Increased expression of interleukin-1 receptor type 1 in active endometriotic lesions

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Abstract

The establishment and progression of ectopic endometrial implants are dependent upon their interaction with and responsiveness to the stimuli present in their new environment. According to our and other previous studies, immune cells-derived cytokines, such as IL-1, may alone or in concert with estrogens, enhance the capability of ectopic endometrial cells to implant and develop into the host tissue. In the present study, immunohistochemical and dual immunofluorescence analyses showed that the functional signaling interleukin-1 receptor type 1 (IL-1RI) is expressed in endometriotic tissue, particularly in the glands, and identified endothelial cells, macrophages, and T-lymphocytes as cells having marked expression of IL-1RI. The highest concentrations of IL-1RI protein in endometriotic tissue, as evaluated using histological score (HSCORE) and measured by ELISA, were found in red endometriotic lesions as compared with typical black-blue or white lesions. Western blotting showed a significant increase in the levels of the 50 kDa band, whose apparent molecular weight corresponds to the soluble form of IL-1RI. RT-PCR analysis of IL-1 mRNA levels showed a pattern of expression comparable to that of the protein. Interestingly, IL-1RI expression was more significant in the proliferative than in the secretory phase of the menstrual cycle. Marked expression of IL-1RI, the functional signaling receptor that mediates cell activation by IL-1, in red endometriotic implants, which are highly vascularized and represent the earliest and most active forms of the disease, point to a higher cell receptivity for IL-1 in these lesions, a relationship with the activity of the disease and a possible involvement in the early steps of endometriotic tissue growth and development.

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Introduction

Endometriosis is a common gynecological disease that affects approximately 10–20% of women of reproductive age and is defined by the presence and the growth of endometrial-like tissue outside the uterus, mainly in the peritoneal cavity (Olive & Schwartz 1993). Common sites of growth include the ovary, the bladder, the intestine, and the pelvic peritoneum. Endometriotic implants resemble eutopic endometrium, histologically, and can in their active forms undergo endometrial-like cyclic menstrual changes with periodic bleeding. Although endometriosis is considered a benign proliferative disease, both mild and severe forms are frequently associated with abdominal pain, dysmenorrhea, dyspareunia, and infertility causing profound physical and psychological distress. The etiology of endometriosis is still not clearly elucidated, but appears to involve a complex interplay of multiple genetic, environmental, and immunological

factors (Lebovic *et al.* 2001a, Barlow & Kennedy 2005, Ulukus & Arici 2005).

Numerous endometriosis-associated immunological changes were detected in the eutopic endometrium and the peripheral blood (Giudice *et al.* 1998, Giudice 2003, D'Hooghe *et al.* 2004, Sharpe-Timms 2005), but were particularly observed locally in the peritoneal cavity where the disease often develops.

The peritoneal fluid of endometriosis patients was shown to contain elevated levels of autoantibodies, proinflammatory mediators, and inflammatory cells, especially activated macrophages. These cells are known to secrete growth factors and proinflammatory stimuli which may exacerbate the inflammatory reaction observed in the peritoneal cavity, but may also have a direct effect on ectopic endometrial cell growth and phenotype (Harada *et al.* 2001, Taylor *et al.* 2002, Bedaiwy & Falcone 2003, Wu & Ho 2003).

Inflammation around and within active endometrial implants, and increased leukocyte infiltration and cytokine secretion as compared to eutopic endometrial tissue have widely been described (Halme *et al.* 1987, Hill *et al.* 1988, Tseng *et al.* 1996, Jolicoeur *et al.* 1998, Jones *et al.* 1998, Witz 2002, Wu & Ho 2003). The secretion of proinflammatory and mitogenic proteins by endometriotic lesions and associated immune cells into the peritoneal environment may contribute to a cascade of events favoring tissue remodeling and invasion of the host tissue, angiogenesis and cell proliferation in the growing lesions and further chemoattraction of leukocytes to these foci of peritoneal inflammation. Cytokines are believed to participate in the induction and effector phases of immune and inflammatory reactions and to play an important role in the development of the endometriosis-related chronic inflammatory processes and tissue remodeling (Lebovic *et al.* 2001a, Taylor *et al.* 2002, Wu & Ho 2003).

Interleukin (IL)-1 is part of a family of polypeptides composed of IL-1 α ; IL-1 β ; and an inhibitor, IL-1 receptor antagonist (IL-1ra). These components interact with two main receptors: interleukin-1 receptor type I (IL-1RI), the functional signaling receptor which is found primarily on T cells, endothelial cells, and macrophages, transduces signals in concert with the co-receptor IL-1 receptor-accessory protein (IL-1RAcP) and is crucial for IL-1-mediated cell activation, and IL-1 receptor type II (IL-1RII), which is a decoy receptor with no signaling properties (Subramaniam *et al.* 2004).

