Chemokine bioactivity of RANTES in endometriotic and normal endometrial stromal cells and peritoneal fluid

D.Hornung1,2, F.Bentzien2, D.Wallwiener1, L.Kiesel3 and R.N.Taylor2,4

1Department of Obstetrics and Gynecology, 72076 Tübingen, Germany, 2Center for Reproductive Sciences, University of California, San Francisco, CA 94143, USA and 3Department of Obstetrics and Gynecology, 48149 Münster, Germany

4To whom correspondence should be addressed. E-mail: rtaylor@socrates.ucsf.edu

Endometriotic lesions secrete chemokines that recruit immune cells into the peritoneal cavity. The accumulation of these immune cells, especially activated macrophages and T lymphocytes, is thought to mediate inflammatory symptoms associated with endometriosis. Previous studies have demonstrated that RANTES (regulated on activation, normal T cell expressed and secreted) is synthesized by endometriotic stromal cells and circulates in peritoneal fluid, commensurate with the stage of endometriosis. In the current studies, we used the human monocytic cell line, U937, to assay chemotactic activity in cell culture conditioned media and peritoneal fluid from patients with endometriosis and normal controls. We demonstrated expression of the human RANTES receptors CCR-1 and CCR-5 in U937 cells and peritoneal macrophages. Over a range of 0–1000 pg/ml recombinant human RANTES had a direct, linear effect on monocyte migration. Conditioned media and peritoneal fluid induced dose-dependent effects on monocyte migration that were correlated with concentrations of immunoreactive RANTES (as measured by enzyme-linked immunosorbent assay) and the severity of endometriosis. Heat denaturation of the RANTES protein or addition of anti-human RANTES antibodies neutralized the chemoattractant effects of conditioned media and peritoneal fluid. RANTES stimulation of monocyte recruitment may be an important pathogenetic target for the treatment of infertility and pain associated with endometriosis.

Key words: chemokine/endometriosis/endometrium/peritoneal fluid/RANTES

Introduction

Inflammatory cells and their secretory products have been recognized recently as important mediators of the pathophysiology of endometriosis (Garcia-Velasco et al., 1999; Lebovic et al., 2000). A variety of cytokines, including tumour necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ) have been described in the peritoneal fluid of affected patients (Anderson and Hill, 1987; Halme, 1989; Khorram et al., 1993; Rier et al., 1994; Ryan et al., 1995; Chiang and Hill, 1997) and some of these have been shown to be produced by endometriotic implants. In general, the concentrations of cytokines in peritoneal fluid are correlated with the clinical stage of the disease.

Among these inflammatory mediators, our laboratory has investigated the expression and accumulation of a specific chemokine, RANTES (regulated on activation, normal T cell expressed and secreted). This 8 kDa protein is a chemoattractant for both monocytes and activated T cells (Schall et al., 1990), the two predominant leukocytes in peritoneal fluid of women with endometriosis (Halme et al., 1987; Khorram et al., 1993). Our findings support the hypothesis that, among several redundant chemokines, RANTES synthesis and secretion from endometriotic implants provides one of the important chemotactic stimuli for peritoneal leukocyte infiltration. Although endometrial and endometriotic stromal cells do not secrete RANTES under basal conditions, they can be induced to express RANTES mRNA and protein in vitro following stimulation with TNF-α and IFN-γ (Hornung et al., 1997; Arima et al., 2000). Furthermore, we have observed that RANTES concentrations in peritoneal fluid are highest in patients with advanced stages of endometriosis (Khorram et al., 1993).

In the current study, we have extended our hypothesis by examining the chemotactic activity of RANTES in conditioned media from endometriotic and normal endometrial stromal cells and in peritoneal fluid from subjects with and without endometriosis. Like primary peritoneal macrophages, a human monocytic cell line, U937, was shown to express RANTES receptors (CCR-1 and CCR-5), both biochemically and functionally, and this latter cell line was used to quantify chemokine bioactivity in specimens derived from patients. Our results indicate that RANTES is an important mediator of leukocyte recruitment in the microenvironment of endometriotic lesions and, hence, should be considered as a potential target for future medical therapies for this syndrome.
D. Hornung et al.

