Monocyte chemotactic protein-1 expression in human corpus luteum

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Invasion of the corpus luteum by macrophages is a characteristic of luteal regression. Monocyte chemotactic protein-1 (MCP-1), a chemokine that recruits macrophages, is expressed in the rat corpus luteum where it increases in amount during luteolysis. In this study we examined the temporal and spatial expression of MCP-1 and changes in macrophage concentration in the human corpus luteum. Corpora lutea (n = 39) were grouped according to menstrual cycle phase and were examined by immunohistochemistry for MCP-1 and macrophages, and by Northern blot for MCP-1 mRNA. We found increasing amounts of macrophages with progressing luteolysis (P < 0.001). Staining for MCP-1 was stronger in the regressing corpora lutea compared with the staining in corpora lutea of early luteal phase (P < 0.05). MCP-1 was more prominent in blood vessel walls surrounding the corpus luteum than in vessels located far from it. The mean MCP-1 mRNA expression in regressing corpora lutea was higher than that observed in corpora lutea of the early and mid-luteal phase (P = 0.003). In conclusion, we found that MCP-1 expression and the number of macrophages increase with regression of the corpus luteum. MCP-1 is mostly expressed in blood vessel walls surrounding the corpus luteum and may play a role in the recruitment of macrophages to the corpus luteum during its regression.

Key words: corpus luteum/luteolysis/macrophages/monocyte chemotactic protein-1

Introduction

The corpus luteum is a transient endocrine gland formed from the ovarian follicle following ovulation and is essential for the maintenance of early pregnancy. It grows, develops and reaches structural and functional maturity by mid-luteal phase and then begins to regress in a non-fertile cycle. Regressive changes continue until the corpus luteum is completely absorbed into the ovarian stroma. The corpus albicans is the terminal stage of the corpus luteum before incorporation into the ovarian stroma. Local interactions between immune and steroidogenic cells may be involved in the control of luteal function. Macrophages, T-lymphocytes, monocytes and natural killer cells are all detected in the human corpus luteum (Wang et al., 1992). However, the predominant type of leukocyte in the human corpus luteum is the macrophage subpopulation. The invasion of the corpus luteum by macrophages is a well-known characteristic of luteal regression in a variety of animals including the guinea pig (Paavola, 1977, 1979), rabbit (Bagavandoss et al., 1990), pig (Hehnke et al., 1994), cow (Lobel and Levy, 1968), sheep (Murdoch and McCormick, 1993), rat and human (Lei et al., 1991; Wang et al., 1992; Brannstrom et al., 1994). There is convincing evidence that the numbers of these macrophages increase as luteolysis proceeds (Suzuki et al., 1998). Various functions have been attributed to macrophages in the corpus luteum including phagocytosis of cells and cell remnants (Paavola, 1979), the production of cytolytic cytokines (Bagavandoss et al., 1990; Benyo and Pate, 1992) and oxygen radicals (Riley and Behrman, 1991; Sawada and Carlson, 1991), and the production of factors that potentially influence steroidogenesis (Benyo and Pate, 1992).

In most tissues, macrophages are recruited as monocytes from the systemic circulation and subsequently undergo a process of differentiation and activation. Monocyte chemoattractant protein-1 (MCP-1), a 76 amino acid basic protein, is a chemokine that plays a role in the recruitment of monocyte/macrophages and is secreted by a variety of cell types including endothelial cells, fibroblasts, and macrophages (Rollins et al., 1991). It has been recently shown that MCP-1 is expressed in the pig and rat corpus luteum with increasing amounts during luteal regression (Hosang et al., 1994; Bowen et al., 1996; Townson et al., 1996). In the present study, we investigated the temporal expression of MCP-1 and macrophage recruitment during luteal regression in the human corpus luteum.

Materials and methods

Collection of tissues

Human corpora lutea were obtained from cycling women (n = 39; age range: 35–48 years) undergoing abdominal hysterectomy for benign gynaecological conditions at Yale–New Haven Hospital. Written informed consent was obtained from each woman prior to surgery using consent forms and protocols approved by the Human Investigation Committee of Yale University. The ovaries were macroscopically inspected for corpora lutea and specimens were
submitted for histological examination and RNA extraction. The day of the menstrual cycle was established from the women’s menstrual history and confirmed by endometrial and luteal histology using published criteria (Visfeldt and Starup, 1974; Noyes et al., 1975).

The indications for the surgery were as follows: leiomyomata (32 patients); endometriosis (five patients); pelvic relaxation (two patients). Samples were grouped according to menstrual cycle phases: early follicular (days 1–5 of the cycle; n = 12), mid-follicular (days 6–10; n = 5), late follicular (days 11–14; n = 8), early luteal (days 15–18; n = 5), mid-luteal (days 19–23; n = 3), and late luteal (days 24–28; n = 6).

