Monocyte chemotactic protein-1 in the follicle of the menstrual and IVF cycle

Pernilla Dahm-Kähler¹, Eva Runesson², Anna Karin Lind and Mats Brännström

Department of Obstetrics and Gynecology, The Sahlgrenska Academy at Göteborg University, Sahlgrenska University Hospital, Göteborg, Sweden

¹To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, The Sahlgrenska Academy at Göteborg University, S-413 45 Göteborg, Sweden. E-mail: pernilla.dahm-kahler@vgregion.se

²Present address: Lundbergs Laboratory for Orthopedic Research, Department of Orthopedics, The Sahlgrenska Academy at Göteborg University, Sahlgrenska University Hospital, S-413 45 Göteborg, Sweden

Ovulation constitutes an inflammatory-like process, with macrophages migrating into the follicle. This study evaluates the production of two macrophage-specific chemokines, monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α), in the human follicle at ovulation. Blood samples, follicular fluids and follicular cells were collected during menstrual and IVF cycles. Levels of MCP-1 and MIP-1α were measured in follicular fluid, blood plasma and cultured media (granulosa, theca and granulosa–lutein cells [GLCs]). Cells were cultured with or without LH, FSH, interleukin (IL)-1α, IL-1β, tumour necrosis factor (TNF) α, progesterone or oestradiol. The levels of MCP-1 were markedly higher in follicular fluid as compared with blood plasma in both menstrual and IVF cycles. The difference in MCP-1 levels between follicular fluid and plasma in menstrual cycles increased from the follicular phase (three-fold difference) to the late ovulatory phase (25-fold). Levels of MIP-1α were low in plasma and follicular fluid of both menstrual and IVF cycles. Theca cells from follicles of menstrual cycles secreted both MCP-1 and MIP-1α under basal conditions, and the secretion was increased by addition of IL-1β (MCP-1 and MIP-1α) and IL-1α (MCP-1). GLCs secreted MCP-1 under basal conditions and also MIP-1α after IL-1β stimulation. The macrophage-specific chemokine MCP-1 is highly expressed and is induced by IL-1 in the theca layer of the human follicle at ovulation.

Key words: chemokine/human/MCP-1/MIP-1α/ovulation

Introduction

Ovulation constitutes an inflammation-like reaction (Espey, 1980; Cavender and Murdoch, 1988) with active participation of leucocytes and their inflammatory mediators. During the ovulatory process, the leucocytes extravasate in the periphery of the preovulatory follicle and are then activated. In humans, the neutrophilic granulocytes and the monocytes/macrophages are the major leucocyte subtype that migrate to the preovulatory follicle (Best et al., 1996; Brannstrom et al., 1994). We have previously shown that leucocytes play active roles in the events leading to follicular rupture by the demonstration of increased ovulation rate in the in vitro perfused rat ovary after addition of leucocytes to the perfusates (Hellberg et al., 1991) and decreased ovulation rate in rats that were leucocyte depleted (Brannstrom et al., 1995).

It has been suggested that the macrophage is the most essential leucocyte subtype in ovulation, since there is a large invasion of macrophages into the follicle at ovulation (Brannstrom et al., 1993a, 1994). Moreover, mice with low monocyte/macrophage counts due to a mutation in the macrophage-colony-stimulating factor (M-CSF) gene exhibit reduced ovulation rates, which is restored by exogenous administration of M-CSF (Cohen et al., 1997). Administration of M-CSF-neutralizing antibodies (Nishimura et al., 1995) or clodronate liposome into the ovarian bursae of eCG/HCG-primed rats to deplete the ovary of macrophages (Van der Hoek et al., 2000) decrease the ovulation rate.

