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Interleukin-37 in endometriosis

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ABSTRACT

Interleukin-37 (IL-37) has been identified as a novel anti-inflammatory cytokine. The present study aimed to evaluate the expression of IL-37 in serum and in peritoneal fluid to determine its clinical significance in endometriosis. Enzyme-linked immunosorbent assay (ELISA) was performed to examine serum IL-37 levels in patients with endometriosis and healthy controls. Peritoneal fluid IL-37 mRNA and NFκB expression were quantified by real-time reverse transcription polymerase chain reaction assays. The association of IL-37 levels with clinical factors and prognosis of endometriosis was analysed. We found that IL-37 levels in PF and in serum were significantly higher in patients with endometriosis compared to women without endometriosis ($P = 0.0005$). IL-37 levels were highly expressed in PF [132.38 ± 34.62 pg/mL; $P < 0.0001$] than in serum [74.10 ± 13.49 pg/mL] in endometriosis patients. IL-37 mRNA expression contrasted with NFκB mRNA expression in PF from patients with endometriosis. A significant inverse correlation was observed between IL-37 mRNA and NFκB mRNA expression. IL-37 expression correlates with endometriosis severity. The affected NFκB mRNA expression in endometriosis contributed the to exhibited increase of IL-37. The increased levels of IL-37 may dampen NFκB activation in endometriosis patients.

1. Introduction

Endometriosis is a benign gynaecological disorder that affects women of reproductive age. It is characterized by the presence of ectopic endometrial cells and stroma in various locations outside the endometrium. Endometriosis shares similarities with several autoimmune diseases, which include elevated levels of cytokines, decreased apoptosis, and cell-mediated abnormalities. Immunological factors and angiogenesis play a key role in the pathogenesis of endometriosis which may affect the woman's susceptibility to exfoliated endometrial cells implantation [1].

The immunological alterations associated with endometriosis are both local and systemic. Most studies reported that the potential link between cytokines and endometriosis was limited in terms of the cytokines profile levels, either in the PF or in the serum. Chronic inflammation activates host immune responses, leading to humoral and cell-mediated inflammation and involving both helper T cell subsets (Th1 and Th2) [2]. Analysis of the cytokine responses occurring in endometriosis has shown a deregulation of cytokine expression affecting interleukins, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), that suggests a possible shift toward a Th2-mediated immune response [2,3].

Recently we depicted the role of IL-33 in endometriosis, a cytokine of the interleukine-1 family with dual function [4]. IL-33 was involved in the trafficking of immune cells to inflammatory sites [5]. Recently IL-37 was reported to be present in eutopic and ectopic endometrium of women with ovarian endometriosis, which might be involved in the inflammatory process [6]. NF-κB was mentioned as constitutively activated in peritoneal endometriosis in women [7]. NF-κB activation and ICAM-1 expression in endometriosis confirm the extensive inflammatory pattern in endometriosis.

The interleukin (IL)-1 family is the largest family of interleukins. It comprises 11 members, including 7 pro-inflammatory agonists (IL-1α, IL-1β, IL-18, IL-33, IL-36α, IL-36β, IL-36γ) and 4 defined or putative antagonists (IL-1R antagonist (IL-1Ra), IL-36Ra, IL-37, and IL-38) exerting anti-inflammatory activities. IL-37 inhibits both innate and adaptive immunity by decreasing expression of pro-inflammatory cytokines (IL-6, TNF-α, IL-17) [8,9]. Anti-inflammatory activity of IL-37 requires IL-18Ra and IL-1R8 to function together [10]. IL-37 could decrease NF-κB expression. The

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suppressed effect of IL-37 on inflammatory mediators may be due to its inhibition on NF-κB [11].

To our knowledge, the pathophysiological role of IL-37 in endometriosis is still unclear and scarce. This study was conducted to investigate gene and protein expression levels of IL-37 and NFκB in endometriosis.

2. Materials and methods

2.1. Study subjects

Thirty women undergoing laparoscopy for unexplained infertility were included in the study. None of the included women had active pelvic inflammatory disease or any autoimmune disease. None had been on hormonal medication. None of the women had been pregnant in the previous 6 months and all tested negative for Chlamydia, routinely screened as part of infertility work up. The local Ethics Committee of our Medicine University approved the study, and written informed consent was obtained from each patient. All the laparoscopies were performed under general anaesthesia and in the luteal phase of the menstrual cycle. Endometriosis was diagnosed and staged according to the revised American Fertility Society (rAFS) classification by visual inspection of the pelvis. Among 30 endometriosis patients studied, 17 were on minimal/mild stage (stage I–II) and 13 on moderate/severe stage (stages III–IV) of the disease. In twenty women used as healthy controls, laparoscopic examination demonstrated normal status of pelvic organs.

