Expression and regulation of CCR1 in peritoneal macrophages from women with and without endometriosis

CCR1 is a CC chemokine receptor with high affinity for RANTES (regulated upon activation, normal T cells expressed and secreted). CCR1 protein and mRNA concentrations in native peritoneal cells were twofold greater, in cultured peritoneal cells threefold greater, in patients with endometriosis compared to patients without endometriosis, as determined by Western blotting fluorescence activated cell sorting analysis, reverse transcription-polymerase chain reaction, and in situ hybridization. (Fertil Steril® 2005;83:1878–81. ©2005 by American Society for Reproductive Medicine.)

Endometriosis is a chronic inflammatory disease affecting 10% to 15% of women in the Western world. An excessive number of leukocytes, with a preponderance of macrophages and T cells (1, 2) has been identified in the peritoneal fluid (PF) surrounding endometriotic implants and within the lesions themselves (3, 4). Chemokines, small molecular weight cytokines, and their cognate chemokine receptors (CCR) are essential for the selective recruitment of different leukocyte subsets to inflammatory sites (5, 6). Peritoneal fluid of women with endometriosis is replete with chemokines that attract immune cells including RANTES (regulated upon activation, normal T cell expressed and secreted chemokine; also referred to as CCL5), a CCR1 and CCR5 ligand. RANTES has potent monocyte chemotactic activity both in vivo and in vitro (7) and is responsible for ~70% of the monocyte chemotactic activity in peritoneal fluid of women with endometriosis (8).

CCR1 is a CC chemokine receptor with high affinity for RANTES (5). TNF-α is known to stimulate RANTES expression in endometriotic stromal cells (9), which is associated with increased chemotaxis of leukocytes into the peritoneal cavity. However, data on cytokine regulation of the CCR1 RANTES receptor in peritoneal macrophages are lacking. Based on the clinical application of CCR1 inhibitors in other inflammatory diseases (10), we were prompted to investigate expression of CCR1 receptors in peritoneal macrophages of women with and without endometriosis.

Thirty-four healthy ovulatory women, who had not received hormones or GnRH agonist therapy for at least 6 months before laparoscopy, provided written informed consent under a study protocol ethically approved by the University of Tübingen and the University of Schleswig-Holstein, Campus Lübeck, Germany, and the University of California, San Francisco. Ovulation was verified by determination of serum P and LH levels. Symptomatic women with endometriosis (n = 19; chronic pelvic pain, n = 11; ovarian cyst, n = 3; chronic pelvic pain and ovarian cyst, n = 5) were staged intraoperatively according to a modification of the revised American Fertility Society system (stage I, n = 4; stage II, n = 3; stage III, n = 5; stage IV, n = 7). Women undergoing surgery for subserosal leiomyomata (n = 6) or requesting tubal ligation (n = 9) served as controls. To minimize variability, all women underwent surgery during the proliferative phase of the menstrual cycle. Peritoneal fluid was aspirated immediately upon entering the peritoneal cavity during laparoscopy. Bloody fluids, possibly contaminated by peripheral blood, were excluded from our study (20% of the endometriosis patients, 12% of the control women). Leukocytes were enriched by centrifugation over a Ficoll gradient. Some of these were subjected to fluorescence-activated cell sorting (FACS), some were frozen at −70°C for further analysis, and some were established in cell culture using macrophage medium (PAA Laboratories, Linz, Austria) free of cytokines. To ascertain the cytokine responsiveness of the peritoneal macrophages cultured from the two groups of subjects, maximally stimulating concentrations of tumor necrosis factor alpha (TNF-α, 5.9 nmol/L; Sigma, München, Germany) and interferon-gamma (IFN-γ, 4.2 nmol/L; Sigma) were added on day 2 of culture.

Characterization of the dispersed peritoneal cells was performed by FACS, using CD64-FITC and CCR1-PE (Becton Dickinson, Erembodegem-Aalst, Belgium) labeling for 30 minutes on ice. For each preparation 150,000 to 250,000 events were acquired and stored on a FACS Calibur flow cytometer (Becton Dickinson) and analyzed using Cellquest software (Becton Dickinson).
Western blotting for CCR1 protein was performed in fresh and cultured peritoneal macrophages using mouse monoclonal antibodies raised against human CCR1 (R&D Systems, Minneapolis, MN). Antibodies were used at a concentration of 2 μg/mL. Quantification of Western blot bands was established by densitometry using the AIDA software program (German Resource Center for Genomics, Berlin, Germany). A constant amount of cellular lysate (100 μg protein) was loaded into each gel lane, and results are expressed as mean ± SD relative density units (rdu).

