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ORIGINAL ARTICLE

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Expression of monocyte chemoattractant protein-1 in peritoneal endometriotic cells

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Abstract It is well known that the number of peritoneal macrophages is increased in patients with pelvic endometriosis. We measured the concentration of monocyte chemoattractant protein-1 (MCP-1) using an enzyme-linked immunosorbent assay (ELISA) in the peritoneal fluid of women with and without endometriosis. The expression of MCP-1 in pelvic endometriotic lesions obtained from the peritoneum was also examined using immunohistochemistry and nonradioactive in situ hybridization. The mean concentration of MCP-1 in the peritoneal fluid was significantly higher in the patients with endometriosis ($P<0.05$). The most significant elevation, compared with non-endometriosis patients, was found in stage I of the disease ($P<0.05$). However, no statistically significant difference was found among endometriosis stages I, II, III, and IV. Immunohistochemical staining revealed that MCP-1-positive cells were localized in the glandular epithelium of the endometriotic lesions and in the stromal macrophages distributed in those lesions, but normal peritoneal cells were negative. The in situ hybridization method demonstrated expression of MCP-1 mRNA on the endometriotic glandular epithelium and stromal macrophages. These findings suggest that MCP-1 may be involved in the histogenesis and early development of peritoneal endometriosis.

Keywords Endometriosis · Monocyte chemoattractant protein-1 · Macrophages · Immunohistochemistry · In situ hybridization

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Introduction

Endometriosis, defined as the presence of ectopic endometrial glands and stroma, is a common benign gynecologic condition, but its pathogenesis remains controversial. In all three main theories – the implantation theory, the metaplastic theory, and the combination theory – peritoneal macrophages are considered to play a key role. This is based on previous studies showing an increased number and activation of macrophages in the peritoneal fluid of patients with pelvic endometriosis [6, 8, 9, 10, 11, 16, 25] as well as elevated levels of macrophage-related cytokines and growth factors [22, 23]. Certain cytokines and growth factors, such as RANTES [15], transforming growth factor-β (TGF-β) [21], tumor necrosis factor-α (TNF-α) [7], and monocyte chemoattractant protein-1 (MCP-1) [2, 5] have been reported to induce infiltration of blood monocytes or macrophages into the peritoneal cavity. MCP-1, one of the most potent monocyte/macrophage chemoattractants, is a 76-amino acid protein produced by many types of cells including monocytes, lymphocytes, fibroblasts, and smooth muscle cells [26], and it is detected in various pathological conditions [14, 26]. Recently, some investigators have reported a higher concentration of MCP-1 in the peritoneal fluid in women with endometriosis than in those without endometriosis [2, 5]. Akoum et al. described the preliminary data: MCP-1 is produced by cultured endometriotic fibroblast-like and epithelial cells in response to cytokines [1]. Arici et al. reported that nontreated mesothelial cells in culture produce and secrete MCP-1 and that the production is increased when they are treated by interleukin-1α (IL-1α) or TNF-α [5]. However, the distribution and localization of MCP-1 have never been shown in endometriotic tissues.

To better understand the pathophysiologic mechanisms underlying the recruitment of peritoneal macrophages in this disorder, we investigated whether MCP-1 is expressed in peritoneal endometriotic lesions in vivo by using in situ hybridization as well as immunohistochemical staining.

Materials and methods

Patients

Seventy-seven subjects were recruited for this study at Kumamoto University Hospital between June 1995 and February 1997. Informed consent was obtained from each patient, and the study was performed according to the guidelines of the ethics committee of the Kumamoto University School of Medicine. Endometriosis was diagnosed during laparoscopy or laparotomy. The severity of the endometriosis was determined according to the revised classification of the American Fertility Society [3]: stage I=25 patients, stage II=6, stage III=10, stage IV=6. None of the patients had undergone hormonal treatment during the 3 months prior to laparoscopy or laparotomy. The control subjects consisted of 30 infertile patients with no evidence of endometriosis at laparoscopy or laparotomy. The day of the menstrual cycle was determined based on basal body temperature, regularity of the cycle, and menstrual history. All subjects were divided into a proliferative phase group (22 endometriosis patients, 19 controls) and a secretory phase group (25 endometriosis patients, 11 controls).