**Immunohistochemistry**

The corpus luteum samples (n = 39) were snap-frozen in OCT (Tissue Tek, Sakura, Torrance, CA, USA). Serial 6–8 µm cryosections were placed on poly-l-lysine coated glass microscope slides and fixed at 4°C in acetone for 10 min for staining the macrophage marker, CD68 and in 4% paraformaldehyde for 15 min for staining MCP-1. Sections were rinsed twice in phosphate-buffered saline (PBS) for 5 min each and in PBS with bovine serum albumin (BSA) (PBS–BSA; 0.1%, wt/vol) for 5 min. Slides were then incubated with 4% blocking horse serum (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature in a humidified chamber. Excess serum was drained and primary antibodies [murine monoclonal anti-human MCP-1 antibody; PharMingen, San Diego, CA, USA], immunoglobulin G1 (IgG 1 ), clone: 5D3-F7, 500 µg/ml, 1/250 dilution in PBS and CD68, murine monoclonal anti-human macrophage antibody, (Dako, Carpinteria, CA, USA), IgG1 clone: EBM11, 395 µg/ml, 1/100 dilution in PBS–BSA] were added to the sections. Sections were incubated overnight at 4°C in a humidified chamber. The specificity of MCP-1 antibody was validated by incubation with an excess amount of recombinant human MCP-1 that eliminated the staining. For the negative control, non-specific mouse IgG was used at the same concentrations. Endogenous peroxidase activity was quenched with 0.6% H2O2 in PBS (vol/vol) for 15 min. The sections were rinsed. Then, biotinylated horse anti-mouse antibody (1.5 mg/ml, Vector Laboratories) was added at a 1:150 dilution for 45 min at room temperature. The antigen-antibody complex was detected by using an avidin–biotin–peroxidase kit (ABC, Vector Laboratories). Diaminobenzidine (3,3 diaminobenzidine tetrahydrochloride dihydrate, Aldrich Chemical Co, Milwaukee, WI, USA); hydrogen peroxide (0.5 mg in 0.03% H2O2 in PBS) was used as the chromogen, and sections were counterstained with haematoxylin. Finally, the sections were mounted with Permount (Fisher Chemicals, Springfield, NJ, USA).

Macrophages stained with CD68 antibody were counted using an Olympus microscope with a special ocular grid. Ten different fields at ×400 magnification were evaluated for both macrophage count and for the analysis of immunohistochemical MCP-1 staining. The MCP-1 staining was scored in a semiquantitative fashion that incorporated both the intensity and the distribution of specific staining (Bacus et al., 1988). The evaluations were recorded as percentages of positively stained target cells of all type in each of four intensity categories which were denoted as 0 (no staining), 1+ (weak but detectable above control), 2+ (distinct), 3+ (intense). For each tissue, an HSCORE value was derived by summing the percentages of cells that stained at each intensity multiplied by the weighted intensity of the staining [HSCORE = S Pi (i+1), where i is the intensity score and Pi is the corresponding percentage of the cells]. Vascularization, size of luteal cells, infiltration of theca cells and cavity organization were also evaluated. Three observers blinded to cycle data performed the HSCORE and the inter-individual variation was 14% for MCP-1 staining and 9% for macrophage staining.

**Northern analysis**

Corpus luteum tissues used for RNA extraction (n = 19) were frozen in liquid nitrogen and stored at –80°C prior to isolation of RNA. Total RNA was extracted using Trizol (Gibco BRL, Grand Island, NY, USA) and was size-fractionated (10 µg per lane) by electrophoresis on 1% formaldehyde-agarose gels. The RNA was transferred electrophoretically to Hybond-N+ membrane (Amersham, Arlington Heights, IL, USA), and cross-linked to the membrane using UV light. Pre-hybridization was conducted for 5 h at 65°C in a buffer composed of NaCl (0.9 mol/l), Tris–Cl (90 mmol/l, pH 8.3), EDTA (6 mmol/l), 5× Denhart’s solution, sodium dodecyl sulphate (SDS; 0.1% wt/vol), sodium pyrophosphate (0.1% wt/vol), and salmon sperm DNA (0.2 mg/ml). Hybridizations were conducted for 16 h at 65°C in a buffer that contained a 30-mer oligo DNA probe (5’T-TTG GTT TTG CTT GTC GTG GTC CAT GGA-3’) radiolabelled with [α-32P] ATP complementary to MCP-1 mRNA. Thereafter, blots were washed once with 6× sodium chloride/sodium citrate (SSC) and SDS (0.1% wt/vol) for 15 min at room temperature, once with 2× SSC and SDS (0.1% wt/vol) for 15 min at room temperature, and once for 20 min at 65°C. Autoradiography of the membrane was performed at –80°C using Kodak X-Omat AR film (Eastman Kodak, Rochester, NY, USA). Using a cDNA probe (Clontech Laboratories, Palo Alto, CA, USA), the amount of RNA in each lane was normalized by the analysis of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA. The autoradiographic bands were quantified using a laser densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

