0013-7227/02/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 87(12):5785–5792

doi: 10.1210/jc.2002-020106

Estradiol and Interleukin-1β Exert a Synergistic Stimulatory Effect on the Expression of the Chemokine Regulated upon Activation, Normal T Cell Expressed, and Secreted in Endometriotic Cells

ALI AKOUM, ANDRÉ LEMAY, AND RODOLPHE MAHEUX

Unité d'Endocrinologie de la Reproduction, Centre de Recherche, Hôpital Saint-François d'Assise, Centre Hospitalier Universitaire de Québec, Faculté de Médecine, Université Laval, Québec, Canada G1L 3L5

Endometriosis, commonly associated with intraperitoneal inflammation, is estrogen dependent. Possible links between the immunoinflammatory and endocrine changes observed in endometriotic women have been poorly understood. In this study, we report that estradiol (E_2) and IL-1β exert a synergistic stimulatory action on RANTES (regulated upon activation, normal T cell expressed, and secreted) expression by endometriotic cells. Treatment of endometriotic cells with IL-1β had a dose-dependent effect on RANTES protein secretion and mRNA steady state levels, whereas cell treatment with E_2 or progesterone had no detectable effect. Interestingly, treatment of endometriotic cells with E_2 before stimulation with IL-1β resulted in a further increase in RANTES protein secretion and mRNA steady state levels, compared with IL-1β alone, whereas treatment with progesterone did not significantly affect cell responsiveness to IL-1β. Assessment of RANTES mRNA half-life revealed that cell pretreatment with E_2 enhanced RANTES mRNA stability and increased gene transcription as shown by run-on analysis. Immunohistochemical analysis of RANTES in endometriotic tissue showed immunostaining to be predominant in the stroma with no noticeable differences in tissues from the proliferative and secretory phase of the menstrual cycle. This appears to be consistent with the cell culture data and indicates that RANTES expression in endometriotic tissue is not subject to cyclic variation. These findings reveal a new regulatory mechanism by which IL-1β produced by activated macrophages can in synergy with ovarian and locally produced E_2 lead to enhanced macrophage and T-lymphocyte recruitment, thereby exacerbating the local immunoinflammatory process. Furthermore, the findings provide a further evidence for a close relationship between the endocrine and immunological changes observed in endometriosis. (*J Clin Endocrinol Metab* 87: 5785–5792, 2002)

TUBAL REFLUX LEADING to the presence of endometrial tissue in the peritoneal cavity is common to almost all menstruating women (1). In some of them, however, and according to Sampson's theory (2), endometrial tissue adheres to the peritoneal lining and develops into endometriotic lesions.

Besides genetic, hormonal, and environmental factors reported to be involved in the development of endometriosis, immunological factors may account for the susceptibility of endometrial tissue to escape immunosurveillance and implant in ectopic locations (3). Endometriosis is often associated with immunoinflammatory changes observed in the peritoneal fluid, which bathes the organs of the pelvic cavity, as well as in endometriotic implants (4, 5). One of the hallmarks of such a local inflammatory reaction is the increased number of activated peritoneal leukocytes, particularly macrophages (6–9). Moreover, these cells show evidence of activation in endometriosis patients and were also found to infiltrate endometriotic tissue (5, 7–11). Paradoxically, instead of eliminating misplaced endometrial tissue, activated macrophages appear to be involved in endometrial tissue adhesion, implantation, and growth. In fact, peritoneal macrophages from women with endometriosis seem to resist apoptosis (12) and have a reduced capacity to eliminate misplaced endometrial tissue (13). In contrast, macrophages from women with endometriosis were shown to secrete growth factors for endometrial cells (14–16) and promote angiogenesis directly by secreting angiogenic factors (17) and indirectly through the ability of macrophage-derived cytokines such as TNFα (18, 19) and IL-1 to induce angiogenic phenotype in endometriotic cells (20). Local inflammatory reaction is also thought to be a possible cause of endometriosis-associated pain and infertility (8, 21), and increased numbers of activated macrophages have been found in the peritoneal fluid of infertile women with endometriosis (7–9).

Increased recruitment of macrophages into the peritoneal cavity can at least in part be due to cyclic stimulation by refluxed endometrial tissue (22). However, data from our and other laboratories showed that chemokines produced by endometriotic tissue may contribute to a feed-forward cascade of events that accentuate the recruitment of leukocyte into the peritoneal cavity of endometriosis patients (23–27).

Regulated upon activation, normal T cell expressed, and secreted (RANTES) is a chemokine reported to have a potent ability to chemoattract monocytes and T cells (28). Elevated concentrations of this mediator have been detected in the peritoneal fluid of endometriotic women, and RANTES levels correlated with the stage of disease (29). This suggests that RANTES may be involved in leukocyte recruitment into the

Abbreviations: DTT, Dithiothreitol; E_2, estradiol; FBS, fetal bovine serum; HBSS, Hanks' balanced salt solution; MCP-1, monocyte chemotactic protein-1; P, progesterone; RANTES, regulated upon activation, normal T cell expressed, and secreted; SSC, saline sodium citrate.

endometriosis implantation sites. Although expression of RANTES was first thought to be limited to activated T cells, it is now known to be secreted by a variety of tissue types including macrophages and endometrial cells and has been reported to be expressed by endometriotic tissue (26, 30). Furthermore, recent studies indicate that RANTES expression by endometriotic cells is up-regulated by IL-1β, a proinflammatory cytokine produced mainly by monocytes/macrophages (31). Our present study corroborated these data and further revealed a synergism between estradiol (E_2) and IL-1, resulting in an increased RANTES secretion by endometriotic cells. Interestingly, E_2 appeared to enhance IL-1-mediated RANTES expression both at the transcriptional and posttranscriptional levels. This may be of interest in view of the critical role of E_2 in the development and maintenance of endometriosis (32–34) and its abnormal production locally in ectopic endometrial tissue (35).

Materials and Methods

Source and handling of tissue

Tissue specimens used in this study were obtained from healthy ovulating women who signed a written informed consent for a research project approved by the Saint François d'Assise Hospital Ethics Committee on Human Research. Endometriosis was diagnosed during laparoscopy and staged according to the revised American Fertility Society classification system (36). Women recruited into the study had no endometrial hyperplasia or neoplasia and had not received any antiinflammatory or hormonal medication during a period of at least 3 months before surgery. The cycle phase (proliferative or secretory) was determined according to the patients' cycle history and serum progesterone (P). The mean age was 35.0 ± 7.5 yr (Table 1).

Endometriotic biopsies were immediately placed at 4 C in sterile Hanks' balanced salt solution (HBSS) containing 1% antibiotics-antimycotics (Invitrogen, Burlington, Ontario, Canada), snap frozen in liquid nitrogen with Tissue-Tek OCT compound (Miles Inc., Elkhart, IN), and stored at −80 C until analyzed by immunohistochemistry or were directly used for cell culture experiments. Some endometriotic specimens were fixed in Tissufix no. 2 solution (Laboratoire Gilles Chaput Inc., Montreal, Quebec, Canada) for 24 h. They were then placed in a Tissue-Tek VIP apparatus (Miles Scientific, Etobicoke, Ontario, Canada) for another 12 h before being embedded in paraffin (TissuePrep, Fisher Scientific, Fair Lawn, NJ) for pathological investigations.

