

Suppression of macrophage activation by peritoneal fluid from patients with endometriosis

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Abstract

To investigate the effects of peritoneal fluid from patients with endometriosis on mouse peritoneal macrophages (M ϕ), peritoneal fluid from endometriosis patients ($n = 15$) were added to a monolayer of C3H/HeJ mouse peritoneal M ϕ . Tumor necrosis factor-producing activity was measured by the L929 assay activated with FK-23 (a preparation of heat-killed *Enterococcus faecalis*). Tumor necrosis factor-producing activity of C3H/HeJ mouse peritoneal M ϕ incubated with peritoneal fluid was suppressed in 14 endometriosis patients. Interestingly, in nine endometriosis patients, tumor necrosis factor-producing activity was much lower than seen with mouse peritoneal M ϕ incubated with corticosterone. Peritoneal fluid contains suppressive properties for the activation of peritoneal M ϕ , which might allow the implantation of free endometrial cells or the metaplastic phenomena stimulated by retrograde menstruation. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

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1. Introduction

Endometriosis is widely known to be associated with infertility and pelvic pain. But the pathophysiology of endometriosis is poorly understood. In recent hypotheses the etiology and process of this disease have been ascribed to abnormalities of the immune system as follows.

Oosterlynck et al. reported that natural killer (NK)-mediated cytotoxicity was significantly decreased in women with endometriosis [1].

Gleicher et al. demonstrated that more than two thirds of endometriosis patients exhibited evidence of abnormal polyclonal B cell activation, which resulted in significant autoantibody abnormalities [2].

Not only lymphoid cells but also monocytes or macrophages (M ϕ) have been thought to play a major role in maintaining homeostasis within the peritoneal cavity with endometriosis, in which the existence of local inflammation, as seen by the increase in peritoneal fluid, adhesion formation and hemorrhagic changes, were found [3,4].

The total number of peritoneal macrophages was significantly increased in these patients [5]. More recently, Braun et al. showed that the cytotoxic func-

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tion of peritoneal M ϕ in patients with endometriosis was inversely correlated with the severity of the disease [6].

Therefore, it is very important to investigate the regulatory mechanisms of peritoneal macrophages in order to understand the pathogenesis of endometriosis.

The objective of this study was to evaluate the effects of peritoneal fluid (PF) from patients with endometriosis as a biological modifier of M ϕ functions. Included in the variety of macrophage functions are production of an inflammatory cytokine, tumor necrosis factor (TNF), recognized as one of the most important steps in the sequential biologic reaction in vivo [7]. We focused on the inhibitory effects of PF on TNF production by murine macrophages which are a well-established experimental system as a model of macrophage activation [8].

2. Materials and methods

2.1. Patient characteristics

PF was obtained from 15 women during laparoscopy in the follicular phase by the Department of Obstetrics and Gynecology, Teikyo University School of Medicine. These preparations were obtained with prior consent from all patients undergoing laparoscopic examination. The mean age of the patients was 29 years (range 23–42). Endometriosis was staged according to the revised American Fertility Society Classification (Re-AFS) [9], which revealed six patients with stage I, two with stage II, five with stage III and two with stage IV.

As a control, PF was obtained from a woman who was suspected to have endometriosis from her clinical history showing severe dysmenorrhea and unexplained infertility. However, she had no endometriosis lesions.

2.2. Preparation of PF

PF was aspirated under laparoscopic examination from the cul-de-sac before surgical treatment. After recording the PF's volume and color, PF was transferred immediately to the laboratory for preparation. The PF was centrifuged at $400 \times g$ for 20 min

at 4°C and the supernatant was stored at -80°C until assay.

TNF- α activity in each PF was measured with a commercial enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Assay Research Center, Tokushima, Japan) (Lot No. IA94). Protein concentration in each PF was measured by Bradford's method [10].

In order to estimate the molecular size of the substances with inhibitory activity, a PF of an endometriosis patient (case 14, T.S., Re-AFS stage IV) was fractionated by ultrafiltration with Microcon-10 (Grace Japan Co.), which is able to fractionate to a molecular mass of 10^4 Da. Each filtrated PF was stored at -80°C until assay.

2.3. Preparation and activation of mouse peritoneal macrophages

Male C3H/HeJ mice (6–8 weeks old) were injected intraperitoneally with 3 ml of 3% thioglycollate as described previously [8]. Four days later, peritoneal exudated cells (PEC) were collected and washed with phosphate-buffered saline (PBS) (pH 7.3–7.65). These peritoneal cells were rinsed twice with Roswell Park Memorial Institute (RPMI 1640) medium containing 5% fetal calf serum (FCS) and adjusted to 1.0×10^6 cells ml^{-1} . 100 μl of the cell suspension was seeded into each well of a 96-well flat-bottomed microtiter plate. Then, peritoneal cells were incubated for 2 h in a 5% CO_2 incubator at 37°C.

