

Follicular fluid of women with endometriosis stimulates the proliferation of endometrial stromal cells

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The peritoneal environment in endometriosis is known to have growth-promoting effects on endometrial cells. To investigate whether follicular fluid, a contributor to the peritoneal fluid, stimulates endometrial cell proliferation, we incubated endometrial stromal cells in culture with various dilutions of follicular fluid obtained from women with or without endometriosis undergoing oocyte retrieval for in-vitro fertilization. Cell proliferation assays were performed using follicular fluid from 28 women (without endometriosis, $n = 13$; with endometriosis, $n = 15$) in eight different endometrial stromal cell culture set-ups. Cell proliferation was assessed by a colorimetric method. Maximum cell proliferation was detected when endometrial cells were incubated with 50% dilution of follicular fluid for 48 h. Follicular fluid from women with endometriosis induced significantly higher cell proliferation than follicular fluid from women without endometriosis ($P < 0.05$). Our findings indicate that follicular fluid contents may contribute to the growth-promoting factors in the peritoneal fluid of women with endometriosis.

Key words: cell proliferation/endometriosis/endometrial stromal cell/follicular fluid

Introduction

Endometriosis, the presence of endometrial tissue outside the uterus, is one of the most common benign diseases of reproductive age women. The incidence of endometriosis is estimated to be 5% with an overall prevalence of 10% (Strathy *et al.*, 1982). Many aspects of the pathogenesis of endometriosis are still unknown. Retrograde menstruation is the most commonly accepted theory. On the other hand, it is not clear why endometriosis develops in only a subgroup of women, although retrograde menstruation is nearly a universal phenomenon (Halme *et al.*, 1984). One explanation is that endometriosis only develops if the peritoneal environment favours the implantation and growth of endometrial cells.

Peritoneal fluid is an important constituent of the peritoneal environment. It is derived from different sources: plasma

transudate, ovarian exudate, tubal fluid, retrograde menstruation, and macrophage secretions. In women the peritoneal fluid volume depends on follicular activity, corpus luteum vascularity, and hormone production and fluctuates throughout the menstrual cycle, reaching its maximum level after ovulation. The average peritoneal fluid volume is ~ 5 ml and may increase to 20 ml after ovulation (Syrop and Halme, 1987). Although there are contradictory findings, most investigators agree that peritoneal fluid volume is elevated in women with endometriosis compared with that of women without endometriosis. The peritoneal fluid of women with endometriosis induces proliferation of endometrial stromal cells in culture (Surrey and Halme, 1990). Peritoneal fluid contains growth factors and cytokines, many of which are shown to be increased in women with endometriosis, and may be involved in the implantation and growth of ectopic endometrial tissue (Giudice *et al.*, 1994; Oral *et al.*, 1996).

Follicular fluid becomes part of the peritoneal fluid after ovulation. Follicular fluid contains growth factors such as vascular endothelial growth factor (Lee *et al.*, 1997), insulin-like growth factors-I and -II (Ramasharma and Li, 1987; van Dessel *et al.*, 1996), and tumour necrosis factor- α (Punnonen *et al.*, 1992), and cytokines such as interleukin (IL)-1 (Chen *et al.*, 1995), IL-6 (Buyalos *et al.*, 1992), IL-8 (Arici *et al.*, 1996), monocyte chemotactic protein-1 (Arici *et al.*, 1997b), leukaemia inhibitory factor (Arici *et al.*, 1997a) as well as prostaglandins, and steroid hormones. Follicular fluid levels of certain cytokines and growth factors differ between women with or without endometriosis.

We postulated that the follicular fluid may be involved in the development of endometriosis by promoting the proliferation of ectopic endometrial cells. To investigate this hypothesis we evaluated the effect of follicular fluids obtained from women with or without endometriosis on endometrial stromal cell proliferation *in vitro*.

