Endometriotic cells are resistant to interferon-γ-induced cell growth inhibition and apoptosis: a possible mechanism involved in the pathogenesis of endometriosis

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In order to evaluate the involvement of cell proliferation and apoptosis in the pathogenesis of endometriosis, we investigated the effects of interferon-γ (IFN-γ) on cell growth inhibition and apoptosis of cultured ovarian endometriotic cyst stromal cells (ECSC), eutopic endometrial stromal cells with endometriosis (ESCwE) and normal endometrial stromal cells (NESC) by modified methylthiazoletetrazolium assay, 5-bromo-2'-deoxyuridine incorporation assay and internucleosomal DNA fragmentation assay. The expression of apoptosis-related molecules and IFN-γ receptor 1 was also examined in ECSC, ESCwE and NESC using western blot analysis. IFN-γ significantly inhibited cell proliferation and DNA synthesis of ESCwE and NESC, and induced apoptosis of these cells. In contrast, IFN-γ did not show apparent effects on the viable cell number, DNA synthesis, or apoptosis of ECSC. An up-regulated expression of Bcl-2 and Bcl-XL proteins was observed in ESCwE in comparison with ECSC and NESC, whereas the levels of Bax, Bad, Fas and Fas ligand proteins in ECSC were similar to those in ESCwE and NESC. IFN-γ receptor 1 expression was detected in ECSC, ESCwE and NESC. Enhanced expression of anti-apoptotic molecules in the ectopic endometrial cells may contribute to the development of endometriosis by conferring resistance to cytokine-induced apoptosis and increasing the chance that these cells will survive and implant outside the uterus. Further investigations on the regulation of cell proliferation in both the endometriotic and the normal endometrium may be important for the elucidation of the pathogenesis of endometriosis.

Key words: apoptosis/Bcl-XL/Bcl-2/endometriosis/interferon-γ/pathogenesis

Introduction

Endometriosis, a disease affecting 3–10% of women of reproductive age, is characterized by the ectopic growth of endometrial tissue (Olive and Schwartz, 1993). The most widely accepted theory about the pathogenesis of endometriosis is that it is a consequence of implantation of viable endometrial tissues in the pelvis via retrograde menstruation (Halme et al., 1984; Bartosik et al., 1986; Kruitwagen et al., 1991; Groothuis et al., 1999). Retrograde dissemination of the endometrial cells has been demonstrated repeatedly in a number of clinical studies (Halme et al., 1984; Bartosik et al., 1986; Kruitwagen et al., 1991; Vercellini et al., 1997). However, this event appears to be a physiological phenomenon that occurs in all women irrespective of the presence of endometriosis (Halme et al., 1984; Bartosik et al., 1986; Kruitwagen et al., 1991).

It has been suggested that in healthy women, endometrial cells expelled during menstruation do not survive in ectopic locations because of programmed cell death, while decreased apoptosis may lead to ectopic survival and implantation of these cells and the development of endometriosis (Harada et al., 1996; Suganuma et al., 1997; Dmowski et al., 1998; Gebel et al., 1998; Jones et al., 1998; Meresman et al., 2000). It has been demonstrated that endometrial apoptosis in the eutopic endometrium is lower in women with endometriosis than in controls and is further decreased in the ectopic endometrium (Dmowski et al., 1998; Gebel et al., 1998). Endometrial cells from women with endometriosis have enhanced proliferation and an increased ability to implant and survive in ectopic locations. Impaired sensitivity of the endometrial tissue to spontaneous apoptosis contributes to the abnormal implantation and growth of the endometrium at ectopic sites. Both the inability of endometrial cells to transmit a ‘death’ signal and the ability of endometrial cells to avoid cell death have been associated with an increased expression of anti-apoptotic factors [e.g. B cell lymphoma/leukaemia-2 (Bcl-2)] and a decreased expression of pro-apoptotic factors [e.g. Bax] (Watanabe et al., 1997; Jones et al., 1998; Eresman et al., 2000). However, most of these findings are based on immunohistochemical studies and the alterations of endometrial cells in the regulation of apoptosis during the development of endometriosis are largely unknown.