Peritoneal fluid

Peritoneal fluid was obtained from the above-mentioned 77 patients at the time of laparoscopy or laparotomy. The fluid was aspirated from the uterovesical space and posterior cul-de-sac before any surgical intervention in order to minimize contamination by blood and was anticoagulated with 100 U of sodium heparin per specimen. Immediately after collection, the samples were centrifuged at 1,500 rpm for 5 min at 4°C, and the supernatants were aliquoted and stored at –80°C.

Tissue samples

Endometriotic specimens were obtained from typical blueberry-spot lesions of 23 of the 47 endometriosis patients at laparotomy. They included 16 cases in the proliferating phase and seven in the secretory phase. All samples were embedded immediately in Tissue-Tek O.C.T. compound (Hiles Inc., Elkhart, Ind., USA), frozen quickly in liquid nitrogen, and stored at –80°C. Serial 6-µm cryostat sections were made from each frozen block, then air-dried and fixed in acetone for 10 min. The first slide was stained with hematoxylin and eosin for histological evaluation, and all of the samples revealed an endometrium-like epithelium and stroma by light microscopy. Peritoneal and endometrial tissues were also collected from 13 patients with uterine myoma who showed a normal pelvic peritoneum. Ten of the patients were in the proliferative phase, and the other three were in the secretory phase.

Monoclonal antibodies

Two antibodies were used for immunohistochemistry: AM-3K, a mouse monoclonal antibody against human tissue macrophages, and F9, a mouse monoclonal antibody against human MCP-1. In several anti-human MCP-1 mouse monoclonal antibodies, a clone F9 may recognize the region of MCP-1 that binds to the receptor and gives the best immunostaining reaction [27]. F9 [27] and AM-3K [28] were prepared in the Department of Pathology, Kumamoto University School of Medicine. F9 was also used as the first antibody for enzyme-linked immunosorbent assay (ELISA).

MCP-1 sandwich ELISA

Concentration of human MCP-1 in the peritoneal fluid was measured using the sandwich ELISA [14] with a rabbit anti-human MCP-1 polyclonal antibody and a mouse anti-human MCP-1 monoclonal antibody (F9). MCP-1 concentration was calculated using interpolation from the standard curve. The detection limit of this assay was 1.6 ng/ml, with intraassay and interassay coefficients of variation <6%. All samples were examined in triplicate.

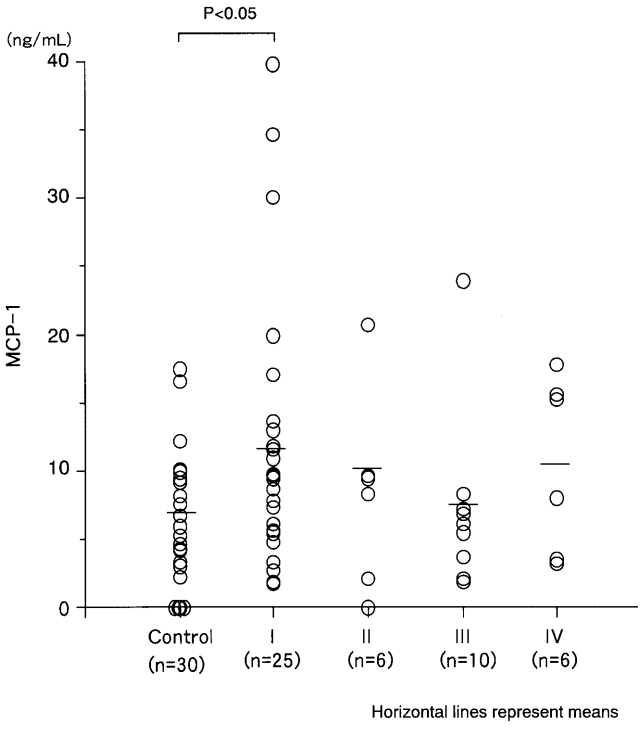

Fig. 1 Concentration of monocyte chemoattractant protein-1 (MCP-1) in peritoneal fluid of endometriosis and non-endometriosis patients. The distribution of MCP-1 concentrations in the peritoneal fluids of controls and endometriosis patients according to the stage of the disease is shown. *Horizontal lines* represent means. The mean MCP-1 level in stage I is significantly higher than in the control group ($P<0.05$)