Materials and methods

Follicular fluid and endometrial tissue collection

Follicular fluids were obtained from women (aged 24–43 years) undergoing in-vitro fertilization (IVF)–embryo transfer at the Yale University IVF programme. Written informed consent was obtained from each woman before the procedure. Consent forms and protocols were approved by the Human Investigation Committee of this university. A standard IVF protocol was used as described elsewhere (Arici *et al.*, 1996). Briefly, after removal of the cumulus–oocyte complexes, samples of follicular fluid were centrifuged at 600 g for 20 min at room temperature. The cell-free supernatants were then aliquoted into polypropylene microcentrifuge tubes and stored at -80°C until assayed.

Table I. The age distribution of women from whom follicular fluids were obtained

	Control (<i>n</i> = 13)	Endometriosis	
		Minimal-mild (<i>n</i> = 6)	Moderate-severe (<i>n</i> = 9)
Age ^a (years) (mean ± SD) (range)	36.1 ± 1.4 (28–46)	34.0 ± 1.6 (29–38)	35.0 ± 1.0 (30–39)

^a*P* = not significant.

Table II. The proliferation of endometrial stromal cell cultures after treatment with 50% follicular fluid for 48 h

Group	Mean ± SEM
Serum-free media	0.16 ± 0.02 ^b
Control	0.17 ± 0.01 ^a
Minimal-mild endometriosis	0.22 ± 0.02
Moderate-severe endometriosis	0.23 ± 0.01 ^{a,b}

^{a,b}*P* < 0.05

Of 28 follicular fluid samples studied, 13 were obtained from women without endometriosis (male factor infertility only) and 15 were from women with endometriosis. Endometriosis was diagnosed and staged according to the revised AFS classification (American Fertility Society, 1985) during laparoscopy and was confirmed by biopsy of the lesion in all patients. All of the control patients included in this study underwent diagnostic laparoscopy as part of their infertility work-up and were free of endometriosis implants by visual assessment. Table I summarizes the age distribution of women from whom follicular fluids were obtained. Nine of the 15 women with endometriosis had minimal to mild endometriosis and the remaining six women had moderate to severe endometriosis.

Endometrial tissue was obtained from uteri after hysterectomy or endometrial biopsies done for reasons other than endometrial disease in reproductive age women. Informed consent in writing for the use of these tissues was obtained from each woman before the surgical procedure. Consent forms and protocols were approved by the Human Investigation Committee of this university. Endometrial tissue was placed in Hank's balanced salt solution and immediately transported to the laboratory for cell separation and culture of endometrial stromal cells. Cultures from eight different women were used in the experiments. The phase of the menstrual cycle and the absence of endometrial pathology were confirmed by histological evaluation. Samples were equally distributed between proliferative (*n* = 4) and secretory (*n* = 4) phases. Reasons for surgery were leiomyomata (*n* = 6), adenomyosis (*n* = 1), and cervical intra-epithelial neoplasia III (*n* = 1).

Isolation and culture of human endometrial stromal cells

Endometrial stromal cells were separated and maintained in monolayer culture as described previously (Arici *et al.*, 1993). Immunohistochemical analyses of isolated cells were conducted using factor VIII as a marker of endothelial cells, cytokeratin as a marker of epithelial cells, vimentin as a marker of stromal cells, and 3C10 as a marker of macrophages. In primary cultures endothelial cells, epithelial cells, and macrophages accounted for 10%, 17%, and 2% of the cells; the remaining were stromal cells. Stromal cells, after the first passage, were plated on 96-well plates in phenol red-free Ham's F-12: Dulbecco's minimal essential medium supplemented with 1% anti-

biotic-antimycotic and 10% charcoal-stripped calf serum, and were allowed to replicate to approximately 60–70% confluence prior to the commencement of each experiment. At that stage, by immunohistochemistry, endothelial cells were not present, epithelial cells accounted for 0–7% of the cells, and macrophages accounted for 0.2% of the total. When cells reached the desired confluence, they were incubated with serum-free media for 24 h before initiation of treatment with follicular fluid.

Proliferation assay

Cellular proliferation was determined by a colorimetric assay using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Mosmann, 1983). MTT is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions that lack phenol red. Dissolved MTT is converted to the coloured product formazan in active mitochondria, and then can be solubilized using acid-isopropanol mixture (1 N HCl:isopropanol; 4:96 v/v). Dual-end optical density reading at 570–650 nm is directly proportional to the number of cells.