Immunohistochemistry

Immunohistochemistry was performed according to a previously described procedure (25), which was modified for RANTES. Briefly, immunostaining was performed on 4- to 5-μm tissue sections using a monoclonal mouse antihuman RANTES antibody (15 μg/ml in PBS containing 1% BSA; R&D Systems, Minneapolis, MN), a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA), and diaminobenzidine (Sigma, St. Louis, MO) as chromogen. Hematoxylin was used for counterstaining. Sections incubated without the primary antibody or with normal mouse IgGs used at the same concentration as the primary antibody were included as negative controls in all experiments. Slides were viewed using a microscope (mikroskopie und systeme GmbH, model DMRB), and photomicrographs were taken with 100 ASA film (Kodak, Rochester, NY).

Tissue dissociation and cell culture

Endometriotic cells were prepared and cultured according to a well-established and routinely used protocol (23). Briefly, endometriotic tissue was washed with cold HBSS, minced into small species, and digested with 1 mg/ml collagenase in HBSS for 1 h at 37 C. After a Ficoll-Paque gradient (Amersham Biosciences Corp., Baie d'Urfé, Quebec, Canada) to eliminate leukocytes, cells were cultured in DMEM-F12 medium containing 10% fetal bovine serum (FBS), 1.7 μM insulin, 6.2 μM transferrin, and 1% antibiotics-antimycotics (Invitrogen). Cultures using this protocol are free of leukocytes and contain predominantly stromal fibroblastlike cells. Epithelial and stromal cells were not separated because cell responsiveness to sex steroid hormones appeared to depend on stromal-epithelial cell interaction (37, 38).

Culture stimulation and RANTES synthesis

Cells were grown in 25-cm^2 culture flasks in DMEM medium containing 10% dextran-coated charcoal-treated FBS. At confluence, the

TABLE 1. Clinical characteristics of patients, sources of endometriotic tissue, laparoscopic findings, and type of experiment performed on each biopsy

Patient no.	Age (yr)	Cycle phase	Stage of endometriosis	Location of endometriotic tissue	Immunohisto-chemistry	Cell culture (passage no.)
1	35	Proliferative	III	Uterosacral ligament (*blackish* lesions)	No	Yes (P6)
2	35	Secretory	II	Peritoneum (*red* vesicles)	No	Yes (P3)
3	31	Secretory	III	Peritoneum (?)	No	Yes (P3)
4	29	Secretory	I	*Blue-black* lesion (uterine horn)	No	Yes (P4)
5	32	Proliferative	III	?	No	Yes (P1)
6	36	Proliferative	III	Cul-de-sac (*brownish* lesion)	No	Yes (P4)
7	17	?	I	Uterosacral ligament (deep lesions)	No	Yes (P3)
8	39	Secretory	III	Ovary (endometrioma)	No	Yes (P4)
9	32	Secretory	III	Ovary (vesicle)	No	Yes (P3)
10	46	Secretory	IV	Ovary (endometrioma)	Yes	No
11	25	Proliferative	IV	Ovary (endometrioma)	Yes	No
12	35	Proliferative	IV	Peritoneum (*red* lesion)	Yes	No
13	45	Proliferative	III	Ovary (*black-blue* lesion)	Yes	No
14	44	Secretory	IV	Peritoneum (*brownish* lesions)	Yes	No
15	46	Proliferative	IV	Ovary (endometrioma)	Yes	No
16	37	Proliferative	IV	Ovary (endometrioma)	Yes	No
17	33	Proliferative	IV	Fallopian tube (*blue* lesion)	Yes	No
18	44	Proliferative	II	Uterosacral ligament (?)	Yes	No
19	32	Secretory	II	Cul-de-sac (vesicle)	Yes	No
20	40	Proliferative	I	Cul-de-sac and uterosacral ligament (*white* vesicular lesions)	Yes	No
21	32	Proliferative	IV	Ovary (*black* lesion)	Yes	No
22	42	Proliferative	I	Ovarian fossae and cul-de-sac (?)	Yes	No
23	26	Secretory	II	Cul-de-sac (*red* lesions)	Yes	No
24	39	Secretory	III	Ovarian fossae (deep lesions)	Yes	No
25	24	Secretory	I	Cul-de-sac (small and superficial *red* lesions)	Yes	No

complete medium was discarded and replaced overnight with FBS-free medium, and cells were cultured for additional periods of time (0–24 h) with fresh FBS-free medium containing different concentrations of IL-1β (0–600 pM). In some experiments, cultures were stimulated with IL-1β (6 pM) in the presence of 355 μM cycloheximide (Sigma), an inhibitor of protein synthesis.

The effect of ovarian steroids [P: 4-pregnen-3,20-dione; E_2: 1,3,5,(10)-estratrien-3, 17β-diol-3-benzoate] (Sigma) was evaluated using an established long-term cell culture treatment protocol in which hormones were added to endometriotic cells at the onset of culture (25) and maintained for 7–8 d (until confluence). At confluence, cells were washed with serum-free medium, and incubation with hormones [E_2, P or E_2 + P] further continued in this medium for 42 h before stimulation or not with IL-1β. Ovarian steroid doses (10^{-8} M for E_2 and 10^{-6} M for P) were chosen on the basis of previous studies showing that ovarian steroid concentrations in the peritoneal fluid are higher than those normally found in the peripheral blood (17, 39).

RANTES protein secretion was evaluated in the culture medium using ELISA, whereas RANTES mRNA steady state level in endometriotic cells was evaluated by Northern blot.

RANTES ELISA

RANTES concentrations were measured using an ELISA procedure developed in the laboratory. Briefly, this technique uses a capture mouse monoclonal antihuman-RANTES antibody (500 ng/well in 96-well plates) (R&D Systems), a goat polyclonal antihuman-RANTES antibody for detection (0.5 μg/ml in PBS/0.5% BSA) (R&D Systems), alkaline phosphatase-conjugated goat antirabbit IgGs peroxidase-conjugated AffiniPure rabbit antigoat IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) (1:4000 dilution in PBS/0.5% BSA) (Chemicon International Inc., Temecula, CA), and 3,3′, 5,5′,-tetramethylbenzidine-peroxidase (Bio-Rad Laboratories, Inc. Ltd., Mississauga, Ontario, Canada) as substrate. The OD was read at 450 nm, and RANTES concentrations were extrapolated from a standard curve using recombinant human RANTES (gift from Dr. Shaun McColl, Department of Molecular Biosciences, University of Adelaide, Adelaide, Australia). The sensitivity limit of the assay was 13 pM, with intraassay and interassay coefficients of variation less than 5%.