Chemokines are small (8–12 kDa) secretory proteins, which by mediating leucocyte migration, play crucial roles in inflammatory reactions. The two major subfamilies of chemokines are the CXC chemokines, specific mainly for neutrophils, and the CC chemokines acting primarily upon monocytes/macrophages and T cells. The CC chemokine monocyte chemotactic protein-1 (MCP-1) has chemotactic activity on monocytes, T lymphocytes and basophils. The activation of macrophages by MCP-1 involves degranulation and respiratory burst, while activation of basophils results in histamine release (Mukaida et al., 1992). Some data have suggested that MCP-1 may be involved in the cyclic changes within the ovary. Thus, this chemokine is expressed in human ovarian stromal cells, and the levels in follicular fluid of IVF cycles are increased by HCG (Arici et al., 1997b). Moreover, there is an upregulation of MCP-1 in the preovulatory rat follicle (Wong et al., 2002), and MCP-1 regulates macrophage influx into the corpus luteum during luteolysis (Townson et al., 1996, 2002; Senturk et al., 1999).

Macrophage inflammatory protein-1α (MIP-1α) is constitutively expressed at low levels in several subclasses of immune cells and vascular cells, but induced by cytokines (IL-1β), interferon [IFN-γ] and by monocyte–endothelial interactions (Menten et al., 2002). This CC chemokine is mainly chemoattractant for monocytes/macrophages and to a lesser extent for B- and T lymphocytes. It potentiates monocyte/macrophage activation induced by IFN-γ (Fahey et al., 1992).
The aim of this study was to examine the two macrophage specific chemokines, MCP-1 and MIP-1α, regarding their local follicular production and their control in both menstrual and IVF cycles to gain further knowledge on the complex intraovarian regulation of the human ovulatory process.

Materials and methods

General and hormones

The study was approved by the local ethics committee of Sahlgrenska University Hospital, and the patients had given their informed consent. Recombinant human LH was from Serono (Rome, Italy); recombinant human FSH from Organon (Oss, Netherlands); interleukin-1α (IL-1α), IL-1β and tumour necrosis factor α (TNF-α) from Peprotec (London, UK) and progesterone as well as estradiol (E2) were from Sigma-Aldrich (Steinheim, Germany).

Samples from IVF cycles

Follicular fluids, blood samples and Granulosa–lutein cells (GLCs) were obtained from patients (n = 17; mean age 35 years, range 27–41 years) undergoing in vitro fertilization and embryo transfer (IVF-ET) procedure. The patients were down-regulated with GnRH agonist (1.2 mg/day; Suprefact, Hoechst, Frankfurt, Germany) and were subsequently stimulated with FSH (75–150 IU/day; Fertinorm, Serono, Geneva, Switzerland). HCG (10 000 IU, Profasi; Serono, Rome, Italy) was injected when the size of the leading follicle was ≥18 mm. Follicle puncture and aspiration of follicular fluid from the follicles which were >15 mm in diameter was performed 36–38 h later. We excluded follicular fluid with blood contamination. The fluids were centrifuged (200 g, 10 min), and supernatants were frozen at –70°C. Blood samples were drawn at the same time from a peripheral vein, and blood plasma was separated and stored at –70°C. GLCs were collected and prepared for culture as described previously (Runesson et al., 2000). The proportions of immune cells, evaluated by immunohistochemistry against CD45 (pan leucocyte antigen) and CD68 (macrophage antigen), are around 11–13 and 8–15%, respectively. Briefly, cells were centrifuged on isotonic Percoll, washed in medium and counted. The cells were then seeded in 24-well plates with 0.5 ml medium M199 (Gibco, Paisley, UK) supplemented with NaHCO3 (0.026 M), gentamicin (50 µg) and 10% fetal bovine serum (FBS; Life Technologies, Paisley, UK).

Samples of peripheral blood from IVF cycles of four patients (mean age = 31 years, range 27–37 years) were taken at the first day of FSH administration and subsequently once at days 3–5, days 6–8, day of HCG and at oocyte aspiration.

Samples from menstrual cycles

Tissue samples and follicular fluids from women (n = 15; mean age 38 years, range 30–52 years) undergoing surgery for benign non-ovarian diseases were collected. Only women who, based on menstrual data, hormonal data (see below) and intraoperative findings, were operated during the follicular or ovulatory phases were included in the study. The women were not taking any medications (NSAIDs, hormones) that would possibly have affected the menstrual cycle or the ovulatory process.

