Interleukin-1 receptor accessory protein is constitutively expressed in human endometrium throughout the menstrual cycle

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Interleukin-1 (IL-1) is one of the principal cytokines that participate in endocrine and local regulation of many endometrial and reproductive functions. The cellular response to IL-1 principally implicates receptor type 1 (IL-1R tI) and, according to recent data, an accessory protein (IL-1R-AcP) that seems to play an essential function in signal transduction. In the present study, we examined the expression of IL-1R-AcP in the endometrium of 39 normal fertile women throughout the menstrual cycle. As studied by immunohistochemistry, IL-1R-AcP was detected across endometrial tissue, but more noticeably in the glands and luminal epithelium. The intensity of IL-1R-AcP immunostaining was consistently high throughout the menstrual cycle, and this was confirmed by Western blot analysis of the protein and corroborated by reverse transcription-polymerase chain reaction analysis of the mRNA. To our knowledge, this is the first report showing that IL-1R-AcP is expressed in endometrial tissue, and without any noticeable variation throughout the menstrual cycle. This suggests that the accessory protein, whose co-expression is critical for IL-1R tI-mediated cell activation, is, in contrast to the functional receptor, constitutively expressed and not subject to similar cycle-dependent regulation.

Key words: endometrium/interleukin-1/IL-1R-AcP/menstrual cycle

Introduction

Interleukin-1 (IL-1) is an important pro-inflammatory cytokine that plays a critical role in the generation of inflammatory responses and the initiation of many normal biological events (Dinarello, 1996). This cytokine is involved in several reproductive functions, and plays an important role in the numerous changes occurring in the human endometrium during the menstrual cycle (Tabibzadeh, 1991a,b). This is not surprising because these changes are similar to those that occur during inflammatory and reparative processes. The IL-1 family comprises three members: two agonists, IL-1α and IL-1β, and one antagonist, the IL-1 receptor antagonist (IL-1ra). These ligands bind to two main receptors: IL-1R type I (IL-1R tI), which is found primarily on T cells, endothelial cells and macrophages, and is crucial for IL-1-mediated signalling events (Sims et al., 1988, 1993) and the IL-1R type II (IL-1R tII), which is a decoy receptor not required for signal transduction (Horuk and McCubrey, 1989; Colotta et al., 1993). Other studies have identified the 66 kDa IL-1R accessory protein (IL-1R-AcP), which does not itself recognize the ligand (Greenfeder et al., 1995), but increases the receptor affinity for IL-1 and appears to be an essential second subunit of the functional IL-1R tI (Greenfeder et al., 1995). In an IL-1 non-responsive cell line expressing IL-1R tI but not IL-1R-AcP, cell responsiveness to IL-1 was restored following transfection with IL-1R-AcP cDNA (Wesche et al., 1996, 1997; Korherr et al., 1997). The extracellular domain of IL-1R-AcP contains 340 amino acid residues divided into three immunoglobulin domains. The transmembrane and intracellular domains contain 29 and 181 amino acids respectively. A soluble form of IL-1R-AcP (sIL-1R-AcP) has also been found and appears to arise from alternative splicing of the IL-1R-AcP gene (Jensen et al., 2000). The association between IL-1R tI and IL-1R-AcP forms a high affinity receptor for IL-1α and IL-1β. It is suggested that IL-1R tI and IL-1R-AcP become associated only after IL-1 binds to IL-1R tI and may involve a permissive conformational change that facilitates recruitment of IL-1R-AcP or down-stream signalling components (Jensen et al., 2000). IL-1R-AcP also seems to act as a stabilizer of the IL-1 receptor signalling complex (Wesche et al., 1998) and appears to be essential for both IL-1-mediated NFkB activation and
recruitment of IL-1 receptor-associated protein (IRAK) to the IL-1R complex (Huang et al., 1997).

