



## Monocyte chemotactic protein-1 expression in human preovulatory follicles and ovarian cells

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### Abstract

There is a considerable population of macrophages (5–15% of the cells) within the human ovarian follicle at the time of ovulation. Macrophages are also present within the ovarian stroma, mostly near perifollicular capillaries. We hypothesized that macrophage migration in and around the preovulatory follicle is hormonally regulated and that regulation of macrophage migration occurs through local modulation of monocyte chemotactic protein-1 (MCP-1) that chemoattracts and activates monocytes/macrophages. In this regard, we investigated the expression and regulation of MCP-1 in human follicular fluid and in ovarian stromal and granulosa-lutein cell cultures. The concentration of MCP-1 in follicular fluid samples obtained from women prior to the administration of hCG was ( $n = 4$ )  $90 \pm 27$  (mean  $\pm$  S.E.) pg/ml; in samples obtained 12 h after the hCG administration it was ( $n = 3$ )  $135 \pm 23$  pg/mL; in follicular fluids obtained 34 h after the hCG administration it was ( $n = 126$ )  $322 \pm 46$  pg/mL ( $P = 0.007$  vs. pre-hCG). The mean ratio of follicular fluid/serum MCP-1 levels was 4.18. There was a correlation between follicular fluid MCP-1 levels and follicular fluid or serum progesterone levels ( $r = 0.21$ ,  $P = 0.02$ ;  $r = 0.29$ ,  $P = 0.03$ , respectively). MCP-1 mRNA and the protein were expressed in ovarian stromal and granulosa-lutein cells in culture and were increased by interleukin-1 $\alpha$  and tumor necrosis factor- $\alpha$  in a time- and concentration-dependent manner. LH/hCG induced higher levels of MCP-1 mRNA expression and protein production in both cell cultures. We propose that regulation of MCP-1 in ovarian stromal and granulosa-lutein cells by cytokines may play a role in the physiology of periovulatory events. © 1997 Elsevier Science Ireland Ltd.

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## 1. Introduction

It is becoming increasingly evident that immune-ovarian cell interactions are essential components of the ovarian cycle including ovulation (Adashi, 1990). Ovulation has many features in common with inflammatory reactions, including the participation of leukocytes and classical inflammatory mediators such as eicosanoids, histamine, and bradykinin (Espey, 1994). The number and type of leukocytes in the ovary varies in a predictable manner with the ovulatory cycle. Just prior to the time of ovulation there is an infiltration of leukocytes into the area surrounding the leading follicle, which has prompted some to consider ovulation as an inflammatory-like reaction (Brannstrom et al., 1994). The addition of leukocytes to the perfusate significantly increases the number of LH-induced ovulations in the rat ovary in vitro, thus these cells may play an active role in the ovarian physiology (Hellberg et al., 1991).

Macrophages are present as resident cells in the ovary and they may have a role in ovulation, luteinization, and luteolysis, as they are well equipped for these events given their capacity to secrete cytokines, eicosanoids, vasoactive amines, and tissue remodeling enzymes. Throughout the follicular phase, macrophages are present within human ovarian stroma mostly near perifollicular capillaries (Katabuchi et al., 1989). There is a considerable population of macrophages (5–15% of the cells) within the human ovarian follicle at the time of ovulation (Castilla et al., 1990; Loukides et al., 1990). In rat ovary, monocyte/macrophages are primarily located in the medullary region and also in the thecal layer of the preovulatory follicle, where they increase 5-fold at the time of ovulation (Brannstrom et al., 1993). The presence of these inflammatory cells in the apical region of the ovarian follicle, suggests a role in the mechanisms of follicular rupture. Studies on human ovarian tissue have also shown the presence of large numbers of macrophages in the follicle wall at ovulation (Brannstrom et al., 1994). An active role for monocytes/macrophages in ovulation is suggested by the ability of these cells to migrate within the follicle and by their presence in the follicular fluid at the time of oocyte retrieval for in vitro fertilization (Castilla et al., 1990; Loukides et al., 1990). In addition, interleukin-1 (IL-1), a major secretory product of macrophages, has been shown to stimulate ovulation-associated phenomena such as prostaglandin biosynthesis in rat ovarian tissue (Kokia et al., 1992), and to induce ovulation and oocyte maturation in the in vitro perfused rabbit ovary (Takehara et al., 1992). Involvement of macrophage-derived products in

ovarian steroidogenesis such as the enhancement of pre-ovulatory progesterone production is also possible. Macrophage products have been shown to stimulate progesterone production by human granulosa-lutein cells (Halme et al., 1985) and by murine granulosa cells (Kirsch et al., 1981). There are several candidate chemoattractants that may recruit macrophages in and around the follicle. One of them is MCP-1, a 76-amino-acid basic protein, that specifically chemoattracts and activates monocytes/macrophages (Wuyts et al., 1994). MCP-1 is secreted by a number of cell types including endothelial cells (Sica et al., 1990), fibroblasts (Yoshimura and Leonard, 1990), monocytes (Yoshimura et al., 1989a), lymphocytes (Yoshimura et al., 1989b), and mesangial cells (Rovin et al., 1992).