Northern blot analysis

Total RNA was extracted from cells with TRIzol reagent according to the manufacturer's instructions (Invitrogen). RNA was size fractionated by electrophoresis on 1% agarose gels containing 10% formaldehyde and transferred to a Hybond-N^+ membrane (Amersham, Oakville, Ontario, Canada). The membrane was then dehydrated at 37 C for 30 min, prehybridized with a hybridization buffer composed of 5× saline sodium citrate (SSC), 5× Denhardt's solution, 50 mM NaH_2PO_4, 0.5% SDS, 200 μg/ml salmon sperm DNA, and 50% formamide, hybridized with ^{32}P-labeled RANTES cDNA (also a gift from Dr. Shaun McColl) in the hybridization buffer and washed with 1× SSC, 0.2× SSC, and 0.1% SDS, respectively, before being exposed to x-ray film (BioMax, Eastman Kodak Co.) at −80 C for about 18 h. Staining with ethidium bromide (Invitrogen) and hybridization with 28S cDNA probe (American Type Culture Collection, Manassas, VA) were performed to ensure equal loading of RNA. Data were analyzed as ratios of the density of the hybridization signals of RANTES to 28S, as determined by computer-assisted densitometry (BioImage, Visage 110s, Genomic Solutions Inc., Ann Arbor, MI).

mRNA stability and half-life experiments

Cells were treated with hormones, as described earlier, and incubated with IL-1β (6 pM) for 6 h. Transcription was then stopped with actinomycin D (8 μM), and cells were harvested after different times (0–4 h) of incubation with actinomycin D for RNA extraction and Northern blot analysis.

Nuclear run-on assay

Cell culture and treatment with hormones and IL-1β were performed as mentioned above and then homogenized using a Dounce homogenizer in a lysis buffer containing 0.25 M sucrose, 10 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and 0.1% (vol/vol) Triton X100. Nuclei were pelleted and washed twice in fresh buffer by centrifugation at 600 × *g* for 5 min at 4 C, and stored in 80 μl glycerol storage buffer [50 mM HEPES (pH 8.0), 40% (vol/vol) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, and 2 mM DTT]. For transcription assays, nuclei were incubated in 200 μl reaction buffer containing 20 mM HEPES (pH 8.0); 5 mM $MgCl_2$; 90 mM NH_4Cl; 0.5 mM $MnCl_2$; 16% (vol/vol) glycerol; 0.04 mM EDTA; 2 mM DTT; 0.4 mM each of ATP, CTP, and GTP (Invitrogen); and 0.25 mCi of [α-^{32}P]UTP (3000 Ci/mmol); the reaction was stopped by the addition of 100 μg/ml RNase-free Dnase I (Invitrogen). After treatment with proteinase K (100 μg/ml) (Invitrogen) in the presence of 10 mM $CaCl_2$ and 25 μg yeast tRNA (Roche Diagnostics, Laval, Quebec) for 20 min at 37 C, RNA was extracted twice with phenol/chloroform (1:1, vol/vol), precipitated overnight at −20 C with 100% ethanol (2:1, vol/vol) in the presence of 7.5 M ammonium acetate (1:2, vol/vol), pelleted by centrifugation at 12,000 rpm at 4 C for 15 min, and dissolved in 850 μl hybridization buffer containing 50 mM PIPES (pH 7.0), 0.5 M NaCl, 2 mM EDTA, 0.4% (wt/vol) SDS, and 33% (vol/vol) formamide. The radioactive RNA was used to probe alkali-denatured plasmid DNA (5 μg) or insert DNA (1 μg) immobilized on nylon membranes using a slot blot apparatus (Hoefer, San Francisco, CA). Hybridization was carried out for 3 d at 42 C using 5 million to 10 million cpm/ml hybridization buffer. Membranes were washed four times with 2× SSC, 0.1% SDS at 65 C for 30 min, incubated with 10 μg/ml RNase A, and 100 μg/ml proteinase K for 30 min at 37 C, respectively, washed twice again with 2× SSC, 0.1% SDS at 65 C for 30 min, and finally exposed to x-ray films (BioMax, Kodak) at −80 C.

Statistical analysis

Data were analyzed using one-way ANOVA, and the Tukey test was used *post hoc* for multiple comparisons. Differences were considered as statistically significant for P values less than 0.05.

Results

RANTES expression in endometriotic lesions

RANTES immunoreactivity was found throughout endometriotic tissue but was predominant in stromal cells (Fig. 1A). Control experiments performed on serial sections of endometriotic tissue confirmed the specificity of RANTES immunoreactivity because there was no noticeable immunostaining with normal mouse IgGs (Fig. 1B), with the primary antibody following preabsorption with an excess of RANTES or when the primary antibody was omitted (data not shown). Similar pattern of staining was observed whether endometriotic tissues were from the inner lining of ovarian endometriomas or from peritoneal lesions. Furthermore, no apparent difference related to the menstrual cycle phase was noted.

Effect of IL-1β and ovarian hormones on RANTES expression

The absence of apparent variations in RANTES immunostaining in relation with the menstrual cycle phase, prompted us to examine endometriotic cell responsiveness to ovarian hormones and assess whether these latter may have any regulatory effect on RANTES expression. Thus, endometriotic cell cultures were exposed to ovarian hormones at concentrations similar to those found in the peritoneal fluid of endometriosis patients (17, 39). As shown in Fig. 2, unstimulated endometriotic cells had no detectable expression of RANTES mRNA, as assessed by Northern blot, and did not release any amount of RANTES protein in the culture medium detectable by ELISA. In addition, endometriotic cell treatment with E_2, P, or a combination of these hormones had

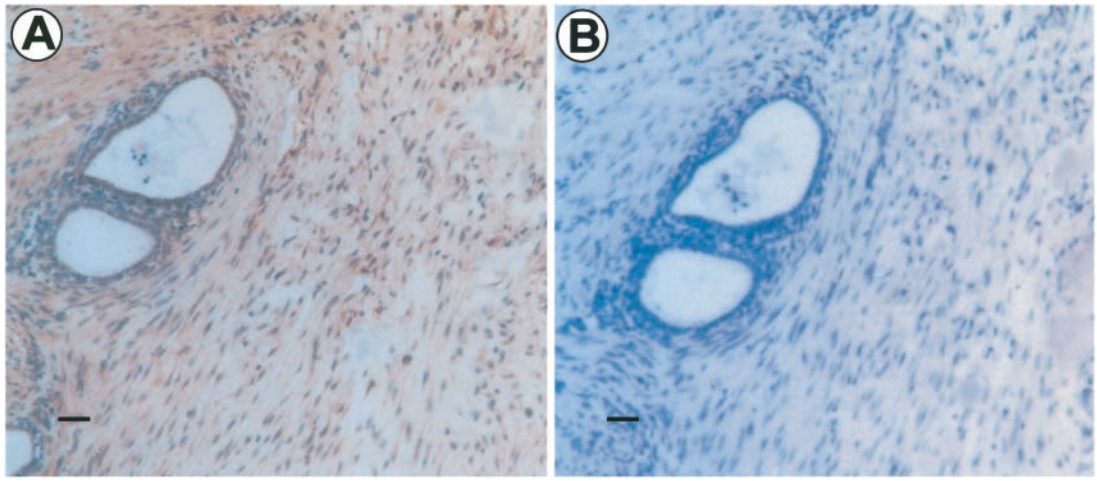

FIG. 1. Immunohistochemical analysis of RANTES expression in endometriotic tissue (biopsy of a *black* endometriotic lesion from a 32-yr-old woman with stage IV endometriosis). Note positive *brown* immunostaining throughout endometriotic tissue, particularly in stromal cells, in the presence of a mouse monoclonal anti-RANTES antibody (A) and the absence of immunostaining in the presence of mouse IgGs used instead of the primary antibody (B) (negative control). *Scale bar,* 30 μm.