Materials and methods

Patient recruitment and characterization

Healthy ovulatory women, who had not received hormones or gonadotrophin-releasing hormone (GnRH) agonist therapy for at least 6 months before surgery, were recruited after they had provided written informed consent under a study protocol approved by the Committee on Human Research at the University of California, San Francisco, CA, USA. Women with endometriosis (n = 19) were staged intraoperatively according to a modification of the revised American Fertility Society system (Ryan et al., 1995). Control subjects (n = 9) were women with subserosal leiomyomata or without pelvic pathology requesting tubal ligation. Patient selection was not based on the presence or absence of infertility or pain. Rather, the final diagnostic groups were identified solely on the absence or degree of laparoscopically visible endometriotic lesions (classical and subtle variations) by experienced gynaecologists.

Sources of tissues and peritoneal fluid

Tissue specimens and peritoneal fluid samples were obtained from patients undergoing laparoscopy or laparotomy. Endometrial and endometriosis biopsies were collected under sterile conditions for cell culture. Peritoneal fluid was aspirated immediately on entering the peritoneal cavity, cells were removed by centrifugation at 2000 g, and 0.1 μg/ml aprotinin was added to the supernatant prior to freezing at −70°C. Primary peritoneal macrophages were recovered from the pelvic fluid by centrifugation using lymphocyte separation medium (Cappel, Aurora, OH, USA).

Human endometrial and endometriosis cell cultures

Primary endometrial and endometriotic cell cultures were prepared from biopsies as described previously (Hornung et al., 1997). Glandular epithelial cells were separated from stromal cells and debris by filtration through narrow gauge sieves. Stromal cells were subcultured twice to eliminate contamination by macrophages or other leukocytes. Extensive characterization of cell cultures prepared using this protocol confirmed that they were >95% pure and retained functional markers of their endometrial and endometriotic origin in vivo (Ryan et al., 1994). At the end of the experiment, cells were counted using the acid phosphatase colorimetric assay (Ueda et al., 1994).

Cytokine treatment of endometrial and endometriotic cell cultures

When the primary cell cultures approached confluence, the complete medium was removed and replaced with fresh α minimal essential medium (MEM) containing 2.5% fetal calf serum (FCS) and antibiotics and the cells were cultured for an additional 48 h with a combination of TNF-α (100 ng/ml; Sigma Chemical Co, St Louis, MO, USA) and IFN-γ (100 ng/ml; Sigma Chemical Co). Previous studies indicated an additive effect of these two cytokines on macrophage chemotaxis (Hornung et al., unpublished).

RANTES receptors CCR-1 and CCR-5 identification in macrophages

Primary peritoneal macrophages and U937 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM)/Ham’s-21 (H-21)/F-12 50% mix containing 10% FCS and antibiotics. These cells were stimulated for 48 h with TNF-α (100 ng/ml, Sigma Chemical Co) and IFN-γ (100 ng/ml, Sigma Chemical Co) or not stimulated. 50 μg of protein lysates from peritoneal macrophages and U937 cells and their conditioned media were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and the proteins were blotted to nitrocellulose paper as described (Hornung et al., 1997). Western blotting for the RANTES receptors CCR-1 and CCR-5 were performed using mouse immunoglobulin G (IgG) monoclonal antibodies raised against these human RANTES receptors CCR-1 (2 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and CCR-5 (2 μg/ml; R&D Systems, Minneapolis, MN, USA).