**Statistical analysis**

The amounts of MCP-1 mRNA and immunohistochemistry scores in the corpus luteum samples were normally distributed (tested by Kolmogorov-Smirnov test) throughout the menstrual cycle. Analysis of variance (ANOVA) and the Tukey test were used for statistical analysis and pairwise multiple comparisons, respectively. Statistical calculations were performed using the Statistical Package for Social Sciences (SPSS) for Windows, version 6.0 (SPSS, Chicago, IL, USA) and Sigmostat for Windows, version 2.0 (Jandel Scientific Corporation, San Rafael, CA, USA).

**Results**

**Immunohistochemical localization of MCP-1 and macrophages in the corpus luteum**

All 39 samples were evaluated by immunohistochemistry. CD68 antibody was used to identify macrophages. Specific dark-brown staining was observed in the cytoplasm of these cells (Figure 1A and 1C). These cells were morphologically different from granulosa or theca cells. The number of macrophages increased with the luteal regression (Figure 1C). In newly formed corpus luteum, macrophages were smaller with elongated cytoplasm that stained densely (Figure 1A). With the progression of luteal regression, larger macrophages that contained large vacuoles in their cytoplasm were seen. The number of macrophages was highest in samples from the mid-follicular phase of the cycle when the corpus luteum regresses (P < 0.001) (Figure 2).

Immunohistochemical staining for MCP-1 was stronger in the regressing corpora lutea in comparison to the staining in newly formed corpora lutea (Figure 1B and 1D). The levels of MCP-1 staining were highest in corpora lutea obtained from women in the mid-follicular phase of the menstrual cycle
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Figure 1. Frozen sections were stained for macrophages using CD68 antibody in samples from early (A) and regressing (C) corpus luteum. There was an increasing number of macrophages with progressing luteal regression. Frozen sections were also stained using monoclonal mouse anti-human MCP-1 antibody in samples from in early (B) and regressing (D) corpus luteum. Open arrows: luteal cells; filled arrows: macrophages; long arrows: endothelial cells. Bars = 75 µm.

Northern blot analysis of MCP-1 in corpus luteum

Corpus luteum samples \((n = 19)\) from 39 patients were evaluated with Northern blot analysis: early follicular \((n = 5)\), mid-follicular \((n = 2)\), late follicular \((n = 3)\), early luteal \((n = 4)\), mid-luteal \((n = 2)\), and late luteal \((n = 3)\). MCP-1 mRNA expression was lowest in the samples from the early and mid-luteal phases of the cycle. These phases correspond to the formation of fresh corpora lutea (Figure 4). The levels of MCP-1 mRNA were highest in corpora lutea obtained from the early follicular phase of the menstrual cycle. These samples were from regressing corpora lutea of the previous menstrual cycle. Expression of MCP-1 mRNA decreased at late follicular
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Figure 2. Number of macrophages per high power field (×400) in corpus luteum according to the menstrual cycle phase and regression in corpus luteum. Bars represent mean ± SEM. *P < 0.001 mid-follicular phase of the cycle (i.e. regressing corpora lutea) versus other phases.

Figure 3. The distribution of immunostaining intensity (HSCORE) in corpus luteum samples with murine anti-human MCP-1 antibody according to the menstrual cycle phase and luteal regression. Staining for MCP-1 was stronger in the regressing corpora lutea (from late luteal and early and mid-follicular phase of the following cycle) in comparison with newly formed corpora lutea of early and mid-luteal phase. Bars represent mean ± SEM. *P < 0.05 early follicular phase compared with early, mid- and late luteal phases of the menstrual cycle (i.e. fresh corpora lutea).

Figure 4. Northern blot analysis of MCP-1 mRNA in human corpus luteum tissues. Total RNA (10 µg per lane) isolated from corpora lutea was evaluated. Samples are arranged according to the menstrual cycle phase corresponding to the regression in corpora lutea. Graphic bars represent the mean value of densitometric units for each group normalized to G3PDH. *P = 0.003 early follicular phase (i.e. regressing corpora lutea) compared with early, mid- and late luteal phases of the menstrual cycle (i.e. fresh corpora lutea).

Discussion

The results of this study indicate that MCP-1 is expressed in the human corpus luteum. The expression of MCP-1 and the infiltration of macrophages increased with the natural regression of the corpus luteum. In recent studies, this has been demonstrated in the rat corpus luteum during natural luteolysis (Bowen et al., 1996) and in ovine and bovine corpora lutea with prostaglandin $F_2\alpha$-induced luteolysis (Tsai et al., 1997).