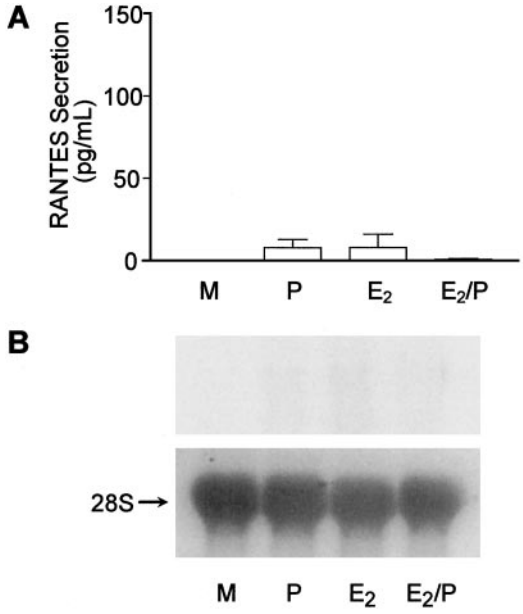

FIG. 2. Effect of E_2 (10^{-8} M) and P (10^{-6} M) on RANTES expression by endometriotic cells. Cells were continuously incubated with E_2 and/or P from the culture onset, as described in *Materials and Methods.* A, RANTES protein secretion in the culture medium as determined by ELISA (pM). B, RANTES mRNA steady state levels and ribosomal 28S RNA in endometriotic cells as analyzed by Northern blot. M, Medium only (control).

no detectable effect on RANTES mRNA expression and resulted in a slight increase in RANTES secretion.

In view of these data, we used IL-1β as stimulus to induce the synthesis of RANTES by endometriotic cells and assessed whether ovarian hormones may regulate the IL-1-induced RANTES expression. IL-1, one of the major proinflammatory cytokines found in elevated concentrations in the peritoneal fluid of patients with endometriosis (40, 41), has been reported to induce RANTES production by endometriotic cells (31). IL-1 is mainly secreted by activated macrophages (42), which have been shown to be actively recruited into the peritoneal cavity of patients and infiltrate endometriotic lesions (5–11).

Thus, endometriotic cells were first incubated with increasing concentrations of IL-1β for different time periods to determine optimal stimulation conditions. Our data showed that IL-1β stimulated RANTES mRNA steady state levels and protein secretion in a dose- and time-dependent manner. Detectable levels of RANTES mRNA and protein were observed following stimulation with 0.6 pM IL-1β, and maximal stimulation occurred in the presence of 60 pM for a 6-h period of treatment (Fig. 3). The protein secretion increased 6 h after stimulation with 6 pM IL-1β and rose gradually during the 24 h of treatment, whereas mRNA levels peaked after 6 h of stimulation and declined afterward over time (Fig. 4). Incubation of endometriotic cells with cycloheximide (355 μM) to inhibit protein synthesis completely abolished RANTES protein secretion in response to IL-1β (6 pM) but led to a marked increase in RANTES mRNA steady state levels (Fig. 3). Thus, IL-1β exerts a direct effect on endometriotic cell steady state mRNA expression that does not necessarily require *de novo* protein synthesis.

Endometriotic cells were then exposed to E_2, P, or to E_2 and P in combination before being exposed to 6 pM IL-1β for 6 h. A 6-h period of treatment and a concentration of 6 pM IL-1β were therefore used in the subsequent experiments to detect any possible effect of ovarian hormones. Data depicted in Fig. 5 show that pretreatment with E_2 increased RANTES mRNA steady state levels in endometriotic cells in response to IL-1β and stimulated as well RANTES protein secretion in the culture medium. However, pretreatment with P, either alone or in combination with E_2, had no significant effect on RANTES mRNA or protein secretion. Results from three independent experiments were analyzed. For RANTES secretion, the increase over control (cells stimulated only with IL-1β) was statistically significant for E_2 (56.8% $\pm$ 11.3%, $P < 0.001$) and E_2 + P (47.4% $\pm$ 4.8%, $P < 0.01$), but P effect (−20.9% $\pm$ 9.0%) was not statistically significant. For RANTES mRNA, steady state levels expressed as the ratio of RANTES hybridization signal density to that of 28S had increased by 79.9% $\pm$ 8.9% ($P < 0.05$) after the addition of E_2, 72.8% $\pm$ 13% ($P < 0.05$) after the addition

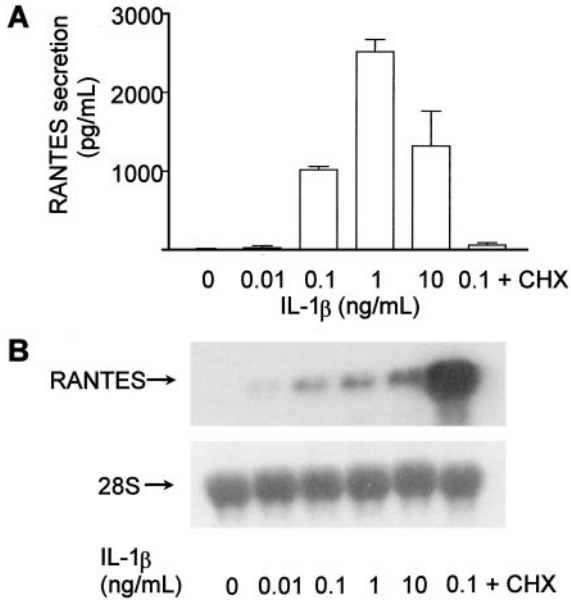

FIG. 3. Dose effect of IL-1β on RANTES expression by endometriotic cells. Cells grown to confluence were incubated for 6 h with increasing concentrations of IL-1β (0–600 pM). At the end of the incubation period, cells and culture media were recovered to evaluate RANTES protein secretion by ELISA and mRNA steady state levels by Northern blot. A, RANTES protein secretion (pM). B, RANTES mRNA as observed by autoradiography following hybridization of RANTES mRNA with ^{32}P-labeled RANTES cDNA probe. The 28S ribosomal RNA demonstrated approximately equal RNA loading. Cycloheximide (CHX; 355 μM), added together with IL-1β (6 pM), inhibited RANTES protein secretion (A) and markedly increased mRNA steady state levels (B).