Interleukin-1, a principal macrophage-derived and major proinflammatory cytokine, may play a central role in the integrated inflammatory cascade associated with endometriosis and in propagating endometriotic implants through a proinflammatory stimulus, synthesis of chemokines, growth, and angiogenic factors (Akoum *et al.* 1995, 1996, 2001, Lebovic *et al.* 2001b). Increased concentrations of the cytokine were found in the peritoneal fluid of women with endometriosis (Fakih *et al.* 1987, Mori *et al.* 1992, Taketani *et al.* 1992) as well as in ectopic endometrial implants (Bergqvist *et al.* 2001). In our previous studies, endometriotic cells showed a marked secretion of monocyte chemotactic protein-1 (MCP-1), regulated on activation normal T-cell expressed and secreted (RANTES), IL-6 and IL-8 following exposure to IL-1, and displayed a remarkable sensitivity for IL-1 (Akoum *et al.* 1995, 1996, 2001, 2002). In keeping with our findings, data from Lebovic *et al.* (2000) suggest that the ability of IL-1 β to activate an angiogenic phenotype in endometriotic stromal cells, but not in stromal cells from normal endometrium, is mediated by the functional signaling receptor IL-1RI.

The present study was undertaken to assess the expression of IL-1RI in endometriotic lesions, to identify the sites of expression, and to investigate whether such an expression varies according to the stage and the activity of the disease. Our study revealed a marked

expression of IL-1RI in the red highly vascularized lesions, which are believed to represent the earliest and most active forms of the disease, as compared with the typical black-blue and the white lesions. Furthermore, our data showed more significant expression of IL-1RI in proliferative phase than in secretory phase endometriotic lesions, thereby revealing cycle phase-dependent cell receptivity for IL-1 and a close relationship with the activity of the disease.

Materials and Methods

Subjects

Endometriotic tissue specimens used in this study were obtained from women ($n=53$) who provided informed consent for a research protocol approved by Saint-François d'Assise Hospital Ethics Committee on Human Research. These patients were found to have endometriosis during laparoscopy or laparotomy, had no endometrial hyperplasia or neoplasia, and had not received any anti-inflammatory or hormonal medication during a period of at least 3 months before surgery. Endometriosis was staged according to the revised American Fertility Society classification system (Anonymous 1997). The cycle phase (proliferative or secretory) was determined according to the cycle history and confirmed by histological examination of endometrial biopsies using the criteria of Noyes *et al.* (1975). The mean age was 32.0 ± 5.0 years.

Collection of endometriotic biopsies

Endometriotic biopsies were immediately placed at 4 °C in sterile Hank's balanced salt solution (HBSS; GIBCO Invitrogen Corp.) containing 100 U/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin, transported to the laboratory at which they were washed in HBSS at 4 °C, snap frozen on dry ice, and kept at -80 °C in eppendorf tubes for western blot, ELISA, and RT-PCR analyses or embedded in paraffin for immunohistochemical studies.

For ELISA, 30 endometriotic biopsies were included. These biopsies were from women with endometriosis stage I (six red, five typical, and four white), stage II (two red, seven typical, and two white), and stages III and IV (one red, one typical and two white).

For immunohistochemical studies, 18 endometriotic biopsies were included. These biopsies were from women with endometriosis stage I (one red, one typical, and three white), stage II (five red and three typical), and stages III and IV (one red, two typical, and two white).

For western blot analysis, 25 endometriotic biopsies were included. These biopsies were from women with endometriosis stage I (two red and two typical), stage II (four red, seven typical, and two white), and stages III and IV (one red, five typical, and two white).

For RT-PCR analysis, 30 endometriotic biopsies were included. These biopsies were from women with endometriosis stage I (five red, three typical, and five white), stage II (three red, six typical, and five white), and stages III and IV (one red and two white).

Immunohistochemistry

Paraffin-embedded tissue sections of 5 µm were mounted on poly-L-lysine-coated microscope glass slides, deparaffinized in toluene, rehydrated through graded solutions of ethanol and water, and washed in PBS. Sections were then permeabilized in PBS-1% Triton X-100 for 20 min at room temperature and treated with 0.3% H₂O₂ in methanol for 20 min at room temperature to eliminate endogenous peroxidase. Thereafter, sections were incubated for 30 min at room temperature with normal rabbit serum (1.5% in PBS containing 0.2% BSA and 0.01% Tween 20) to block non-specific binding, then 1 h at 37 °C in a humidified chamber with a polyclonal goat anti-human IL-1 receptor type I (primary antibody; R&D Systems, Minneapolis, MN, USA) used at 15 µg/ml in the PBS/BSA/Tween 20 solution. After three rinses with PBS/0.1% Tween 20, tissue sections were incubated for 45 min at room temperature with a biotin-conjugated rabbit anti-goat IgG (H+L; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA; 1:1000 dilution in PBS/BSA/Tween 20), rinsed with PBS/0.1% Tween 20, then incubated for 30 min at room temperature with peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc.; 1:500 dilution in PBS/BSA/Tween 20). After a final PBS rinse, the sections were stained for 20 min with 3,3'-diaminobenzidine (DAB; Sigma-Aldrich Canada Ltd; 0.5 mg DAB/0.03% H₂O₂ in PBS), rinsed in water, counterstained with hematoxylin and mounted in Mowiol (Calbiochem, La Jolla, CA, USA). Sections incubated with an equivalent concentration of normal goat immunoglobulins instead of the primary antibody were included as negative controls in all experiments. Normal endometrial tissue, which according to our previous studies, expresses IL-1RI receptor (Bigonnesse *et al.* 2001), was used as positive control. All sections were examined on a blind basis using a Leica microscope (mikroskopie und systeme GmbH, model DMRB, Leica Corp., Postfach, Wetzlar, Germany) with 40× objective. IL-1RI immunostaining was evaluated using a semi-quantitative histological score (HSCORE) which takes into account the distribution and the intensity of staining. The HSCORE was calculated with the following equation: $HSCORE = \sum Pi(i+1)$, where i = intensity of staining with a value of 1, 2, or 3 (weak, moderate, or strong respectively) and Pi is the percentage of stained cells for each intensity, varying from 0 to 100%. It is of note that endometriotic tissue sections showing no endometrial-like glandular structures were not included (four tissues out of 22, including one red, two typical, and one white lesions).