In the present study we investigated the anti-apoptotic properties of endometriotic cyst stromal cells (ECSC), eutopic endometrial stromal cells with endometriosis (ESCwE) and normal endometrial stromal cells (NESC) against interferon-γ (IFN-γ) and the expression of apoptosis-related proteins in these cells in relation to cytokine-induced apoptosis using primary cultures of the ovarian ECSC, ESCwE and NESC. We also discuss the pathological role of the anti-apoptotic property of endometriotic and endometrial cells during the development of endometriosis.
Materials and methods

ECSC, ESCwE and NESC isolation procedure and cell culture conditions

ECSC were obtained from pre-menopausal patients who had undergone salpingo-oophorectomy or excision for ovarian endometrioid cysts (n = 8). ESCwE (n = 7) were obtained from pre-menopausal patients who had undergone hysterectomies for endometriosis. NESC were obtained from pre-menopausal patients who had undergone hysterectomies for leiomyoma and had no evidence of endometriosis (n = 8). All patients were free of any hormonal treatment prior to the operation. All the specimens were diagnosed as being in the late secretory phase using a standard histological examination of the endometrial tissues. This study was approved by the institutional review board of the Faculty of Medicine, Oita University.

ECSC were isolated from ovarian endometriotic tissues by enzymatic digestion as previously described (Iwabe et al., 1998; Fukuda et al., 2004). Briefly, the tissues were minced in Hank’s balanced salt solution and digested with 0.5% collagenase (Gibco-BRL, Gaithersburg, MD, USA) in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL) at 37°C for 40 min. The dispersed cells were filtered through a 70µm nylon mesh to remove the undigested tissue pieces. The filtrated fraction was further separated from epithelial cell clumps by differential sedimentation at unit gravity as follows. The cells were resuspended in 2 ml of culture medium and layered slowly over 10 ml of the medium in a centrifuge tube. Sealed tubes were placed in an upright position at 37°C in 5% CO2 in air for 30 min. After sedimentation, the top 8 ml of the medium was collected. Finally, the medium containing stromal cells was filtered through a 40µm nylon mesh. Final purification was achieved by allowing stromal cells, which rapidly attached to plates, to adhere selectively to culture dishes for 30 min at 37°C, followed by the removal of non-adhering epithelial cells. ESCwE and NESC were also isolated by digesting the endometrial tissue fragments with 0.5% collagenase as previously described (Nasu et al., 2001). Isolated ECSC, ESCwE and NESC were cultured in DMEM supplemented with 100 IU/ml of penicillin (Gibco-BRL), 50 mg/ml of streptomycin (Gibco-BRL) and 10% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL) at 37°C in 5% CO2 in air.

ECSC, ESCwE and NESC in monolayer cultures after the third passage were >99% pure as analysed by immunocytochemical staining with antibodies against vimentin (V9; Dako, Copenhagen, Denmark), CD10 (SS2/36; Dako) (Potlog-Nahari et al., 2004), cytokeratin (Dako), factor VIII (Dako) and leukocyte common antigen (2B11 + PD7/26, Dako) and were used for the following experiments. Cells isolated from each individual patient were used for one experiment at a time. Each experiment was performed in triplicate and repeated at least four times.

Assessment of cell proliferation and cell viability of ECSC, ESCwE and NESC after IFN-γ treatment

Cell proliferation and cell viability of ECSC, ESCwE and NESC after IFN-γ treatment were determined in 96-well plates by a modified methylthiazol tetrazolium (MTT) assay using WST-1 (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer’s protocol. DMEM supplemented with 10% fetal calf serum (FBS) having 5 x 10^5 cells of ECSC, ESCwE and NESC was distributed into each well of a 96-well flat-bottomed microplate (Corning, New York, NY, USA) and incubated overnight. The medium was then removed and the cells were incubated for 48h with 200 µl of experimental medium containing various concentrations of IFN-γ (0.1–100 ng/ml) (R&D Systems, Minneapolis, MN, USA). Thereafter, 20 µl of WST-1 dye was added to each well and further incubated for 4 h. Cell proliferation was evaluated by measuring the absorbance at 540 nm. Data were calculated as the percentage compared to the untreated controls.

