

Drug-induced apoptosis was markedly attenuated in endometriotic stromal cells

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BACKGROUND: The survival of endometriotic cells in the ectopic site has been investigated from the aspect of susceptibility of endometriotic tissues to apoptosis. In order to investigate the nature of abnormal survival of endometriotic cells in ectopic locations, we compared drug-induced apoptosis in endometrial and endometriotic cells. **METHODS:** Endometrial stromal cells were obtained from normal endometrium in 11 patients who underwent hysterectomy for leiomyoma without endometriosis. Endometriotic cells were isolated from the chocolate cyst linings of the ovary in 13 patients who underwent laparoscopic surgery. Cells were cultured in the presence or absence of staurosporine. Apoptotic cell death was evaluated by staining nuclei with propidium iodide and phosphatidylserine (a marker of early apoptotic events) with Annexin V as well as by DNA fragmentation assay. The number of viable cells was estimated by modified MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide WST-8] assay. **RESULTS:** After 3 h of exposure to staurosporine, >50% of the endometrial stromal cells became Annexin V positive. In contrast, >30% of the endometriotic cells were Annexin V positive. DNA fragmentation was not clearly induced in the endometriotic cells. Less than 20% of the endometrial cells survived after staurosporine exposure, while >40% of the endometriotic cells survived. Cell death induced by staurosporine was partially blocked by incubation with the caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)fluoromethyl-ketone (ZVAD-fmk), suggesting that a caspase cascade may play a role in the cell death process. **CONCLUSIONS:** Attenuated susceptibility to apoptosis in endometriotic stromal cells may be associated with abnormal survival in ectopic sites in an environment that is probably unfavourable. These results may be implicated in the pathophysiology of endometriosis.

Key words: apoptosis/endometriosis/pathophysiology/staurosporine

Introduction

Endometriosis is characterized by the presence and growth of endometrial tissue in locations other than the uterine cavity. Despite decades of clinical experience and experimental research, endometriosis remains an enigma and its pathogenesis is still controversial. Sampson's theory of implantation (Sampson, 1927), which describes endometrial cells in retrograde menstruation that may attach, implant, and grow, seems plausible because peritoneal lesions are most frequently found in the ovaries and the posterior cul-de-sac where regurgitated menstrual material pools (Jenkins *et al.*, 1986).

Peritoneal fluid (PF), which contains various local factors, increases in volume in women with endometriosis (Harada *et al.*, 2001). Surrey and Halme (1990) demonstrated a direct stimulatory effect of the cell-free fraction of peritoneal fluid samples derived from patients with endometriosis on the proliferation of normal uterine endometrial cells, indicating that factors within the PF are involved in the progression of endometriosis.

Our previous study revealed that PF levels of IL-8 significantly enhanced the proliferation of stromal cells derived from ovarian endometriomas (Iwabe *et al.*, 1998). We also demonstrated that elevated levels of tumour necrosing factor (TNF)- α in the PF of patients with endometriosis might contribute to the progression of endometriosis by inducing production of interleukin (IL)-8 through the transcription factor NF- κ B activation (Iwabe *et al.*, 2000; Sakamoto *et al.*, 2003). These studies suggest that cytokines in peritoneal fluid might promote progression of endometriosis and contribute to the survival of endometriotic cells in ectopic locations.

The survival of endometriotic cells in the ectopic site has also been investigated from the aspect of susceptibility of endometriotic tissues to apoptosis (Gebel *et al.*, 1998; Harada *et al.*, 2004). Apoptosis, or programmed cell death, is an actively initiated process by which multiple cell types are eliminated during embryogenesis and in fully developed adult multicellular organs. Apoptosis, which occurs under both physiological and pathological conditions, is involved in a diverse group of processes, including reproduction (Izawa and Yeh, 1997).

Accumulated evidence suggests that apoptosis is directly involved in the regulation of the menstrual cycle, by eliminating senescent cells from the functional layer of the uterine endometrium during the late-secretory and menstrual phases (Hopwood and Levison, 1976; Kokawa *et al.*, 1996). Reports indicate that spontaneous apoptosis of ectopic and eutopic endometrial tissues are impaired in patients with endometriosis (Gebel *et al.*, 1998; Dmowski *et al.*, 2001), suggesting that decreased susceptibility of endometrial tissue to apoptosis (abnormal survival) contributes to the aetiology or pathogenesis of endometriosis.