Total RNA from peritoneal macrophages was isolated using Trizol (Invitrogen Life Technologies, Karlsruhe, Germany). One μg of each RNA was reverse transcribed in a 20-μL reaction containing: 5 mmol/L MgCl₂, 1× polymerase chain reaction (PCR) buffer (Invitrogen Life Technologies), 1 U reverse transcriptase, 1 mmol/L each of dATP, dCTP, dGTP, and dTTP, and 2.5 μmol/L random hexamers. The entire 20-μL reaction was then subjected to PCR amplification. PCR reaction conditions were similar to above, with Taq Taqstart antibody (0.5 U Taq equivalent, according to instructions from R&D Systems) replacing reverse transcriptase. The final volume of the PCR reaction was 100 μL. The oligonucleotide primer sequences for GAPDH were described previously (11). The primers used to amplify CCR1 cDNA were 5′-GTCACTTAAATACGACTCATATAGGGAAAGCCGTGAACAGGAAAGACAGGTCAAGAA-3′ (sense) and 5′-AACTCCAGTGCCAGAAGGTGAAAGCAGAG-3′ (antisense). Cycle parameters for PCR amplification were: 94°C, 5 min; 34 cycles 94°C, 45 s; 56°C, 45 s; 72°C, 45 s. A final extension round (72°C, 10 min) was used to maximize complete product formation. Ten μL of PCR products were separated on 4% NuSieve agarose gels (SeaKem, Hessisch Oldendorf, Germany), stained with ethidium bromide, and photographed.

In situ hybridization was performed on macrophages cultured in Lab-Tek chamber slides (Nunc International, Naperville, IL) fixed in 95% ethanol. Antisense and sense [35S]cRNA was transcribed from CCR1 templates using T7 RNA polymerase following the protocol described previously (12). Slides were dipped in NTB-2 nuclear tract emulsion and exposed for 4 weeks at 4°C before developing. Reduced radioactive grains in the emulsion of sense probe (background) were subtracted from antisense probe (signal) grains to determine CCR1 mRNA expression.

All experiments were repeated a minimum of three times and the results are presented as the mean ± SD. Kolmogorov-Smirnov analyses demonstrated that the distribution of the results was Gaussian and did not differ between normal and endometriosis cases. The data were analyzed by unpaired t tests or analysis of variance (ANOVA) between groups with Fisher post hoc tests for multiple comparisons. Significant differences were accepted when two-tailed analyses yielded 𝑃<.05. Based on an expected twofold difference between CCR1 protein levels among the two groups of patients and the observed standard deviations, 10 subjects in each of the two groups provided 90% power to detect that difference, assuming 𝛼=.05.

FACS analysis demonstrated that over 95% of CD64-positive peritoneal macrophages from both patient populations expressed the CCR1 receptor protein. The amount of CCR1 detected by Western immunoblotting in freshly isolated peritoneal macrophages from women with endometriosis (305 ± 68 rdu) was twice the level observed in fresh macrophages from control subjects (148 ± 42 rdu; 𝑃<.05).
Western immunoblot analysis of peritoneal macrophages cultured for two days revealed a threefold higher level of CCR1 concentration in cells from patients with endometriosis (274 ± 85 rdu) compared to cells from patients without endometriosis (81 ± 46 rdu; P<.05) (Fig. 1). When cultured peritoneal macrophages were incubated with maximally stimulating concentrations of TNF-α (5.9 nmol/L) and IFN-γ (4.2 nmol/L), CCR1 expression showed a rapid but not significant up-regulation compared to unstimulated cells (75 ± 32 rdu) within 10 minutes (102 ± 22 rdu; P>.05) and a significant up-regulation with more than a doubling in CCR1 by 48 hours (158 ± 34 rdu; P<.05).

To verify that CCR1 expression in peritoneal macrophages was mediated at a genomic level, the expression of CCR1 mRNA in these cells was confirmed by RT-PCR and in situ hybridization. Using in situ hybridization, an approximate doubling of CCR1 mRNA transcripts were detected in cells derived from women with endometriosis compared to women without evidence of the disease.

CCR1 belongs to a family of G-protein coupled heptahelical receptors that are differentially expressed on leukocyte subpopulations (5, 6). In humans, CCR1 expressing monocytes and macrophages have been implicated in arthritis, allergic encephalomyelitis, inflammatory renal diseases, transplant rejection, and multiple sclerosis (13–17). The experiments reported here suggest that CCR1 up-regulation in peritoneal macrophages of women with endometriosis represents another inflammatory manifestation involved in this disease.

In this study we showed an increased constitutive expression of CCR1 in peritoneal macrophages of women with endometriosis that may play a contributory role in the pathogenesis of endometriosis. Increased cytokines, particularly TNF-α and IFN-γ, in the PF of women with endometriosis that are known to increase chemokine expression also appear to have CCR1-modulating properties (18–22). We verified this in cultured peritoneal macrophages of women with and without endometriosis.

In addition to previously established findings that chemokines are elevated in the setting of endometriosis, our data indicate that macrophage sensitivity to chemokines also is up-regulated in this condition. We postulate that the combination of enhanced cytokine and chemokine production, in the face of augmented chemokine sensitivity, contribute to a feed-forward loop of peritoneal inflammation in women with endometriosis. Strategies targeted to suppress these inflammatory cascades, including the application of CCR1 antagonists, are likely to provide clinical benefits to women who suffer from this disorder.

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REFERENCES