Cell proliferation of ECSC, ESCwE and NESC after IFN-γ treatment was also determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation using cell proliferation enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics GmbH). DMEM supplemented with 10% FBS having 1 x 10^5 cells of ECSC, ESCwE and NESC was distributed into each well of a 96-well flat-bottomed microplate and incubated overnight. The medium was then removed and the cells were incubated for 48h with 100 µl of experimental medium containing various concentrations of IFN-γ (0.1–100 ng/ml).

Thereafter, 10 µl of BrdU (10 mM) was added to each well and further incubated for 2h. BrdU incorporation was then evaluated according to the manufacturer’s protocols. Cell proliferation was evaluated by measuring the absorbance at 450 nm.

Assessment of DNA fragmentation and apoptosis in ECSC, ESCwE and NESC after IFN-γ treatment

Internucleosomal DNA fragmentation in ECSC, ESCwE and NESC after IFN-γ treatment was evaluated by a Quick apoptotic DNA ladder detection kit (BioVision Research Products, Mountain View, CA, USA), DMEM (10 ml) supplemented with 10% heat-inactivated FBS and 1 x 10^5 cells of ECSC, ESCwE and NESC was plated on 100mm culture dishes (Corning) and cultured overnight. The supernatant was replaced with fresh culture medium containing recombinant human IFN-γ (10 ng/ml). Twenty-four hours after stimulation, DNA was extracted from these cells according to the manufacturer’s protocols. DNA fragmentation was analysed by electrophoresis on an agarose gel (1.2%). The DNA bands were visualized by staining with ethidium bromide and photographed under UV light using a transilluminator.

In addition, IFN-γ-induced apoptosis of ECSC, ESCwE and NESC was quantified by direct determination of nucleosomal DNA fragmentation with a cell death detection ELISA (Roche Diagnostics). The assay used specific monoclonal antibodies directed against histones from fragmented DNA, allowing the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Briefly, 1 x 10^5 cells were plated on 24-well culture plates (Corning) in 1 ml of culture medium with 10% heat-inactivated FBS and cultured overnight. The supernatant was replaced with fresh culture medium containing various amounts of recombinant human IFN-γ (0.1–100 ng/ml). Twenty-four hours after stimulation, the cells were lysed according to the manufacturer’s manual, followed by centrifugation (200 g, 5 min). The mono- and oligonucleosomes in the supernatants were determined using an anti-histone-biotin antibody. The concentration of nucleosomes-antibody was determined photometrically at a wavelength of 405 nm using 2,2′-azino-di(3)-ethylbenzthiazolin-sulphonate as substrate.

Assessment of apoptosis-related proteins and IFN-γ receptor 1 (IFN-γR1) in ECSC, ESCwE and NESC

The expression of apoptosis-related proteins [Bcl-2, Bcl-XL, Bax, Bad, Fas and Fas ligand (FasL)] and IFN-γR1 in untreated ECSC, ESCwE and NESC were investigated by western blot analysis. ECSC, ESCwE and NESC were cultured on 100mm dishes until confluent. Then, the cells were washed with phosphate-buffered saline, and whole cell extracts were prepared by lysing the cells in lysis buffer (50 mM Tris–HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM ethylenediaminetetraacetic acid, 50 mM NaF and 0.1% phenyl-methylsulphonylfluoride). The suspension was centrifuged at 15,000 g for 15 min at 4°C, and the supernatant was collected. The total protein concentration was quantified using the Coomassie protein assay reagent (Pierce, Rockford, IL, USA). The whole cell protein extract was resolved with sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel under reduced conditions. After transfer to Immobilon-P transfer membrane (Millipore, Bedford, MA, USA), the protein was stained with Ponceau S (Sigma–Aldrich, St Louis, MO, USA) to verify uniform loading and transfer. Membranes were blocked with 5% skim milk (Becton–Dickinson, Sunnyvale, CA, USA) in Tris-buffered saline with Tween 20 (50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) (TBS-T) overnight and subsequently incubated with primary antibodies [Bcl-2, Bcl-XL, Bax, Bad, Fas, FasL (BD Biosciences, San Jose, CA, USA), IFN-γR1 (R&D Systems) and glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX, USA)] at appropriate dilution for 1 h at room temperature. The membrane was washed three times with TBS-T and incubated with the appropriate horse-radish peroxidase-conjugated secondary antibody for 1 h at room temperature. Subsequently, the membrane was washed three times with TBS-T and analysed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Chicago, IL, USA).