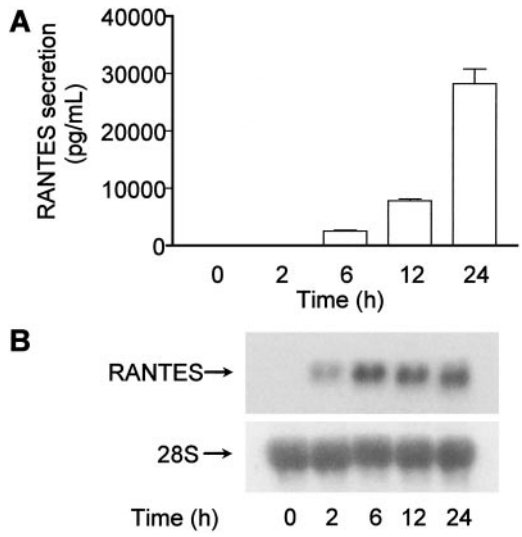

FIG. 4. Time course of IL-1β-induced RANTES secretion and mRNA synthesis by endometriotic cells. Confluent cultures were treated with IL-1β (6 pM) or control medium for different time periods. The culture supernatant was recovered at each time point to measure RANTES protein secretion by ELISA (pM), and total cellular RNA was extracted to evaluate RANTES mRNA expression by Northern blot. A, RANTES protein secretion. B, RANTES mRNA steady state levels and 28S ribosomal RNA.

of P to E_2-treated cells, and 10.7% ± 21.8% ($P > 0.05$) after the addition of P alone.

To determine whether the effect of E_2 was exerted at the

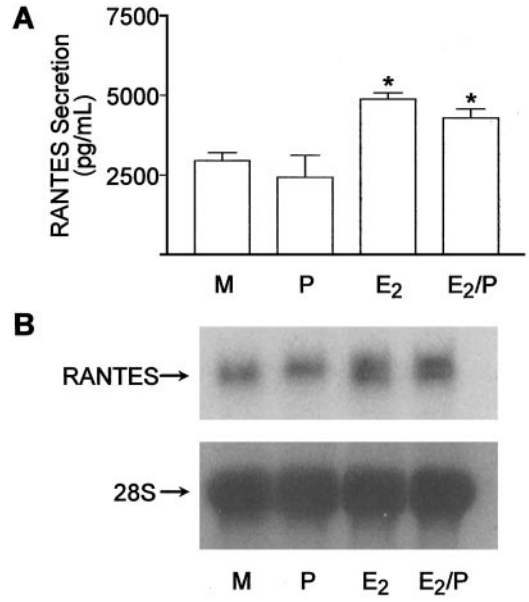

FIG. 5. Effect of cell pretreatment with E_2 (10^{-8} M) and P (10^{-6} M) on IL-1β-mediated RANTES expression. Cells were treated with E_2 and/or P from the culture onset until stimulation with IL-1β (6 pM for 6 h), as described in *Materials and Methods*. A, RANTES protein secretion in the culture medium as determined by ELISA. B, RANTES mRNA steady state levels in endometriotic cells as analyzed by Northern blot. M, Medium only (control). *, Significant increase of RANTES secretion, compared with control (cells exposed to IL-1β without prior treatment with ovarian steroids, $P < 0.05$).

transcriptional and/or the posttranscriptional levels, we evaluated RANTES mRNA half-life and gene transcription. Cells were pretreated or not with E_2 (10^{-8} M) before stimulation with 6 pM IL-1β for 6 h, as previously described. Figure 6 shows that pretreatment with E_2 retarded RANTES mRNA degradation following arrest of *de novo* RNA transcription by actinomycin D (8 μM). Without pretreatment with E_2, the half-life of RANTES mRNA was approximately 3.6 h, whereas in E_2-pretreated cells, it was approximately 6.7 h (data from three separate experiments). Nuclear run-on analysis showed that E_2 pretreatment also increased RANTES nuclear transcription in response to IL-1β (Fig. 7). The mean increase ± SEM in RANTES gene transcription as determined by densitometric analysis of data from three experiments was 61.7% ± 11.1%.

Discussion

Endometriosis is dependent on estrogen for its development and evolution. The disease arises at menarche, regresses after menopause, and can be present in postmenopausal women with elevated estrogen levels (32, 33). Animal models proved estrogen plays a critical role in the establishment and growth of endometriotic implants (32). Recent studies uncovered an autocrine mechanism that favored abnormal production of estrogen in endometriotic tissue (35). However, in spite of these facts, the mechanisms by which estrogen contributes to the pathogenesis of endometriosis are still poorly understood.

The present study revealed that E_2 and IL-1β act in synergy to stimulate RANTES production by endometriotic cells. In

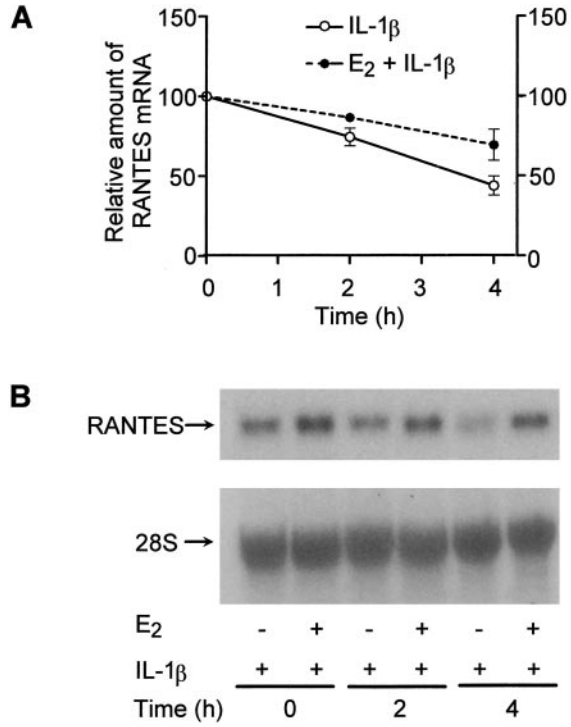

FIG. 6. Effect of E_2 on RANTES mRNA stability. Cells were pretreated with E_2 (10^{-8} M) and stimulated with 6 pM IL-1β for 6 h. Actinomycin D (8 μM) was added to stop *de novo* RNA synthesis and cells were harvested after 0, 2, 4, and 8 h of incubation with actinomycin D for RNA extraction and Northern blot analysis. A, Kinetics of RANTES mRNA degradation. The ratio of RANTES mRNA steady state level to that of 28S RNA, as quantified by densitometric analysis of RANTES and 28S hybridization signals, was determined at each time point, and data were expressed as the percentage ± SEM of RANTES mRNA level in endometriotic cells at time 0. B, An autoradiogram showing a representative experiment.

