

Tumour necrosis factor- α up-regulates macrophage migration inhibitory factor expression in endometrial stromal cells via the nuclear transcription factor NF- κ B

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BACKGROUND: A series of controlled changes including proliferation, secretion and menstrual shedding occur in the human endometrium during every normal menstrual cycle. Macrophage migration inhibitory factor (MIF), a multifunctional cytokine with numerous proinflammatory, immunomodulatory and angiogenic properties, appears to be expressed in the human endometrium and to follow a regulated cycle phase-dependent expression, but the mechanisms underlying endometrial MIF expression remain to be fully elucidated. **METHODS AND RESULTS:** Results from enzyme-linked immunosorbent assay (ELISA) demonstrated a significant dose- and time-dependent increase in MIF secretion by human endometrial cells in response to tumour necrosis factor-alpha (TNF- α) (0.1–100 ng/ml). This increase was also observed at the mRNA level as shown by reverse transcription (RT)–PCR. Curcumin (10^{-8} mol/l), a known nuclear factor (NF)- κ B inhibitor, inhibited the TNF- α -induced pI κ B phosphorylation as shown by western blotting, NF- κ B translocation into the nucleus as shown by electrophoretic mobility shift assay, and MIF synthesis and secretion as measured by ELISA and RT–PCR. The expression of a dominant-negative NF- κ B inhibitor (I κ B) significantly decreased the TNF- α -induced MIF promoter activity as analysed by transient cell transfection. **CONCLUSIONS:** These results indicate clearly that TNF- α up-regulates the expression of MIF in endometrial stromal cells. This took place possibly through NF- κ B activation, and may play an important role in the physiology of the human endometrium.

Key words: endometrium/MIF/NF- κ B/TNF- α

Introduction

Human endometrium undergoes a series of controlled changes including proliferation, secretion and menstrual shedding during every normal menstrual cycle. These dynamic processes require a network of hormonal, growth and immunoinflammatory factors in which cytokines locally produced within the endometrial tissue play a crucial role (Tabibzadeh, 1996; 1998; Lebovic *et al.*, 2000; von Wolff *et al.*, 2000; Bigonnesse *et al.*, 2001; Boucher *et al.*, 2001; Critchley *et al.*, 2001). Of particular interest is the role that appears to be played by tumour necrosis factor-alpha (TNF- α) in human endometrial physiology.

TNF- α was first identified as a cytokine secreted by endotoxin-activated macrophages that induced the necrosis of tumours (Carswell *et al.*, 1975). TNF- α is now known as a pluripotent cell mediator and angiogenic cytokine that promotes the production of other cytokines in various cells (Feldmann and Maini, 1999). The human endometrium is characterized by a variety of cell types, including fibroblasts, immune cells, vascular cells and

epithelial cells, all of which express TNF- α (Hunt *et al.*, 1992; Philippeaux and Pigué, 1993; Tabibzadeh *et al.*, 1995; Chegini *et al.*, 1999; von Wolff *et al.*, 1999; Bergqvist *et al.*, 2000; 2001). Despite discrepancies in the cycle expression patterns, these studies suggest a local role for TNF- α in a variety of normal endometrial functions. Increased expression of this cytokine was shown to cause pathophysiological effects reflected by its involvement in implantation failure (Hazout, 1995), abortion (Giacomucci *et al.*, 1994) and endometriosis (Zhang *et al.*, 1993).

Our previous studies identified macrophage migration inhibitory factor (MIF) as a potent mitogenic factor for human endothelial cells released by ectopic endometrial cells (Yang *et al.*, 2000) in women with endometriosis (Kats *et al.*, 2002b). Subsequent studies from our laboratory showed a cycle phase-dependent expression of MIF during the menstrual cycle, with a particular increase occurring in the late secretory phase, suggesting a tight regulation and perhaps different roles for this factor in the reparative, reproductive and inflammatory-like processes that occur in human endometrium during the normal

menstrual cycle (Kats *et al.*, 2005). MIF, one of the first described cytokines, was identified for its ability to inhibit macrophage migration within the inflammatory sites (Bloom and Bennett, 1970; David, 1966). More recent studies have shown, however, that MIF is rather a multifunctional cytokine/hormone endowed with a multitude of proinflammatory, immunomodulatory, angiogenic and tissue remodelling effects (Metz and Bucala, 1997; Nishihira, 1998; Calandra and Roger, 2003; Nishihira *et al.*, 2003). Accordingly, we believe that MIF may in different ways influence human endometrial tissue functions and be involved in its normal cyclic changes.