Many studies have demonstrated the implication of the IL-1 system in human endometrium, particularly during the implantation process and in the inflammatory-like processes taking place at the end of the menstrual cycle when implantation fails or does not occur. IL-1 has been shown to be secreted by the human embryo and is thought to act as embryonic signal (De Los Santos et al., 1996). Successful implantation after IVF has been correlated to high concentrations of both IL-1α and IL-1β in the culture media of human embryos (Sheth et al., 1991). IL-1β mRNA has been detected in the secretory phase, beginning on day 23 of the menstrual cycle (Kauma et al., 1990). Moreover, IL-1R tI has been detected in total human endometrium (Simon et al., 1993) and was shown to have a triphasic expression throughout the menstrual cycle (Bigonnesse et al., 2001). Normal reproduction in transgenic mice lacking a functional IL-1R tI has been reported (Abbondanzo et al., 1996). However, blockade of maternal endometrial IL-1R tI with IL-1ra has been shown to prevent implantation in the mouse by interfering with embryonic adhesion through a direct effect on epithelial plasma membrane transformation at the time of implantation (Simon et al., 1998). Altogether these studies suggest that IL-1R-AcP may be present in human endometrium and participate in endometrial and reproductive functions.

The objective of the present study was to examine the protein and mRNA expression levels of IL-1R-AcP in the endometrium throughout the menstrual cycle of normal

Figure 1. Immunohistochemical staining of interleukin-1 receptor accessory protein (IL-1R-AcP) in human endometrium. The immunoreaction was developed by the avidin-biotin-peroxidase complex using diaminobenzidine as a chromogen, and the sections were counterstained with haematoxylin (blue staining). Note the intense positive brown staining obtained with mouse monoclonal anti-IL-1R-AcP in the glandular and luminal epithelium (A) (day 6). No immunostaining was observed in control sections incubated with mouse immunoglobulins of the same isotype and concentration as the primary antibody (B). Representative illustrations of IL-1R-AcP immunostaining in the endometrial tissue throughout the menstrual cycle are shown: (C) day 10; (D) day 14; (E) day 20; (F) day 26. Note the brown positive immunostaining throughout endometrial tissue, particularly in glandular and luminal epithelium. g = gland; s = stroma; se = surface epithelium; v = blood microvessel. Scale bar = 20 µm.
and treated with 0.3% H2O2 in methanol for 20 min at room temperature, then permeabilized with Triton X-100 [1% in phosphate (Fisher Scienti, Nepean, ON, Canada)] for 20 min at room temperature, fixed in 10% buffered formalin xed in 10% buffered formalin, then permeabilized with Triton X-100 [1% in phosphate buffered saline (PBS) for 20 min at room temperature] and treated with 0.3% H2O2 in methanol for 20 min at room temperature to block endogenous peroxidase activity. Immunostaining was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). After blocking with normal horse serum (1.5% in PBS) for 30 min at room temperature, the sections were incubated for 120 min with monoclonal mouse anti-human IL-1R-AcP (primary antibody) (Transduction Laboratories, Lexington, KY, USA) used at 5 µg/ml in PBS containing 1% bovine serum albumin (BSA) and 0.05% Tween 20. Different concentrations of the primary antibody (3, 4, 5 and 6 µg/ml) were tested to determine the optimal concentration. This experiment was performed three times on three different series of biopsies taken from the different phases of the menstrual cycle. The concentration 5 µg/ml was selected as it allowed an optimal ratio of specificity (minimal background) and sensitivity (detectable positive signal). After three rinses with PBS, 0.05% Tween 20, tissue sections were incubated for 60 min at room temperature with a biotinylated horse anti-mouse polyclonal antibody, rinsed with PBS, 0.05% Tween 20, then incubated for 45 min at room temperature with the avidin-biotinylated horseradish peroxidase complex. After a final PBS rinse, the sections were stained for 10 min with 3,3′-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO, USA) (0.5 mg DAB/0.03% H2O2 in PBS), rinsed in water, counterstained with haematoxylin, and mounted in Mowiol (Calbiochem-Novabiochem Corp., La Jolla, CA, USA). Sections incubated with an equivalent concentration of normal mouse immunoglobulins instead of the primary antibody were included as negative controls in all experiments. Slides were viewed using a Leica microscope (Leica Mikroskopie und systeme GmbH, Model DMRB, Postfach, Wetzlar, Germany) and were photographed with Kodak 100 ASA film (Kodak, Toronto, ON, Canada). The resulting staining was quantified visually using an arbitrary scale (0 = absent, 1 = light, 2 = moderate and 3 = intense) by two independent observers who had no information regarding the menstrual cycle day and the gynaecological status of patients. High concordance between the two observers was found as determined by the kappa (κ) measure of agreement for IL-1R-AcP expression score in epithelial cells (κ = 0.94).