Dual immunofluorescent staining

Paraffin-embedded tissue sections were treated, then incubated for 30 min at room temperature with PBS/5% BSA (blocking solution) and incubated for 1 h at 37 °C with a polyclonal goat anti-human IL-1 receptor type I antibody (R&D Systems), at 15 µg/ml in PBS/BSA/Tween as described earlier (immunohistochemistry section). After a PBS/0.05% Tween 20 rinse, sections were incubated at room temperature for 90 min with one of the following antibodies: mouse monoclonal anti-human CD68 (DAKO Diagnostics Canada, Inc., Mississauga, ON, Canada; diluted 1:50 in PBS/BSA/Tween) to detect macrophages; mouse monoclonal anti-human CD3 (a gift from Dr W Mourad, Laval University, Quebec, Canada; diluted 1:100 in PBS/BSA/Tween) to detect T-lymphocytes; and mouse monoclonal anti-human von Willebrand factor (vWF; DAKO Diagnostics Canada, Inc.; diluted 1:25 in PBS/BSA/Tween) to detect endothelial cells. After a subsequent wash in PBS/0.05% Tween 20, tissue sections were incubated simultaneously for 60 min at room temperature in the dark with fluorescein isothiocyanate-conjugated donkey anti-goat antibody (Jackson ImmunoResearch Laboratories, Inc.; diluted 1:50 in PBS/BSA/Tween) and Alexa Fluor 594 labeled goat anti-mouse antibody (Molecular Probes Inc., Eugene, OR, USA; diluted at 2 µg/ml in PBS/BSA/Tween). After a final wash in PBS/0.05% Tween 20, slides were mounted in Mowiol containing 10% para-phenylenediamine (Sigma-Aldrich Canada Ltd), an anti-fading agent, and observed under the microscope (Leica Corp.) equipped for fluorescence with a 100 W u.v. lamp and connected to an image analysis system (ISIS, Meta-systems, Altlußheim, Germany).

IL-1RI ELISA

Proteins were extracted from frozen endometrial tissues. The tissues were directly homogenized with a micro-scale tissue grinder (Kontes, Vineland, NJ, USA) in a buffer containing 0.5% Triton X-100, 10 mmol/l HEPES (pH 7.4), 150 mmol/l NaCl, 2 mmol/l ethyleneglycoltetraacetic acid, 2 mmol/l ethylenediaminetetraacetic acid, 0.02% NaN₃, and a mixture of anti-proteases composed of 5 µmol/l aprotinin, 63 µmol/l leupeptin, and 3 mmol/l phenylmethylsulfonylfluoride (PMSF). Tissue homogenate was then incubated at 4 °C for 45 min under gentle shaking, and centrifuged at 11 000 g for 30 min to recover the soluble extract, whose total protein concentration was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories Ltd). IL-1RI concentrations were measured using an ELISA procedure developed in the laboratory. This assay uses a mouse monoclonal anti-human IL-1RI antibody (capture antibody) that does not cross-react with rhIL-1RII (R & D Systems) and a goat polyclonal anti-human IL-1RI antibody (detection antibody) that shows 5–10%

cross-reactivity with rhIL-1RII (R&D Systems). Briefly, 96-well plates were coated overnight at 4 °C with the mouse monoclonal anti-human IL-1RI antibody. Plates were washed four times with PBS containing 0.01% Tween 20 (washing buffer) and incubated 90 min at 37 °C with different concentrations of human recombinant soluble IL-1RI (0–6.4 ng/ml; R&D Systems), or with endometriotic tissue protein extracts diluted in PBS/0.5% BSA. The plates were then washed and incubated for 60 min at 37 °C with PBS/3% BSA (blocking solution). After washing, the plates were incubated with a goat polyclonal anti-human IL-1RI for 1 h at 37 °C, washed again and incubated for 60 min at 37 °C with a peroxidase-conjugated rabbit anti-goat antibody (Jackson ImmunoResearch Laboratories Inc.). After a final wash, 100 µl TMB (3,3',5,5'-tetramethylbenzidine)-peroxidase substrate (Bio-Rad Laboratories Ltd) was added to each well, the enzymatic reaction was terminated by the addition of 50 µl 2 N H₂SO₄ and the optical density was determined at 450 nm. IL-1RI concentrations were calculated by interpolation from the standard curve. The sensitivity limit of the assay based on rhIL-1RI was 20–25 pg/ml, and no detection of rhIL-1RII was noted.

