Follicular fluid as a favourable environment for endometrial and endometriotic cell growth *in vitro*

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Follicular fluid from women with endometriosis has been shown to induce a higher endometrial cell proliferation than that derived from women without the disease. To elucidate this issue further, the aims of the present study were to compare the ability of follicular fluid and peritoneal fluid to stimulate both endometrial and endometriotic cell proliferation and to verify whether the mitogenic effect was merely sex steroid-dependent. Endometrial and endometriotic cells were cultured in follicular fluid or peritoneal fluid diluted in serum-free media; the growth induced in these conditions was compared with that obtained by culturing these cells in medium supplemented with charcoal stripped calf serum and a correspondent content of 17-β-oestradiol and progesterone. Follicular fluid was able to induce significantly higher cell proliferation than peritoneal fluid from controls, patients with endometriosis stage I–II and women with endometriosis stage III–IV ($P < 0.05$). Moreover, the growth in control media containing a corresponding amount of steroid hormones was significantly lower than that obtained with follicular or peritoneal fluids. This finding indicates that the stimulating effect is not simply related to the concentrations of 17-β-oestradiol and progesterone present in these fluids. Finally, based on these results and on other previous observations, the hypothesis that follicular fluid may be involved in the development of endometriotic ovarian cysts is discussed.

Key words: endometrioma/endometrium/follicular fluid/peritoneal fluid

Introduction

The pathogenesis of ovarian endometriotic cysts is still controversial. At least three different explanations have been offered to explain the development of ovarian cystic endometriosis. First, endometriomas may be formed by invagination of the ovarian cortex (Hughesdon, 1957; Brosens *et al.*, 1994). According to this hypothesis, the process would originate from a free superficial implant that is in contact with the ovarian surface and is sealed off by adhesions; the menstrual shedding and bleeding of this small implant would result in progressive invagination of the ovarian cortex and formation of the endometrioma. Alternatively, endometriotic cystic lesions could originate by metaplasia of the coelomic epithelium that lines the small cystic inclusions frequently found in ovaries (Nisolle and Donnez, 1997). This hypothesis, recently emphasized by Nisolle and Donnez (Nisolle and Donnez, 1997), is essentially based on the reported considerable metaplastic potential of the ovarian mesothelium (Serov *et al.*, 1973). Finally, in the original article by Sampson, endometriomas were considered to arise as a result of invasion of functional cysts by surface implants (Sampson, 1921). In more recent years,

this theory has been further supported by Nezhat *et al.* who suggested that the secondary involvement of functional ovarian cysts by endometriosis is crucial to the development of large ovarian endometriomas (Nezhat *et al.*, 1992).

Notwithstanding the aetiopathogenetic model considered, there are only few and limited observations regarding the role played by the ovulatory ovarian activity in the development of endometriotic cysts (Bahtiyar *et al.*, 1998). In this study, we postulate that ovarian follicles may be involved in the pathogenesis of endometrioma, at least in promoting their growth. To investigate this hypothesis, we evaluated the ability of follicular fluid to stimulate endometrial and endometriotic cell proliferation *in vitro*.

Materials and methods

Subjects

All subjects enrolled in this study were <38 years and had regular menstrual cycles. Women with previous autoimmune or neoplastic disorders were excluded from the study. The extent of endometriosis was staged according to the Revised American Fertility Society Classification (American Society for Reproductive Medicine, 1997).

Approval for this study was granted by the local Human Institutional Investigation Committee. Written consent was obtained from each woman before the procedures.