Monocyte chemotaxis assay

Chemotaxis was assayed in Boyden chambers containing permeable 3-μm pore size polyethylene terephthalate (PET) track-etched membranes (Becton Dickinson, Franklin Lakes, NJ, USA). In this assay, we used a human histiocyte cell line (U937) that can be induced to display monocyctic differentiation and chemotactic responsiveness after treatment with 1 mmol/l 8-bromo-cAMP (Kay et al., 1983). U937 cells were cultured at 37°C with 5% CO₂ in 50% DMEM H-21/50% F-12 medium supplemented with 10% FCS and penicillin G (100 IU/ml), streptomycin (100 μg/ml) and gentamicin (50 μg/ml; UCSF Cell Culture Facility) and incubated with 1 mmol/l 8-bromo-cAMP (Sigma) every 24 h for a total of 48 h. Supernatants from cytokine stimulated endometrial and endometriotic stromal cells or peritoneal fluids were prepared. Samples were diluted in phosphate-buffered saline (PBS) with 0.1% bovine serum albumin (BSA) and placed in the bottom wells of the Boyden chambers (600 μl per well). PET track-etched membranes were then fixed in place in 24-well plates to separate bottom from top compartments and 5 × 10⁵ U937 cells in 200 μl of PBS containing 0.1% BSA were added to the upper wells. The loaded chambers were incubated at 37°C in humidified air with 5% CO₂ for 120 min. Non-migrating cells were removed by several washes with PBS and migrating cells were fixed to the membrane with absolute methanol overnight at 4°C and stained with Crystal Violet (Sigma). Two independent assays were used to quantify monocyte chemotaxis: (i) optical absorption of the filters at a wavelength of 570 nm; and (ii) microscopic quantification of cells traversing the Boyden chambers. In the latter analysis the filters were visualized at ×100 magnification and six random, non-overlapping fields (accounting for 67% of each filter) were scored. The coefficients of variation of the direct cell counts ranged from 9–15%. Both methods were highly correlated (r = 0.99, P < 0.001). N-Formyl-methionyl-leucyl-phenylalanine (FMLP; 0.1 mmol/l Sigma), a known chemotactic peptide, was dissolved in PBS with 0.1% BSA and used as a positive control.

To evaluate the monocyte chemotactic activity due to RANTES in conditioned media and peritoneal fluid, samples were incubated with neutralizing dilutions of specific anti-RANTES antibodies (VL2, 0.8 μg/ml, Callag, Burlingame, CA, USA, or AF-278-NA, 2 μg/ml, R&D Systems) for 30 min at 37°C before incubation with U937 cells, and the chemotactic activity was measured as described above. These antibodies are highly specific for RANTES and do not cross-react with monocyte chemotactic proteins (MCP) 1–3, MIP 1α or β, vascular endothelial growth factor (VEGF) or many other human chemokines (R&D Systems Technical Bulletin). Alternatively, blockade of RANTES receptors was effected using anti-CCR-1 (Santa Cruz Biotechnology) and anti-CCR-5 antibodies (R&D Systems).

Statistical analyses

All experiments were repeated a minimum of three times and results were expressed as the mean ± SD. Normally distributed data were analysed using a paired t-test or analysis of variance (ANOVA). Non-parametric data were analysed by the Kruskal–Wallis and Mann–Whitney statistics. Linear regression analysis was performed to determine the degree of correlation between variables. The conservative, non-parametric Spearman statistic (ρ) was used. Significant differences were accepted when two-tailed analyses yielded P < 0.05.
Chemokine activity in endometriosis and endometrium

Figure 1. (A) Western blot of U937 cells and peritoneal macrophages, stained for human RANTES (regulated on activation, normal T cell expressed and secreted) receptor CCR-1, demonstrated the expected 41 kDa band in both cell types (lanes 1 and 2). We were unable to detect soluble forms of CCR-1 in conditioned media in any of these cells (lanes 3 and 4). (B) Western blot, stained for human RANTES receptor CCR-1, in tumour necrosis factor α (TNF-α) and interferon-γ (IFN-γ) stimulated (lane 2) and unstimulated (lane 1) U937 cells and stimulated (lane 4) and unstimulated (lane 3) peritoneal macrophages. An increase in CCR-1 protein was noted in both cell types after cytokine stimulation.

Results

CCR-1 and CCR-5 expression in peritoneal macrophages and U937 cells

Cells of the monocyte lineage are known to be primary targets of RANTES. Western blotting demonstrated a 41 kDa protein band, consistent with the known molecular weight of human RANTES receptor CCR-1 (Su et al., 1996), in U937 cells (Figure 1A, lane 1) and peritoneal macrophages (Figure 1A, lane 2). We were unable to detect soluble forms of CCR-1 in conditioned media from any of these cells (Figure 1A, lanes 3 and 4 respectively). A marked increase of CCR-1 protein was noted in TNF-α and IFN-γ stimulated U937 cells (Figure 1B, lane 2) and stimulated peritoneal macrophages (Figure 1B, lane 4) also showed increased CCR-1 compared with the unstimulated cells (Figure 1B, lanes 1 and 3 respectively). Similar results were observed in both cell types using specific antibodies against human CCR-5 (data not shown).