According to menstrual data and the levels of E2, LH and progesterone in serum, the women were grouped into mid-follicular phase (MF; cycle day –6 to –4 in relation to predicted LH surge (day 0) with E2 <0.6 nmol/l, LH <8 IU/l and progesterone <6 nmol/l), late follicular/early ovulatory phase (LFO; cycle day –3 to –0 with E2 >0.6 nmol/l, LH >8 IU/l and progesterone <10 nmol/l), late ovulatory phase (LO; cycle day 0 to +1; LH >8 IU/l and progesterone >10 nmol/l) and post-ovulatory phase (PO; cycle day +2 to +5, LH >8 IU/l and progesterone <6 nmol/l). All follicles from MF were >9 mm in diameter on cycle day +7, to ascertain that the material was from a dominant follicle (Pache et al., 1990).

Follicles were removed as the initial procedure during surgery, put in ice-chilled phosphate-buffered saline (PBS) and then immediately brought to the laboratory. Follicular fluid was aspirated and centrifuged (200g, 10 min) to collect granulosa cells (GCs), and fluid was stored at –70°C. The follicle was then cut open, and the inside of the follicular wall was gently scraped with a Strabismus hook (PMS GmbH, Tuttingen, Germany) to further harvest the GC which was attached to the interior of the follicle as previously described (Bergh et al., 1993; Runesson et al., 2000). GCs were then washed (M199 four times, and cell viability was examined using Trypan Blue exclusion test. GC viability was around 60% in all samples. The theca cell (TC) layer was pulled away from the surrounding stroma using watchmaker’s forcesps. TCs were isolated after enzymatic treatment as described previously (Bergh et al., 1993; Runesson et al., 2000). TCs were washed four times in M199. Cell viability of TCs was determined by Trypan Blue exclusion and was 80–95% in all experiments. The proportion of immune cells in theca cultures was 3–4% (CD45 positive) and around 2% (CD68 positive), whereas no immune cells were detected in GCs (Runesson et al., 2000).

Six volunteers (mean age = 27 years, range 19–33 years) with normal menstrual cycles provided serum samples at day 0 (first day of menstruation), day 5, day 9 and then every other day until the day of LH surge and every day for 3 days after the LH surge followed by every fifth day until menstruation. The day of LH surge was detected in urine samples by the use of Clearplant® stick (Unipath Ltd, Bedford, UK).

Cell cultures

GCs and TCs from menstrual cycles (3 x 104 cells/well) were seeded in 0.5 ml M199 supplemented with 10% FBS on a 24-well plate and cultured for 24 h to allow attachment of the cells. After change of culture media, the cells were exposed to either E2, progesterone, LH or FSH (10 ng/ml) or the cytokines IL-1α, IL-1β and TNF-α (3 ng/ml) for 48 h. These concentrations were chosen as several studies have shown that they would produce near maximal response on cells of follicles in culture (Bergh et al., 1993; Machelon et al., 2000; Kawano et al., 2004a), and furthermore, the concentrations of these cytokines are similar in human follicular fluid (Buscher et al., 1999). GCs were cultured under the same conditions with or without IL-1β (3 ng/ml). The medium was then collected aliquoted and stored at –70°C until analysed. All cultures were performed on cells from individual follicles and performed in duplicate. Each data point is the mean of these two values.

Assays

Immunoreactive MCP-1 and MIP-1α in blood, follicular fluid and conditioned media were measured using ELISA kits (Amersham Int., Buckinghamshire, UK and R&D systems, Abingdon, UK). The tests were validated for measurements of follicular fluid levels and conditioned media of the cell cultures by test of recovery and linearity. E2 and LH in serum were analysed with a microparticle enzyme immunoassay (MEIA; Abbot Laboratories, Abbot Park, IL, USA) with sensitivities of 25 and 0.5 IU/l, respectively. Serum progesterone was analysed with a direct immunofluorescent kit (DELFIA; Wallac Oy, Turku, Finland) with a sensitivity of 1 nmol/l. Inter- and intra-assay coefficients of variations of all assays were <10% and <5%, respectively.