We postulated that MCP-1 may be an important modulator of periovulatory events by attracting and activating macrophages that play a role in timely follicular rupture. Our hypothesis is that monocyte/macrophage migration in and around the preovulatory follicle is hormonally regulated and that regulation may occur through local modulation of MCP-1 expression. In the present study, we measured MCP-1 concentrations in human follicular fluid. Using granulosa-lutein cells and ovarian stromal cells in culture we also investigated the regulation of MCP-1 gene expression by cytokines and hormones.

## 2. Materials and method

### 2.1. Follicular fluid and tissue collection

Follicular fluids and cells for granulosa-lutein cell culture were obtained from patients (age 24–43) undergoing in vitro fertilization-embryo transfer (IVF-ET) at Yale University School of Medicine IVF Program. Written informed consent was obtained from each woman before the procedure; consent forms and protocols were approved by the human investigation committee of the university. Patients were selected at random from a group undergoing IVF. Etiologic factors for infertility included 37% tubal factor, 33% male factor, 11% endometriosis, and 19% unexplained infertility. A standard IVF protocol was used. Briefly, GnRH-agonist (leuprolide acetate [Lupron], Tap Pharmaceuticals, Deerfield, IL) was administered 1 mg/day SC, starting in the midluteal phase of the preceding cycle or first day of the stimulation cycle. Stimulation with hMG (Pergonal; Serono Laboratories, Norwell, MA) or FSH (Metrodin; Serono Laboratories, Norwell, MA) was initiated when there was no sonographic evidence of ovarian follicular activity and the serum E2 level was < 50 pg/ml (conversion factor to SI unit, 3.671). Stimulation was continued until E2 levels reached 500 pg/ml or

greater and at least two follicles of 17 mm or larger in diameter were present. At that time 10 000 mIU hCG (Profasi; Serono Laboratories, Norwell, MA) was administered and Lupron and hMG were discontinued. Oocyte retrieval by transvaginal ultrasound guidance was performed at approximately 34 h after hCG administration. After removal of the cumulus-oocyte complexes, follicular fluids were centrifuged at  $600 \times g$  for 20 min. The cell-free supernatants were then stored at  $-80^{\circ}\text{C}$  until assayed. Cell pellets were used for culture of granulosa-lutein cells.

The four samples of follicular fluid collected before hCG administration and three samples of follicular fluid collected after 12 h of hCG administration were obtained from King Faisal Specialist Hospital and Research Center, Riyadh, Kingdom of Saudi Arabia and shipped on dry ice and stored at  $-80^{\circ}\text{C}$  until assayed together with other samples. All samples were handled by the same individual (A. Arici). Consent forms and protocols were approved by the human investigation committee of that institution. These samples were obtained from patients undergoing controlled ovulation stimulation with GnRH-agonist and hMG for timed intrauterine insemination. Patients underwent ultrasound guided follicular reduction (of follicles of 16 mm or larger in diameter) to prevent ovarian hyperstimulation syndrome and/or multiple gestation.

Blood samples were collected from all women on the day of hCG administration and from 15 other women on the day of oocyte retrieval. The blood was centrifuged to separate serum, which was stored at  $-80^{\circ}\text{C}$  until assayed.

Ovarian tissue was obtained from women of reproductive age undergoing hysterectomy with oophorectomy for reasons other than ovarian disease. Written informed consent for the use of these tissues was obtained from each woman prior to surgery. The consent forms and protocols used were approved by the human investigation committee of this university. The ovarian tissues were placed in culture medium and transported to the laboratory for dissection of the visible follicles and culture of ovarian stromal cells.

## *2.2. Isolation and culture of granulosa-lutein cells*

In all the experiments, granulosa-lutein cells were obtained approximately 34 h after hCG administration. After the centrifugation of follicular fluids, cell pellets were resuspended in Hank's balanced salt solution (HBSS) and the cell suspension was gently layered over 6.2% (wt./vol.) Ficoll (LSM, Organon Teknika, Durham, NC), then centrifuged at  $500 \times g$  for 20 min to pellet red blood cells. Cells at the interface were removed with a Pasteur pipette and resuspended in HBSS, centrifuged at  $500 \times g$  for 10 min.

Finally, the cell pellet was resuspended in Ham F-12/DMEM (1:1, vol./vol.) that contained antibiotics-antimycotics (1%, vol./vol.) and FBS (10%, vol./vol.). The average viability of cells was 85% as assessed by dye exclusion using trypan blue (Gibco BRL, Grand Island, NY). Dispersed cells were counted using a hemocytometer; cell count varied from  $10 \times 10^6$  to  $30 \times 10^6$  cells per patient. The cell suspension was then diluted accordingly to a concentration of  $5 \times 10^5$  viable cells/ml and plated in 6-well plates. The plates were incubated for 1–6 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air with daily replacement of medium.

In some experiments, granulosa-lutein cells were treated with the monoclonal anti-CD45 antibody coupled with magnetic immunobeads (Amac, Westbrook, ME) to remove white blood cells. Isolated granulosa-lutein cells were suspended in 2 ml of medium that contained 30% fetal calf serum and incubated for 10 min at room temperature with anti-CD45 immunomagnetic beads. The suspension was then placed into a magnetic test tube rack (Bio-Mag, Lexington, MA) for 10 min at room temperature, resulting in the removal of immunobead-bound white blood cells from the cell suspension. After careful removal of the supernatant, the process was repeated twice under identical conditions and 'purified' granulosa-lutein cells were centrifuged, and then resuspended in Ham F-12/DMEM. The viability was 95% as assessed by the dye exclusion test using trypan blue. The granulosa-lutein cells were plated in 24-well plates at  $5 \times 10^4$  cells/well density for experiments. Some cells were plated on 22 mm<sup>2</sup> cover slips in 35 mm wells for histologic examination by immunohistochemistry that confirmed the absence of white blood cells.