Table 1 Peritoneal fluid concentration of monocyte chemoattractant protein-1 (MCP-1) in endometriosis and non-endometriosis patients, mean(SD)

	Patients (*n*)	MCP-1 concentration (ng/ml)
Non-endometriosis	30	6.37±3.24 (0.00–17.52)*
Endometriosis	47	10.56±7.47 (0.00–39.90)*
I	25	12.02±7.84 (1.72–34.71)*
II	6	11.72±5.78 (0.00–20.80)
III	10	6.73±4.50 (2.00–23.94)
IV	6	9.67±4.59 (3.27–17.91)
Non-endometriosis		
Proliferative phase	19	5.50±2.49 (0.00–12.20)
Secretory phase	11	7.89±4.11 (0.00-16.68)
Endometriosis		
Proliferative phase	22	10.77±7.10 (1.92-39.94)
Secretory phase	25	10.37±6.06 (0.00-34.70)

*$P<0.05$

Immunohistochemistry

Cryostat sections were stained using the indirect immunoperoxidase method, as described previously [4], after inhibition of

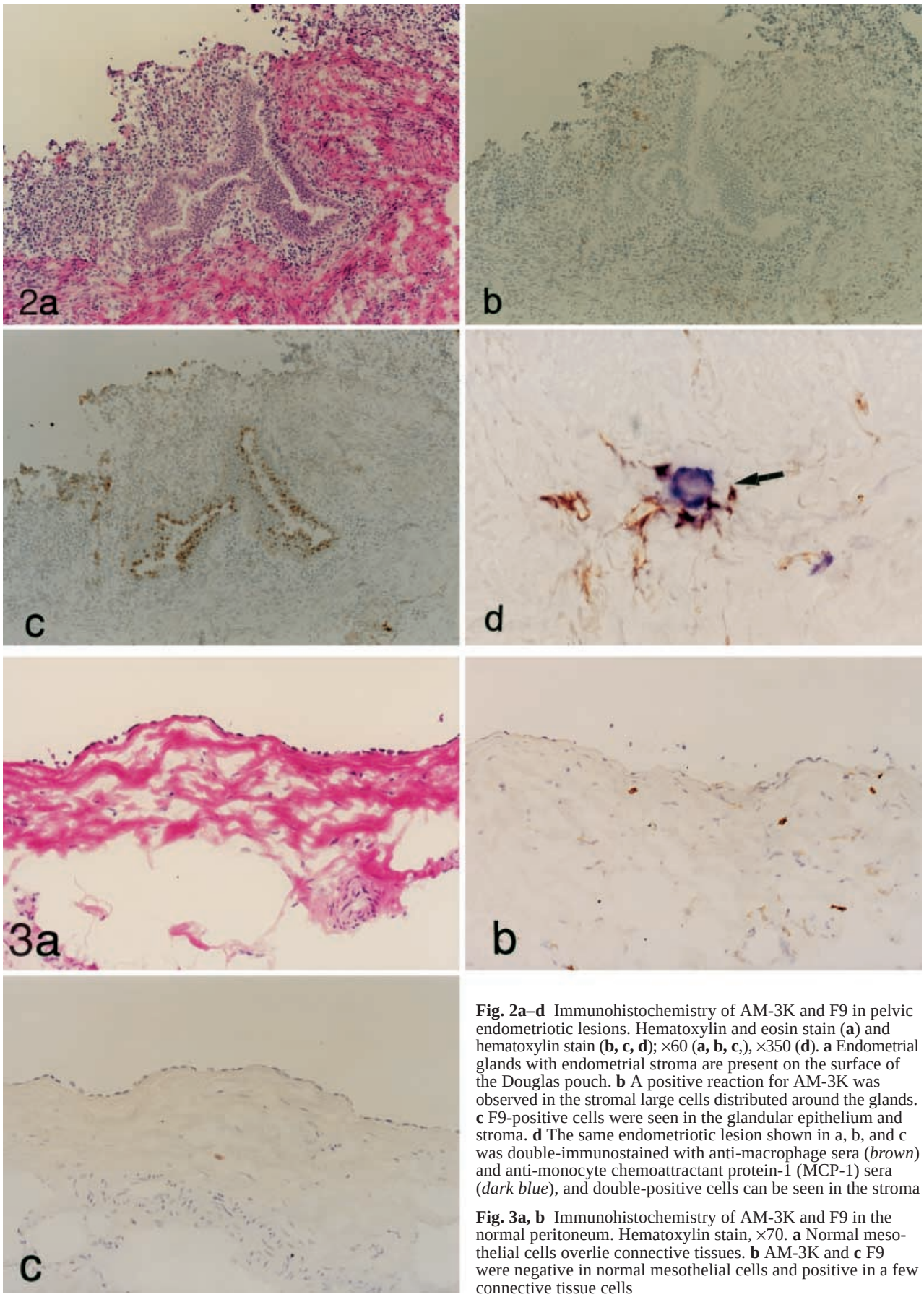

Fig. 2a–d Immunohistochemistry of AM-3K and F9 in pelvic endometriotic lesions. Hematoxylin and eosin stain (**a**) and hematoxylin stain (**b, c, d**); ×60 (**a, b, c**,), ×350 (**d**). **a** Endometrial glands with endometrial stroma are present on the surface of the Douglas pouch. **b** A positive reaction for AM-3K was observed in the stromal large cells distributed around the glands. **c** F9-positive cells were seen in the glandular epithelium and stroma. **d** The same endometriotic lesion shown in a, b, and c was double-immunostained with anti-macrophage sera (*brown*) and anti-monocyte chemoattractant protein-1 (MCP-1) sera (*dark blue*), and double-positive cells can be seen in the stroma

Fig. 3a, b Immunohistochemistry of AM-3K and F9 in the normal peritoneum. Hematoxylin stain, ×70. **a** Normal mesothelial cells overlie connective tissues. **b** AM-3K and **c** F9 were negative in normal mesothelial cells and positive in a few connective tissue cells