Statistical analysis

All analyses were performed on absolute values, and the data is presented as medians and individual values. Statistical analysis was performed by the non-parametric Mann–Whitney test and by Wilcoxon signed ranks test when paired data was analysed (data of Figures 2, 3 and 6). Significance was assigned at P < 0.05.

Results

MCP-1 in peripheral blood during the menstrual cycle and IVF-cycles

The levels of MCP-1 in the serum of all samples from the menstrual cycle (median 388 pg/ml, range 232–628) were similar to serum levels from all samples of the IVF cycles (median 370 pg/ml, range 197–480). No significant variation in circulating MCP-1 was detected throughout the menstrual or the IVF cycle (Figure 1A and B).

Chemokines in blood plasma and follicular fluid

The levels of MCP-1 were about four-fold higher in follicular fluid (median 201 pg/ml) than in plasma (median 57 pg/ml) of IVF cycles at oocyte aspiration (Figure 2). In menstrual cycles, the levels of MCP-1 in follicular fluid from MF and LF/EO phase were approximately
three-fold higher than in plasma (Figure 3). The plasma/follicular fluid difference in MCP-1 levels further increased (approximately 25-fold difference) in the LO phase. The follicular fluid MCP-1 levels were around 10-fold higher in LO phase than at MF stage, but there were no differences in plasma levels when comparing these timepoints (Figure 3). MIP-1α was not detected in plasma or follicular fluid from either IVF or menstrual cycles.

Chemokines in cultures of TCs, GCs and GLCs

The TCs secreted both MCP-1 and MIP-1α (Figure 4), and the levels in the media of MCP-1 under basal conditions (median 1053 pg/ml) were about 40-fold higher than that of MIP-1α (median 58 pg/ml). Presence of gonadotrophins (LH, FSH) or steroids (progesterone, E₂) did not change the levels of MCP-1 or MIP-1α in the conditioned media of TC cultures (data not shown). The presence of IL-1α and IL-1β increased the levels of MCP-1 around three-fold (Figure 5A). MIP-1α was increased around two-fold by IL-1β (Figure 5B). The presence of TNF-α did not affect MCP-1 (Figure 5A) nor MIP-1α (Figure 5B) secretion from TCs.

In conditioned media of GCs obtained from menstrual cycles, MCP-1 and MIP-1α were undetectable. The presence of IL-1α or IL-1β resulted in low but detectable levels of MCP-1 in two out of five cultures (145 and 116 pg/ml). In the media of cultured GLCs, there were detectable levels of MCP-1 (median 102 pg/ml) during unstimulated conditions (Figure 6). The MCP-1 levels were increased about six-fold (median 693 pg/ml) by the presence of IL-1β.
which also induced MIP-1α secretion (Figure 6) to detectable levels (median 259 pg/ml).

**Discussion**

Interactions between immune cells and somatic cells of the ovary are important in most physiological processes within the ovary (Brannstrom and Norman, 1993b). The localization of specific subtypes of immune cells in the theca layer and in the stroma around the follicle alters with the differentiation stage of the follicle (Brannstrom et al., 1993a, 1994). In all the tissues of the body, the presence and distribution of immune cells are regulated by locally produced chemotactic factors such as chemokines, with the ability to attract leucocytes from the vascular system through the process of chemotaxis (Adams and Shaw, 1994). In line with this, it was previously recognized that human follicular fluid exerts chemotactic activity on immune cells, and the levels of this chemotactic activity seemed to be correlated to the maturity of the follicle (Herriot et al., 1986).