At the end of each experiment, culture media were collected and frozen at –80°C for quantification of MCP-1 by ELISA. Attached cells were dissolved in 0.5 N NaOH and protein concentration was measured using the Bio-Rad protein assay (Bio-Rad laboratories, Hercules, CA) according to the manufacturer's instructions. This approach allowed us to check that the attached cells were equally distributed in all wells. Experiments were repeated at least three times using cells collected from different patients.

### *2.3. Isolation and culture of ovarian stromal cells*

Ovarian tissue cleared from visible follicles was digested by incubation of tissue minces in HBSS that contained Hepes (25 mM), penicillin (200 U/ml), streptomycin (200 mg/ml), collagenase (1 mg/ml, 15 U/mg), and DNase (0.1 mg/ml, 1500 U/mg) for 2 h at 37°C with agitation. The dispersed cells were filtered through a wire sieve (73 µm diameter pore) to remove undigested tissue pieces. Ovarian stromal cells were plated in Ham F12/DMEM (1:1, vol./vol.) that contained antibiotics-antimycotics (1%, vol./vol.) and FBS

(10%, vol./vol.). Cells were plated in plastic flasks (75 cm<sup>2</sup>), maintained at 37°C in a humidified atmosphere (5% CO<sub>2</sub> in air), and allowed to replicate to confluence. Thereafter, the stromal cells were passed by standard methods of trypsinization and plated in culture dishes (100 mm diameter or 24-well plates) as appropriate for the experimental design, and allowed to replicate to confluence. Experiments were commenced 1–3 days after confluence was attained. Because we have previously shown in other cell types that serum stimulates MCP-1 production (Arici et al., 1995), the confluent cells were treated with serum-free media for 24 h before initial treatment with test agents.

At the end of each experiment, the culture media were collected and frozen at –80°C for quantification of MCP-1 by ELISA. Cells were used for quantification of total protein or for isolation of RNA. Experiments were repeated at least three times using cells collected from different patients.

#### *2.4. Immunocytochemical analyses of granulosa-lutein and ovarian stromal cells*

Immunocytochemical analyses of granulosa-lutein and ovarian stromal cells were conducted using factor VIII as a marker of endothelial cells, low molecular weight cytokeratin as a marker of epithelial cells, vimentin as a marker of mesenchymal cells, aromatase as a marker of estrogen producing cells, and HAM 56 as a marker of monocyte/macrophages. Freshly isolated granulosa-lutein cells and cultured ovarian stromal cells at first passage were plated on 22 mm<sup>2</sup> cover slips in 35 mm wells. Cells were fixed in 4% paraformaldehyde and stored at –20°C. Cells were exposed to monoclonal murine anti-human antibodies (for factor VIII (Dako, Carpinteria, CA, 1:100 dilution); for low molecular weight cytokeratin (Dako, 1:500 dilution); for vimentin (BioGenex, San Ramon, CA, 1:30 dilution); for HAM 56 (Dako, 1:150 dilution); for aromatase (generously donated by Dr. N. Harada, Fujida Health University, Toyoake, Aichi, Japan, 1:2000 dilution)). An avidin-biotin developing system (Vectostain ABC kit, Vector Labs) was used as described. The percentage of cells in granulosa-lutein and ovarian stromal cells was calculated by counting stained cells and unstained granulosa cells in three random high-power fields ( $\times 100$ ) in which at least 100 cells were present.

In the granulosa-lutein cell cultures, macrophage-marker-positive cells, endothelial cells, and cytokeratin-positive cells accounted for 6–9%, 1–2 and 0%, respectively. Nearly all the cells (95–100%) were positive for vimentin. Macrophage-marker-positive cells represented less than 1% after treatment with anti-CD45 immunobeads. In confluent ovarian stromal cell cultures after first passage, macrophage-marker-positive cells, endothelial

cells, and cytokeratin-positive cells accounted for 2–4, 1–2 and 5–7%, respectively. Nearly all the cells (95–100%) were positive for vimentin. Approximately 10–15% of the cells were positive for aromatase. These data were compiled from preparations of cells from three different samples.