After incubation, non-adherent cells were discarded. These monolayers, containing more than 90% M ϕ as confirmed by methods described previously [8], were preincubated with PF for 1 h. Then, these M ϕ were incubated in the presence of 100 μg ml^{-1} of a preparation of heat-killed *Enterococcus faecalis* (FK-23), which is a bacterial TNF inducer [11], for an additional 3 h. As controls for inhibitory effects, peritoneal M ϕ were incubated with or without corticosterone (10^{-6} M). Corticosterone is a glucocorticoid with strong inhibitory effects on the TNF production of M ϕ as previously described [7]. After incubation with FK-23, the medium of each well was collected. These supernatants, obtained by centrifugation, were stored in aliquots at -80°C until the L929 cytotoxicity assay was performed as described below. Activity of PF samples was shown by the

TNF-producing ratio which was calculated as [TNF produced by FK-23-stimulated M ϕ in the presence of PF/in the absence of PF]. We previously reported the accuracy of this method by masking exclusively with an anti-TNF polyclonal antibody [12].

2.4. Preparation and activation of human peripheral monocytes

20 ml of peripheral blood was drawn from a healthy male donor (blood type AB, Rh positive). Peripheral mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation. Mononuclear cells (2×10^5 cells well $^{-1}$) were cultured in RPMI 1640 medium containing 5% FCS in 96-well microtiter plates for 2 h at 37°C in 5% CO $_2$. The non-adherent cells were discarded. These monolayers that were rich in monocytes were incubated with the PF for 1 h and then incubated with FK-23 for an additional 17 h. After incubation, the supernatant from each well was collected and immediately centrifuged. These preparations were stored in aliquots at -80°C until the L929 cytotoxicity assay.

2.5. L929 cytotoxicity assay

In vitro cytotoxicity against L929 cells was assayed by the methods of Ruff and Gifford as described in detail elsewhere [13]. Briefly, cultures of 8×10^5 cells in 100 μl of Eagle's minimum essential medium (EMEM) with 5% calf serum (CS) were established in 96-well flat-bottomed microtiter plates and in a 5% CO $_2$ incubator at 37°C for 3 h. 50 μl of the sample and a final concentration of 1 $\mu\text{g ml}^{-1}$ actinomycin D were added to each well and incubated for 18 h in a 5% CO $_2$ incubator. After incubation, these L929 cells were stained with crystal violet and examined under an optical microscope. The absorbance at 540 nm was measured photometrically and the survival ratio was calculated as previously described [14].

2.6. Statistical analysis

Statistical evaluation of the data was done with Student's *t*-test for differences among groups or ANOVA (Kruskal-Wallis for nonparametric evaluation) for correlation.

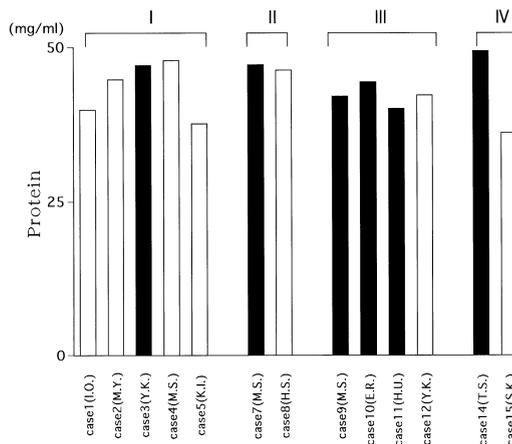


Fig. 1. Protein concentrations in the PF from 13 patients with endometriosis. I, II, III and IV show the Re-AFS classification. Open and closed bars represent PF in which TNF- α measured by ELISA was negative and positive, respectively.

3. Results

3.1. Protein concentration and TNF- α activity in PF

The concentration of protein in PF obtained from 13 patients with endometriosis was determined. The mean protein concentration of PF was 44.2 mg ml^{-1} , ranging from 37.7 to 49.4 mg ml^{-1} (Fig. 1). There was no significant correlation between protein concentration and Re-AFS classification (ANOVA, $P=0.0530$). As shown by the solid bars in Fig. 1, significant levels of TNF- α ($>25 \text{ pg ml}^{-1}$) were detected in six cases of 13 (46%), ranging from 56.0 to $1200.0 \text{ pg ml}^{-1}$. No correlation was observed between TNF- α concentration and Re-AFS classification.