Statistical analysis

Data were calculated as percentages of untreated controls, presented as the means ± SD, and were analysed by the Bonferroni/Dunn test with StatView
4.5 (Abacus Concepts, Berkeley, CA, USA). $P < 0.05$ was accepted as statistically significant.

Results

The effects of IFN-$\gamma$ on cell proliferation and cell viability of ECSC, ESCwE and NESC were investigated by modified methylthiazoletetrazolium assay. As shown in Figure 1, IFN-$\gamma$ did not have any apparent effect on the viable cell number of ECSC. On the other hand, the number of viable ESCwE and NESC was significantly decreased by the addition of increasing amounts of IFN-$\gamma$. Similar results were obtained in all repeated experiments.

To further assess the effects of IFN-$\gamma$ on cell proliferation, DNA synthesis of ECSC, ESCwE and NESC after IFN-$\gamma$ treatment was evaluated by BrdU incorporation. As shown in Figure 2, BrdU incorporation of ECSC was not affected by IFN-$\gamma$ treatment. IFN-$\gamma$

\begin{figure}[h]
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\caption{The effect of IFN-$\gamma$ on cell viability of ECSC, ESCwE and NESC as assessed by modified MTT assay. ECSC, ESCwE and NESC were treated with IFN-$\gamma$ (0.1–100 ng/ml) for 48 h. The data are presented as percentages relative to non-treated controls. Representative results are shown. Open bars, ECSC; closed bars, ESCwE; hatched bars, NESC. $*P < 0.0005$, $**P < 0.0001$ versus untreated controls.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{The effect of IFN-$\gamma$ on the BrdU incorporation of ECSC, ESCwE and NESC as assessed by modified MTT assay. ECSC, ESCwE and NESC were treated with IFN-$\gamma$ (0.1–100 ng/ml) for 48 h. The data are presented as percentages relative to non-treated controls. Representative results are shown. Open bars, ECSC; closed bars, ESCwE; hatched bars, NESC. $*P < 0.0005$, $**P < 0.0001$ versus untreated controls.}
\end{figure}

treatment showed significant inhibition of BrdU incorporation of ESCwE and NESC in a dose-dependent manner.

Next, the apoptotic effects of IFN-$\gamma$ on ECSC, ESCwE and NESC were assessed by evaluating the presence of internucleosomal DNA fragmentation with a cell death detection ELISA. The assay used specific monoclonal antibodies directed against histones from fragmented DNA, allowing the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. ECSC, ESCwE and NESC were treated with IFN-$\gamma$ (0.1–100 ng/ml) for 24 h. The data are presented as percentages relative to non-treated controls. Representative results are shown. Open bars, ECSC; closed bars, ESCwE; hatched bars, NESC. $*P < 0.0025$, $**P < 0.0005$ and $***P < 0.0001$ versus untreated controls.

\begin{figure}[h]
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\caption{The effect of IFN-$\gamma$ on the DNA fragmentation of ECSC, ESCwE and NESC. DNA was extracted from ECSC, ESCwE and NESC cultured for 24 h with or without IFN-$\gamma$ (10 ng/ml), and analysed on agarose gel electrophoresis. Representative results are shown.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{The effect of IFN-$\gamma$ on the apoptosis of ECSC, ESCwE and NESC as assessed by direct determination of nucleosomal DNA fragmentation with a cell death detection ELISA. The assay used specific monoclonal antibodies directed against histones from fragmented DNA, allowing the determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. ECSC, ESCwE and NESC were treated with IFN-$\gamma$ (0.1–100 ng/ml) for 24 h. The data are presented as the percentages relative to non-treated controls. Representative results are shown. Open bars, ECSC; closed bars, ESCwE; hatched bars, NESC. $*P < 0.0025$, $**P < 0.0005$ and $***P < 0.0001$ versus untreated controls.}
\end{figure}