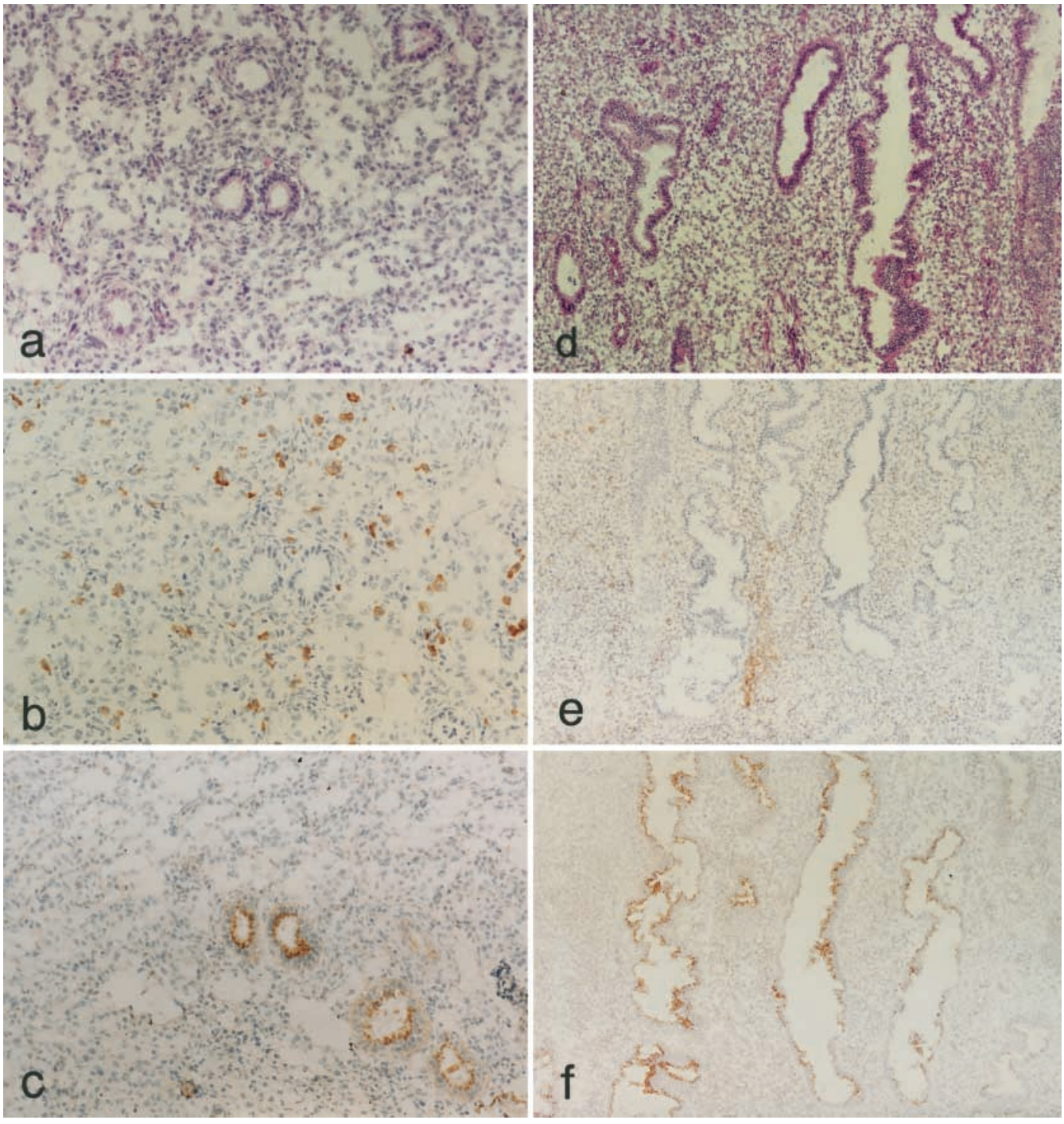

Fig. 4a–f Immunohistochemistry of the normal endometrium in the proliferative and the secretory phases. Hematoxylin and eosin stain (**a, d**) and hematoxylin stain (**b, c, e, f**). **a–c,** ×120, **d–f**, ×60. In **a** proliferative phase and **d** secretory phase, positive anti-human macrophage mouse monoclonal antibody (AM-3K) reactions were selectively observed in **b**, **e** the stroma. **c**, **f** F9-positive cells were seen in the glandular epithelium in both phases

endogenous peroxidase activity according to the method of Isobe et al. [13]. In the first step, the sections were incubated with each antibody for 90 min and then washed three times with phosphate-buffered saline (PBS, pH 7.2). In the second step, the sections were incubated with peroxidase-conjugated sheep anti-mouse immunoglobulin $F(ab')_2$ (Amersham, Little Charfont, UK) for 60 min. Following visualization with 3,3'-diaminobenzidine (Dojindo, Kumamoto, Japan), the sections were counterstained with hematoxylin.

Double immunostaining was performed with F9 and AM-3K to identify the specific cell types involved in MCP-1 production. At first, after inhibition of endogenous peroxidase activity, the frozen sections were incubated with AM-3K for 90 min, and, following three washes with PBS, the sections were incubated with peroxidase-conjugated sheep anti-mouse immunoglobulin $F(ab')_2$ (Amersham) for 60 min. Peroxidase was visualized by using 3,3'-diaminobenzidine (Dojindo) as a substrate to stain the sites brown. The sections were then rinsed in 0.1 mol/l glycine–hydrochloride (HCl) buffer (pH 2.2) for 40 min to remove the first and second