Monocyte chemotactic activity in conditioned media of normal endometrial and endometriotic stromal cells

As the yield and availability of primary peritoneal macrophages are limited to clinical cases, we established a relevant and reproducible model of human monocyte chemotaxis using 8-bromo-cAMP activated U937 cells, which express the same RANTES receptors. The following controls were used: U937 cells migrating toward a 0.1 µmol/l concentration of FMLP in PBS with 0.1% BSA was set at an arbitrary level of 100% chemotaxis (positive control), whereas PBS containing 0.1% BSA revealed the background activity (negative control). FMLP showed similar activity in the presence of 10% FCS.

Under these conditions, recombinant human RANTES over a range of 0–1000 pg/ml had a direct, linear effect on monocyte migration ($\rho = 0.95$, $P < 0.001$, Figure 2). At concentrations of >1000 pg/ml RANTES, we did not see a further increase in chemotactic activity (data not shown). Conditioned media from cytokine-treated normal endometrial (NE) stromal cells contained 250 pg/ml immunoreactive RANTES, as determined by enzyme-linked immunosorbent assay (ELISA), whereas identically treated endometriosis implant (EI) stromal cells secreted 1000 pg/ml RANTES (D.Hornung, I.P.Ryan and R.N.Taylor, unpublished data). The monocyte chemotactic activity of these conditioned media was highly correlated with the immunoreactive RANTES concentration ($\rho = 0.87$, $P < 0.001$).

Figure 3 shows photomicrographs of the underside of the Boyden chamber filters, demonstrating the RANTES (regulated on activation, normal T cell expressed and secreted) concentration-dependent migration of U937 cells across the porous membrane. Conditioned media from cytokine-treated normal endometrial stromal cells secreted 250 pg/ml immunoreactive RANTES (determined by enzyme-linked immunosorbent assay). Identically treated endometriotic implant stromal cells secreted 1000 pg/ml RANTES. The monocyte chemotactic activity was well correlated with the immunoreactive RANTES concentration. The negative control with 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) is shown on the left.

Monocyte chemotaxis by the EI cell conditioned media was inhibited >95% by heating the sample to 95°C for 5 min, suggesting that the activity was proteinaceous in nature (Figure 4, lane 2). Neutralizing anti-RANTES IgG decreased chemotaxis by 75 ± 5%, indicating that the majority of the chemotactic activity in the conditioned media was secondary to RANTES (Figure 4, lane 3). Both treatments differed significantly from control conditioned media ($P < 0.05$, paired t-tests, Figure 4). In contrast, anti-RANTES antibodies failed to significantly
Monocyte chemotactic activity of the conditioned media was inhibited >95% by heating the sample to 95°C for 5 minutes (lane 2). Neutralizing anti-RANTES (regulated on activation, normal T cell expressed and secreted), immunoglobulin G (IgG) inhibited the majority of chemotactic activity (75 ± 5%, lane 3). Lane 1 shows untreated conditioned media, normalized to 100% (*P < 0.05).

Having demonstrated the ability of normal and ectopic endometrial stromal cells to secrete bioactive RANTES, we proceeded to investigate whether this phenomenon also occurs in vivo. Peritoneal fluid specimens were evaluated by measuring their ability to induce monocyte chemotaxis using the same U937 cell bioassay. Peritoneal fluids of healthy controls (n = 9), patients with minimal or mild endometriosis (n = 11) and patients with moderate or severe endometriosis (n = 8) were tested. Consistent with our prior demonstration of elevated RANTES levels in women with advanced stages of endometriosis (Khorram et al., 1993), we observed increased monocyte chemotactic activity in endometriosis stage III and IV (72 ± 23%) compared with endometriosis stage I and II (45 ± 33%) and unaffected controls (31 ± 26%). These measurements were made relative to 0.1 µmol/l FMLP-induced chemotaxis. The difference between healthy controls and patients with advanced endometriosis was statistically significant (*P < 0.05, Kruskal–Wallis with post-hoc Mann–Whitney tests, Figure 5).

To estimate the contribution of RANTES to the total monocyte chemotactic activity of the peritoneal fluid, we added specific RANTES-neutralizing monoclonal antibodies. These IgG antibodies inhibited ~70% of the chemotactic activity (*P < 0.05, paired t-test, Figure 6).