Western blotting
Frozen endometrial tissues were directly homogenized with the use of a microscale tissue grinder (Kontes, Vineland, NJ, USA) in a buffer containing 0.5% Triton X-100, 10 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM ethylene glycol tetra-acetic acid (EGTA), 2 mM ethylene diamine tetra-acetic acid (EDTA), 0.02% NaN3 (Tabibzadeh et al., 1995) and a mixture of antiproteases composed of 5 µg/ml aprotinin, 63 µg/ml leupeptin, and 3 µmol/l phenylmethyl-sulphonylfluoride. Tissue homogenates were 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, Mississauga, ON, Canada). Proteins (100 µg) from each extract were heated in a boiling bath for 5 min in 5× sodium dodecyl sulphate (SDS) sample buffer (1.25 mol/l Tris–HCl pH 6.8, 50% glycerol, 25% β-mercaptoethanol, 10% SDS and 0.01% bromophenol blue), separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% acrylamide slab gels and transferred onto 0.45 µm nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA) using an electrophoretic transfer cell (Bio-Rad). Equal loading in each lane was confirmed by staining the 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 for 60 min at 37°C with a monoclonal mouse anti-human IL-1R-AcP (Transduction Laboratories) (0.1 µg/ml in PBS containing 1% BSA and 0.1% Tween 20) or with normal mouse immunoglobulins (IgG) (R&D Systems, Minneapolis, MN, USA) at equivalent concentration. Thereafter, the strips were incubated for 1 h at 37°C with Fe-specific peroxidase-labelled goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA).
Figure 3. Western blot analysis of interleukin-1 receptor accessory protein (IL-1R-AcP) expression in the endometrium throughout the menstrual cycle. Equal amounts of proteins obtained from the entire endometrial tissue (A) (100 µg/lane) or from separated stromal and glandular epithelial cells (B) (15 µg/lane) were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis and Western blotting. (A) Lane 1, day 7; lane 2, day 14; lane 3, day 17; lane 4, day 21; lane 5, day 23; lane 6, day 27; lane 7, positive control (total lysate from a human endothelial cell line); lane 8, negative control (normal mouse IgG). (B) Lanes 1 and 2, day 3; lanes 3 and 4, day 10; lanes 5 and 6, day 13; lanes 7 and 8, day 16; lanes 9 and 10, day 22; lanes 11 and 12, day 28; stromal cells, uneven lane number; epithelial cells, even lane number. The detected band has an estimated apparent mol. wt of ~60 kDa.

RT-PCR

Total RNA was extracted from endometrial tissues and separated cells (see below) with TRIzol reagent according to the manufacturer's instructions (Gibco BRL). cDNA was synthesized using 500 ng of total cellular RNA and 2.5 µmol/l random hexamers in 20 µl of a solution containing 50 mmol/l KCl, 10 mmol/l Tris–HCl, 5 mmol/l MgCl₂, 1 mmol/l each of dNTPs, 20 IU RNase inhibitor, and 50 IU 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 20% of the reverse transcription (RT) reaction volume as template in a final volume of 50 µl with 25 pmol of each IL-1R-AcP primer (forward primer, 5’CAC TTC TGT GTG GTG TAG TGA3’; reverse primer, 5’AAT GCA ACT TGT CTA CAA TAT G3’; amplimer size 378 bp), 0.2 mmol/l dNTPs, and 1 IU Vent DNA Polymerase (New England Biolabs, Beverly, MA, USA). Amplification was performed for 35 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 10, 25, 50 and 75% of the RT reaction volume.