The first column of each 96-well plate did not contain any cells and was used as a blank. Four hours before the end of each experiment, MTT solution was added into all wells (10 µl/100 µl medium per well), and plates were incubated at 37°C. At the end of the incubation period, acid-isopropanol mixture was added into each well (100 µl/well), and plates were read within 30 min with a multiwell plate reader (Thermomax; Molecular Devices Corporation, Menlo Park, CA, USA). Proliferation assays were performed on eight separate endometrial stromal cell cultures obtained from eight different women.

Statistical analysis

Statistical analysis was carried out using SigmaStat for Windows version 2.0 (Jandel, San Rafael, CA, USA). Normality test was performed with the Kolmogorov–Smirnov distance test. Statistical comparisons of the groups were performed using Student's *t*-test, one-way analysis of variance with post-hoc Bonferroni correction for independent variables. Values were presented as mean ± SEM.

Results

Endometrial stromal cells in culture were treated with 10, 25, 50, 75, and 100% follicular fluid diluted in serum-free media. The rate of cell proliferation increased as the concentration of follicular fluid in media increased up to 50% dilution. Then a decrease in the cell proliferation rate was observed with 75% and undiluted (100%) follicular fluid samples (Figure 1). Although the increase in cell proliferation at 50% dilution was not statistically significant, the remaining experiments were performed using this dilution. Thereafter, endometrial stromal cells in culture were treated with 50% follicular fluid for 24, 48, and 72 h. Maximal cell proliferation was observed at 48 h. After 48 h, inconsistent proliferative rates were observed (Figure 2).

Follicular fluid from women with endometriosis induced higher cell proliferation than follicular fluid from women without endometriosis (*P* < 0.05). Follicular fluid oestradiol concentrations were not different between these groups. When compared separately, follicular fluid from women with moderate or severe endometriosis increased cell proliferation significantly compared with that of follicular fluid from women without endometriosis or media alone (Table II) (*P* < 0.05). The difference between moderate-severe and minimal-mild

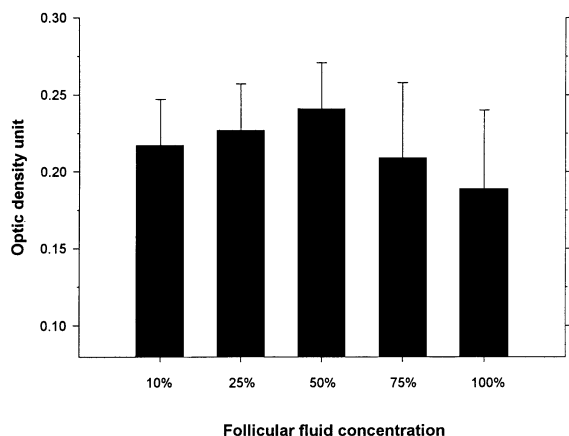


Figure 1. Concentration-dependent stimulation of proliferation of endometrial stromal cells in culture by follicular fluid. Highest proliferation was observed at 50% dilution of the follicular fluid.

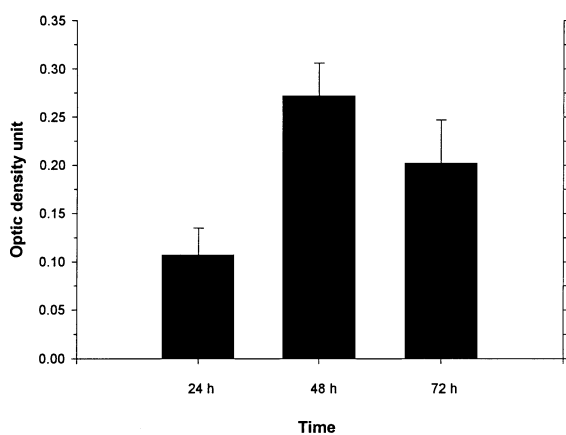


Figure 2. Endometrial stromal cells in culture were treated with 50% follicular fluid for 24, 48, and 72 h. Highest cell proliferation was observed after 48-h treatment.

endometriosis groups, however, was not statistically significant (Figure 3).