The mechanisms underlying MIF expression in the human endometrial tissue remain unknown. Herein we report that TNF- α had a direct stimulatory effect on MIF expression by human endometrial stromal cells. Cell treatment with TNF- α caused a significant increase in MIF protein secretion and mRNA synthesis, which appeared to be likely mediated by the activation of the nuclear transcription factor NF- κ B. Interaction between TNF- α and MIF, owing to the properties of these two major and multifunctional cytokines, may play a considerable role in the physiology of the human endometrium.

Materials and methods

Subjects and tissue handling

Endometrial biopsies were obtained during laparoscopy for tubal ligation from eight normal fertile women who had not received hormonal or anti-inflammatory therapy for at least 3 months before surgery (mean age \pm SD, 30.71 \pm 4.03 years). Four women were in the proliferative phase of the menstrual and four in the secretory phase. Written informed consent was obtained from these women under a study protocol approved by the Ethical Committee on Human Research at Laval University, Quebec, Canada. Biopsies were obtained by aspiration with the use of a Pipelle (Unimar Inc., Prodimed, Neuilly-En-Tchelle, France), immediately placed at 4°C in sterile Hank's balanced salt solution containing 100 U/ml penicillin, 100 μ g/ml streptomycin and 0.25 μ g/ml amphotericin B (Invitrogen Life Technologies, Burlington, ON, Canada) and transported to the laboratory.

Cell culture and stimulation

Endometrial stromal cells were obtained and characterized according to our previously described procedure (Boucher *et al.*, 2000). Cells were subcultured to eliminate contamination by macrophages or other leukocytes and used between passages 3 and 5. Extensive characterization of cell cultures prepared using this protocol previously confirmed >95% purity with cells retaining cytoskeletal markers of their endometrial stromal origin. For enzyme-linked immunosorbent assay (ELISA) and reverse transcription (RT)-PCR studies, endometrial cells were plated in 12-well plates in Dulbecco's minimal essential medium F-12 medium, supplied with 10% fetal bovine serum (FBS), 5 μ g/ml insulin, 5 μ g/ml transferrin and 1% antibiotics-antimycotics. Medium was changed every 48 h. When cells proliferated to confluence, the culture medium was replaced overnight with serum-free medium, then with serum-free and phenol red-free medium containing various concentrations of TNF- α (0–100 ng/ml) (R & D systems, Minneapolis, MN, USA) for different time periods (0–24 h). In some cultures, curcumin (10⁻⁸ mol/l) (Sigma Chemical Co., St Louis, MO, USA), an extract from plant known for inhibiting NF- κ B (D'Acquisto *et al.*, 2002; Ghosh and Karin, 2002) was added 1 h before TNF- α . The culture supernatants were collected for MIF ELISA, whereas cells were collected for mRNA or nuclear protein extraction.

Immunocytofluorescence

Endometrial stromal cells were plated on chamber slides (Nalge Nunc International, Naperville, IL, USA). At confluence, cells were incubated overnight with serum-free medium, and then with TNF- α at 1 ng/ml for 6 h. Cells were then fixed in formaldehyde [3.5% v/v in phosphate buffered saline (PBS)] for 15 min at room temperature, rinsed in PBS–0.01% Tween 20, incubated with a goat anti-human MIF antibody [0.66 mg/ml in PBS/0.2% bovine serum albumin (BSA)/0.01% Tween 20] (R & D Systems) for 90 min at room temperature, rinsed in PBS–0.01% Tween 20, incubated with fluoresceine isothiocyanate (FITC)-conjugated anti-goat antibody (1/50 dilution in PBS–BSA–Tween) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), rinsed in PBS–0.01% Tween 20 and mounted in Mowiol containing 10% para-phenylenediamine (Sigma). Cells incubated without the primary antibody or with goat immunoglobulins (Sigma) at the same concentration as the primary antibody were included as negative controls. Slides were observed under a microscope equipped for fluorescence (Leica mikroskopie und systeme GmbH, Model DMRB; Postfach, Wetzlar, Germany) and photographed.

ELISA

MIF ELISA was performed using a mouse monoclonal anti-human-MIF antibody (R & D Systems) as a capture antibody, a rabbit polyclonal anti-human-MIF antibody for detection with an alkaline phosphatase-conjugated goat anti-rabbit antibody and para-nitrophenyl phosphate (Sigma) as substrate (Calandra *et al.*, 1995). The optical density was measured at 405 nm and MIF concentrations were extrapolated from a standard curve using recombinant human MIF. The sensitivity limit of the assay was 300 pg/ml, with intra- and inter-assay coefficients of variation <4%.

RT-PCR

Briefly, following appropriate treatment with TNF- α , cells were washed with ice-cold PBS and extraction of total RNA was performed with Trizol reagent according to the manufacturer's instructions (Invitrogen). RNA was reverse transcribed in the presence of random primers, and the resulting cDNA was amplified with oligonucleotide primers specific to human MIF (amplimer size 255 bp) and to human glyceraldehyde phosphate dehydrogenase (GAPDH) used as internal control (amplimer size 240 bp). The PCR reaction products were then separated on 1.2% agarose gel by electrophoresis for qualitative analysis of mRNA expression as described previously (Kats *et al.*, 2002b).