To analyse the underlying mechanisms of the above findings, we evaluated the expression of apoptosis-related proteins and IFN-$\gamma$R1 in untreated ECSC, ESCwE and NESC. As shown in Figure 5, up-regulated expression of Bcl-2 and Bcl-X$_L$ proteins was observed in all samples of ECSC ($n = 5$) in comparison with ESCwE ($n = 4$) and NESC ($n = 5$). However, the levels of Bax, Bad, Fas and FasL proteins in ECSC were similar to those in ESCwE and NESC. Significant expression of IFN-$\gamma$R1 was observed in ECSC, ESCwE and NESC.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5.png}
\caption{The expression of apoptosis-related proteins and IFN-$\gamma$R1 in untreated ECSC, ESCwE and NESC. $n = 5$ for ECSC, ESCwE and NESC. $*P < 0.0005$, $**P < 0.0001$ versus untreated controls.}
\end{figure}
Discussion

Ectopic and eutopic endometria of women with endometriosis show some fundamental differences compared to the normal endometria of women without endometriosis. These include a variety of anomalies in structure, proliferation, immune components, adhesion molecules, proteolytic enzymes and their inhibitors, steroid and cytokine production and responsiveness, gene expression and protein production (Sharpe-Timms, 2001; Nishida et al., 2004). These differences could contribute to the survival of endometrial cells regurgitated into the peritoneal cavity and the development of endometriosis. Cells and tissue elements derived from such an altered eutopic endometrium and shed into the peritoneal cavity have been proposed to have a higher potential for implantation and growth on peritoneal surfaces and development into endometriosis (Noble et al., 1997; Jolicoeur et al., 1998).

In the present study, a high apoptotic index together with low proliferation was observed in IFN-γ-treated ESCwE and NESC. In contrast, IFN-γ did not show apparent effects on the cell growth and apoptosis of ECSC. Interestingly, IFN-γ receptor was detected in all three cell types. These characteristics of ECSC may be involved in the development of endometriosis. Considering the theory that endometriosis arises due to retrograde menstruation and peritoneal implantation, our results suggest that normal endometrial cells misplaced in the peritoneal cavity may become apoptotic, may not implant, may be removed immediately by the peritoneal immune system, and may not develop into endometriotic lesions. On the other hand, endometrial cells that become resistant to apoptosis may survive, implant and invade the peritoneal cavity and contribute to the development of endometriosis. Clarification of the mechanisms underlying these differences is important for the elucidation of this disease.

Apoptosis in the cyclic endometrium is a physiological mechanism to regulate the remodelling of this tissue by eliminating unwanted or dysfunctional cells during implantation or menstruation and is thereby an important regulator of cell homeostasis in human endometrium (Otsuki et al., 1994; Harada et al., 1996; Watanabe et al., 1997). Expression of apoptosis regulatory molecules has been observed in the human endometrium, and these molecules are believed to fine-tune this process. With respect to the regulation of cell growth, endometriosis can be recognized as a condition in which endometrial cells exhibit abnormal proliferative and apoptotic properties. It has been demonstrated that the levels of apoptosis are significantly lower in the endometriotic tissue, suggesting ectopic pre-selection of apoptosis-resistant cells (Gebel et al., 1998).