Western blotting

Frozen endometriotic tissues were directly homogenized with the use of a microscale tissue grinder (Kontes) in a buffer (pH 7.4) containing 0.5% Triton X-100, 10 mM HEPES, 150 mM NaCl, 2 mM ethyleneglycoltetraacetic acid (EGTA), 2 mM ethylenediaminetetraacetic acid (EDTA), 0.02% NaN₃, and a mixture of anti-proteases composed of 5 µM aprotinin, 63 µM leupeptin, and 3 mM PMSF. Tissue homogenate was then incubated at 4 °C for 45 min under gentle shaking, and centrifuged at 11 000 g for 30 min to recover the soluble extract. Total protein concentration was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories Ltd). Proteins (50 µg) from each extract were heated in a boiling bath for 5 min in 5× SDS sample buffer (1.25 M Tris-HCl, 50% glycerol, 25% β-mercaptoethanol, 10% SDS, and 0.01% bromophenol blue, pH 6.8), separated by SDS-PAGE in 10% acrylamide linear gradient slab gels and transferred onto 0.2 µm nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) using electrophoretic transfer cell (Bio-Rad Laboratories Ltd). Human recombinant soluble IL-1RI (R&D Systems) was used as a positive control.

Equal loading in each lane was confirmed by staining blots with Ponceau S (2%). Nitrocellulose membranes were then immersed in PBS containing 5% skimmed milk and 0.1% Tween 20 (blocking solution) for 1 h at 37 °C, cut into strips and incubated overnight at 4 °C with a mouse monoclonal anti-human IL-1 receptor type I (R&D Systems; 2 µg/ml in PBS containing 1% BSA and 0.1% Tween 20) or with normal rabbit immunoglobulins

(IgGs; R&D Systems) at equivalent concentration. Thereafter, the strips were incubated for 45 min at 37 °C with peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.; 1:3000 dilution in PBS/BSA/Tween 20). Membranes were also blotted with an anti-α-actin antibody (1:1000 dilution in PBS/BSA/Tween 20; Iowa University developmental studies hybridoma bank (DSHB). After three washes (10 min each in PBS/0.1% Tween 20), the strips were incubated for 1 min with an ECL system: BM chemiluminescence blotting substrate (POD; Roche Diagnostics) and exposed to Biomax film (Kodak) from 30 s to 1 min for an optimal detection (all bands visible but not overexposed).

Reverse transcriptase (RT)-PCR

Total RNA was extracted from endometriotic tissue with TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies). Complementary deoxyribonucleic acid (cDNA) was synthesized using 500 ng total cellular RNA and 2.5 µM random hexamers in 20 µl solution containing 50 mM KCl, 10 mM Tris-HCl, 5 mM MgCl₂, 1 mM each of dNTPs, 20 U RNase inhibitor, and 50 U Reverse Transcriptase using Gene Amp PCR Core Kit (Perkin-Elmer, Foster City, CA, USA). The reaction was incubated at 25 °C for 15 min, 42 °C for 30 min, and 99 °C for 5 min.

For PCR analysis, we used 10% of the RT reaction volume as template in a final volume of 50 µl with 25 pmol each IL-1RI primer (forward primer, 5' GAT TCA GGA CAT TAC TAT TGC G 3'; reverse primer, 5' CTG GGA TCC CAA GTC TAC TTC C 3'; amplicon size 464 bp), 0.2 mmol/l dNTPs, and 1 U Vent DNA Polymerase (New England Biolabs Ltd, Mississauga, ON, Canada). Amplification was performed for 30 cycles of 30 s denaturation (95 °C), 30 s annealing (50 °C), and 1 min primer extension (72 °C). These optimal conditions were determined following linearity tests using 2.5, 5, 10, and 20% of RT reaction volume.

Amplification of genomic DNA with these primers did not produce a signal, suggesting that the amplification sites crossed at least one intron/exon boundary. Twenty percentage of the PCR volume was then analyzed on a 1.8% agarose gel in the presence of ethidium bromide and transferred to a 0.45 µm Biodyne B transfer membrane from Pall Life Sciences (VWR International Ltd, Montreal, QC, Canada). The membrane was then dehydrated at 37 °C for 30 min, prehybridized with a buffer comprising 5× SSC, 5× Denhardt's solution, 50 mM NaH₂PO₄, 0.5% SDS, 200 µg/ml salmon sperm DNA, and 50% formamide, hybridized with ³²P-labeled type 1 IL-1R cDNA in the same buffer (without Denhardt's solution) and washed with 1× SSC, 0.2× SSC, and 0.1% SDS respectively, before being exposed to X-ray film (Kodak) for about 1 h.