### *2.5. Preparation of total RNA and northern analysis*

Total RNA from cells in culture was extracted using Trizol (Gibco BRL, Grand Island, NY) (Chomczynski and Sacchi, 1987). Total RNA (5 or 10 µg per lane) was size-fractionated by electrophoresis on 1% formaldehyde-agarose gels, transferred electrophoretically to Hybond-N<sup>+</sup> membrane (Amersham; Arlington Heights, IL), and cross-linked to the membrane by use of UV light. Prehybridization was conducted for 5 h at 42°C in buffer containing 5 × standard saline citrate (SSC), 5 × Denhardt's solution, formamide (50%, vol./vol.), dextran sulfate (5%, wt./vol.), NaH<sub>2</sub>PO<sub>4</sub> (50 mM), and salmon sperm DNA (0.5 mg/ml). Hybridizations were conducted for 16 h at 42°C in buffer composed of 5 × SSC, 2 × Denhardt's solution, formamide (50%, vol./vol.), dextran sulfate (10%, wt./vol.), NaH<sub>2</sub>PO<sub>4</sub> (20 mM), and salmon sperm DNA (0.1 mg/ml) with a DNA (cDNA) probe (5–15 µCi) complementary to MCP-1 mRNA radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP by random hexamer priming. This probe was kindly provided to us by Dr. T. Yoshimura (NCI, Frederick, MD). After hybridizing, the blots were washed with 1 × SSC and SDS (0.1%, wt./vol.) for 15 min at room temperature, and once with 0.1 × SSC and SDS (0.1%, wt./vol.) for 15 min at room temperature, and once for 20 min at 65°C. Autoradiography of the membranes was performed at –80°C using Kodak X-Omat AR film (Eastman Kodak, Rochester, NY). The presence of equal amounts of total RNA in each lane was verified by visualization of ethidium bromide-stained 28S and 18S ribosomal RNA subunits and by analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA, using a cDNA probe (Clontech Laboratories, Palo Alto, CA) radiolabeled with [ $\alpha$ -<sup>32</sup>P]dCTP by random hexamer priming. The autoradiographic bands were quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Each MCP-1 band was normalized using the value for the corresponding G3PDH mRNA, thus correcting for any variation in amounts of RNA applied to each lane. Similar experiments were conducted on three different occasions with cells prepared from different patients.

### *2.6. MCP-1 immunoassay*

Immunoreactive MCP-1 in follicular fluid samples and culture supernatant was quantified using an ELISA (R & D Systems, Minneapolis, MN).

According to the manufacturer, there is no measurable cross-reactivity with other known cytokines in this assay. The sensitivity for MCP-1 was 4.7 pg/ml in media and 18.1 pg/ml in serum. All of the follicular fluid samples were evaluated in a duplicate assay. Validation of its use for human follicular fluid was also performed: recombinant MCP-1 was diluted in assay buffer and pooled follicular fluid, and parallelism was observed between the standard curve of buffer and follicular fluid dilutions. Each experiment was done using three replicate wells for each condition and supernatant from each well is tested in a single ELISA assay. Each experimental setup was repeated on three or more occasions using cells obtained from three different patients. The intraassay and interassay coefficients of variation were 4.9 and 5.9%, respectively.

### *2.7. Progesterone and estradiol immunoassays*

Immunoreactive progesterone in follicular fluid and serum was quantified by RIA (Progesterone MAIA, Biodata Diagnostics, Rome, Italy). According to the manufacturer, there is less than 1% cross-reactivity with other steroid hormones, the sensitivity for progesterone is 0.022 ng/ml and the intraassay and interassay coefficients of variation are 8.06 and 7.71%, respectively.

Immunoreactive estradiol in follicular fluid and serum was quantified by RIA (Estradiol MAIA, Biodata, Rome, Italy). According to the manufacturer, there is less than 1.8% cross-reactivity to other steroid hormones, the sensitivity for estradiol is 5 pg/ml and the intraassay and interassay coefficients of variation are 7.17 and 9.85%, respectively. Due to high concentrations of steroids in follicular fluid, the samples were diluted 1:500 in steroid-free zero standard solution as recommended by the manufacturer.

### *2.8. Statistical analyses*

Because the levels of MCP-1 in follicular fluids were not normally distributed, they were analyzed with nonparametric analysis of variance by ranks (Kruskal–Wallis). Individual groups were compared post hoc with the nonparametric Mann–Whitney test. Data from the ELISA assays were evaluated by analysis of variance with Bonferroni post hoc analysis for multiple comparisons. Correlation analyses were performed using Pearson or Spearman rank tests as appropriate. Statistical calculations were performed using Statistical Package for Social Sciences (SPSS) Version 6.0 for Windows (SPSS, Chicago, IL).



## 2.9. Reagents

Culture media, antibiotics-antimycotics, estradiol, progesterone, human recombinant hCG, and FBS were from Sigma (St. Louis, MO). IL-1 $\alpha$ , TNF- $\alpha$  (both recombinant) were from the R&D Systems (Minneapolis, MN). Human LH and FSH were obtained from the National Hormone and Pituitary Program, NIH.

## 3. Results

### 3.1. Immunoreactive MCP-1 in follicular fluid

The mean diameter of aspirated follicles was 17 mm (range 15–25 mm). Concentration of MCP-1 in follicular fluid samples obtained from women ( $n = 4$ ) prior to the administration of hCG and prior to any detectable LH surge was  $90 \pm 27$  pg/ml. Concentration of MCP-1 was  $135 \pm 23$  pg/ml in follicular fluid samples obtained from women ( $n = 3$ ) 12 h after hCG administration. In follicular fluid samples obtained from women ( $n = 126$ ) 34 h after the hCG administration concentration of MCP-1 was  $322 \pm 46$  pg/ml which was significantly higher than pre-hCG levels ( $P = 0.007$ ) (Fig. 1). When we measured MCP-1 levels in individual follicles according to the size (between 15 and 25 mm diameter) from 6 women 34 h after the hCG administration; the mean concentration of MCP-1 from these follicles did not differ significantly.

