing ER\(\beta\) showed a statistically significant increase of MIF mRNA synthesis compared with cells transfected with the empty expression vector and exposed to E2 (\(P < 0.01\)). To further assess the involvement of ER\(\alpha\) and ER\(\beta\) in E2-mediated MIF production, endometriotic stromal cells were cultured for 48 hours with ER\(\alpha\)-specific agonist PPT or ER\(\beta\)-specific agonist DPN. Our data showed a significant induction of MIF secretion in cells treated with \(10^{-7}\) M PPT (\(P < 0.05\)) or \(10^{-8}\) and \(10^{-7}\) M DPN (\(P < 0.05\) and \(P < 0.01\), respectively) (Figure 3E).

Estrogen is abnormally synthesized in ectopic endometrial implants and excessive local production of estrogen may play an important role in the development of endometriosis. Therefore, our next studies were to assess whether overproduction of MIF may in turn have any direct impact on the expression of aromatase, a key enzyme in estrogen synthesis. Our data indicate that MIF upregulates aromatase expression in ectopic endometrial cells. Cell exposure to MIF led to a significant dose-dependent upregulation of aromatase mRNA steady-state levels in cells exposed to 10 (\(P < 0.05\)) and 50 ng/mL MIF (\(P < 0.01\)), as observed by real-time PCR (Figure 4A). However, no statistically significant effect of MIF on aromatase mRNA expression in endometrial stromal cells from women with endometriosis was noted (Figure 4B).

Analysis of aromatase expression at the protein level showed an obvious increase in aromatase immunostaining in endometriotic stromal cells (Figure 4C), whereas Western blotting and densitometric analysis of aromatase bands showed a statistically significant increase in aromatase expression in these cells following incubation.
Interestingly, E2 treatment significantly increased aromatase mRNA expression in endometriotic stromal cells. As shown in Figure 5A, no significant effect of MIF on aromatase promoter activity was detected, whereas forskolin (25 μmol/L), used as positive control and known for inducing aromatase transcription, significantly increased aromatase promoter activity. This would suggest that the regulation of aromatase expression by MIF is not exerted at the transcriptional level. However, real-time PCR analysis of aromatase mRNA following arrest of mRNA de novo transcription by actinomycin D (10 μg/mL) showed a significant increase in aromatase mRNA stability in cells exposed to MIF (Figure 5B). The half-life of aromatase mRNA was approximately 0.9 ± 0.1 hour, whereas in MIF-treated cells, it was significantly higher 2.0 ± 0.3 hours (P < 0.01).

We next evaluated whether inhibition of endogenous MIF could influence aromatase expression in endometriotic cells. As shown in Figure 6, MIF-specific siRNA significantly inhibited MIF mRNA expression in endometriotic cells treated with E2 (P < 0.001) (Figure 6A). Interestingly, E2 treatment significantly increased aromatase mRNA in nontransfected cells and in cells transfected with negative control siRNA (P < 0.01). However, inhibition of MIF by siRNA resulted in a significant down-regulation of the E2-induced aromatase expression (P < 0.05) (Figure 6B). To further assess the involvement of estrogen receptors ERα and ERβ in the E2-induced aromatase expression, endometriotic stromal cells were treated with ERα and ERβ specific agonists PPT and DPN. As shown in Figure 6C, a significant induction of aromatase mRNA expression in cells treated with 10^{-8} and 10^{-7} M PPT (P < 0.01 and P < 0.05, respectively) or with 10^{-7} M DPN (P < 0.01) was observed.

Discussion

Endometriosis, a disease occurring in women of reproductive age, is estrogen dependent. Estrogens are believed to be essential to the maintenance and growth of ectopic endometrium and may play a major role in the disease-associated biological changes and clinical manifestations. Among these, a chronic pelvic inflammatory reaction has been described with consistency in women suffering from endometriosis. However, despite these facts, the mechanisms underlying estrogen actions and abnormal production in endometriosis have not been yet clearly elucidated.

The current study revealed for the first time the presence of a positive feedback loop by which E2 acts directly on ectopic endometrial cells to upregulate the expression of MIF. In turn, MIF displays the capability of inducing the expression of aromatase, also called estrogen synthetase, the key and rate-limiting enzyme for estrogen synthesis.

Table 1. Cells were also treated with E2 (10^{-9} M) or DPN, a specific ER agonist (10^{-6} and 10^{-7} M) for 48 hours. Data are mean ± SD from three different endometriotic lesions (patients 11, 12, and 13; Table 1). *P < 0.05 compared with corresponding cells incubated with the culture medium alone without E2. **P < 0.05 compared with cells transfected with negative control siRNA and incubated with E2.
First, our results indicate that ectopic endometrial stromal cells respond to E2 by up-regulating MIF protein synthesis and MIF mRNA steady-state levels. Such an effect appeared to be specific, as pretreatment of ectopic endometriotic cells with Fulvestrant, a pure estrogen receptor antagonist, significantly blocked the E2-induced MIF expression at both the protein and the mRNA levels. Second, transient cell transfection analysis further showed a significant activation of hMIF promoter by E2, which strongly supports the above-described data and suggests a transcriptional activation of MIF gene by E2. However, our study did not show any significant effect of E2 on MIF expression in eutopic endometrial stromal cells from women with or without endometriosis. According to our previous studies, MIF expression displays a menstrual cycle phase-dependent pattern in the human endometrium and is abnormally elevated in women with endometriosis. Endometrial stromal cells express E2 receptors according to previous reports. Therefore, our present data seems to rule out any direct effect of E2 on the endometrial expression of MIF, at least in stromal cells; however, further studies with endometrial epithelial or endometrial explants will be necessary.