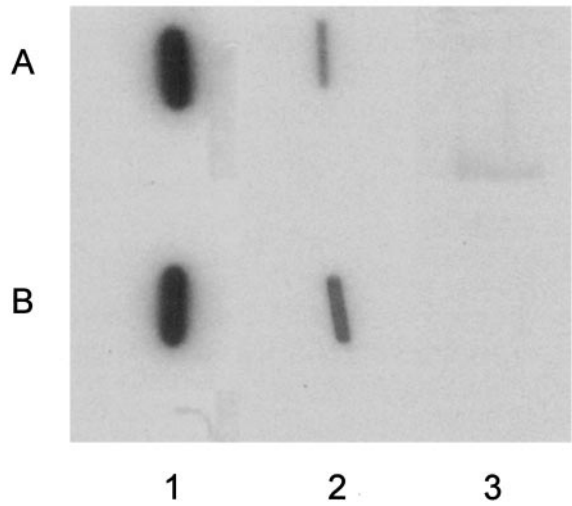

FIG. 7. Nuclear run-on analysis of RANTES gene expression by endometriotic cells following pretreatment with E_2 and stimulation with 6 pM IL-1β. DNA samples immobilized onto nylon membranes are as follows: 1, 28S cDNA; 2, RANTES cDNA; 3, pUC18 plasmid DNA. Radioactive transcripts were from cells having not been submitted to any hormonal pretreatment before exposure to IL-1β (6 pM) (A) and cells pretreated with E_2 (10^{-8} M) before stimulation with IL-1β (B).

fact, E_2 alone slightly affected RANTES protein secretion and had no detectable effect on RANTES mRNA expression. These data are in accordance with those of Hornung *et al.* (26, 30), who reported that sex steroids had no apparent effect on RANTES expression by endometriotic stromal cells. However, the present study showed that E_2 enhances endometriotic cell responsiveness to IL-1β and significantly increases RANTES protein secretion and mRNA steady state levels in response to the proinflammatory cytokine. Further analysis of E_2 effect showed that the hormone acts both at the transcriptional and the posttranscriptional levels by increasing RANTES gene transcription and mRNA stability. However, although one would expect these two mechanisms to be additive, the fact that the mean increase in RANTES mRNA transcription rate following E_2 treatment (61.7% over control) and the augmentation in RANTES mRNA stability (86.1% over control) are not fully reflected by the increase in RANTES mRNA steady state levels induced by E_2 (77.9% over control) is difficult to explain with certainty. This might presumably be due to interpatient variations and/or differences between lesions regarding RANTES expression by endometriotic cells and cell sensitivity to E_2 and IL-1.

These findings may have an interesting significance. First, they indicate that E_2 may, by enhancing endometriotic cell secretion of RANTES in response to IL-1β, indirectly contribute to the enhanced leukocyte recruitment into the peritoneal cavity of endometriosis patients and endometriotic tissue itself. In fact, one of the most known property of RANTES is that of T-lymphocyte and monocyte/macrophage chemoattraction (28). These cells, which were shown to infiltrate endometriotic lesions and be present in increased numbers in the peritoneal environment (5–11), are believed through their secretion of proteases, growth factors, reactive oxygen species, prostaglandins, and inflammatory and embryotoxic cytokines to contribute to endometriotic tissue growth and endometriosis-associated pain and infertility (14–17, 22, 42). This strongly suggests that in synergy with proinflammatory cytokines, such as IL-1β, E_2 may exacerbate the immunoinflammatory process taking place in the disease locally in the peritoneal cavity and within endometriotic implants. This is all the more plausible because E_2 appeared to be abnormally synthesized in endometriotic tissue (35) and may therefore act by endocrine and autocrine mechanisms. It is noteworthy that E_2 was also found to stimulate vascular endothelial growth factor production by peritoneal fluid macrophages (17) and uterine endometrial stromal cells of women with endometriosis (43) and favor endometriosis growth by potentiating angiogenesis.

Second, our data give further support to the emerging concept tying the endocrine and immune dysfunctions in endometriosis pathophysiology. In fact, endometriotic epithelial cells were induced to secrete the chemokine eotaxin after treatment with cytokines and ovarian steroids (44). According to our previous data, E_2 has an enhancing effect on IL-1-mediated IL-8 and monocyte chemotactic protein-1 (MCP-1) production by ectopic endometrial cells (24, 25). Interestingly, although E_2 appeared to enhance MCP-1 gene transcription (25), its effect on RANTES expression was also manifest at the posttranscriptional level, which is suggestive of different and cytokine-dependent regulatory mechanisms.

It is of interest to note that estrogen effects appear to be cell type dependent and vary according to the pathological state. Thus, although E_2 enhances cytokine production in endometriosis, it was reported to decrease cytokine production in some pathological states and increase it in others. For instance, estrogen was found to up-regulate IL-1β-induced IL-6 production in fibroblastlike synoviocytes from rheumatoid arthritis patients (45) and prolong T-cell survival in systemic lupus erythematosus (46). On the other hand, estrogen appeared to decrease MCP-1 production by monocytes and coronary artery smooth muscle cells and is believed to play a protective effect against atherosclerosis (47, 48). However, the mechanisms underlying such opposite actions remain completely unknown.

RANTES expression by endometriotic cell cultures was not significantly modified following exposure to P alone or in the presence of E_2 as in the secretory phase of the menstrual cycle. These *in vitro* data are consistent with immunohistochemical analysis of RANTES expression in endometriotic tissue, which has not revealed any noticeable difference between tissues from the proliferative and secretory phase of the menstrual cycle.

RANTES expression in the normal endometrium has previously been reported (26, 30). According to these studies, the chemokine is expressed throughout the ovulatory cycle *in vivo*, and sex steroids had no apparent effect on its expression by endometrial cells *in vitro*. Recent data from the same group (49) have, however, shown that long-term treatment with medroxyprogesterone acetate resulted in a significant decrease in RANTES secretion or mRNA synthesis by endometrial cells. Interestingly, Attia *et al*. (50) showed that the stimulatory P receptor isoform B is quite well expressed in the eutopic but is decreased or absent only in the ectopic endometrial tissue. This may relate to defective P sensitivity associated with endometriosis, as indicated by our study, and also suggests that eutopic endometrial cells have the capability of responding to P. In contrast, the responsiveness of endometrial cells to E_2 alone or in combination with IL-1 remains to be elucidated.