Recently, certain proteins, such as Bcl-2 (Koh et al., 1995; Tabibzadeh et al., 1995), Bcl-X (Otsuki et al., 1994; Tabibzadeh et al., 1995; Watanabe et al., 1997), Bax (Otsuki et al., 1994; Koh et al., 1995; Tabibzadeh et al., 1995), Fas (Garcia-Velasco et al., 1998; Yamashita et al., 1999; Song et al., 2002) and FasL (Garcia-Velasco et al., 1998; Yamashita et al., 1999; Song et al., 2002) have been shown to be associated with endometrial apoptosis. Increased expression of Bcl-2 (Jones et al., 1998; Mersman et al., 2000) and decreased expression of Bax (Meresman et al., 2000) in endometriotic lesions has been reported. It has been suggested that endometriotic cells may resist apoptotic signals and proliferate in the peritoneal cavity as a result of the activation of anti-apoptotic genes and the inhibition of pro-apoptotic genes. In the present study, we demonstrated the up-regulated expression of anti-apoptotic factors of the Bcl-2 family (Bcl-2 and Bcl-XL) in ECSC, which is consistent with previous reports. However, we could not find any difference in the expressions of Fas/FasL or the pro-apoptotic factors of the Bcl-2 family (Bad and Bax). This discrepancy between our results and previous reports may be related to differences in the experimental procedures used. That is, most previous reports employed immuno-histochemistry, whereas we used an in vitro culture system, which may not correspond to the conditions in vivo.

Although the precise mechanism of resistance of ECSC against IFN-γ-induced apoptosis is unknown, the presence of IFN-γ receptor in ECSC suggests the dysregulation of subsequent intracellular signalling pathways in these cells. It has been demonstrated that IFN-γ induces apoptosis of a variety of cell types by regulating the expression of apoptosis-related proteins (Zhang et al., 2003; Awasthi and Wagner, 2004). Although we did not examine the changes in apoptosis-related protein levels after IFN-γ treatment, up-regulated expression of anti-apoptotic factors of the Bcl-2 family
may be involved in the acquisition of anti-apoptotic properties of ECSC.

Recent studies have reported that a variety of cytokines, such as interleukin-1 (Fakhii et al., 1987), IFN-γ (Ho et al., 1996) and tumour necrosis factor-α (Halme, 1989), form complex networks and play important roles in the growth and expansion of endometriotic lesions. IFN-γ, a product of activated T lymphocytes and natural killer cells, is known as a potent modulator of cell growth and differentiation, as well as immune defences (Kalvakolanu, 2000). Clayton et al. (2004) examined the anti-proliferative effect of IFN-γ on a primary culture of NESC, and obtained results similar to those of the present study. They speculated that IFN-γ might exert similar properties on the ECSC. However, as shown in the present study, endometriotic stromal cells are resistant to IFN-γ-induced apoptosis. Thus it is not unreasonable to suspect that ECSC and NESC may react in a similar fashion.

Interestingly, in the present study, ESCwE were sensitive to IFN-γ-induced cell growth inhibition and apoptosis. However, recent studies have demonstrated that the number of apoptotic cells in the eutopic endometrium of women with endometriosis was significantly reduced compared to that of healthy control subjects without endometriosis (Dmowski et al., 2001). Taking these observations into account, it is considered that, in patients with endometriosis, the endometrial cells in the uterine cavity have already become resistant to apoptotic signals, and thus are already prepared for survival and implantation outside the uterus when they are transported into the peritoneal cavity by retrograde menstruation. It is speculated that the eutopic endometrium with endometriosis may contain relatively small cell populations of endometrial stromal cells with the ability to develop endometriosis and the endometrial stromal cells without anti-apoptotic properties might be selected during isolation of the cells in our present study.

In summary, we demonstrated that ECSC are resistant to IFN-γ-induced apoptosis, whereas, NESC became apoptotic upon IFN-γ treatment. The expressions of anti-apoptotic factors of the Bcl-2 family (Bcl-2 and Bcl-XL) were up-regulated in untreated ECSC. These findings may explain how misplaced endometrial cells in the peritoneal cavity are able to survive and be implanted in ectopic locations. Further studies on the cell proliferation and apoptosis of ECSC, NESC and ectopic endometrial stromal cells isolated from these women are necessary to obtain evidence of the dysregulated expression of CXC chemokines in endometriotic stromal cells: a possible mechanism for the altered immunological functions in endometriosis. In addition, as demonstrated in the present report, our experimental system is suitable for the investigation of the cell proliferation and apoptosis of ECSC. This culture system could be used for testing new drugs and cytokines for the treatment of endometriosis.

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