Discussion

Cellular and soluble factors in the peritoneal fluid have been implicated in the pathophysiology of endometriosis. Many growth factors and cytokines are increased in the peritoneal fluid of women with endometriosis and some are known to stimulate endometrial cell proliferation. Peritoneal fluid from women with endometriosis stimulates the proliferation of endometrial cells (Surrey and Halme, 1990). Although a recent study failed to confirm these findings, this inconsistency is probably due to the difference in experimental designs (Overton *et al.*, 1997).

Follicular fluid is an important constituent of the peritoneal fluid and contains growth factors and cytokines, as well as oestrogen, progesterone, and prostaglandins. The composition of follicular fluid is different in women with endometriosis (Abae *et al.*, 1994). These data suggest that follicular fluid with its numerous constituents may be involved in the growth of ectopic endometrial tissue. To test this hypothesis, we

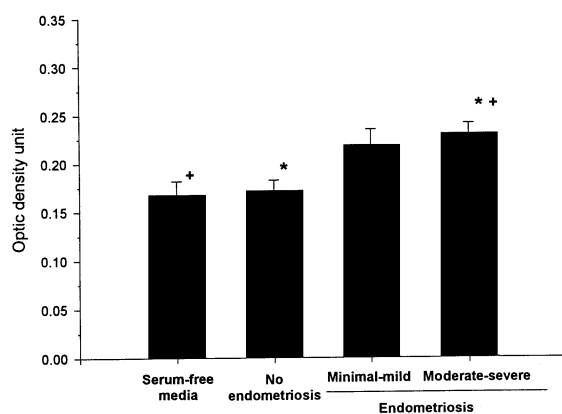


Figure 3. The effect of follicular fluid on endometrial stromal cell proliferation at 50% concentration for 48 h. Treatments with media only, follicular fluid from women without endometriosis and from women with minimal-mild and moderate-severe endometriosis were compared. + = Serum-free media versus follicular fluid of women with moderate-severe endometriosis; $P < 0.001$. * = Follicular fluid of women without endometriosis versus follicular fluid of women with moderate-severe endometriosis; $P < 0.05$.

compared the effects of follicular fluids from women with or without endometriosis on endometrial cell proliferation.

Given the difficulty of obtaining adequate amounts of endometriotic tissue and its contamination by surrounding tissues (e.g. ovary or peritoneum), we used eutopic endometrial cells to assess the cell proliferation. It has been shown previously that eutopic and ectopic endometrial cells are morphologically and biochemically similar in culture (Matthews *et al.*, 1992). Eutopic endometrial cells cultured *in vitro* may thus serve as a model for the investigation of factors involved in the proliferation of ectopic endometrial cells.

Our central hypothesis is that the follicular fluid may be involved in the proliferation of ectopic endometrial cells. We are not claiming that the follicular fluid is playing a role in the initial attachment of the pieces of the endometrium which arrive at the peritoneal cavity by retrograde menstruation 2 weeks before the ovulation. We propose that, once attached, endometrial implants may be stimulated further to proliferate by repetitive exposure to the follicular fluid.

We first determined the optimum follicular fluid concentration and time course for cell proliferation. We found that a 48-h incubation at 50% dilution of follicular fluid induced the highest proliferation rate. Follicular fluid also contains, besides nutrients and growth factors, waste products. The balance between the stimulation of cell proliferation by growth factors and the inhibition by metabolites seems to reach the optimum level at 50% concentration. The decrease in the proliferation rate after 48 h may be due to further accumulation of metabolites.

We showed in this study that follicular fluid from women with endometriosis induced higher cell proliferation than follicular fluid from women without endometriosis. The proliferative effect did not differ significantly between follicular fluids from women with minimal-mild or moderate-severe endometriosis. In conclusion, we found that *in-vitro* incubation of endometrial stromal cells with follicular fluid from women with endometriosis increased their proliferation. Our data

indicate that repetitive release of follicular fluid with its yet to be identified factors may play a role in the growth of ectopic endometrial implants.

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