Discussion

The importance of peritoneal macrophage accumulation has been recognized for two decades, when it was noted (Haney et al., 1981) that they were the most abundant leukocytes in the pelvic fluid of women with endometriosis. Subsequent studies indicated that the total concentration (Hill et al., 1988) and activation status (Olive et al., 1991) of macrophages were specifically increased in endometriosis. On the basis of these observations, we postulated that chemotactic activities for monocyte-derived cells were present in peritoneal fluid and initiated our studies of RANTES (Khorram et al., 1993). This 8 kDa protein was among the first monocyte chemokines identified by the cloning of T cell cDNAs (Schall et al., 1988).

Presently many cytokines with macrophage chemotactic or stimulating activities have been described and several of these are detectable in endometriotic implants or show elevated peritoneal fluid concentrations in endometriosis. Macrophage chemotactic activity with the characteristics of an immunophilin-like protein has been demonstrated in pelvic fluid (Weil et al., 1997). Detection of the chemokine, MCP-1 has been reported in endometrial cell cultures (Arici et al., 1995) and in pelvic fluid (Akoum et al., 1996) and immunostaining for granulocyte-macrophage colony stimulating factor (GM-CSF) was seen to be increased in secretory phase endometriotic lesions (Sharpe-Timms et al., 1994). Moreover, several macrophage-derived cytokines, e.g. interleukin (IL)-1, platelet-derived growth factor (PDGF), TNF-α and IL-6, have been found to be increased in pelvic fluid from endometriosis.
subjects (Eisermann et al., 1988; Hill and Andersen, 1989; Surrey and Halme, 1991; Rier et al., 1995).

In addition to these findings, other chemokines, including those responsible for neutrophil, IL-8 (Ryan et al., 1994; Arici et al., 1995), and eosinophil, eotaxin (Hornung et al., 2000) recruitment, have been discovered in peritoneal fluid. It appears that multiple types of inflammatory cells are recruited from the circulation and accumulate in the peritoneal cavity and within endometriotic implants themselves (Klein et al., 1994). Our studies, which identified macrophages and T cells as the most abundant pelvic leukocytes in endometriosis, led us to examine RANTES, a chemokine known to recruit cells of these two lineages (Khorram et al., 1993).

The current investigation corroborates the biological activity of RANTES as a monocyte chemokine secreted by cultured endometrial, and particularly endometriotic stromal cells, and present in the peritoneal fluid conditioned by endometriotic implants in situ. Using the U937 chemotaxis assay in Boyden chambers, we established that RANTES accounts for the majority (75 ± 5%) of the monocyte chemotactic activity in endometriotic stromal cell conditioned media and ~70% of that activity in peritoneal fluid. Other monocyte chemokines (e.g. MCP-1, eotaxin) are likely to contribute the remaining 30% activity observed in vitro and to the overall in vivo regulation of macrophage recruitment in endometriosis (Arici et al., 1995; Hornung et al., 2000). In addition, we established that CCR-1 and CCR-5, the two highest affinity G-protein coupled RANTES receptors (Su et al., 1996), are expressed in U937 cells and in primary peritoneal macrophages. In both cell types, expression of CCR-1 was up-regulated after exposure to TNF-α and IFN-γ, cytokines which are present in peritoneal fluid of women with endometriosis. Similar results were reported recently in other leukocytes (Durig et al., 1998). Thus, peritoneal factors associated with endometriosis potentially enhance monocyte recruitment in this clinical condition.

Our findings confirm that RANTES is a biologically relevant protein in endometriosis. Although we have focused our studies on the effects of this single chemokine and a single target leukocyte, ultimate elucidation of the inflammatory environment in endometriosis requires investigators to recognize that RANTES and monocytes do not act alone, but rather participate within a network of peritoneal cytokines and immune cells (Ryan et al., 2000). A better understanding of these cascades should lead to new therapeutic strategies for the treatment of endometriosis.

Acknowledgements
These studies were supported by Fortune-Project F1241133, the Deutsche Forschungsgemeinschaft (DH), and a grant from the NIH/NICHD, through co-operative agreement U54-HD37321, as part of the Specialized Cooperative Centers Program in Reproduction Research (RNT and DH).

References


D. Hornung et al.


Received on August 16, 2000; accepted on November 30, 2000