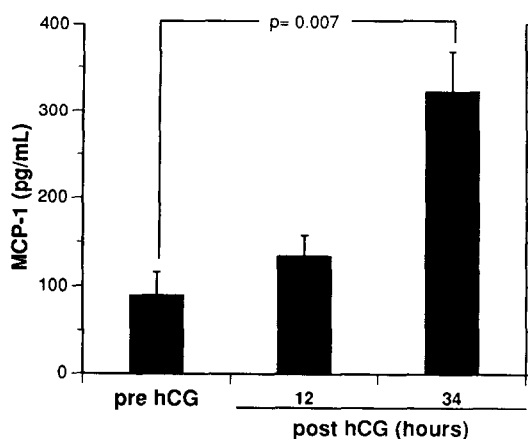


Fig. 1. Immunoreactive MCP-1 concentrations in follicular fluid obtained before ( $n = 4$ ), 12 h ( $n = 3$ ), and 34 h after ( $n = 126$ ) hCG administration. Values are mean (S.E.,  $P = 0.007$  prehCG vs. 34 h posthCG).

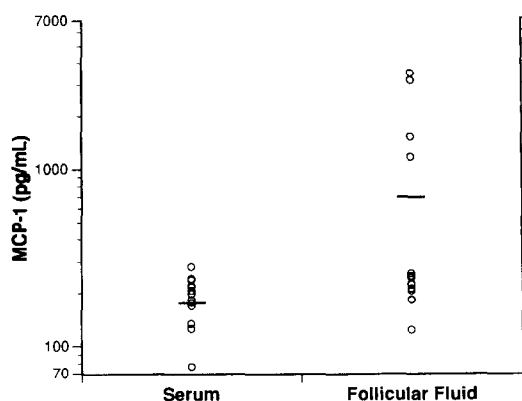


Fig. 2. Immunoreactive MCP-1 concentrations in serum and follicular fluid samples ( $n = 15$ ), same patient's values are connected by a line;  $P = 0.047$  between medians of each group.

In 15 women from whom blood samples were taken on the day of follicular aspiration (34 h after hCG administration), we observed higher (mean: 4.2-fold) follicular fluid MCP-1 levels ( $712 \pm 262$  pg/ml) than serum MCP-1 levels ( $181 \pm 13$  pg/ml). There was no correlation between the follicular fluid and serum MCP-1 levels. In 11 out of 15 matched pairs follicular fluid MCP-1 levels were higher than serum MCP-1 levels ( $P = 0.047$ , Wilcoxon signed-ranks test) (Fig. 2). Two distinct clusters of MCP-1 concentrations in follicular fluid were observed. There were no differences in age, diagnosis, follicular fluid or serum estradiol or progesterone levels between these two groups.

In follicular samples where progesterone levels were measured ( $n = 107$ ), there was a correlation between follicular fluid MCP-1 and progesterone levels from individual patient samples ( $r = 0.21$ ;  $P = 0.02$ ). The same correlation was observed between follicular fluid MCP-1 levels and serum progesterone levels ( $n = 56$ ) which were obtained on the day of oocyte retrieval ( $r = 0.29$ ;  $P = 0.03$ ). There was no correlation between follicular levels MCP-1 levels and follicular fluid estradiol ( $r = -0.01$ ;  $P = 0.34$ ) or serum estradiol ( $r = -0.01$ ;  $P = 0.89$ ) levels.

### 3.2. Cytokine modulation of MCP-1 production and mRNA expression in granulosa-lutein and ovarian stromal cells

Before anti-CD45 purification, granulosa-lutein cells secreted MCP-1 into the culture medium (Fig. 3). Cells treated with IL-1 $\alpha$  (10–100 U/ml) and TNF- $\alpha$  (10 ng/ml) alone or in combination produced significantly higher levels of MCP-1 than untreated cells (by 24 h,  $P = 0.01$ ). In cultures

performed after purification with anti-CD45 to eliminate leukocytes, granulosa-lutein cells did not produce detectable MCP-1. Treatment of these cells with IL-1 $\alpha$  (10–100 U/ml) and TNF- $\alpha$  (10 ng/ml) alone or in combination in three separate experiments did not induce production of detectable MCP-1 protein.

Ovarian stromal cells secreted MCP-1 ( $126 \pm 12$  ng/mg of total protein by 8 h, mean  $\pm$  S.E. for three replicates). Treatment of ovarian stromal cells with IL-1 $\alpha$  (10 U/ml) resulted in increased accumulation of MCP-1 in the media ( $316 \pm 20$  ng/mg of total protein by 8 h) ( $P = 0.03$ ). Similar findings were also observed following treatment with lower concentrations of IL-1 $\alpha$  (0.1 and 1 U/ml) and TNF- $\alpha$  (0.1 and 10 ng/ml) by 8 h. The level of MCP-1 mRNA ( $\sim 0.7$  kb) was low but detectable in stromal cells but increased markedly in response to IL-1 $\alpha$  and TNF- $\alpha$  treatment alone or in combination (Fig. 4). The increase in the levels of MCP-1 mRNA in stromal cells was dependent upon the concentration of IL-1 $\alpha$  (0.01–10 U/ml) or TNF- $\alpha$  (0.01–1 ng/ml) (Fig. 5, panel A and B). Increase of MCP-1 mRNA levels was evident by 15 min (data not shown), and high levels of MCP-1 mRNA were present by 4–6 h after initiation of either treatment, after which they began to decrease (Fig. 6A, B).