Peritoneal fluid samples were collected under direct vision from the pelvis before any operative manipulation to minimise blood contamination. Patients with blood contaminated PF were excluded from the study. The PF was centrifuged immediately at $400 \times g$ for 10 min to collect cell pellet. Supernatants were removed and stored in aliquots at −80 °C, until IL-37 assay.

2.2. ELISA for human IL-37

Patients and controls donated 10 mL of blood. Peripheral blood mononuclear cells were isolated using Percoll separation (Amersham, Castle Hill, NSW, Australia). IL-37 levels were measured in serum and PF by commercial sandwich ELISA (Gen-Way Biotech, San Diego, USA), following the manufacturer's instructions and as we have recently reported [12,13].

2.3. RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was isolated from mononuclear cells, using RNeasy Micro kit (Qiagen N.V., Venlo, The Netherlands). cDNA synthesis was performed using a BioRad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time quantitative PCR was done on a BioRad MyiQ real time PCR Detection System, using iQ SYBR Green Supermix kit (BioRad). The following primers were used: IL-37, forward primer: 5′-CTCC TGGGGGTCTCTAAAGG-3′ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward: AGAAG GCTGGGGCTCATTT; GAPDH reverse: GAGGCATTG CTGATGATCTTG. The amount of each mRNA was normalized to the amount of GAPDH in the same sample. Relative increases in mRNA expression were calculated using the $2-^{\Delta\Delta}$ ct method, as described previously [14].

2.4. NF-κB DNA-binding activity assay

NF-κB DNA-binding activity was analysed using the Trans AMNF-κB p65 transcription factor assay kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions and as previously described [15,16]. Briefly, nuclear extracts were prepared from freshly isolated PBMCs. Protein levels of the nuclear extracts were quantified with the Bradford assay (Pierce Chemicals, Rockford, IL) and 10 μg was incubated in a 96-well plate coated with oligonucleotide containing the NF-κB consensus-binding sequence 5′-GGGACTTTCC-3′. Bound NF-κB was then detected by a p65-specific primary antibody. An HRP-conjugated secondary antibody was then applied to detect the bound primary antibody and provided the basis for colorimetric quantification. The enzymatic product was measured at 450 nm with a reference wavelength of 650 nm by a microplate reader. To quantify the amount of NF-κB, serial dilutions of purified p65 recombinant protein (20–0.16 ng) were measured to provide a calibration curve between p65 binding and absorbance. The specificity of the assay was further tested by the addition of wild type or mutated NF-κB consensus oligonucleotide in the competitive or mutated competitive control wells before the addition of nuclear extracts. The addition of the wild-type NF-κB consensus oligonucleotide completely abolished NF-κB binding.

2.5. Statistical analysis

The significance of differences was assessed by ANOVA test, allowed by Bonferroni's multiple comparisons test for normally distributed values. Data with non-Gaussian distribution were analyzed by Kruskal Wallis test followed by Dunn's multiple comparisons test. Paired samples were analyzed using the Wilcoxon matched pairs test. A difference between groups was considered to be significant if $P < 0.05$. We used Spearman's rank correlation coefficient to assess relationships between two variables. All statistical analyses were performed using GraphPad Prism version 6 (GraphPadsoft-ware).

3. Results

We compared PF and blood samples from 30 women with endometriosis to those obtained from 20 women without endometriosis acting as controls. There was no significant difference in age between patients (mean ± SD: 32.0 ± 7.5 years) and controls (30.9 ± 8 years). Among 30 endometriosis patients studied, 17 had minimal/mild stage (stage I + II), 13 had moderate/severe stage (stage III + IV).

3.1. IL-37 expression in the serum and in the peritoneal fluid

Serum IL-37 levels were increased in patients with endometriosis compared to controls (132.38 ± 34.62 pg/mL versus 74.10 ± 13.49 pg/mL; $P < 0.0001$). The increase was more pronounced in moderate/severe cases [143.88 ± 35.71 pg/mL] than in milder ones [116.53 ± 27.17 pg/mL $P = 0.029$] (Fig. 1).

Similarly, PF from endometriosis patients exhibited higher levels of IL-37 [164.26 ± 61.14 pg/mL] compared to controls [68.90 ± 13.21 pg/mL; $P = 0.0001$] (Fig. 2). This increase of the IL-37 levels was dependent on disease severity (mild stage [130.82 ± 42.70 pg/mL] *v.s.* moderate/severe stage [208.0 ± 54.28 pg/mL; $P = 0.0002$]) (Fig. 2).

3.2. IL-37 gene expression in peritoneal fluid cells

Peritoneal fluid cells were used to assess IL-37 mRNA expression. As shown in Fig. 3, increased levels of IL-37 mRNA transcripts were detected in patients with endometriosis compared to controls [$P < 0.0001$].

To examine possible mechanisms of elevated IL-37 in PF of endometriosis patients, we quantified the activation of the transcription factor NF-κB, which has been shown previously to interact with interleukin-1 family cytokines [17]. NF-κB DNA binding activ-