In the present study, we focused on possible follicular mediators of chemotaxis of macrophages, since this seems to be the most important leucocyte subtype to promote ovulation (Cohen et al., 1997; Van der Hoek et al., 2000; Wu et al., 2004). The main findings of the present study were that the macrophage-specific chemokine MCP-1, but not MIP-1α, is present at higher concentrations in follicular fluid of ovulating follicles of both menstrual and IVF cycles in comparison with that of blood plasma. Markedly elevated levels of follicular fluid MCP-1 were found during LO phase of the menstrual cycle compared with earlier phases. There were no cycle-related fluctuations in MCP-1 levels in the blood of either menstrual or IVF cycles. The ovarian cellular source of both MCP-1 and MIP-1α in the menstrual cycle was the TCs, which produced detectable levels under basal conditions, with a further induction by IL-1. GCs of follicles obtained during menstrual cycles did not secrete detectable amounts of these chemokines, but GLCs secreted MCP-1 under basal conditions with an induction by IL-1β.

A major finding of the present study was the very high MCP-1 levels in follicular fluid. It should be noted that the factors found in follicular fluid are either filtered from the blood through the capillaries of the theca interna or secreted directly from the GCs. Thus, substances that are produced in high amounts by the GCs such as E2 and progesterone are found at extremely high concentrations in follicular fluid as compared with blood (McNatty et al., 1976; McNatty et al., 1979). High follicular fluid levels of MCP-1 (Arici et al., 1997b) and another CC chemokine (MIP-3α) (Kawano et al., 2004b) have previously been demonstrated in IVF cycles. The CXC chemokines IL-8 (Arici et al., 1996; Runesson et al., 1996; Runesson et al., 2000) and growth-regulated oncogene α (Oral et al., 1997; Karstrom-Encrantz et al., 1998; Zeineh et al., 2003) also show marked follicular fluid to blood concentration gradients. In the present study, we have looked closely at ovarian MCP-1 by evaluating MCP-1 production in the different follicular compartments of the menstrual as well as the IVF cycle during different cytokine and hormonal stimulations.
The possible follicular source of MCP-1 and MIP-1α in the humans has not previously been investigated in cell cultures of human follicular cells, although human ovarian stromal cells were found to be a source of MCP-1 (Arici et al., 1997b). Our results point towards the fact that the TC is the major cell type that produces MCP-1 since there was a high basal secretion and an induction by IL-1 in TC cultures. The TC cultures naturally contain fibroblasts and endothelial cells, since this is a compartment with fibrous elements and with also dense capillary network. Thus, contributions from these cell types have to be also considered, but nevertheless they are a natural component of the TC layer. The immune cells of the follicle also have to be considered as possible cellular sources of chemokine. However, it is likely that the production of MCP-1 seen in the TC cultures represents a major production in these cells and not production from the immune cells in the tissue, since GLCs with a three- to four-fold higher immune cell contamination as compared with TCs produced much less MCP-1. Moreover, it was shown in ovarian expression experiments in the mouse that chemokines and chemokine receptors in the ovary were present and that the major contributor was ovarian tissue (Zhou et al., 2004), although there was a minor contribution from peripheral blood leukocytes. In the present study, the secretion of both MCP-1 and MIP-1α from TCs increased by the presence of IL-1α or IL-1β but not by TNF-α. A similar upregulation of MCP-1 by IL-1 was seen in human ovarian stromal cells (Arici et al., 1997b). Since the ovarian stromal cells are derived from the TCs, the similarity in cytokine regulation of MCP-1 in these cell types was expected. The regulation by IL-1 is of interest in the context of IL-1 as a putative central and early facilitator of the ovulatory cascade (Brannstrom, 2004).

Since the present study included samples both of the menstrual and IVF cycle, we were able to compare the follicular environment during a physiological state and a state influenced by exogenous gonadotrophin stimulation. It was found that the levels of MCP-1 were elevated to the same extent in follicular fluid of IVF cycle at oocyte retrieval as in menstrual cycle at mid-follicular phase. The previous study on MCP-1 in follicular fluid of IVF patients found comparable levels (Arici et al., 1997b) as found in IVF follicular fluid of the present study. Interestingly, there were 10 times higher MCP-1 levels in follicular fluid from LO phase of the menstrual cycle compared with the MCP-1 levels in follicular fluid of IVF cycles. These two sampling times are at a comparable stage of the ovulatory process, since they are both within a few hours before follicular rupture.