antibodies. Secondly, the same sections were incubated with F9 for 90 min, followed by washing with PBS. They were next incubated with rabbit anti-mouse immunoglobulin $F(ab')_2$ (alkaline phosphatase anti-alkaline phosphatase (APAAP kit, Dako) for 30 min. The sections were washed with Tris-buffered saline (pH 7.6) and reacted with APAAP complex (APAAP kit) for 30 min. Next, the sections were incubated with a mixture of 0.2 mmol/l naphthol AS-MX phosphate, 1 mmol/l fast blue BB salt, and 1 mmol/l levamisole (Sigma Chemical Company, St. Louis, Mo., USA) in 50 mmol/l Tris–HCl buffer (pH 8.7) for 10 min to color the reaction blue, and then mounted without counterstaining. Negative control staining was performed in the same manner except that the first antibody used in the first and/or second step was omitted. As positive controls, adequate autopsy specimens were submitted to the same procedures. All immunohistochemical staining procedures were performed in a humidified box at room temperature.

In situ hybridization

A digoxigenin-labeled single-strand RNA probe was prepared by using a DIG RNA labeling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Human MCP-1 probe, a 0.7 kb *Eco*R I fragment that covers the full length of human MCP-1 complementary DNA, was kindly supplied by Dr. T. Yoshimura (Immunology Section, National Cancer Institute, Frederick, Md., USA). Hybridization was performed according to the method described elsewhere [12]. A sense probe was used as a negative control in every run.

Statistical analysis

A logarithmic transformation was performed on the concentration of MCP-1 in the peritoneal fluid of the endometriosis patients and controls. Subsequently, using an unpaired *t*-test, the MCP-1 levels were compared between the endometriosis group and the non-endometriosis group, and between the proliferative phase group and the secretory phase group. Values were evaluated using one-way analysis of variance (ANOVA) with the Bonferroni/Dunn procedure for multiple comparisons. For all analyses, differences were considered statistically significant for P values less than 0.05.

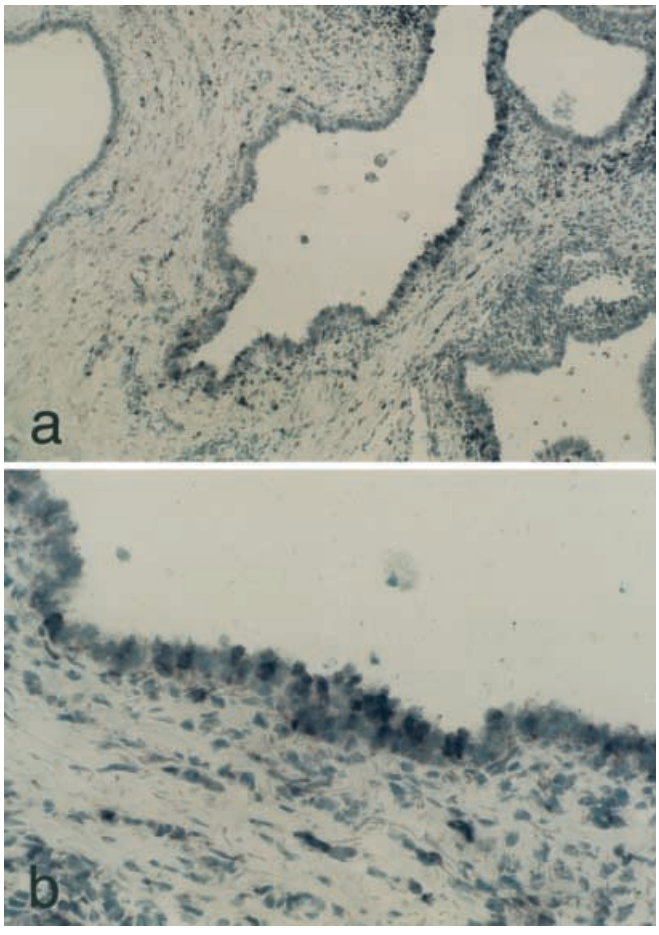

Fig. 5a, b In situ localization of MCP-1 mRNA in pelvic endometriotic lesions. The sections were hybridized with an MCP-1 anti-sense probe. Hematoxylin stain; **a**, ×70; **b**, ×150. **a**, **b** MCP-1 mRNA was expressed in some glandular cells and stromal large cells in endometriotic tissue