Quantitative real-time PCRs were carried out in an ABI 7000 Thermal Cycler (Applied Biosystems). Each standard PCR reaction contains 2 ml of RT product, 0.5 ml of each primer (final concentration, 5 pmol/l), 12.5 ml SYBR Green PCR Master Mix consisting of Taq DNA polymerase reaction buffer, dNTP mix, SYBR green I, MgCl₂ and Taq DNA polymerase. Following a 10 min denaturation at 95°C, the reactions were cycled 40 times with a 15 s denaturation at 95°C and a 60 s annealing at 59 or 60°C for MIF and GAPDH, respectively. MIF primers (forward, 5'-GCCCCGACAGGGTCTACA-3'; reverse, 5'-CTTAGGCGAAGGTGGAGTTGTT-3'; amplimer size 125 bp) (Boeuf *et al.*, 2005), and GAPDH primers (forward, 5'-CAGGGCT-GCTTTTAACTCTGG-3'; reverse, 5'-TGGGTGGAATCATATT-GGAACA-3'; amplimer size 102 bp) designed with Primer Express™, version 2.0 (Applied Biosystems), span intron–exon boundaries to avoid amplification of genomic DNA, and were selected so as to have compatible *T_m* values (59–61°C). Quantitation of MIF mRNA was performed using a relative quantitation method. For each experimental sample, MIF mRNA levels were normalized to GAPDH mRNA levels. After each run, melting curve analysis (55–95°C) was performed to verify the specificity of the PCR. All samples were tested in

duplicate, and each run included no-template and no-reverse transcription controls.

Western blotting

Briefly, 80 μ l lysis buffer (50 mmol/l Tris-HCl, 125 mmol/l NaCl, 0.1% Nonidet-P40, 5 mmol/l ethylenediamine tetraacetic acid, 50 mmol/l NaF, 0.1% phenylmethylsulfonylfluoride and protease inhibitors) was used to extract cytoproteins after collecting cells with cold PBS supplied with phosphorylation inhibitors. Proteins (20 μ g) were electrophoretically separated using 10% SDS-PAGE, then transferred onto polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA) overnight at 4°C. After incubation with anti-human p-I κ B (Active Motif, Carlsbad, CA, USA) in 0.1% Tween 20-PBS for 1.5 h at room temperature, membranes were incubated with horseradish peroxidase (HRP)-labelled anti-mouse IgGs for 1 h, and a chemiluminescence kit was used for detection following the manufacturer's instructions (Roche, IN, USA). Membranes were stripped and reblotted with a monoclonal antibody specific to tubulin (Sigma) (1:50 000 dilution in Tween 20-PBS) used as an internal control for protein loading and transfer.

Extraction of nuclear proteins and EMSA

Nuclear proteins were extracted using a nuclear extract kit according to the manufacturer's instructions (Active Motif) and protein concentration was determined using the Bio-Rad DC Protein assay kit (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). Nuclear extracts were stored at -80°C until use.

DNA binding of NF- κ B was examined using the consensus oligonucleotide of NF- κ B (5'-AGT TGA GGG GAC TTT CCC AGG C-3'). The oligonucleotide was end-labelled with (³²P) γ -ATP (Amersham Pharmacia Biotech) using T4 polynucleotide kinase (Promega, Madison, WI, USA). The labelled probe was purified using a Probe-Quant G-50 micro-column (Amersham Biosciences, Baie d'Urfe, QC, Canada) (3000 r.p.m., 1 min) and recovered in Tris-EDTA buffer pH 8.0. Binding reactions included 5 μ g of nuclear proteins in incubation buffer containing 50 mmol/l Tris-HCl pH 7.5, 250 mmol/l NaCl, 2.5 mmol/l dithiothreitol, 2.5 mmol/l EDTA, 5 mmol/l MgCl₂, 0.25 mg/ml poly(dI-dC)poly(dI-dC) and 20% glycerol. After 10 min, the labelled oligonucleotide (4 \times 10⁵ c.p.m.) was added and the mixture was incubated for 20 min at room temperature in a final volume of 10 μ l. When indicated, controls for specific or non-specific competitions were performed using unlabelled NF- κ B which was added 20 min before the incubation with the labelled oligonucleotide. Immediately after binding, the nucleoprotein-oligonucleotide complexes were separated from unbound oligonucleotide by electrophoresis on a non-denaturing 4% acrylamide gel at 100 V for 4 h using 0.5 mol/l Tris-borate-EDTA (TBE) buffer. The gel was then dried and exposed overnight at -80°C to x-ray films (BioMax; Eastman Kodak, Rochester, NY, USA).