The human corpus luteum is composed of various regions. Steroid secreting cells (granulosa–luteal cells) are the main part of the corpus luteum. In the centre, there is a region of loose connective tissue that increases in size rapidly as luteolysis proceeds. Around granulosa–luteal cells is the thecal zone in which there are several cell layers that also secrete steroids. The predominant sites of white cell distribution are in the junction between the theca–luteal and granulosa–luteal areas and in the loose connective tissues. The predominant type of leukocyte in the human corpus luteum is the macrophage (Wang et al., 1992).

In the human ovarian stroma there are relatively few macrophages, but their number increases rapidly in the developing follicle and immediately prior to ovulation. There is also a preovulatory rise in the values of follicular fluid MCP-1 that may play a role in the chemotaxis of macrophages (Arici et al., 1997). Thus, human follicular fluid contains a significant number of macrophages (Loukides et al., 1990). In the human corpus luteum, macrophages comprise at least 10% of the total cells, distributed equally between the theca–luteal and granulosa–luteal areas (Wang et al., 1992). A significant number of macrophages could also be detected in the central loose connective tissue area of the corpus luteum.

Using immunohistochemistry, we first confirmed the invasion of human corpus luteum by macrophages during natural luteolysis, a result that is consistent with previous studies (Lei et al., 1991; Wang et al., 1992; Brannström et al., 1994). In fresh corpus luteum samples, there were a few small macrophages with elongated cytoplasm and dense staining. With natural luteolysis, macrophages increased in number and enlarged in size, containing large vacuoles in their cytoplasms.
Macrophages may be involved in luteal regression in a number of ways such as phagocytosis, secretion of cytokines and oxygen radicals, or release of substances that inhibit steroidogenesis (Townson et al., 1996).

We have found the value of MCP-1 mRNA expression to be lower in samples from the early and mid-luteal phases of the cycle, i.e. from newly formed, active corpus luteum samples. However, the level of MCP-1 mRNA expression was higher in samples from the early follicular phase of the cycle, consistent with the regression of corpus luteum. We have shown the same pattern for MCP-1 protein levels using immunohistochemistry. It is apparent that MCP-1 mRNA expression and MCP-1 production increase with the natural luteolysis in the human corpus luteum. In addition, blood vessels surrounding corpora lutea were rich in cells that stained strongly for MCP-1 but not for the CD68 macrophage marker. We postulate that luteal endothelial cells may express MCP-1 mRNA, probably in response to factors produced by steroidogenic luteal cells. Recent ovine and bovine studies were not able to demonstrate MCP-1 mRNA expression in large luteal cells (Tsai et al., 1997). Thus, the increases we observed in MCP-1 mRNA expression and MCP-1 production are unlikely to be the result of a direct expression in large luteal cells. Rather, these increases may be due to large luteal cell-derived products such as reactive oxygen species that act on another cell type, i.e. luteal endothelial cells. Indeed, we observed that endothelial cells stain strongly for MCP-1 but not for the CD68 macrophage marker. In addition to reactive oxygen species, cytokines such as interleukin-1, tumour necrosis factor-α, interferon-γ and growth factors such as epidermal growth factor and platelet-derived growth factor may be other agents that induce MCP-1 production (Oppenheimer et al., 1991; Miller and Krangel, 1992; Mukaida et al., 1992).

Moreover, the recruited macrophages from the systemic circulation during luteolysis in corpus luteum may be the secondary source for MCP-1 production, and this production may recruit more macrophages to the site of luteolysis. These immune cells also secrete cytokines that have been shown to decrease luteal cell steroidogenesis and viability (Benyo and Pate, 1992). In addition, direct stimulatory effects of MCP-1 on fibroblast collagen and transforming growth factor-β have been reported, findings that suggest a potential role for MCP-1 in luteolysis. This may represent another role for MCP-1 in luteolysis, independent of its role through immune cell recruitment and activation (Gharaei-Kermani et al., 1996).

In conclusion, we have found that MCP-1 expression and the number of macrophages increase with the regression of the human corpora lutea. Immunostaining for MCP-1 is also prominent in the blood vessel walls surrounding the corpus luteum. The expression of MCP-1 is associated with, or precedes, the accumulation of monocytes/macrophages in the regressing corpus luteum. We postulate that MCP-1 is mostly expressed in the endothelial cells of the blood vessels surrounding the corpus luteum, and MCP-1 production in these vessels is in response to various agents secreted by luteal cells. Thus, MCP-1 may play a role in the recruitment and activation of monocyte/macrophages to the human corpus luteum during its regression.

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References


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