Glyceraldehyde-phosphate dehydrogenase (GAPDH) amplification was used as control. PCR used 25% of the RT reaction volume as template in a final volume of 50 μ l with 25 pmol each primer (forward primer, 5' TGA TGA CAT CAA GAA GGT GGT GAA G3'; reverse primer, 5' TCC TTG GAG GCC ATG TGG GCC AT 3'; amplicon size 240 bp), 0.2 mmol/l dNTPs, and 1 U Vent DNA Polymerase. Amplification was performed for 30 cycles of 30 s denaturation (95 °C), 30 s annealing (60 °C), and 1 min primer extension (72 °C). These optimal conditions were determined following linearity tests using 10, 25, and 50% of the RT reaction volume.

Specificity of the amplification process was verified by Southern blot hybridization. A negative control (PCR in absence of cDNA) as well as a positive control (cDNA preparation from human endometrial adenocarcinoma Ishikawa cells expressing IL-1RI) were included in each series of IL-1RI or GAPDH amplification. The quantity of the PCR products was determined by densitometric analysis of the intensity of the hybridization signal. The relative level of IL-1RI mRNA normalized to GAPDH mRNA was calculated, and the results were expressed as a percentage of the control value (positive control).

Statistical analysis

IL-1RI HSCORE, protein levels as measured by ELISA and western blotting, and mRNA levels as measured by RT-PCR followed a parametric distribution and were therefore analyzed using one-way ANOVA and the Bonferroni's *post hoc* test for multiple comparisons. Comparison of two groups was performed using the unpaired *t* test. All analyses were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). Differences were considered as statistically significant for *P* value <0.05.

Results

The first objective of this study was to assess the presence of IL-1RI in endometriotic lesions. Immunohistochemical analysis of IL-1RI expression showed a specific brownish immunostaining localized to specific compartments of endometriotic tissue. IL-1RI was found to be strongly expressed in glandular epithelial cells and stromal cells. Incubation of tissue sections with normal goat IgGs used at concentration equivalent to that of the primary goat polyclonal anti-human-IL-1RI antibody (negative control) did not result in any non-specific immunostaining (Fig. 1A). Human endometrial tissue from normal women, which according to our previous studies expresses IL-1RI (Bigonnesse *et al.* 2001), was used as positive control (data not shown). The intensity and distribution of IL-1RI immunostaining in endometriotic glands and stroma was then evaluated using HSCORE. As shown in Fig. 1B, HSCORE was signi-

Figure 1 Immunohistochemical analysis of IL-1RI expression in endometriotic tissue. (A) Note the intense brown immunostaining in the glands and cell aggregates throughout the stroma in the presence of a polyclonal goat anti-human IL-1RI antibody (a) and the absence of such staining in the presence of goat IgGs used at concentration equivalent to that of the primary antibody (b; negative control); scale bar, 30 μ m. (B) HSCORE in red (*n*=7), typical (*n*=6), and white (*n*=5) endometriotic lesions. G, gland, S, stroma; *, † and ‡ significant difference with red lesions (*P*<0.05, <0.01, and <0.001 respectively) as analyzed by ANOVA and the Bonferroni's test for multiple comparisons.

ficantly higher in red endometriotic lesions as compared with typical black-blue or white endometriotic lesions, both in the glandular (*P*<0.05 and <0.001 respectively) and the stromal (*P*<0.01 and <0.01 respectively) compartments. Further analysis of these data did not show any statistically significant difference between endometriosis stages I, II and, III and IV. However, IL-1RI HSCORE was significantly higher in the proliferative than in the secretory phase of the menstrual cycle in endometriotic glands (*P*<0.05). A tendency for a higher IL-1RI HSCORE in the proliferative compared with the secretory phase was also observed in the stroma, but the difference did not reach the level of statistical significance (*P*=0.14).

To identify cells expressing IL-1RI in the stroma, dual immunofluorescent staining was performed using antibodies specific to IL-1RI, CD3, CD68, and vWF. Representative photomicrographs exhibited in Fig. 2 show a marked expression of IL-1RI in CD3-positive T-lymphocytes, CD68-positive macrophages, and vWF-positive endothelial cells.