Transfections and luciferase assays

Transfections were performed in triplicate in 24-well plates using Plus and LipofectAMINE Reagents (Invitrogen) as described by the manufacturer. Cells were transiently co-transfected with 0.5 μ g of human MIF promoter construct in the pGL3 luciferase reporter vector (pGL3-MIF) and 1 μ g of human I κ B dominant-negative cDNA (S32A/S36A) in the pUSE vector (pUSE-I κ B) (Upstate, Charlottesville, VA, USA), or with the appropriate control vectors pGL3 and pUSE. Cells were then washed with PBS and incubated in the culture medium containing 10% FBS overnight before they were stimulated with 1 ng/ml TNF- α for 24 h. Cell extracts were assayed for firefly and renilla luciferase activities using the dual luciferase reporter assay system (Promega) as instructed by the manufacturer.

Statistical analysis

Data were expressed as mean \pm SEM. An unpaired *t*-test was used for comparing two means, and one-way ANOVA followed by the Dunnett's test was performed for multiple comparisons using GraphPad Software, Prism 3.0 (GraphPad Software, San Diego, CA, USA). Differences were considered as statistically significant whenever a *P*-value <0.05 occurred.

Results

Analysis of MIF expression in endometrial stromal cells in response to TNF- α was first examined by immunocytofluorescence using anti-MIF antibody. Only a weak staining was observed in cells incubated with the culture medium alone without stimulus (Figure 1A). However, cell incubation with TNF- α (1 ng/ml) for 24 h markedly increased MIF immunostaining (Figure 1B). Staining was virtually absent in cells incubated with goat IgGs instead of anti-MIF antibody or with anti-MIF antibody pre-absorbed with an excess rhMIF (2 μ g/ml) (data not shown).

MIF secretion by endometrial stromal cells in response to TNF- α was then quantified by ELISA. As shown in Figure 2, TNF- α induced a dose- and time-dependent increase in MIF concentration in the culture medium. There was no significant increase in MIF secretion in response to TNF- α after 2 h of stimulation. Treatment with 0.1 ng/ml resulted in a progressive increase in MIF secretion, although with no statistical significance. MIF secretion was statistically significant after 6, 12 and 24 h of stimulation with 1 and 10 ng/ml TNF- α (*P* < 0.05), and appeared to reach a plateau after 6 h of treatment, whereas higher TNF- α concentration (100 ng/ml) induced a statistically significant increase in MIF secretion after 24 h of stimulation (*P* < 0.05).

The effects of TNF- α on MIF gene expression in endometrial stromal cells were further analysed by RT-PCR. As shown in Figure 3, endometrial stromal cells express detectable levels of MIF mRNA in culture without stimuli. However, adding TNF- α (0-100 ng/ml) to the culture medium for 24 h considerably increased MIF mRNA levels in a dose-dependent manner. Quantitative analysis of MIF mRNA expression using real-time PCR confirmed these data and further showed a significant increase in MIF mRNA levels in endometrial stromal cells in response to 1 (*P* < 0.01) and 10 (*P* < 0.05) ng/ml TNF- α .

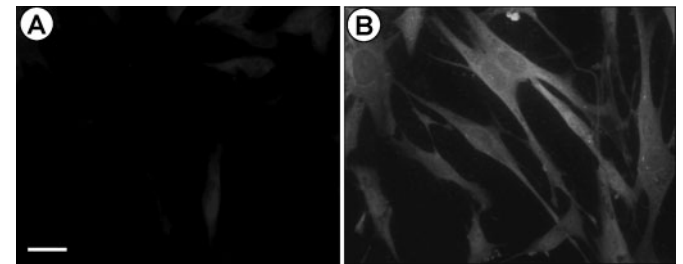


Figure 1. TNF- α increased MIF expression as detected by immunocytofluorescence. Human endometrial stromal cell cultured to confluence in chamber were incubated for 24 h in culture medium without stimuli (A) or with 1 ng/ml TNF- α (B). Cells were fixed and MIF protein was visualized with goat anti-hMIF and FITC-anti-goat IgGs. Scale bar = 30 μ m.