The mechanisms by which E_2 enhances RANTES expression by ectopic endometrial cells in response to IL-1β remain unclear. It is well documented that IL-1β induction of RANTES gene expression in endometriotic stromal cells is associated with the activation of the transcriptional factor nuclear factor κB and depends on a nuclear factor-κB site in the proximal promoter (31). We found no estrogen-responsive element in the known sequence of the RANTES gene proximal promoter (51) using the Transcription Element Search System (www.cbil.upenn.edu/tess/). However, E_2 treatment, without subsequent stimulation with IL-1β, had no detectable effect on RANTES mRNA steady state levels, which makes less probable a direct mechanism involving E_2 receptor binding to the RANTES gene regulatory region. Our nuclear run-on analysis showed an enhancement at the transcriptional level of the IL-1β-induced RANTES gene expression by E_2. This suggests a mechanism by which E_2 may activate a target gene whose products in turn may interact with IL-1β-induced transcription signals. On the other hand, E_2 was also shown to increase RANTES mRNA stability. This may be interesting because cytokines are known to have short-life mRNAs, and by prolonging RANTES mRNA half-life, E_2 shows up a new way by which it may influence endometriosis pathophysiology and contribute to maintain endometriosis-associated local inflammation. The mechanisms of such E_2-mediated actions are currently being investigated in our laboratory.

In summary, the results of the present study show that E_2 up-regulates, although indirectly, the expression of a potent chemotactic factor for monocytes and T lymphocytes by endometriotic cells and that this chemokine is expressed in endometriotic lesions without noticeable cyclic variation. E_2 enhances endometriotic cell responsiveness to the proinflammatory cytokine IL-1β and increases RANTES gene transcription and mRNA stability. These findings may have an interesting significance in view of the biological properties of RANTES whose levels are elevated in the peritoneal fluid of women with endometriosis and the paramount role attributed to E_2 in the pathophysiology of endometriosis, which in addition has been found to be abnormally produced locally in endometriotic tissue. Furthermore, they provide a new insight in the mechanisms of estrogen action in endometriosis and put forward a close relationship between immune and endocrine dysfunctions observed in this disease.

Acknowledgments

We thank Dr. Shaun McColl (Department of Microbiology and Immunology, University of Adelaide, Australia) for providing RANTES human recombinant protein and cDNA; Dr. Michel Marois, head of the Department of Pathology (Hôpital Saint-François d'Assise, Québec, Canada), for pathological investigations on endometriotic tissue biopsies; Drs. Jacques Bergeron and Marc Villeneuve for patient evaluation and providing endometriotic biopsies; and François Bigonnesse, Annie Boucher, Christine Jolicoeur, Monique Longpré, Johanne Pelletier, and Sylvie Pleau for technical assistance.

Received January 28, 2002. Accepted September 12, 2002.

Address all correspondence and requests for reprints to: Ali Akoum, Ph.D., Laboratoire d'Endocrinologie de la Reproduction, Centre de Recherche, Hôpital Saint-François d'Assise, 10 rue de l'Espinay, Local D0–711, Québec, Canada G1L 3L5. E-mail: ali.akoum@crsfa.ulaval.ca.

This work was supported by Grant MOP-14638 from Medical Research Council of Canada (to A.A.). A.A. is a "Chercheur Boursier National" of the Fonds de la Recherche en Santé du Québec (FRSQ).

References

1. **Halme J, Hammond MG, Hulka JF, Raj SG, Talbert LM** 1984 Retrograde menstruation in healthy women and in patients with endometriosis. Obstet Gynecol 64:151–154
2. **Sampson J** 1927 Peritoneal endometriosis due to menstrual dissemination of endometrial tissue into the peritoneal cavity. Am J Obstet Gynecol 14:422–469
3. **Vinatier D, Orazi G, Cosson M, Dufour P** 2001 Theories of endometriosis. Eur J Obstet Gynecol Reprod Biol 96:21–34
4. **Mulayim N, Arici A** 1999 The relevance of the peritoneal fluid in endometriosis-associated infertility. Hum Reprod 14(Suppl 2):67–76
5. **Witz CA, Schenken RS** 1997 Pathogenesis of endometriosis. Semin Reprod Endocrinol 15:199–208
6. **Hill JA, Faris HMP, Schiff I, Anderson DJ** 1988 Characterization of leukocyte subpopulations in the peritoneal fluid of women with endometriosis. Fertil Steril 50:216–222
7. **Haney AF, Muscato JJ, Weinberg JB** 1981 Peritoneal fluid cell populations in infertility patients. Fertil Steril 35:696–698
8. **Halme J, Becker S, Hammond MG, Raj MHG, Raj S** 1983 Increased activation of pelvic macrophages in infertile women with mild endometriosis. Am J Obstet Gynecol 145:333–337
9. **Olive DL, Weinberg JB, Haney AF** 1985 Peritoneal macrophages and infertility: the association between cell number and pelvic pathology. Fertil Steril 44:772–777
10. **Cirkel U, Ochs H, Mues B, Zwadlo G, Sorg C, Schneider HP** 1993 Inflam-