Follicular fluid

Follicular fluids were obtained from 12 women undergoing IVF and embryo transfer procedures at the Reproductive Centre, Reggio Emilia Hospital for tubal factor or male infertility. In these women a preliminary laparoscopy ruled out the presence of endometriosis. A standard IVF protocol was used as described elsewhere (Viganò *et al.*, 1998). Briefly, these women were pretreated with gonadotrophin-releasing hormone analogue (buserelin acetate, Suprefact; Hoechst, L'Aquila, Italy) started in the mid-luteal phase of the preceding cycle and continued for 14 days. Follicle-stimulating hormone (FSH, Metrodin, Serono Laboratories, Inc., Rome, Italy) was started thereafter with dose variations depending on individual responses. Follicular development was monitored by daily measurements of serum oestradiol and by ultrasonographic measurements of follicle diameter. Human chorionic gonadotropin (HCG, 10 000 IU, Profasi; Serono Laboratories, Inc.) was administered when the size of at least two leading follicles reached 15 mm or serum oestradiol levels >1 nmol/mature follicle. Transvaginal ultrasound-guided oocyte retrieval was performed 36 h later and the contents of visible follicles were aspirated. Care was taken to select only follicular fluids that were clear, non-bloody and that did not contain flushing medium. Oocytes were identified and separated. The follicular aspirates obtained were centrifuged at 800 *g* for 10 min at room temperature to separate them from cells and then stored at –20°C. At the time of experiment, samples were thawed and pooled. 17-β-Oestradiol and progesterone concentrations of the pools were measured. Overall, three different pools were obtained using follicular fluids from at least three different women for each pool.

Peritoneal fluid

Samples of peritoneal fluid were collected at the time of laparoscopy from 38 women who, on the basis of the laparoscopic diagnosis, were divided into the following three groups: endometriosis stage I–II (12 women), endometriosis stage III–IV (12 women) and controls (14 women). All the women were in early follicular or late luteal phase of the menstrual cycle in order to avoid contamination of follicular fluid into peritoneal fluid. No woman had received hormones for at least 3 months. In the control group, laparoscopic examination demonstrated normal pelvic organs in four cases, pelvic adhesions in two cases, benign ovarian pathology in six cases and benign uterine pathology in two cases. Peritoneal fluid samples were centrifuged at 800 *g* for 10 min at room temperature to separate them from cells and then stored at –20°C. At the time of experiment, samples were thawed and pooled. Each pool was established so that an equal number of early follicular and late luteal phase samples were used. 17-β-Oestradiol and progesterone concentrations of the pools were measured. Overall, three different pools of peritoneal fluid for each of the three groups of women were obtained.

Collection, isolation and culture of endometrium and endometriotic cyst

Samples of uterine endometrium were obtained at the time of laparoscopy using an endometrial biopsy curette from 11 proliferative phase women affected by benign ovarian pathology but without evidence of endometriosis. Moreover, six samples of ovarian endometriotic cysts were obtained from six proliferative phase women affected by endometriosis stage III–IV. Histopathological examination confirmed the endometriotic character of the cysts and both the phase of the menstrual cycle and the absence of pathological conditions of the endometria. Endometriotic cysts were excised at operation, biopsied and endometriotic tissue was dissected from underlying parenchyma. In previous studies, we have successfully established and employed endometrial cell cultures from endometrial samples and from endometriotic cysts biopsies (Viganò *et al.*, 1993, 2000; Somigliana *et al.*, 1996; Di Blasio *et al.*, 1997). Briefly, tissues were gently minced into small pieces (1–2 mm^3) and washed in fresh medium to remove mucus or debris. Thereafter, they were incubated for 2 h at 37°C in a shaking water bath in 10 ml Ham's F-10 (BioWhittaker, PBI International, Milan, Italy) containing 0.2% collagenase (Boehringer-Mannheim Biochemicals, Milan, Italy). After several washings, the cell suspension was digested in a 0.05% trypsin solution for 3–5 min. Cells, which represent a mixture of both stromal and epithelial components, were washed twice in Ham's F-10 supplemented with 10% fetal calf serum (FCS; Flow Laboratories, Opera, Milan, Italy) and antibiotics and counted using a Cell Counter (Seac, Milan, Italy). Specifically, ~50 000–100 000 cells/well were allowed to adhere selectively to 24-well tissue culture plates and cultured in 1 ml of Ham's F-10 with 10% FCS, 2 mmol/l L-glutamine, antibiotics and 2.5 µg/ml fungizone in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Proliferation assay

Duplicates of endometrial and endometriotic cells cultures established at day –1 were allowed to proliferate in eight different medium conditions from day 0:

> Ham's F-10 with 10% pooled follicular fluids
> Ham's F-10 with 10% pooled peritoneal fluids from controls
> Ham's F-10 with 10% pooled peritoneal fluids from endometriosis stage I–II
> Ham's F-10 with 10% pooled peritoneal fluids from endometriosis stage III–IV

For each of these conditions, control medium was represented by Ham's F-10 with 10% charcoal stripped (steroid depleted) calf serum (Sigma) added with the corresponding concentration of 17-β-oestradiol and progesterone (Sigma) (Table I). Steroids were dissolved in ethanol as concentrated stocks. The final ethanol concentration was below 0.1% in all media. Content of 17-β-oestradiol in follicular fluid pools ranged between 2.2×10^6 and 3.5×10^6 pg/ml, whereas progesterone concentration was between 10×10^6 and 14×10^6 pg/ml. Levels of 17-β-oestradiol and progesterone in pools of peritoneal fluids ranged between 300 and 640 pg/ml and between 18×10^3 and 31×10^3 pg/ml respectively. All media were supplemented with 2 mmol/l L-glutamine, antibiotics and 2.5 µg/ml fungizone and changed every other day. After 8 days of treatment, cells were harvested by a 10 min incubation in 0.05% trypsin-0.02% EDTA solution and counted using a Cell Counter (Seac).

Statistical analysis

Data are expressed as median and range. Statistical analysis was performed using Statview SE+. Differences between groups were determined by Wilcoxon signed-rank test or Friedman test as appropriate. $P < 0.05$ was considered as statistically significant.

Results

Endometrial and endometriotic cells were cultured in follicular fluid and peritoneal fluid diluted in serum-free media; the growth induced in these conditions was compared with that obtained by culturing these cells in medium supplemented with charcoal stripped CS and an equal content of oestrogen and progesterone (control media). In the present study, since

Table I. Endometrial cell proliferation in the presence of follicular fluid and peritoneal fluid from women with and without endometriosis. Experimental media consisted of Ham's F-10 plus 10% follicular fluid or 10% peritoneal fluid obtained from patients with endometriosis stage I–II, from patients with endometriosis stage III–IV and from women without the disease. Control media consisted of Ham's F-10 plus charcoal-stripped call serum added with a content of 17-β-oestradiol and progesterone correspondent to concentrations present in the experimental media

Condition	Experimental medium median/range (10^3 cells)	Control medium median/range (10^3 cells)	P
Follicular fluid	106.5* (48–476)	38.5 (24–203)	0.02
Peritoneal fluid of women without endometriosis	80.5 (45–440)	42.5 (26–216)	0.02
Peritoneal fluid of women with endometriosis stage I–II	77.5 (37–351)	42 (22–182)	0.02
Peritoneal fluid of women with endometriosis stage III–IV	69.5 (42–369)	40 (24–187)	0.02

*$P < 0.05$ compared with the other three conditions.

Table II. Endometriotic cell proliferation in the presence of follicular fluid and peritoneal fluid from women with and without endometriosis. Experimental media consisted of Ham's F-10 plus 10% follicular fluid or 10% peritoneal fluid obtained from patients with endometriosis stage I–Il, from patients with endometriosis stage III–IV and from women without the disease. Control media consisted of Ham's F-10 plus charcoal-stripped calf serum added with a content of 17-β-oestradiol and progesterone correspondent to concentrations present in the experimental media

Condition	Experimental medium median/range (10^3 cells)	Control medium median/range (10^3 cells)	P
Follicular fluid	158* (76–276)	67 (28–116)	0.03
Peritoneal fluid of women without endometriosis	98 (36–177)	65 (20–124)	0.03
Peritoneal fluid of women with endometriosis stage I–II	90 (45–164)	61 (20–128)	0.03
Peritoneal fluid of women with endometriosis stage III–IV	79.5 (49–122)	70 (20–114)	0.14

*$P < 0.05$ compared with the other three conditions.