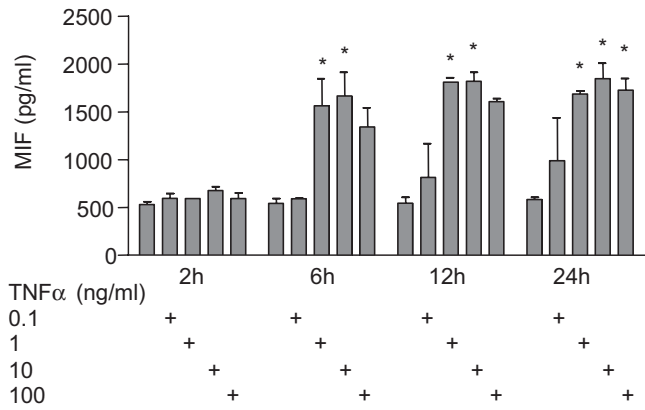


Figure 2. TNF- α induced a dose- and time-dependent increase in MIF protein secretion in the culture medium as analysed by ELISA. The bars represent means \pm SEM; * P < 0.05 versus control (culture medium without stimuli for each incubation time), as analysed by the Dunnett's test.

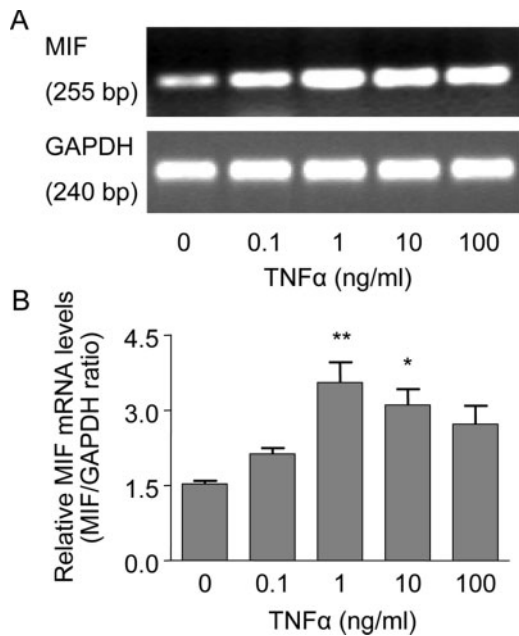


Figure 3. TNF- α increased MIF mRNA steady-state levels in cultured human endometrial stromal cells. Cells were treated with TNF- α (0, 0.1, 1, 10 and 100 ng/ml) for 24 h. Total RNA was extracted and reverse transcribed, then MIF and GAPDH (internal control) cDNAs were amplified by PCR as described in Material and methods. (A) Analysis of amplified cDNAs by electrophoresis and ethidium bromide staining; (B) quantitative real-time PCR analysis. MIF mRNA levels were expressed as MIF to GAPDH mRNA ratio. The bars represent means \pm SEM; * P < 0.05, ** P < 0.01 versus control (culture medium without stimuli), as analysed by the Dunnett's test.

Transcription factor NF- κ B plays a major role in the regulation of the expression of multiple genes that control the immune system, growth and inflammation (Makarov *et al.*, 1997). NF- κ B appeared to play an important role in mediating TNF- α effects in a variety of cell types (Makarov *et al.*, 1997). However, it is still unknown whether NF- κ B is involved in MIF gene transcription. Our data showed that curcumin

(10^{-8} mol/l), an NF- κ B inhibitor (Xu *et al.*, 1997; Bharti *et al.*, 2003), significantly decreased the ability of TNF- α to induce MIF protein secretion (P < 0.05) and mRNA synthesis (P < 0.01) (Figure 4). Analysis of nuclear proteins showed that TNF- α induced expression of the functional p50 component of NF- κ B in endometrial stromal cells, as indicated by binding of radiolabelled p50-specific NF- κ B oligonucleotide and by super-shifting of this complex with an antibody specific to NF- κ B p50. The TNF- α -induced NF- κ B p50 band was reduced or eliminated by curcumin (10^{-8} mol/l) or an excess of unlabelled oligonucleotide (Figure 5). Phosphorylation of the NF- κ B inhibitor, I κ B, inactivates I κ B, and allows NF- κ B to translocate into the cell nucleus where it triggers synthesis of inflammatory cytokines (Makarov *et al.*, 1997). As shown in Figure 6, pI κ B was increased after exposure to TNF- α (1 ng/ml), and the increase was inhibited by curcumin (10^{-8} mol/l) as detected by western blot.

To further determine whether NF- κ B can be involved in MIF gene transcription, the transcriptional activity of the MIF promoter-luciferase construct was evaluated after co-transfection with the pUSE-I κ B dominant-negative construct in the presence and absence of TNF- α . The pUSE-I κ B dominant-negative vector expresses a mutated non-phosphorylatable, non-degradable I κ B. The results showed that the activity of the MIF promoter