Results

Concentration of MCP-1 in peritoneal fluid

Seventy-two of 77 peritoneal fluid samples obtained, whether from endometriosis patients or not, contained detectable levels of MCP-1 at varied concentrations (Fig. 1). Of the five subjects without a detectable level (less than 1.6 ng/ml), only one was an endometriosis patient (Fig. 1). The mean concentration of MCP-1 in the peritoneal fluid was significantly higher in endometriosis patients than in the controls ($P<0.05$, Table 1). Although the MCP-1 level in stage I patients was significantly elevated compared with the controls ($P<0.05$), there was no significant difference between the controls and stage II, III, or IV patients (Table 1, Fig. 1). Although no significant differences were found among the stages of endometriosis, MCP-1 concentration was higher in stage I patients than in stage II, III, or IV patients (Table 1, Fig. 1). No significant differences were shown between the follicular and luteal phase of the menstrual cycle within each patient group (Table 1).

Distribution and localization of macrophages and MCP-1 in endometriotic tissues

In endometriotic tissues, many macrophages were observed in the stroma, clustering around the endometriotic glands (Fig. 2a, b). Positive reactions for MCP-1 were distinctly observed in glandular epithelial cells and in some cells in the stroma of the endometriotic tissues (Fig. 2c). The double-immunostaining technique demonstrated that the MCP-1-positive cells located in the stroma were macrophages (Fig. 2d). In contrast, the normal peritoneum of both the endometriosis and non-endometriosis patients, which consists of a thin layer of mesothelium overlying the connective tissue, was negative with both F9 and AM-3K (Fig. 3a–c). A very small population of macrophages was observed in the connective tissue underlying the peritoneal cells (Fig. 3b). In the proliferative phase of the cyclic endometrium (Fig. 4a), a positive AM-3K reaction was observed in stromal large cells (Fig. 4b), and F9-positive cells were observed in the glandular epithelium (Fig. 4c). No double-positive cells were seen (data not shown). In the secretory phase

Table 2 Distribution of macrophages and expression of monocyte chemoattractant protein-1 (MCP-1) in endometriosis lesions, normal peritoneum, and normal endometrium. *AM-3K* anti-human macrophage mouse monoclonal antibody; *F9* anti-human MCP-1 mouse monoclonal antibody; – negative reaction; + positive reaction; –/+ positive reaction in some cells; *ND* not done

Tissues	Immunohistochemical staining		In situ hybridization MCP-1 mRNA
	AM-3K	F9	
Endometriosis			
Glandular epithelial cells	–	+	+
Stromal cells	–/+	–/+	+
Normal peritoneum			
Mesothelial cells	–	–	–
Mesenchymal cells	–/+	–	–
Normal endometrium			
Proliferative phase			
Glandular epithelial cells	–	+	ND
Stromal cells	–/+	–/+	ND
Secretory phase			
Glandular epithelial cells	–	+	ND
Stromal cells	–/+	–/+	ND

(Fig. 4d), the distributions of macrophages and MCP-1 positive cells were similar to those in the proliferative phase (Fig. 4e,f).

Expression of MCP-1 mRNA in endometriotic tissues

The constitutive expression of MCP-1 mRNA in the endometriotic lesions and normal peritoneum was determined using in situ hybridization. MCP-1 mRNA was expressed in epithelial cells and some large stromal cells in the endometriotic tissues (Fig. 5a, b) independent of the menstrual phase. MCP-1 mRNA was not expressed in any of the cells in the normal peritoneum. The expression pattern of MCP-1 mRNA was consistent with the results of immunostaining of MCP-1 (Table 2). Sense transcript controls obtained by parallel hybridization with the same specimens did not generate any signals.