Estrogens bind to two known ER, ERα, and ERβ. The most recently described receptor ERβ is highly homologous to the classical ER (ERα) and has been shown to modulate transcription of target genes. The most significant disparity between the two receptors lies in their tissues. It appeared that ERβ receptors are predominantly expressed in the ovary and prostate. ERα is a potent endogenous ligand for ERs and binds equally to ERα and ERβ. A clear role for ERβ has been established in the ovary, the cardiovascular system, and the brain as well as in several animal models of inflammation including arthritis, endometriosis, inflammatory bowel disease and sepsis. However, its biological roles in human endometrium and endometriosis are not yet well understood. Several investigators have studied the expression of ERα and ERβ in normal endometria and endometriotic tissues. It appeared that ERα is the predominantly expressed ER subtype in the normal uterine endometrium. According to some studies, the expression of ERs is different in endometriotic lesions. ERβ was shown to be up-regulated in endometriotic lesions of women with endometriosis and selectively up-regulated in endometrial implants at all stages of the disease in a baboon model for endometriosis. Other studies, however, are not consistent with these observations. Our results showed that increasing the expression of either ERα or ERβ enhanced E2-mediated MIF expression in endometriotic cells, but a statistically significant increase in MIF expression in response to E2 was observed only with ERβ. Selective stimulation of cells with ERα specific agonist PPT significantly induced MIF production at 10^{-7} M, whereas DPN, a specific ERβ agonist, significantly induced MIF secretion at 10^{-8} and 10^{-7} M. This suggests a specific receptor activation that seems to be mediated by both E2 receptors in endometriotic stromal cells, but particularly ERβ.

Our study showed that MIF acts directly on ectopic endometrial stromal cells to significantly up-regulate aromatase protein and mRNA levels, which appeared to occur via a posttranscriptional mRNA stabilization mechanism. However, one could postulate that this effect might involve modulation of specific miRNAs in endometriosis. A TargetScan search for miRNAs targeting aromatase showed nine hits, one of which is miR-98; the remaining belong to miR-let-7 family (http://www.targetscan.org). However, the role of these miRNAs in the regulation of aromatase expression or in MIF-induced aromatase expression remains unknown.

Our data further showed that E2 itself can upregulate aromatase expression in endometriotic cells and that inhibiting endogenous MIF significantly inhibits, but does not completely abolish E2-induced aromatase, suggesting the involvement of yet unknown factors. Interestingly, ERα/ERβ agonists significantly upregulated aromatase expression, supporting our data indicating that E2 is capable of inducing aromatase expression in endometriotic cells. Aromatase, the key enzyme in estrogen synthesis, is upregulated in ectopic endometrial tissue. Although challenged by some studies, abnormal aromatase expression in ectopic and eutopic endometrial tissue of women with endometriosis and estrogen production has been confirmed by many other studies. Mechanisms involved in such an aberrant expression of aromatase have been reported. Prostaglandin E2 (PGE2) appeared to be a major direct upregulatory factor, whereas major proinflammatory factors such as IL1, IL6 and TNF, which are increasingly secreted by activated peritoneal fluid macrophages of women with endometriosis, did not appear to exert a direct regulatory action. This, together with the fact that MIF upregulates cyclooxygenase (Cox)–2 and PGE2 secretion in other cell types, suggesting that MIF operates at least via direct and PGE2-dependent indirect mechanisms. Investigations are underway to elucidate these mechanisms and signaling pathways likely involved in the MIF-mediated effects on aromatase expression in endometriosis. Interestingly, ERK and P38 MAPK signaling pathways, which are activated by MIF and contribute to its growth-promoting and tissue remodeling activities, were reported to induce aromatase expression in other cell types.

These findings may have considerable relevance for the pathophysiology of endometriosis, considering the crucial role of estrogen in endometriosis development and the manifestation of clinical symptoms of the disease and the potent inflammatory, tissue-remodeling and growth-promoting effects of MIF. Originally known for its ability to inhibit macrophage migration, MIF appears as a major regulatory factor of immune cells endowed with a wide spectrum of biological properties including inhibition of apoptosis, induction of cell proliferation and stimulation of angiogenesis and tumorogenesis. MIF has been shown to act via CD74 receptor binding and activation of CD44. Enhanced CD44 activation appeared to promote breast cancer cell invasion, whereas inhibition of MIF or MIF-binding receptor CD74 has been shown to attenuate prostate cancer invasion. Furthermore, in an animal model of B cell lymphoma,
administration of MIF monoclonal antibody resulted in markedly inhibited tumor growth and neoangiogenesis. MIF also has the unique ability to stimulate sustained ERK1 and ERK2 MAPK activation in nontransformed cells, leading to enhanced cell proliferation, while comitantly antagonizing the growth arrest and proapoptotic action of the tumor suppressor p53. In view of these novel functions, MIF represents a valuable therapeutic target.

In this study, endometriotic cells were obtained from women with endometriosis stages I, II, and III in the proliferative and secretory phases of the menstrual cycle. Comparing endometriotic cell responsiveness according to endometriosis stage or the menstrual cycle phase was beyond the scope of the present work, and no conclusive observations could be made, as more cultures for each set of experiments would be needed. Nonetheless, our findings showed a significant role for MIF in local estrogen synthesis in endometriotic lesions and the reciprocal capability of E2 to amplify MIF secretion by endometriotic cells revealed a new mechanism by which MIF may favor ectopic endometrial tissue growth and development, through elaborate immunoe–endoctrine interactions. Further studies will be needed to elucidate the underlying down-stream signaling events and the potential use of MIF as a target for treatment of endometriosis.

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