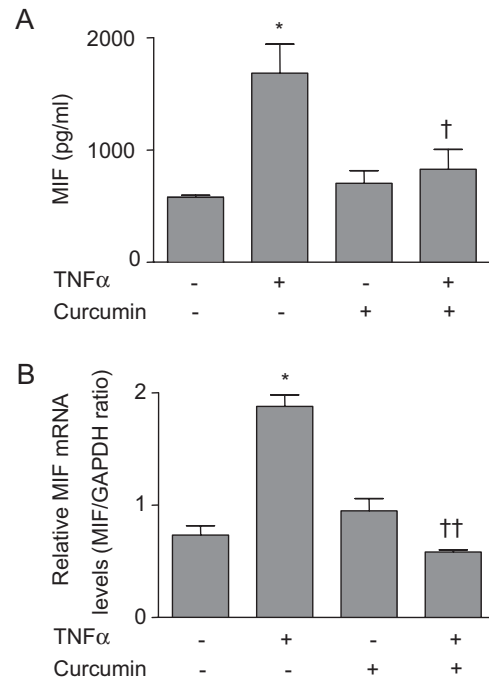


Figure 4. Curcumin suppressed TNF- α -induced increase in MIF expression in endometrial stromal cells. Confluent cultures of endometrial stromal cells were preincubated for 1 h with or without curcumin (10 nmol/l); TNF- α (1 ng/ml) was then added and the cells were incubated for further 24 h. (A) MIF concentration in the culture supernatant as measured by ELISA; (B) MIF mRNA levels as evaluated by real-time PCR. The bars represent means \pm SEM. *Significant stimulation of MIF secretion and mRNA synthesis by TNF- α as analysed by the unpaired t -test (P < 0.05); † P < 0.05 and †† P < 0.01, significant inhibition of TNF- α -induced MIF secretion and mRNA synthesis in the presence of curcumin, as analysed by the unpaired t -test.

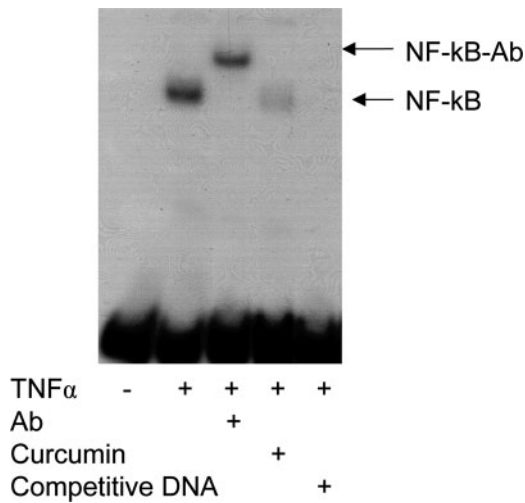


Figure 5. TNF- α induced nuclear translocation of NF- κ B as analysed by electrophoretic mobility shift assay (EMSA). Confluent cell cultures were exposed for 6 h to medium alone, medium with TNF- α (1 ng/ml) or medium with TNF- α (1 ng/ml) following 1 h preincubation with curcumin (10^{-8} mol/l). Nuclear protein extracts (5 μ g) were incubated with radiolabelled NF- κ B oligonucleotide. The resulting complexes were separated on 5% non-denaturing polyacrylamide gel. To test for specificity of NF- κ B binding, supershift analysis with antibody against the p50 subunit of NF- κ B (NF- κ B-Ab) and competition experiments with unlabelled oligonucleotide were conducted.

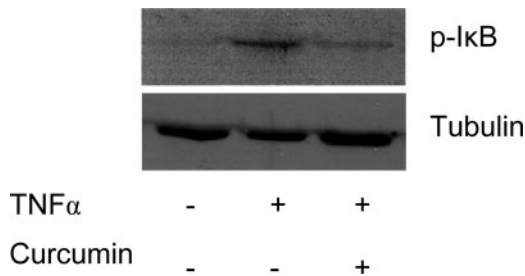


Figure 6. TNF- α induced I κ B phosphorylation in human endometrial stromal cells, as detected by western blot. Confluent cell cultures were exposed to medium without stimuli, 1 ng/ml TNF- α or 1 ng/ml TNF- α for 15 min following 1 h preincubation with 10^{-8} mol/l curcumin. Twenty micrograms of total proteins were separated by electrophoresis on a 10% gradient polyacrylamide gel and immunoblotted using mouse monoclonal anti-pI κ B antibodies and HRP-anti-mouse IgGs (pI κ Ba, 43 kDa). Tubulin levels are shown to demonstrate equal protein loading.

was significantly stimulated with TNF- α ($P < 0.05$). Furthermore, cell co-transfection with the pUSE-I κ B dominant-negative vector resulted in a significant inhibition of the TNF- α -induced MIF promoter activity (Figure 7).