there may be specific interactions between stroma and epithelium that could influence the reciprocal growth, we have specifically decided not to separate these two components. Results are reported in Tables I and II. Peritoneal fluids from controls, women with endometriosis stage I–II and endometriosis stage III–IV patients and follicular fluids displayed a significant increased ability to stimulate endometrial cell proliferation when compared to correspondent control media (Table I). Similar results were obtained for endometriotic cells, with the exception of peritoneal fluid from women with endometriosis stage III–IV in which an increased but not statistically significant ability to stimulate these cells in culture was observed (Table II). Furthermore, follicular fluid induced a higher cellular proliferation than peritoneal fluid from controls, patients with endometriosis stage I–II and women with endometriosis stage III–IV. This significant increase in cellular growth induced by follicular fluids could be observed for both endometrial (Table I) and endometriotic cells (Table II). Conversely, stimulation of cellular proliferation induced by peritoneal fluids obtained from the different groups of patients resulted similar (Tables I and II).

Endometrial cells were also treated with different concentrations of follicular fluid. At the concentrations tested, the rate of cell proliferation increased as the content of follicular fluid

in media increased (Figure 1, upper panel). Furthermore, in preliminary experiments, proliferation of endometrial cells cultured in 10% follicular fluid was also tested for a time-response effect. As shown in Figure 1 (lower panel), an increased proliferation with time was observed.

Discussion

In this study, we demonstrated that both peritoneal and follicular fluids are able to stimulate endometrial and endometriotic cell growth *in vitro*. Moreover, we observed that this effect is much more evident using follicular fluids.

Peritoneal fluid contains many growth factors and cytokines which have been advocated to be involved in the implantation and growth of ectopic endometrial fragments (Koutsilieris *et al.*, 1991). Interestingly, it has been reported that the peritoneal fluid of women with endometriosis would induce an increase in endometrial stromal cells proliferation (Surrey and Halme, 1990). However, results from other studies on this subject are conflicting (Meresman *et al.*, 1997; Overton *et al.*, 1997). In the present study, we failed to detect an increased ability to stimulate endometrial cells proliferation using peritoneal fluids from women with endometriosis. However, this topic was not the main objective of our experimental design
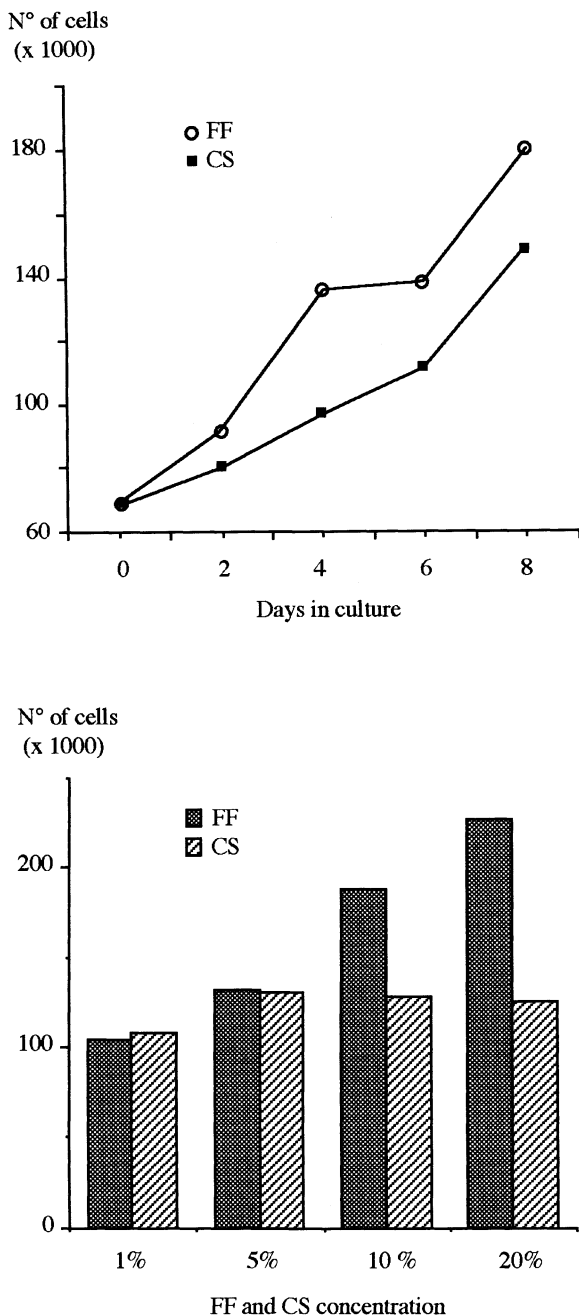
Figure 1. Effect of follicular fluid on endometrial cell proliferation as a function of time (upper panel) and concentration (lower panel). Cells were cultured with 10% follicular fluid, harvested at the indicated time points and counted. Numbers of cells represent the mean of duplicates of a representative experiment (upper panel). Cells were cultured with different concentrations as indicated for 8 days. Numbers of cells represent the mean of duplicates of a representative experiment (lower panel). FF = follicular fluid; CS = charcoal stripped calf serum.