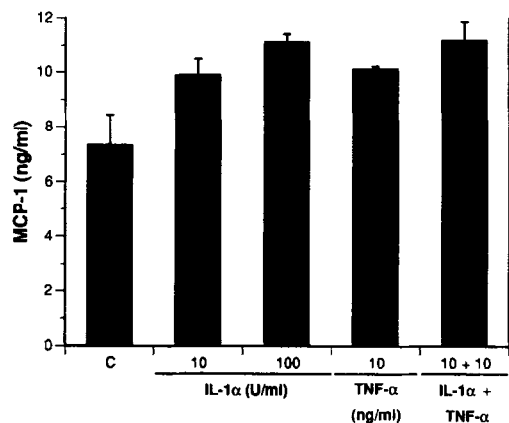


Fig. 3. Stimulation of immunoreactive MCP-1 production by granulosa-lutein cells before elimination of leukocytes in culture by IL-1 $\alpha$  and TNF- $\alpha$ . Granulosa-lutein cells were treated with serum-free medium alone (control) or with medium containing IL-1 $\alpha$  (10–100 U/ml) or TNF- $\alpha$  (10 ng/ml) for 24 h. The culture media were collected, and MCP-1 was quantified by ELISA. Data are mean  $\pm$  SEM for three replicates,  $P = 0.01$  control vs. treatments.

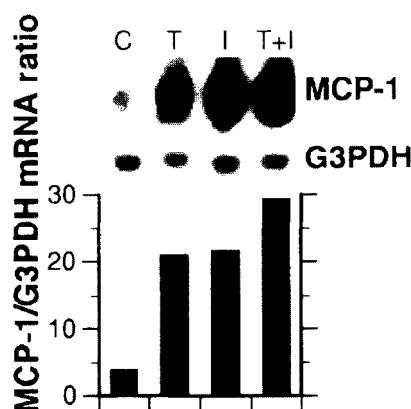


Fig. 4. Northern analysis of MCP-1 mRNA in ovarian stromal cells treated with IL-1 $\alpha$  and TNF- $\alpha$ . Confluent ovarian stromal cells were incubated for 8 h with IL-1 $\alpha$  (10 U/ml) or TNF- $\alpha$  (10 ng/ml) or both. Total RNA (10  $\mu$ g per lane) was evaluated. C: control; I: IL-1 $\alpha$ ; T: TNF- $\alpha$ .

### 3.3. Steroid and trophic hormone modulation of MCP-1 production and mRNA expression in ovarian stromal and granulosa-lutein cells

Treatment of ovarian stromal cells with LH (1–10 000 mIU/ml) for 8 h led to increases (between concentrations of 10 and 10 000 mIU/ml) in MCP-1 mRNA expression and protein secretion into the media that did not appear to be dose-dependent (Fig. 7). Treatment of ovarian stromal and granulosa-lutein cells in culture with hCG led to an increase in the level of MCP-1 mRNA (Data not shown). Treatment of stromal cells with FSH (1–1000 mIU/ml), progesterone (10–1000 ng/ml), and estradiol (0.01–100 ng/ml, up to 24 h of treatment) did not affect MCP-1 mRNA expression and protein production in three separate experiments (Fig. 8 showing treatment with estradiol).

## 4. Discussion

The ovulatory period denotes the time between the preovulatory LH surge and follicular rupture and extrusion of the oocyte. The LH surge initiates the final maturation of the oocyte and simultaneously triggers the cascade of events leading to follicular rupture and luteinization of granulosa cells. This period is characterized by dramatic and rapid changes in the peri- and intra-follicular area. These events are comparable to an inflammatory process because of the striking similarities in the extent of tissue remodeling

and the evident participation of leukocyte cell populations and their products (Espey, 1994). The observation that just prior to the time of ovulation there is an infiltration of macrophages into the area surrounding the leading follicle in rat ovaries suggest a critical role for these leukocytes in peri-ovulatory events (Brannstrom et al., 1993). On the other hand, there is always the possibility that the increase in leukocytes may be a consequence of the inflammatory-like events that lead to ovulation, rather than a cause of this degradative process. There is also evidence that follicular leukocyte infiltration is not an obligatory component of the ovulatory process in sheep, but rather involved in transforming the follicle into a fully functional corpus luteum (Murdoch and McCormick, 1993). Although the regulation of macrophage traffic in and around periovulatory follicles is not clearly understood, the temporal and spatial distribution points to a hormonally dependent event.

One of the candidate cytokines for monocyte/macrophage attraction is MCP-1, a member of the C-C chemokine family, in which the first two cysteine amino acid residues are in juxtaposition with each other (Peveri et al., 1988). MCP-1 has chemotactic specificity for monocytes (Matsushima et al., 1989). Our present study demonstrates the existence of MCP-1 in human pre-ovulatory follicular fluid following ovarian stimulation. If MCP-1 is truly involved in the peri-ovulatory events, the concentration of MCP-1