Discussion

These results indicate, for the first time, that TNF- α up-regulates MIF gene expression and protein synthesis in human endometrial stromal cells and that this effect is mediated by activation of NF- κ B. In quiescent cells, NF- κ B is bound to I κ B in the cytoplasm. Upon stimulation by various agents

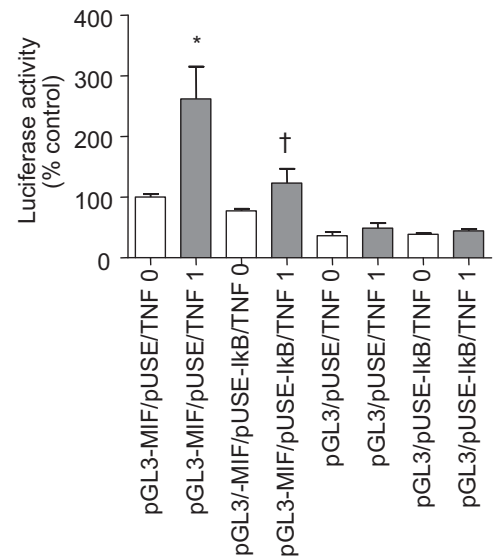


Figure 7. I κ B dominant-negative inhibited TNF- α -induced MIF promoter activity. Human endometrial stromal cells were transiently co-transfected with pGL3-MIF and pUSE-I κ B, or with the appropriate control vectors pGL3 and pUSE. The transfected cells were then incubated for 24 h with and without TNF- α (1 ng/ml) before being harvested. Cell lysates were analysed for firefly and renilla luciferase activities as described in Materials and methods. Data were expressed as percentage of control which represents MIF promoter activity without co-expression of dominant-negative I κ B and stimulation with TNF- α (cells co-transfected with pGL3-MIF and the pUSE vector and incubated with the culture medium). The bars represent means \pm SEM. *Significant increase in MIF promoter activity in response to TNF- α as compared to the culture medium ($P < 0.05$); †significant decrease in the TNF- α -induced MIF promoter activity following co-transfection with dominant-negative I κ B ($P < 0.05$), as analysed by the unpaired *t*-test.

such as TNF- α , I κ B undergoes phosphorylation to form p-I κ B, which subsequently undergoes ubiquitylation and degradation. This process allows NF- κ B to enter the nucleus, where it activates cytokines, adhesion molecules, cyclooxygenase and other target genes (Makarov *et al.*, 1997). Several compounds are now used as NF- κ B inhibitors with different levels of effects. Our data showed that curcumin depressed the ability of TNF- α to increase MIF secretion and inhibited pI κ B phosphorylation and NF- κ B translocation into the nucleus. Furthermore, co-transfection of endometrial cells with MIF promoter and I κ B dominant-negative vectors resulted in a significant inhibition of the TNF- α -induced activation of the MIF promoter. These data suggest that TNF- α activates MIF gene transcription in human endometrial stromal cells, and provide further evidence on the ability of TNF- α to up-regulate MIF expression via NF- κ B.

It is now quite well-known that TNF- α is a pluripotent cell mediator having proinflammatory, growth/apoptotic and angiogenic effects and a wide spectrum of cell targets (Feldmann and Maini, 1999). TNF- α has been detected in various reproductive organs including the ovaries (Roby *et al.*, 1990), the oviduct (Hunt, 1993), the endometrium and preimplantation embryos (Zolti *et al.*, 1991; Hunt *et al.*, 1992; Philippeaux and Piguat, 1993; Sharkey *et al.*, 1995; Tabibzadeh *et al.*, 1995;

Chegini *et al.*, 1999; von Wolff *et al.*, 1999), thus implicating TNF- α in reproduction.

Several studies have shown the menstrual cycle-dependent production of TNF- α in the human endometrium, its expression in different cell types and the presence of TNF receptors (Hunt *et al.*, 1992; Philippeaux and Pigué, 1993; Tabibzadeh *et al.*, 1995; Chegini *et al.*, 1999; von Wolff *et al.*, 1999). Despite discrepancies in the cyclic expression patterns, these studies suggest a local role for TNF- α in a variety of endometrial functions. This is not surprising, since the human endometrium represents one of the most dynamic tissues during the reproductive age, and cytokines appear to have a crucial role in the regulation of the cyclic events of cell proliferation, remodelling, angiogenesis, apoptosis/necrosis, bleeding and menstrual shedding occurring in this tissue (Tabibzadeh, 1996; 1998; Lebovic *et al.*, 2000; von Wolff *et al.*, 2000; Bigonnesse *et al.*, 2001; Boucher *et al.*, 2001; Critchley *et al.*, 2001). Thus, our study showing an up-regulation of MIF expression by TNF- α in human endometrial cells identifies one of the potential mechanisms underlying MIF expression in the human endometrium, and points to a plausible interaction between these two multifunctional cytokines the local expression and biological properties of which are of particular relevance for the physiology of a tissue like the human endometrium.