matory reaction in endometriotic tissue: an immunohistochemical study. Eur J Obstet Gynecol Reprod Biol 48:43–50
11. **Bulmer JN, Rebecca KJ, Searle RF** 1998 Intraepithelial leukocytes in endometriosis and adenomyosis: comparison of eutopic and ectopic endometrium with normal endometrium. Hum Reprod 13:2910–2915
12. **McLaren J, Prentice A, Charnock-Jones DS, Sharkey AM, Smith SK** 1997 Immunolocalization of the apoptosis regulating proteins Bcl-2 and Bax in human endometrium and isolated peritoneal fluid macrophages in endometriosis. Hum Reprod 12:146–152
13. **Dmowski WP, Gebel H, Braun DP** 1998 Decreased apoptosis and sensitivity to macrophage mediated cytolysis of endometrial cells in endometriosis. Hum Reprod Update 4:696–701
14. **Halme J, White C, Kauma S, Estes J, Haskill S** 1988 Peritoneal macrophages from patients with endometriosis release growth factor activity *in vitro.* J Clin Endocrinol Metab 66:1044–1049
15. **Olive DL, Montoya I, Riehl RM, Schenken RS** 1991 Macrophage-conditioned media enhance endometrial stromal cell proliferation *in vitro.* Am J Obstet Gynecol 164:953–958
16. **Rana N, Braun DP, House R, Gebel H, Rotman C, Dmowski WP** 1996 Basal and stimulated secretion of cytokines by peritoneal macrophages in women with endometriosis. Fertil Steril 65:925–930
17. **McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM, Smith SK** 1996 Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest 98:482–489
18. **Iwabe T, Harada T, Tsudo T, Nagano Y, Yoshida S, Tanikawa M, Terakawa N** 2000 Tumor necrosis factor-alpha promotes proliferation of endometriotic stromal cells by inducing interleukin-8 gene and protein expression. J Clin Endocrinol Metab 85:824–829
19. **Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, Kuwano M** 1997 Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol 17:4015–4023
20. **Lebovic DI, Bentzien F, Chao VA, Garrett EN, Meng YG, Taylor RN** 2000 Induction of an angiogenic phenotype in endometriotic stromal cell cultures by interleukin-1beta. Mol Hum Reprod 6:269–275
21. **Klein NA, Pergola GM, Rao-Tekmal R, Dey TD, Schenken RS** 1993 Enhanced expression of resident leukocyte interferon gamma mRNA in endometriosis. Am J Reprod Immunol 30:74–81
22. **Halme J** 1991 Role of peritoneal inflammation in endometriosis-associated infertility. Ann N Y Acad Sci 6:266–274
23. **Akoum A, Lemay A, Brunet C, Hébert J, "Le Groupe d'Investigation en Gynécologie"** 1995 Cytokine-induced secretion of monocyte chemotactic protein-1 by human endometriotic cells in culture. Am J Obstet Gynecol 172: 594–600
24. **Akoum A, Lawson C, McColl S, Villeneuve M** 2001 Ectopic endometrial cells respond to estradiol by up-regulating the interleukin-1-induced interleukin-8 expression *in vitro* and express high levels of interleukin-8 *in vivo* regardless of the menstrual cycle phase. Mol Hum Reprod 7:859–866
25. **Akoum A, Jolicoeur C, Boucher A** 2000 Estradiol amplifies interleukin-1 induced monocyte chemotactic protein-1 expression by ectopic endometrial cells of women with endometriosis. J Clin Endocrinol Metab 85:896–904
26. **Hornung D, Ryan IP, Chao VA, Vigne JL, Shcriock ED, Taylor RN** 1997 Immunolocalization and regulation of the chemokine RANTES in human endometrial and endometriosis tissues and cells. J Clin Endocrinol Metab 82:1621–1628
27. **Tseng JF, Ryan IP, Milam TD, Murai JT, Scheriock ED, Landers DV, Taylor RN** 1996 Interleukin-6 secretion *in vitro* is up-regulated in ectopic and eutopic endometrial stromal cells from women with endometriosis. J Clin Endocrinol Metab 81:1118–1122
28. **Schall TJ** 1991 Biology of the RANTES/SIS cytokine family. Cytokine 3: 165–183
29. **Khorram O, Taylor RN, Ryan IP, Schall TJ, Landers DV** 1993 Peritoneal fluid concentration of the cytokine RANTES correlate with severity of endometriosis. Am J Obstet Gynecol 169:1545–1549
30. **Hornung D, Klingel K, Dohrn K, Kandolf R, Wallwiener D, Taylor RN** 2001 Regulated on activation, normal T-cell-expressed and -secreted mRNA expression in normal endometrium and endometriotic implants: assessment of autocrine/paracrine regulation by in situ hybridization. Am J Pathol 158: 1949–1954
31. **Lebovic DI, Chao VA, Martini JF, Taylor RN** 2001 IL-1β induction of RANTES (regulated on activation, normal T cell expressed and secreted) chemokine gene expression in endometriotic stromal cells depends on a nuclear factor-kB site in the proximal promoter. J Clin Endocrinol Metab 86:4759–4764
32. **Di Zerga GS, Barger DL, Hodgen GD** 1980 Endometriosis: role of ovarian steroids in initiation, maintenance and suppression. Fertil Steril 33:649–665
33. **Meldrum DR** 1985 Management of endometriosis with gonadotropin-releasing hormone agonists. Fertil Steril 44:581–582
34. **Lemay A** 1993 La place de la suppression ovarienne dans le traitement de l'endométriose. Ref Gynecol Obstet 1:243–250
35. **Bulun SE, Zeitoun KM, Takayama K, Sasano H** 2000 Review: estrogen biosynthesis in endometriosis: molecular basis and clinical relevance. J Mol Endocrinol 25:35–42
36. **American Fertility Society** 1985 Revised American Fertility Society classification of endometriosis. Fertil Steril 43:351–352
37. **Metzger DA, Szpak CA, Haney AF** 1993 Histologic features associated with hormonal responsiveness of ectopic endometrium. Fertil Steril 59:83–88
38. **Cunha GR, Bigsby RM, Cooke PS, Sugimura Y** 1985 Stromal-epithelial interactions in adult organs. Cell Differ 17:137–148
39. **De Leon FD, Vijayakumar R, Brown M, Rao CHV, Yussman MA, Schultz G** 1986 Peritoneal fluid volume, estrogen, progesterone, prostaglandin, and epidermal growth factor concentrations in patients with and without endometriosis. Obstet Gynecol 68:189–194
40. **Fakih H, Baggett B, Holtz G, Tsang KY, Lee JC, Williamson HO** 1987 Interleukin-1: a possible role in the infertility associated with endometriosis. Fertil Steril 47:213–217
41. **Taketani Y, Kuo TM, Mizuno M** 1992 Comparison of cytokine levels and embryo toxicity in peritoneal fluid in infertile women with untreated or treated endometriosis. Am J Obstet Gynecol 167:265–270
42. **Nathan CF** 1987 Secretory products of macrophages. J Clin Invest 79:319–326
43. **Shifren JL, Tseng JF, Zaloudek CJ, Ryan IP, Meng YG, Ferrara N, Jaffe RB, Taylor RN** 1996 Ovarian steroid regulation of vascular endothelial growth factor in the human endometrium: implications for angiogenesis during the menstrual cycle and in the pathogenesis of endometriosis. J Clin Endocrinol Metab 81:3112–3118
44. **Hornung D, Dohrn K, Sotlar K, Greb RR, Wallwiener D, Kiesel L, Taylor RN** 2000 Localization in tissues and secretion of eotaxin by cells from normal endometrium and endometriosis. J Clin Endocrinol Metab 85:2604–2608
45. **Kawasaki T, Ushiyama T, Inoue K, Hukuda S** 2000 Effects of estrogen on interleukin-6 production in rheumatoid fibroblast-like synoviocytes. Clin Exp Rheumatol 18:743–745
46. **Evans MJ, MacLaughlin S, Marvin RD, Abdou NI** 1997 Estrogen decreases *in vitro* apoptosis of peripheral blood mononuclear cells from women with normal menstrual cycles and decreases TNF-alpha production in SLE but not in normal cultures. Clin Immunol Immunopathol 82:258–262
47. **Pervin S, Singh R, Rosenfeld ME, Navab M, Chaudhuri G, Nathan L** 1998 Estradiol suppresses MCP-1 expression *in vivo:* implications for atherosclerosis. Arterioscler Thromb Vasc Biol 18:1575–1582
48. **Seli E, Selam B, Mor G, Kayisli UA, Pehlivan T, Arici A** 2001 Estradiol regulates monocyte chemotactic protein-1 in human coronary artery smooth muscle cells: a mechanism for its antiatherogenic effect. Menopause 8:296–301
49. **Zhao D, Lebovic DI, Taylor RN** 2002 Long-term progestin treatment inhibits RANTES (regulated on activation, normal t cell expressed and secreted) gene expression in human endometrial stromal cells. J Clin Endocrinol Metab 87: 2514–2519
50. **Attia GR, Zeitoun K, Edwards D, Johns A, Carr BR, Bulun SE** 2000 Progesterone receptor isoform A but not B is expressed in endometriosis. J Clin Endocrinol Metab 85:2897–2902
51. **Nelson PJ, Kim HT, Manning WC, Goralski TJ, Krensky AM** 1993 Genomic organization and transcriptional regulation of the RANTES chemokine gene. J Immunol 151:2601–2612