Cell separation

Endometrial tissue was minced into small pieces and dissociated with collagenase (Sigma Chemical Co.) to separate epithelial glands from fibroblast-like cells using differential sedimentation and adhesion and Percoll density gradients (Amersham Pharmacia Biotech, Inc., Baie d’Urfe, QC, Canada) according to our previously reported procedure (Akoum et al., 1995). The purity of epithelial or fibroblast-like stromal cells was verified morphologically, and immunocytochemically on coverslip culture using antibodies specific to cytokeratins (epithelial cell marker), vimentin (stromal cell marker), smooth muscle α-actin and factor VIII (endothelial cell marker) as previously described (Akoum et al., 1995). There were no cytokeratin-positive cells detectable in stromal cells, whereas epithelial cells were contaminated by a small number of stromal cells (~1%). Furthermore, flow cytometric analyses have shown that either epithelial or stromal cells contained <0.5% contaminating CD45-positive cells (leukocytes) and factor VIII-positive cells (endothelial cells).
obtained from the whole endometrial tissue (A) or from separated stromal and glandular epithelial cells (B) were reverse transcribed, amplified with IL-1R-AcP or glyceraldehyde dehydrogenase (GAPDH) primers, and hybridized with 32P-labelled corresponding probes, as described in Materials and methods. (A) Lane 1, day 9; lane 2, day 13; lane 3, day 17; lane 4, day 23; lane 5, day 26; lane 6, day 28. (B) Lanes 1 and 2, day 1; lanes 3 and 4, day 9; lanes 5 and 6, day 14; lanes 7 and 8, day 20; lanes 9 and 10, day 27; stromal cells, uneven lane number; epithelial cells, even lane number; IL-1R-AcP amplier size = 378 bp; GAPDH amplier size = 240 bp.

**Statistical analyses**

IL-1R-AcP scores follow an ordinal scale. Therefore, statistical analyses were performed using non-parametric methods. The association and correlation between the day of menstrual cycle and the immunohistochemistry scores were evaluated using Fisher’s exact test and Spearman’s correlation coefficient, respectively. All analyses were performed using statistical analysis system (SAS Institute Inc., Cary, NC, USA). P < 0.05 was considered statistically significant.

**Results**

**Immunohistochemical analysis of IL-1R-AcP expression**

Positive immunohistochemical staining of IL-1R-AcP was detected across endometrial tissue, in both epithelial and stromal compartments. Immunostaining was quite obvious in microvascular endothelial cells which were first prominent in late-secretory phase endometrial tissues, but was particularly intense in glandular and surface epithelia (Figure 1). The brown positive immunoreaction that occurred in the presence of mouse monoclonal anti-human IL-1R-AcP antibody was virtually absent when the latter was replaced by an equal concentration of mouse immunoglobulins of the same isotype (Figure 1). The intensity of epithelial cell staining in endometrial sections from 39 fertile women was scored using an arbitrary scale, and the distribution of immunostaining scores according to day of the menstrual cycle is shown in Figure 2. This graph clearly shows that IL-1R-AcP follows an invariable pattern of expression throughout the menstrual cycle. Statistical analysis of the data using Spearman’s correlation coefficient showed no correlation between immunostaining scores and the day of menstrual cycle (r = -0.0087, P = 0.9583).

**Western blot analysis of IL-1R-AcP expression**

To confirm the immunohistochemical data, total proteins extracted from endometrial tissue and separated stromal and epithelial cells were subjected to SDS-PAGE followed by Western blotting (Figure 3). As shown in Figure 3, only one band having an estimated apparent molecular weight of ~60 kDa was detected by the monoclonal anti-IL-1R-AcP in the whole tissue and separated cells. For the same amount of proteins, the intensity of the 60 kDa band was comparable in tissues from different cycle phases (Figure 3A). Separated glandular epithelial cells clearly showed a higher level of protein expression than did stromal cells, but, consistent with the immunohistochemical data, no cycle-dependent change in the protein expression within each of the two cell populations was noted (Figure 3B). Total lysate from a human endothelial cell line, used as positive control for IL-1R-AcP, showed a band of a similar molecular weight, while no immunoreactive bands were observed when the primary antibody was replaced by an equal concentration of mouse immunoglobulins of the same isotype.

**RT-PCR analysis of IL-1R-AcP expression**

RT-PCR and Southern blot analyses of IL-1R-AcP mRNA expression were first performed on the whole endometrial tissue. As shown in Figure 4A, transcripts for IL-1R-AcP were detected throughout the menstrual cycle and followed a pattern of expression similar to that of the protein. These experiments were then performed on separated stromal and glandular epithelial cells. As shown in Figure 4B, each of these two cell populations was shown to express IL-1RAcP mRNA, although a weaker signal in stromal cells compared to epithelial glandular cells was sometimes observed. The intensity of the signal varied slightly between patients, but did not appear to be dependent on the menstrual cycle periods.