MIF was first reported in 1932 and was described as a cytokine produced by T lymphocytes that inhibited the random migration of microphages during delayed hypersensitivity (David, 1966; Bloom and Bennett, 1970). It is now well documented that MIF is a major multifunctional proinflammatory cytokine that activates a number of immunocompetent cells (Metz and Bucala, 1997; Calandra and Roger, 2003; Nishihira *et al.*, 2003) and overrides the anti-inflammatory effects of glucocorticoids (Calandra and Bucala, 1997; Kitaichi *et al.*, 2000). New evidence, however, showed a wider spectrum of action for MIF and an involvement in angiogenesis and tissue remodelling (Metz and Bucala, 1997; Nishihira, 1998; Calandra and Roger, 2003; Nishihira *et al.*, 2003). For instance, MIF was shown to promote directly endothelial cell proliferation *in vitro* (Chesney *et al.*, 1999; Ogawa *et al.*, 2000; Yang *et al.*, 2000), to stimulate angiogenesis *in vivo* (Chesney *et al.*, 1999; Nishihira *et al.*, 2003) and to induce the synthesis and the secretion of matrix metalloproteinases in different cell types (Meyer-Siegler, 2000; Onodera *et al.*, 2000), including endometrial cells according to our recent data (A.Akoum and M.J.Thériault, unpublished data). Accordingly, it is quite possible that local induction and production of MIF may contribute to active tissue remodelling and angiogenesis that take place in the endometrial tissue and are required for the tissue's growth and regeneration, as well as to the inflammatory-like process of leukocyte invasion and menstrual breakdown occurring at the end of the cycle in the absence of implantation (Critchley *et al.*, 2001).

The findings of the present study may have a particular relevance for the understanding of endometriosis, a common gynecological disorder where an endometrial-like tissue creates lesions outside the uterus (Sampson, 1927; Taylor, 2003). Endometriosis is recognized as an inflammatory-like disorder; a chronic immunoinflammatory process has often been described

in the peritoneal cavity of endometriosis patients (Halme *et al.*, 1987; Taylor, 2003; Wu and Ho, 2003). Peritoneal fluid from patients with endometriosis was shown to contain high levels of leukocytes, particularly macrophages, and inflammatory cytokines. These cytokines could then contribute to the development of peritoneal endometriosis by promoting endometrial cell adhesion, invasion and proliferation (Hammond *et al.*, 1993; Zhang *et al.*, 1993; Barcz *et al.*, 2000; Wu and Ho, 2003).

Several studies have shown a significant role of TNF- α in endometriosis pathophysiology. Elevated concentrations of TNF- α were found in the peritoneal fluid of women with endometriosis (Rana *et al.*, 1996; Barcz *et al.*, 2000; Iwabe *et al.*, 2000; Bedaiwy *et al.*, 2002; Bullimore, 2003; Darai *et al.*, 2003). TNF- α appeared to stimulate metalloproteinase expression (Sillem *et al.*, 2001), to favour cell adhesion to mesothelial cells (Zhang *et al.*, 1993) and to play a considerable role in endometriosis-associated immunoinflammatory changes (Sakamoto *et al.*, 2003). Interestingly, TNF- α appeared to enhance eutopic and ectopic endometrial cell growth (Iwabe *et al.*, 2000; Braun *et al.*, 2002; Sakamoto *et al.*, 2003), but failed to do so in endometrial cells of controls without endometriosis (Braun *et al.*, 2002). This is suggestive of an endometriosis-related intrinsic difference in endometrial cell responsiveness to TNF- α , and in keeping with our previous data showing an increased secretion of monocyte chemoattractant protein-1 (MCP-1) in endometrial epithelial cells of women with endometriosis exposed to TNF- α *in vitro* (Akoum *et al.*, 1995). The effect of TNF- α on MIF secretion by eutopic as well as ectopic endometrial cells of women with endometriosis remains to be elucidated, and it is still to be determined whether these cells had a modified responsiveness to TNF- α with regard to MIF secretion. Nevertheless, our data showing a marked stimulatory effect of TNF- α on endometrial stromal cell secretion of MIF, suggest that peritoneal fluid TNF- α may interact with retrograde endometrial tissue and activate NF- κ B, thereby stimulating MIF secretion and exacerbating inflammation. This is all the more plausible since our previous studies showed a marked increase in MIF expression in ectopic endometrial tissue and the peritoneal fluid of endometriosis patients and a relationship with the disease stage (Kats *et al.*, 2002a; b).

In conclusion, our study provides for the first time evidence that TNF- α increases MIF expression in human endometrial stromal cells and that such an effect is mediated by NF- κ B. This, owing to the properties of these two major and multifunctional cytokines, may play an important role in the cyclic changes occurring in the human endometrial tissue and potentially contribute to endometriosis pathophysiology.

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