Discussion

Macrophages are the predominant cell type observed in the peritoneal cavity of endometriosis patients and are implicated in the clinical manifestations of endometriosis by phagocytising sperm or interfering with ovum capture [19]. Their activation also results in the release of cytokines/growth factors and other secretory products that may be responsible for the alterations in sperm–egg interaction and early embryo development [24]. Since the early 1980s, many studies on the peritoneal fluid of endometriosis patients have shown an increased number of monocyte–macrophage lineage cells [6, 8, 9, 10, 11]. In addition, a high concentration of monocyte/macrophage chemoattractant cytokines and growth factors, i.e., RANTES [15], TGF-β[21], TNF-α[7] or MCP-1 [2, 5], which can induce macrophage infiltration into the pelvic cavity and stimulate the production of macrophage-derived cytokines and growth factors, has also been shown. However, the mechanisms that trigger their presence in the peritoneal cavity remain unresolved. Our present immunohistochemical staining of peritoneal endometriotic lesions always localized MCP-1 protein in the glandular epithelium and stromal macrophages. MCP-1 mRNA was also expressed in peritoneal endometriotic lesions, but not in the normal pelvic peritoneum. This is the first report to demonstrate directly the expression of MCP-1 in peritoneal endometriosis in vivo. It suggests that endometriotic glandular epithelium and stromal macrophages may be important sources of MCP-1 in the early stage of endometriosis development.

Despite more than a century of intensive investigation, the pathogenesis of endometriosis is poorly understood, but there are three major theories: the implantation theory, the metaplastic theory, and the combination theory [23]. Based on their ultrastructural and histological study, Nakamura et al. showed that pelvic endometriosis occurs in a series of changes from normal mesothelial cells [17]. Ohtake et al. demonstrated that endometriotic lesions can arise from human ovarian surface epithelial cells, which are embryologically homologous to peritoneal mesothelial cells, through a process of metaplasia in the presence of 17β-estradiol and endometrial stromal cells [20], suggesting that retrogradely flowing menstrual debris containing endometrial stromal cells may influence the peritoneal metaplastic change to endometriotic tissue.

Recently, in idiopathic pulmonary fibrosis, MCP-1 expression was reported in cuboidal and flattened metaplastic epithelial cells on alveolar walls, in macrophages accumulating in alveolar spaces, and in some vascular endothelial cells [14]. This study suggested that the MCP-1 production in idiopathic pulmonary fibrosis may play an important role in the recruitment of monocyte-macrophage lineage cells and that MCP-1 may be one of the key factors inducing irreversible progression of the disease. This study also indicated the possibility that MCP-1 may be secreted by metaplastic peritoneal cells in patients with pelvic endometriosis.

In 1996, Akoum et al. first reported that the concentration of MCP-1 was increased in the peritoneal fluid of endometriosis patients compared with non-endometriosis patients [2]. They compared the MCP-1 level in stages I and II with stages III and IV [2]. Arici et al., however, observed a stage-dependent elevation in peritoneal fluid MCP-1 concentration [5]. In the present study, we also detected a significant elevation of MCP-1 concentration in the peritoneal fluid of endometriosis patients compared with that of non-endometriosis patients. No statistical correlations of MCP-1 concentration were found with the severity of disease, but it was significantly higher in patients with minimal stage disease than in the control patients. Based on our results, MCP-1 may be involved in the development of endometriosis at the early stage. Our data and those of other investigations provide evidence that MCP-1 is at least an important constituent of the peritoneal fluid of women with pelvic endometriosis.

The results of our study indicate that MCP-1 may exert a causal and consequent influence on peritoneal endometriosis. We postulate that expression of MCP-1 on the metaplastic mesothelial cells is an early event in the development of pelvic endometriosis and that MCP-1 subsequently contributes to the accumulation of peritoneal macrophages, which perpetuate the inflammatory reaction and adversely affect the fertility of endometriosis patients by secreting cytokines/growth factors and cytotoxic compounds. Furthermore, peritoneal macrophages themselves secrete MCP-1 into the pelvic cavity.

Our study provided insights into the role of MCP-1 in the pathogenesis of peritoneal endometriosis. Further studies on the expression of MCP-1 on columnar and ciliated epithelial cells constituting peritoneal infolding, which may be an initial stage of endometriosis [17, 18], should lead to a better understanding of MCP-1 in this disease.

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