To further quantify IL-1RI expression in endometriotic tissue, we measured IL-1RI concentrations in total protein extracts by ELISA. As shown in Fig. 3, IL-1RI protein concentrations were higher in red than in typical black-blue (*P*<0.05) and white (*P*<0.01) endometriotic

Figure 2 Dual-immunofluorescent staining of IL-1RI (A–C) and CD3 (D), CD68 (E), or vWF (F) in endometriotic tissue. Tissue sections were incubated with goat polyclonal anti-human IL-1RI antibody and with mouse monoclonal anti-CD3, mouse monoclonal CD68, or mouse monoclonal anti-human vWF antibody. Sections were then incubated simultaneously with fluorescein isothiocyanate-conjugated donkey anti-goat antibody and rhodamine-conjugated sheep anti-mouse antibody to detect co-expression of IL-1RI with CD3, CD68, or vWF. Note the expression of IL-1RI (green) in CD3-, CD68-, and vWF-positive T-lymphocytes, macrophages, and endothelial cells respectively (red). Superposition of fluorescein (green) and rhodamine (red) signals clearly shows co-expression (yellow signal) of IL-1RI with CD3 (G = A + D), CD68 (H = B + E), and vWF (I = C + F). Scale bars, 30 µm.

lesions. No statistically significant difference in IL-1RI protein expression between endometriosis stages I, II, and III and IV was observed. Analysis of IL-1RI protein expression according to the phase of the menstrual cycle showed higher expression in the proliferative than in the secretory phase ($P < 0.05$).

Western blot analysis of protein extracts from endometriotic tissue using a mouse monoclonal anti-human-IL-1RI antibody showed two specific bands at approximately 90 and 50 kDa, whose molecular weight are comparable to the known molecular weight of the membrane bound and the soluble form of IL-1RI (Fig. 1A). Densitometric analysis of IL-1RI bands in protein extracts from different types of endometriotic lesions showed no significant difference in the intensity of the 90 kDa band between red, typical, and white endometriotic lesions. However, the 50 kDa band was significantly more intense in red endometriotic lesions compared with typical endometriotic lesions ($P < 0.05$). The 50 kDa band was also more intense in red compared with white endometriotic lesions, though the difference was not statistically significant ($P = 0.087$; Fig. 4B).

We further analyzed IL-1RI expression at the mRNA level using RT-PCR and Southern blotting. Figure 5A shows specific amplification of a 464 bp IL-1RI cDNA fragment from control Ishikawa cells and endometriotic tissue. Analysis of Southern blot data from different types of endometriotic lesions showed a more marked expression of IL-1RI mRNA in red than in typical black-blue ($P < 0.05$) and white ($P < 0.01$) lesions (Fig. 5B). Analysis of IL-1RI mRNA expression as to endometriosis stage and the menstrual cycle phase showed here again a more marked expression in the

proliferative than the secretory phase ($P < 0.05$), whereas no statistically significant difference between endometriosis stages I, II, and III and IV was noted.

Discussion

Endometriosis might be a multifactorial disease and its etiology remains hypothetical. The presence of tissue outside the uterine cavity structurally and to some extent functionally comparable with the endometrium suggests that the condition, at least peritoneal endometriosis, results from implantation of exfoliated endometrium following retrograde menstruation (Sampson 1927).

Figure 3 Graphical illustration of IL-1RI concentrations (ng/mg of total proteins) as measured by ELISA in endometriotic tissue. Endometriotic implants were classified according to their appearance at laparoscopy (red, $n = 9$; typical, $n = 13$; and white, $n = 8$); *, † significant difference with red lesions ($P < 0.05$ and < 0.01 respectively) as analyzed by ANOVA and the Bonferroni's test for multiple comparisons.

Figure 4 (A) Representative western blot analysis of IL-1RI expression in endometriotictissue. Human recombinant soluble IL-1RI (lanes 1 and 3) and endometriotictissue protein extracts (lanes 2 and 4) were subjected to SDS-PAGE analysis and western blotting using a mouse monoclonal anti-human IL-1RI antibody (lanes 1–2) or an equivalent concentration of normal mouse IgGs instead of the primary antibody (lanes 3–4). The detected bands had an estimated apparent molecular weight of approximately 90 and 50 kDa. (B) Densitometric analysis of IL-1RI bands normalized to corresponding α -actin bands from endometriotictissues (red, $n=7$; typical, $n=14$; and white, $n=4$); *significant difference with red lesions ($P<0.05$) as analyzed by ANOVA and the Bonferroni's test for multiple comparisons.

Accordingly, the ectopic development of endometrial tissue requires factors that are capable of remodeling the host tissue and sustaining endometrial tissue growth and the genesis of new microvessels.