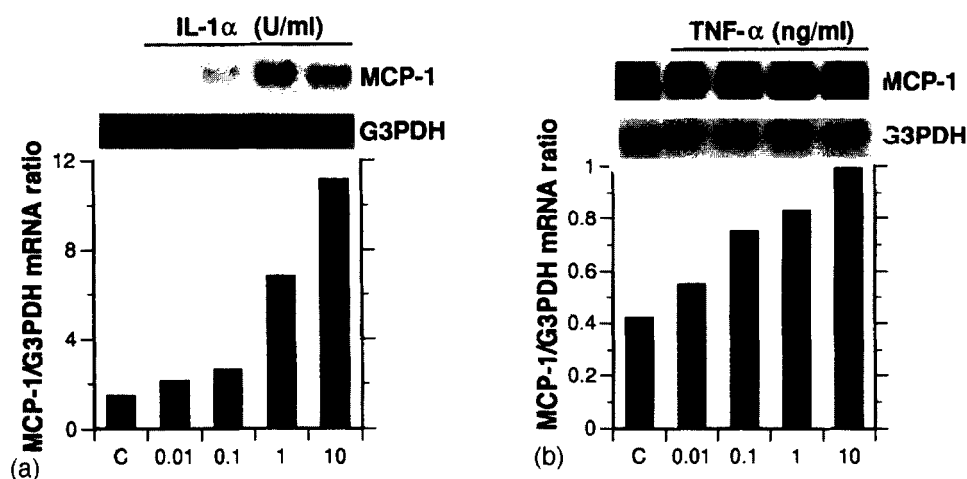


Fig. 5. Induction of MCP-1 mRNA in ovarian stromal cells by IL-1 $\alpha$  and TNF- $\alpha$  dose response. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation for 6 h in culture medium containing various concentrations of IL-1 $\alpha$  (0.01–10 U/ml) (panel A) or TNF- $\alpha$  (0.01–10 ng/ml) (panel B). At the end of the incubation period, total RNA was prepared from the cells. MCP-1 mRNA was evaluated by northern analysis of total RNA (10  $\mu$ g per lane).

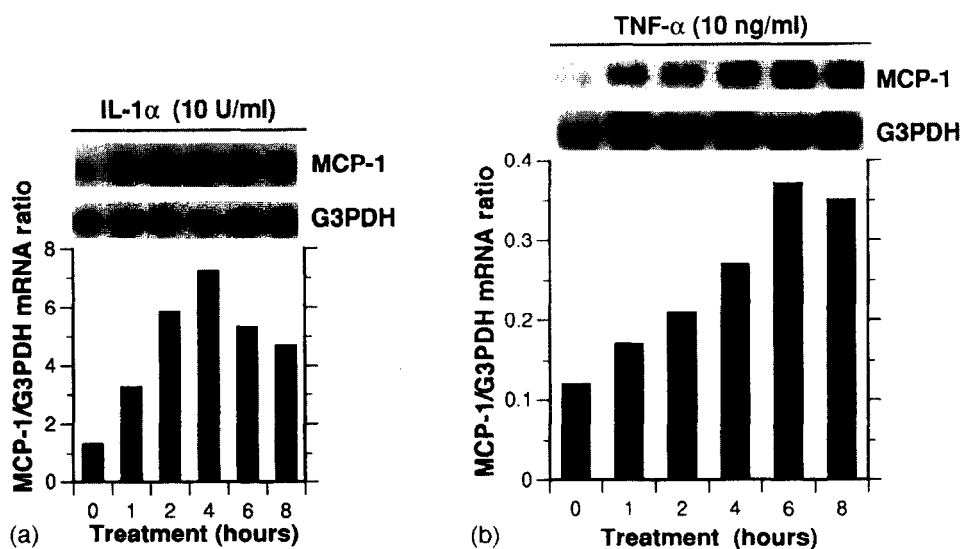


Fig. 6. IL-1 $\alpha$  and TNF- $\alpha$  mediated increases in MCP-1 mRNA levels. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation with culture medium containing IL-1 $\alpha$  (10 U/ml) for 1–8 h (panel A) or TNF- $\alpha$  (10 ng/ml) for 1–8 h (panel B). At the end of the incubation period, the culture media were removed, and total RNA was prepared from the cells. MCP-1 mRNA was evaluated by northern analysis of total RNA (10  $\mu$ g per lane).

should be increased in follicular fluids obtained after the LH surge (or the administration of hCG) when compared to pre-surge follicular fluids. This increase is precisely the finding of this study: MCP-1 is found in greater quantities in follicles following hCG administration.

If MCP-1 is a participant in the chemoattraction of macrophages in and around the peri-ovulatory follicle, then where does this cytokine originate? During the process of aspiration, follicular fluid might be mixed with blood, and monocytes could contaminate the follicular cell population. Thus, peripheral blood contamination is one potential source, but the MCP-1 concentrations observed in follicular fluid are higher than levels observed in serum. Therefore, the markedly elevated MCP-1 concentrations should reflect the true follicular fluid levels and are not secondary to contamination by peripheral blood. On the other hand, our data is obtained from analysis of follicular fluids obtained from women undergoing stimulation cycles and may not represent a physiological process. Although granulosa-lutein cells predominate in follicular fluid, macrophages and monocytes comprise 5–15% of human periovulatory follicular tissue cells and these cells are a known source of MCP-1 (Loukides et al., 1990). We found that after purification of granulosa-lutein cells by use of anti-CD45 immunobeads,

MCP-1 production is markedly diminished. Thus, resident macrophages are one of the contributors to the follicular fluid MCP-1 pool and they may be involved in amplifying the chemoattraction of more leukocytes. Another potential source for MCP-1 is the theca cell from the ovarian stroma. We found that cultured ovarian stromal cells express MCP-1 mRNA and secrete MCP-1 protein. Thus, MCP-1 is abundant in and around the follicle.