**Discussion**

In the present study, we have shown the presence and localization of IL-1R-AcP in the endometrium of fertile women throughout the menstrual cycle. The protein expression, as studied by immunohistochemistry, was detected in endometrial tissue, primarily in epithelial glands and luminal epithelium and less markedly in the stroma. Furthermore, IL-1R-AcP expression was consistently high throughout the menstrual cycle and showed no noticeable cycle-dependent variations. This invariable pattern of expression has been confirmed by analysis of IL-1R-AcP protein in the endometrial tissue and in separated stromal and glandular epithelial cells by Western blot. IL-1R-AcP mRNA, as evaluated by RT-PCR, also followed a similar pattern. These results might be of considerable interest because of the critical role that IL-1R-AcP plays in signal transduction in association with IL-1R tI. To our
knowledge, this is the first report showing that IL-1R-AcP may be constitutively expressed in the endometrium throughout the menstrual cycle.

These findings are consistent with recent studies showing high level of expression for IL-1R-AcP in mouse brain and lack of regulation during inflammatory episodes triggering the IL-1 system (Gabellec et al., 1996b; Ilyin et al., 1998), suggesting that the protein might be constitutively expressed in excessive amounts.

The expression of IL-1R-AcP in the human endometrial tissue contrasts with that of IL-1R. In a previous study, we showed that IL-1R tI followed a triphasic pattern of expression throughout the menstrual cycle in the endometrium of normal fertile women (Bigonnesse et al., 2001). The expression was low in the proliferative phase, moderate between the ovulation and the implantation period, and most intense at the end of the menstrual cycle in the late secretory phase. Our results suggest that the accessory protein, whose co-expression is critical for IL-1R tI-mediated cell activation, is, in contrast to the functional receptor, not subject to similar cycle-dependent regulation. It has been reported that cell stimulation with IL-1 or IL-10 did not affect the level of IL-1R-AcP expression or the proliferation of a Th2 cell line (Yoon and Dianrello, 1998). A high concentration of IL-1 down-regulated the gene expression of IL-1R tI (Ye et al., 1992; Yoon and Dianrello, 1998) but the changes in IL-1R-AcP mRNA were not significant (Yoon and Dianrello, 1998). IL-10 increased IL-1R tI expression, but had no effect on that of IL-1R-AcP (Yoon and Dianrello, 1998). Moreover, injection of lipopolysaccharide (LPS) has been shown to increase IL-1R tI mRNA in mouse brain (Gabellec et al., 1996a,b; Ilyin et al., 1998), but the level of IL-1R-AcP transcripts was not affected. All these data are in keeping with our results and suggest a constitutive and non-regulated expression of IL-1R-AcP in the human endometrium. This may be important as the regulation of endometrial cell responsiveness to IL-1, a cytokine playing a major role in the various remodeling and reproductive processes arising in the human endometrium during the menstrual cycle, does not seem to involve a regulation of IL-1R-AcP, which appears to be steadily available, but rather a regulation of the functional IL-1R tI.

Our study revealed different levels of expression for IL-1R-AcP protein in endometrial stromal and epithelial cells. These latter showed a more intense immunostaining by immunohistochemistry, and for the same amount of total proteins they were found to contain higher amounts of IL-1R-AcP by Western blotting. This is in contrast with IL-1R tI protein expression, which according to our previous studies was comparable in these two cell populations (Bigonnesse et al., 2001). However, the difference between stromal and epithelial cells was less obvious at the level of IL-1R-AcP mRNA, as analysed by RT-PCR. The reasons for the higher expression of IL-1R-AcP protein in epithelial cells compared with stromal endometrial cells remain to be investigated. Furthermore, it would be interesting to determine whether such an elevated expression makes epithelial cells more responsive to IL-1 than stromal cells. Further investigations are also necessary to understand the specific role that IL-1R-AcP plays in the endometrial and reproductive functions.

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References


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