In other studies that have compared cytokine levels in menstrual and IVF cycles, quite different ratios have been found, indicating differential hormonal or immune cell-mediated regulation. Thus, the concentrations of IL-8 in follicular fluid of IVF cycles at oocyte retrieval was in the same range as those of ovulatory phase in menstrual cycles (Runesson et al., 2000), and CSF-1 was higher in follicular fluid from IVF cycles than ovulatory phase of menstrual cycles (Shinetugs et al., 1999). One reason for the difference between menstrual cycles and IVF cycles regarding MCP-1 may be that the effect on MCP-1 production by the natural LH peak is not fully reproduced by HCG in IVF cycles. Previous studies have shown that HCG is involved in immunosuppressive actions by its ability to induce suppressor T lymphocytes (Fuchs et al., 1981). Since the T lymphocyte secretes large quantities of cytokines that can modulate MCP-1 secretion, it may be that HCG by this mechanism negatively regulates MCP-1 secretion from the monocytes/macrophages and from TCs. Another explanation to the difference in follicular fluid levels of MCP-1 in the monovulatory menstrual cycle as compared with the multifollicular IVF cycle may be the disrupted selection/dominance mechanisms in the latter. An indication along this line is the demonstrations of lower MCP-1 (Kawano et al., 2001) and MIP-3α (Kawano et al., 2004b) levels in IVF follicular fluids that contain immature oocytes as compared with those containing mature oocytes.

There was a marked increase in MCP-1 levels between the follicular phase and the LO phase of the menstrual cycle. This indicates a hormonally regulated expression of MCP-1 also in the follicle of the menstrual cycle. In hyperstimulated cycles of humans (Arici et al., 1997b) and rats (Wong et al., 2002), a direct effect of HCG on MCP-1 production has been demonstrated.

The results of the present study with absence of MIP-1α in follicular fluid demonstrate that MIP-1α is not normally produced in significant quantities in the human follicle of the menstrual cycle. Thus, there seems to be a selectivity regarding the presence of the CC family of chemokines in the follicle at ovulation so that only some of these chemokines are induced during ovulation. In a study of chemokine expression in the rat ovary (Wong et al., 2002), it was demonstrated that MIP-1α protein was present in ovarian cells but not upregulated by LH/HCG, and a study in the mouse showed variable MIP-1α mRNA levels in the ovary (Zhou et al., 2004).

We could not find any direct effects of gonadotrophins on chemokine production from any ovarian cell type. In previous work it was demonstrated that cultures of human ovarian stroma cells and GLCs increased the expression of MCP-1 mRNA in response to HCG/LH, whereas stroma cells treated with FSH were not affected (Arici et al., 1997a). It should be pointed out that the present study evaluated protein levels and not mRNA levels of MCP-1, which could explain the difference. However, secretion of MCP-1 protein increases in human immortalized GCs after treatment with IL-1α and TNF-α (Kawano et al., 2004a).

The MCP-1 expression in ovarian cells does not seem to be steroid dependent as shown by the experiments on TCs and GLCs in the present study and previous experiments on ovarian stromal cells (Arici et al., 1997b). However, expression of MCP-1 mRNA in endometrial stroma cells was inhibited both by E2 and progesterone (Arici et al., 1999). A reason for this obvious difference in steroid regulation may be that the MCP-1 mRNA is not expressed as MCP-1 protein in TCs or GLCs or that the protein is quickly degraded.

In conclusion, the results of this study point to chemokine selectivity in the human ovulation and specifically show that MCP-1 is produced by TCs in response to IL-1.
Acknowledgements
This research was supported by grants from the Swedish Research Council (11607 to M.B.), Medical Faculty at the Sahlgrenska Academy, Hjalmar Svensson’s Research Foundation and Göteborg Medical Society.

References

Submitted on October 14, 2005; accepted on December 5, 2005