Beside the intrinsic dysfunctions found in the eutopic endometrial tissue of women with endometriosis that may favor its ectopic development (Sharpe-Timms 2005), the establishment and progression of endometrial implants are dependent upon their interaction with and responsiveness to the stimuli present in their new environment. According to our and other previous studies, immune cells-derived cytokines, such as IL-1, may alone or in concert with estrogens, enhance the capability of ectopic endometrial cells to implant and develop into the host tissue, by inducing an angiogenic phenotype and stimulating the production of proinflammatory, growth, and tissue remodeling factors (Akoum *et al.* 2000, 2001, 2002, Taylor *et al.* 2002, Seli & Arici 2003, Wu & Ho 2003, Osteen *et al.* 2004). Furthermore, our and other previous studies showed

Figure 5 (A) Representative RT-PCR and Southern blot analysis of IL-1RI transcripts. Total RNA obtained from endometriotictissue was reverse transcribed, amplified with IL-1RI (upper lanes) or GAPDH (lower lanes) primers, and hybridized with 32 P-labeled corresponding probes, as described in Materials and Methods. Lane 1, negative control (PCR in the absence of cDNA); lanes 2–4, linearity test with different RT volumes; lane 5, positive control (cDNA preparation from human endometrial adenocarcinoma Ishikawa cell line which expresses IL-1RI). (B) Graphical illustration of IL-1RI mRNA steady-state levels in endometriotictissues as assessed by RT-PCR and Southern blotting. The relative level of IL-1RI mRNA normalized to GAPDH mRNA was determined, and the results were expressed as percentage of control (positive control). Endometriotictissues were classified according to their appearance at laparoscopy (red, $n=9$; typical, $n=9$; and white, $n=12$). *, †Significant difference with red lesions ($P<0.05$ and <0.01 respectively) as analyzed by ANOVA and the Bonferroni's test for multiple comparisons.

augmented sensitivity of endometriotictcells to the biological actions of IL-1 (Akoum *et al.* 1995, 1996, 2001, 2002, Lebovic *et al.* 2000), which may account for their ability to favor their own ectopic growth.

The present study showed that the functional receptor type I of IL-1, which mediates IL-1-induced cell activation, was expressed in ectopic endometrial tissue and that in different cell types. IL-1RI expression was quite obvious in endometriotictglandular and stromal cells. Dual immunofluorescence analysis further identified endothelial cells and inflammatory cells, such as macrophages and T-lymphocytes, as cells markedly expressing IL-1RI in the stroma. These findings are suggestive of a relationship between IL-1RI expression and the implant's activity considering that active endometriotictlesions show a more marked vascularization and leukocyte infiltration (Halme *et al.* 1987, Hill *et al.* 1988, Wiegerinck *et al.* 1993, Kokorine *et al.* 1997, Jones *et al.* 1998, McLaren 2000, Witz 2002, Taylor & Mueller 2004).

Endometriotic lesions can be classified according to their appearance and to their activity. In fact, lesions of the peritoneal lining of the pelvis have various macroscopic appearances, which reflect their age and/or activity. The red subtle lesions are more vascularized and have a higher epithelial mitotic index than the typical, puckered black or bluish peritoneal lesions, whereas the vascularization and the mitotic index are lower in the white lesions. Thus, red lesions are thought to correspond to the first, active stage of early implantation of endometrial glands and stroma, and would evolve toward the typical black or bluish lesion after enclosure beneath the peritoneal lining. The white lesions, which are believed to correspond to fibrotic quiescent lesions, show less vascularization and/or mitotic activity and represent less active forms of the disease (Nisolle *et al.* 1993, Wiegerinck *et al.* 1993, Kokorine *et al.* 1997, Nisolle *et al.* 1997). Interestingly, our present study revealed that IL-1RI expression was significantly higher in red than in typical black-blue or white endometriotic lesions. The protein expression, as assessed by HSCORE in endometriotic glands and stroma and measured by ELISA according to endometriotic lesion type was in keeping with that of mRNA as assessed by RT-PCR. This indicates that reduced expression of IL-1RI in more advanced endometriotic lesions occurs at the mRNA level, and is more likely due to a reduced mRNA synthesis and/or to mRNA instability than to translational and/or post-translational events. Of note is the 50 kDa band, whose molecular weight corresponds to the soluble IL-1RI, which was significantly increased in red endometriotic lesions and markedly more intense than the 90 kDa band corresponding to the membrane-bound receptor. This result is intriguing as soluble IL-1RI may, by interacting with IL-1, prevent cell activation via the membrane-bound signaling receptor. However, such a putative inhibitory property of soluble IL-1RI is hampered by its capability to interact as well with IL-1 receptor antagonist (IL-1ra), which is a potent inhibitor of IL-1 (Boraschi *et al.* 1996). The inhibitory activity of IL-1ra was even shown to be enhanced by soluble IL-1RII and hindered by soluble IL-1RI (Burger *et al.* 1995). According to our and other previous data, endometriotic cells are highly sensitive and responsive to IL-1 (Akoum *et al.* 1995, 2000, 2001, 2002), and secrete increased levels of angiogenic factors compared with endometrial cells from normal women (Lebovic *et al.* 2000). Also, IL-1RI is, in contrast to the decoy inhibitory IL-1RII, slightly released in the culture medium of endometrial and endometriotic cells (personal data). Further studies will be required to elucidate whether the predominant detection of the soluble IL-1RI, compared to the membrane-bound IL-1RI, was rather due to or amplified by the experimental conditions.