and we cannot exclude that increasing the number of peritoneal fluid pools tested would lead to some differences. Indeed, the central purpose of this study was to evaluate the ability of follicular fluid to affect endometrial and endometriotic cell proliferation. To this specific aim, comparison with peritoneal fluid was established, since this milieu is a well-known mitogenic source for endometrial cells (Koutsilieris *et al.*, 1991). In this contest, results from our study suggest that

follicular fluid may represent an extremely favourable environment for endometrial and endometriotic cells growth. Indeed, a marked and statistically significant enhanced ability to stimulate proliferation of these cells was observed using follicular fluids obtained from partially luteinized follicles.

Our study also implies that the follicular fluid-mediated induction of endometrial cell proliferation is not merely due to steroid hormones. Indeed, the control media used in this study did actually contain a concentration of steroid hormones similar to the experimental conditions tested. Therefore, other factors present in follicular fluid may be responsible for this effect. In particular, growth factors such as vascular endothelial growth factor (Lee *et al.*, 1997), insulin-like growth factors I and II (VanDessel *et al.*, 1996), steroidogenesis-inducing protein (Khan *et al.*, 1997) 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 inhibiting factor (Arici *et al.*, 1997a), as well as prostaglandins have been detected in follicular fluid. Interestingly, direct evidences of interaction between growth factors and sex steroids in the regulation of endometrial stromal cell growth have been reported (Irwin *et al.*, 1991). However, factors and molecular interactions that are responsible for the ability of follicular fluid to increase endometrial stromal cell proliferation markedly were not further investigated herein, and are beyond the scope of the present study. Exciting new insights might be drawn from future investigations in this field.

Our results are generally in keeping with those from Bahtiyar *et al.* in terms of reported ability of follicular fluid to stimulate endometrial cell growth (Bahtiyar *et al.*, 1998). Moreover, in that study follicular fluid from women with endometriosis was shown to induce an increased cell proliferation than that obtained using follicular fluid from women without the disease. It should, however, be noted that these authors failed to evaluate the mitogenic ability of follicular fluid relative to other milieus and did not investigate whether this effect was merely steroid-dependent. Interestingly, they postulated that repetitive release of follicular content into the peritoneal fluid at the time of ovulation may play a role in the growth of peritoneal ectopic endometrial implants. Although this hypothesis might have some relevance, it is tempting to speculate that ovarian follicles may be also involved in the growth of ovarian endometriomas. At present, endometriotic cyst formation could probably not be explained by a unique aetiopathogenetic model and this has undoubtedly complicated the research on this topic. Nevertheless, there is a small amount of compelling evidence to support a possible role of ovarian follicles in the pathogenesis of these cysts. First, Nezhat *et al.* found that some large endometriomas also had histological characteristics of luteal or follicular ovarian cysts suggesting that these lesions may develop as a result of secondary involvement of functional ovarian cysts (Nezhat *et al.*, 1992). Moreover, very recently, Jain and Dalton showed by serial transvaginal tracking of ovarian follicles that a chocolate cyst can develop from an ovarian follicle (Jain and Dalton, 1999). In each of the 12 cases reported by these authors, the diagnosis was successively confirmed laparoscopically.

In conclusion, our findings suggest that follicular fluid may be important in the pathogenesis of endometriomas. Results from experimental and clinical future investigations are mandatory to verify this hypothesis. If the role of the follicular fluid would be confirmed, our ability to prevent, diagnose and treat endometriomas could be improved.

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