3.2. Effect of PF on TNF-producing activity of macrophages (C3H/HeJ mouse peritoneal M ϕ and human peripheral monocytes)

Fig. 2 shows the change of TNF-producing activity by serial dilution of PF from an endometriosis patient (case 14, T.S., Re-AFS classification stage IV). These dose-response curves reveal a suppressive effect on TNF-producing activity in relation to the concentration of PF in a dose-dependent manner. Fig. 3 shows that TNF-producing activity in PFs of endometriosis patients (14/15 cases) was signifi-

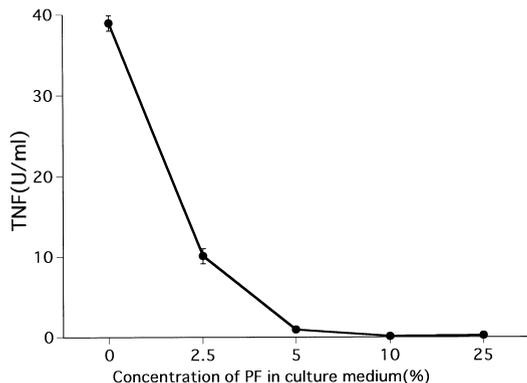


Fig. 2. Suppressed TNF production of murine macrophages in the presence of PF from endometriosis patients. TNF-producing activity in each plot is expressed as the mean \pm S.E.M.

cantly lower than that of the PF of the non-endometriosis patient. Moreover, in nine (of the 14) cases the TNF-producing activities induced by FK-23 were significantly lower than in corticosterone-treated specimens (Fig. 3). However, there was no significant correlation between the inhibitory activity of PF and Re-AFS classification (Fig. 4).

As regards molecular size, the higher molecular fraction ($\geq 10^4$ Da) of PF was significantly more suppressive than the lower molecular fraction ($< 10^4$ Da) (Fig. 5).

The inhibitory effect of PF on human peripheral monocytes was also investigated. Compared with mouse peritoneal M ϕ , TNF-producing activity from

human monocytes which were incubated with FK-23 followed by PF samples was almost identical, since a significant correlation was observed between the values showing the TNF-producing activities obtained from two systems using human monocytes and murine macrophages (Fig. 6).

4. Discussion

Our results show that PF from endometriosis patients markedly suppressed the activation of mouse peritoneal M ϕ stimulated by FK-23. In nine of the 15 samples of PF with endometriosis (60%), a strong suppressive effect on the TNF-producing activity, induced by FK-23, was observed, comparable with that of corticosterone treatment. Abe et al. reported that very low, but physiological concentrations of corticosterone inhibit the cytotoxic activity of TNF against the L929 fibroblast cell line [14]. Glucocorticoids are known to inhibit inflammatory reactions through induction of de novo synthesis of lipocortin, a phospholipase A₂ inhibitor [15,16]. We confirmed also that the inhibitory activity of PF was effective on the TNF production in human monocytes. Therefore, we could assume that PF suppresses TNF production in peritoneal M ϕ in patients with endometriosis.

M ϕ are the cell type found in the greatest quantity in PF in endometriosis. The regulatory mechanisms

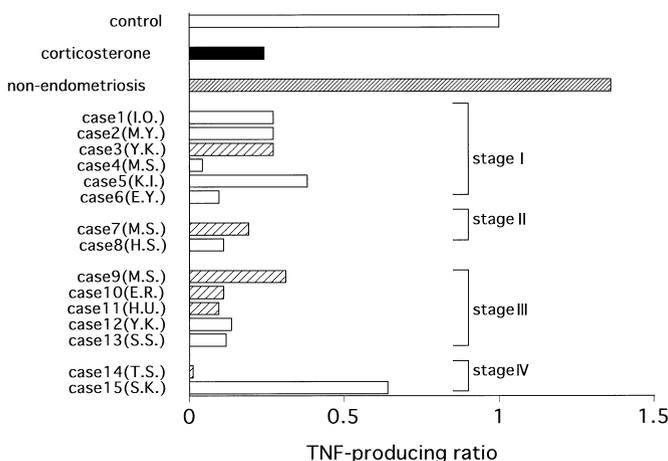


Fig. 3. The inhibitory effect of PF on TNF-producing activity of murine macrophages. Hatched bars represent TNF- α positive PF. I, II, III and IV show the Re-AFS classification.