Macrophages are cells that can produce a number of factors that have been implicated in the paracrine regulation of ovarian functions. The cytokines IL-1 and TNF- $\alpha$  are released in large quantities from activated macrophages and can exert effects on ovarian cells. An intraovarian IL-1 system complete with ligands, receptors, and a receptor antagonist in humans is now well established (Hurwitz et al., 1992). In addition, human follicular fluid was reported to contain considerable IL-1 activity (Barak et al., 1992). We have presented data obtained with treatments using IL-1 $\alpha$  isoform. In some experiments we compared the effects of IL-1 $\beta$  isoform and we obtained identical results. This is somehow expected because despite the low sequence homology, these isoforms bind to the same receptor and have very similar if not identical biological properties. TNF- $\alpha$  also is found in the pre-ovulatory follicular fluid at concentrations used in the present study (Roby et al., 1990; Wang et al., 1992). Moreover, human granulosa cells were demonstrated to be both a source and a target for TNF- $\alpha$  (Zolti et al., 1990). This study presents evidence that the expression of MCP-1 from ovarian stromal and follicular cells is modulated by other cytokines such as

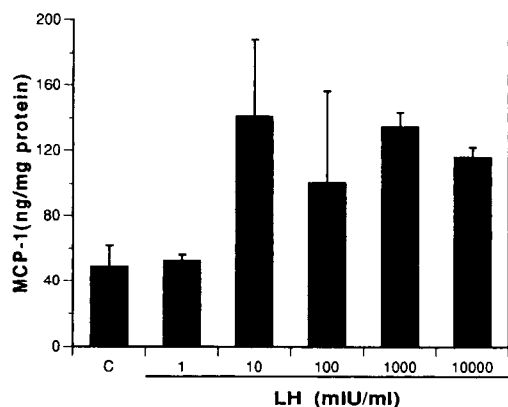


Fig. 7. Stimulation of MCP-1 protein production in ovarian stromal cells by LH. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation for 24 h in culture medium alone or with medium containing LH (1–10 000 mIU/ml). At the end of the incubation period, the culture media were collected, and MCP-1 was quantified by ELISA. Data are mean  $\pm$  S.E. for four replicates.

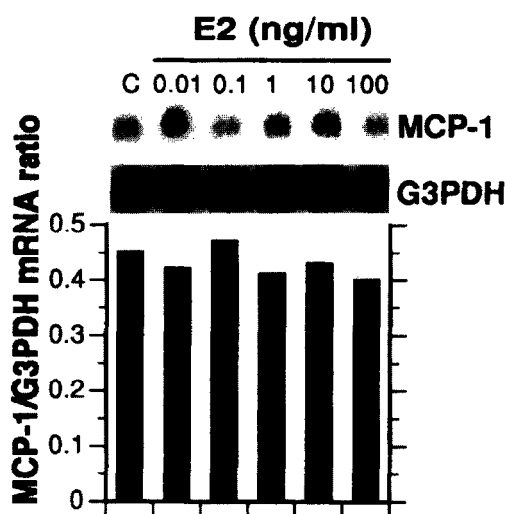


Fig. 8. Induction of MCP-1 mRNA in ovarian stromal cells by E2. Confluent ovarian stromal cells were placed in serum-free medium 24 h before incubation for 6 h in culture medium alone or with medium containing E2 (0.01–100 ng/ml). At the end of the incubation period, total RNA was prepared from the cells. MCP-1 mRNA was evaluated by northern analysis of total RNA (10  $\mu$ g per lane).

IL-1 and TNF- $\alpha$ . Thus, both cytokines may play some role in the constitutive secretion of MCP-1 as well as stimulating enhanced production and secretion.

As our hypothesis is that macrophage migration in and around the peri-ovulatory follicle is hormonally regulated and that regulation occurs through modulation of MCP-1 expression, we also investigated the effect of trophic hormones and sex steroids on the expression of MCP-1. We have found that hCG/LH stimulates MCP-1 mRNA expression and protein production from ovarian stromal cells and granulosa-lutein cells within 6–8 h.

In co-incubation studies, it has been demonstrated that macrophage-derived products stimulate progesterone production in human granulosa-lutein cells (Halme et al., 1985) and in mouse granulosa cells (Kirsch et al., 1981). Furthermore, in preovulatory follicles from the rat and hamster, IL-1 $\alpha$  and TNF- $\alpha$  have been demonstrated to stimulate progesterone production by the theca cells (Nakamura et al., 1990; Roby and Terranova, 1990), implicating macrophage-derived cytokines in the regulation of follicular progesterone production around the ovulation. Recently, it has been shown that IL-1 levels strongly correlates with progesterone levels in human follicular fluid (Barak et al., 1992). In the present study, we found a positive



correlation between follicular fluid MCP-1 levels and follicular and serum progesterone levels. Our finding that MCP-1 levels are correlated with progesterone levels but not with estradiol levels suggest that MCP-1 may not be relevant in earlier follicular events, and its main role is around or following ovulation. One of the limitations of our study is that our experiments are performed on granulosa-lutein cells. To better evaluate the relationship between steroid hormones and MCP-1 expression, experiments on pre-hCG granulosa cells are needed.

In summary, we have found that MCP-1 levels are elevated in periovulatory follicular fluid and ovarian stromal cells express the mRNA and produce the protein. We propose that regulation of MCP-1 in these cells by cytokines may play a role in the physiology of periovulatory events.

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