The findings of the present study may have a great relevance since red endometriotic lesions are believed to

represent the earliest and most active forms of endometriosis. Therefore, increased IL-1RI expression may reflect increased endometriotic cell receptivity to IL-1 in these lesions and a close relationship with the activity of the disease, and point to a process of cell activation, through IL-1 signaling, that takes place in the ectopic endometrial tissue, particularly in its earliest forms. In the present study, no statistically significant difference in IL-1RI expression between endometriosis stages I, II, and III and IV was found. Although more samples are required to ascertain the relationship between the evolution of endometriosis stage and IL-1RI expression, our data suggest that IL-1RI expression is more related to the lesion's type and activity than to endometriosis stage.

Ectopic implantation and growth of endometrial tissue following retrograde menstruation requires new active tissue remodelling and blood supply to enable a successful implantation of the migrating tissue in the peritoneal cavity. Early and most active endometriotic lesions are markedly vascularized and increased vascularization is seen at the implant's surface as well as also in the surrounding peritoneal tissue (Nisolle *et al.* 1993, 1997, Wiegerinck *et al.* 1993, Kokorine *et al.* 1997), suggesting that endometriotic implants are capable of inducing its own neovascularization by deriving local microvasculature. Evidence available to date supports a central role for activated immune cells, particularly macrophages and their secretory products, in the progression of endometriotic implants and offers an immunological explanation for the neovascularization that surrounds these lesions (Taylor *et al.* 2002). IL-1 is a prototypic example of an activated peritoneal macrophage secretory product in endometriosis. Macrophages from women with endometriosis were found to secrete elevated levels of IL-1 (Zeller *et al.* 1987). Elevated concentrations of IL-1 were found in the peritoneal fluid of women suffering from endometriosis (Fakih *et al.* 1987, Mori *et al.* 1992, Taketani *et al.* 1992). IL-1 would have more pronounced effects on adhesion in endometriosis (Sillem *et al.* 1999). Activation and recruitment of immune cells into endometriotic implants can be mediated by other secreted cytokines and chemokines such as MCP-1, RANTES, IL-6, and IL-8, which were found to be up-regulated by IL-1 in endometriotic cells (Akoum *et al.* 1995, 1996, 2001, 2002) and could in turn promote IL-1 secretion by activated immune cells. IL-1 induces secretion of IL-8 and IL-6, which exhibit proangiogenic properties (Akoum *et al.* 1996, 2001). Data from Lebovic *et al.* (2000) showed an enhanced production of angiogenic molecules such as vascular endothelial growth factor (VEGF) and IL-6 in response to IL-1 β in endometriotic cells, as compared with endometrial cells from normal endometrium, and increased expression of IL-1RI mRNA, suggesting a role for IL-1 β ligand binding and signaling via IL-1RI. Therefore, in view of IL-1 biological effects, marked IL-1RI expression in active and early endometriosis lesions may play a

significant role in promoting a successful implantation of endometrial tissue in the earliest steps of endometriosis following retrograde menstruation.

In the present study, we analyzed IL-1RI expression according to the menstrual cycle phase and found that both protein and mRNA expression of the receptor were significantly higher in the proliferative than in the secretory phase of the menstrual cycle. Interestingly, previous studies from our and other laboratories showed that IL-1RI expression in the endometrial tissue was particularly elevated in the late secretory phase of the menstrual cycle (Simon *et al.* 1993, Bigonnesse *et al.* 2001). This suggests that endometrial tissue debris, which migrate into the peritoneal cavity at the end of the menstrual cycle and may develop into endometriosis lesions, express the highest levels of IL-1RI, and may therefore be more responsive to IL-1. This may lead to an exaggerated peritoneal inflammatory response; however, it is still to be determined whether IL-1RI expression in the menstrual endometrial tissue is more elevated in women with endometriosis than in normal women. Moreover, it is quite possible that red hemorrhagic implants, which follow the cyclical changes of the menstrual cycle and express higher levels of IL-1RI in the proliferative phase, would produce higher levels of growth, mitogenic, and proinflammatory factors in response to IL-1, thereby favoring tissue growth and implantation in the host tissue during that phase. In agreement with this hypothesis is the finding that VEGF concentrations in the peritoneal fluid of women with endometriosis are significantly higher in the peritoneal fluid during the proliferative phase compared with the secretory phase (McLaren *et al.* 1996).

In conclusion, the present study showed IL-1RI expression in various cell types throughout endometriotic tissue and its marked expression in lesions representing the earliest and most active stages of the disease. In view of IL-1-mediated changes in endometriotic cells, this may play a significant role in the initial steps of ectopic endometrial tissue growth and the pathophysiology of endometriosis. Future investigations are needed to determine whether the up-regulation of the IL-1RI is a result of endometrial cell interaction with a cytokine-rich environment in the ectopic sites, as IL-1 was shown to up-regulate its own receptor (Simon *et al.* 1994), and/or the successful selection of highly cytokine-sensitive endometrial cells displaced into the peritoneal cavity by retrograde menstruation.

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