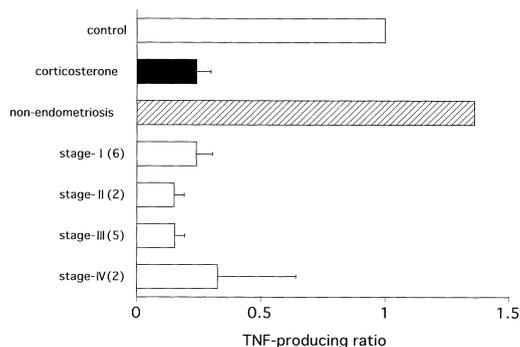


Fig. 4. Summary of the inhibitory effect of PF on TNF-producing activity and classification of Re-AFS. This shows the accumulated data from three experiments. Corticosterone (10^{-6} M) was used as an internal standard for each experiment. Stages indicate class (Re-AFS) of endometriosis and numbers in parentheses indicate number of PF donors tested.

of peritoneal M ϕ might affect the pathogenesis of endometriosis in the peritoneal cavity [3–6]. The relation of the inhibitory activity to various pathological parameters of endometriosis, such as Re-AFS classification, was tested. PF of patients in stage II or III seemed to be more suppressive on TNF production (Fig. 4), but the difference was not statistically significant. Protein concentration, volume and TNF concentration of PF did not seem to be correlated with its inhibitory activity. These relationships

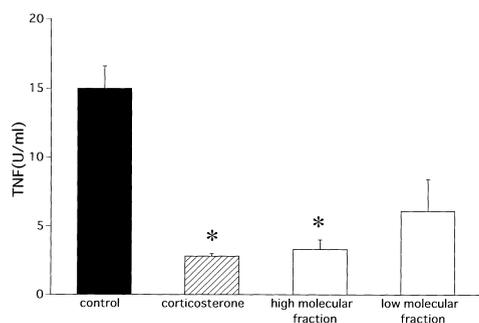


Fig. 5. Inhibitory activity of the high molecular fraction of PF on TNF production of macrophages. PF from an endometriosis patient (case 14, T.S., Re-AFS stage IV) was fractionated by ultrafiltration. High or low molecular fractions correspond to the fractions which were retained or filtrated through the ultrafilters (cut size MW: 10^4 Da), respectively. Each fraction was added to culture medium for macrophages at a concentration equivalent to 5% of original PF. Statistically significant difference from control by Student's *t*-test, * $P < 0.01$.

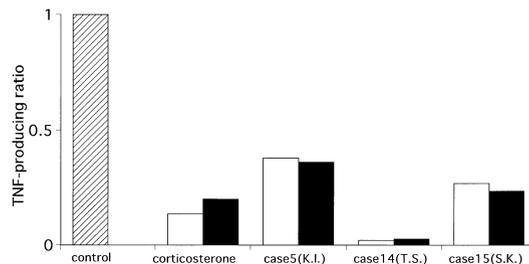


Fig. 6. The inhibitory effects of PF on the TNF-producing activity of human peripheral monocytes and murine macrophages. Open and closed columns represent the TNF production of human monocytes or murine macrophages, respectively. The correlation coefficient between the TNF obtained from the two types of cells was calculated to be 0.91, $P < 0.05$ by ANOVA.

should be discussed after more detailed studies with a larger number of patients.

Thus, PF, which contains suppressive properties for the activation of peritoneal macrophages, might allow the implantation of free endometrial cells or the metaplastic phenomena stimulated by retrograde menstruation in endometriosis.

Oosterlynck et al. demonstrated that lymphocytes preincubated with the PF of women with severe endometriosis had a decreased cytotoxicity toward K562 target cells compared to lymphocytes preincubated with PF of women without endometriosis. So they suggested the presence of a suppressive factor in PF [17].

Ultrafiltration experiments with PF suggested that the molecular size of the inhibitory factors is larger than 1×10^4 Da (Fig. 5). Therefore, candidates for the inhibitory factor seemed to be high molecular mass substances rather than prostaglandins and or steroid hormones, such as estrogen (E_2), progesterone (P) and cortisol. The maintenance of extrauterine ectopic endometrial tissue appears to be under hormonal control with a requirement for E_2 and P [18,19]. The concentrations of E_2 and P in the PF are the same in patients with or without endometriosis [20].

Peritoneal fluid has been shown to have a toxic effect on ovum pick-up, sperm mobility/survival, sperm-oocyte interaction and embryo development. Suginami et al. demonstrated an ovum capture inhibitor for the oviduct fimbriae present in endometriosis PF [21]. Clinically, it is well known that conception often occurs after diagnostic laparoscopy for

unexplained infertility and minimal endometriosis [22]. We can speculate that laparoscopic techniques, such as irrigation with copious fluids and suction removal of PF, might reduce the inhibitory factors from the PF in patients with endometriosis and minimize the abnormal immunologic milieu in the pelvic cavity. It is very important for the treatment of endometriosis to reestablish a normal immunologic homeostasis in the pelvic cavity. Further investigations are necessary for the evaluation of PF in order to better understand the pathophysiology of endometriosis.

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