In order to investigate the nature of abnormal survival of endometriotic cells in ectopic locations, we attempted to establish a drug-induced apoptosis system. We evaluated the relative rate of drug-induced apoptosis in isolated endometrial and endometriotic stromal cells. We also examined the effect of caspase inhibitor on drug-induced apoptosis in the cells.

Materials and methods

Isolation and culture of endometrial and endometriotic stromal cells

Endometrial tissues were obtained from uteri of cycling pre-menopausal women who underwent hysterectomy for uterine leiomyoma during the proliferative ($n = 6$) or secretory phase ($n = 5$). The chocolate cyst lining of the ovaries in patients with endometriosis ($n = 13$) was the source of endometriotic tissue collected during the follicular ($n = 6$) or luteal phase ($n = 7$). The patients received no hormonal treatments before surgery. The menstrual cycle phase was determined by measuring serum estradiol and progesterone levels as well as by histological examination. Informed consent was obtained from all subjects.

Stromal cells were collected from endometrial and endometriotic tissue as previously described (Iwabe *et al.*, 1998, 2000; Sakamoto *et al.*, 2003). The tissues were minced and digested with 0.5% collagenase in Dulbecco's modified Eagle's medium and Ham's F-12 (1:1, vol/vol) at 37°C for 60 min. The dispersed cells were filtered through a 70 μm nylon mesh to remove the undigested tissue pieces containing the glandular epithelium. The filtered fraction was separated further from epithelial cell clumps by differential sedimentation at unit gravity. The medium containing stromal cells was filtered through 40 μm nylon mesh. Final purification was achieved by allowing stromal cells, which attach rapidly to plates, to adhere selectively to culture dishes for 30 min at 37°C in 5% CO_2 in air. Non-adherent epithelial cells were removed. Immunohistochemical analysis of isolated endometrial and endometriotic stromal cells was performed, using antibodies against cytokeratin, vimentin, and factor VIII, to confirm the purification of the stromal cells. The results showed that the purity of stromal cells was >98%.

Induction of apoptosis

Endometrial and endometriotic stromal cells were seeded and maintained in each well of a 96-well plate (5×10^4 cells per well) or in a 60 mm dish (8×10^5 cells per dish) for 24 h, then treated with 0.5 or 1.0 $\mu\text{mol/l}$ staurosporine at appropriate time intervals (Izawa *et al.*, 1999). In experiments where the effect of the caspase inhibitor, *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)fluoromethyl-ketone (ZVAD-fmk; Peptide Institute, Osaka, Japan) was examined, cells were pretreated with 30 $\mu\text{mol/l}$ of ZVAD-fmk for 1 h prior to staurosporine treatment, then maintained in conditioned medium in the presence of Z-VAD-fmk and staurosporine.

Assessment of apoptotic cells

Apoptotic cells were assessed morphologically by staining with propidium iodide (Sigma, St Louis, MO, USA). At the same time, the

number of viable cells was estimated by a modified MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide WST-8 assay; Wako, Osaka, Japan] as described previously (Teramachi and Izawa 2000).

To evaluate the early event of apoptosis, exposure of phosphatidyserine on a cell surface was examined (Fadok *et al.*, 1992, 2000; Schlegel and William, 2001). In brief, cells ($\sim 2 \times 10^5$) plated on a cover slip in a 35 mm dish were treated with staurosporine to induce apoptosis as described above, then stained with Annexin V-tagged enhanced green fluorescent protein EGFP (Medical & Biological Laboratories Co., Ltd, Nagoya, Japan). The cover slip was then removed and fixed in a methanol at -20°C for 5 min. The cover slip was further stained with propidium iodide at room temperature for 5 min. Finally, cells were examined under UV microscopy. The relative number of Annexin V-EGFP positive cells expressed as the percentage of cells was stained with propidium iodide.

As a biochemical marker of apoptosis, DNA fragmentation into a nucleosomal unit was examined. In brief, cells were lysed in an extraction buffer [10 $\mu\text{mol/l}$ Tris-HCl, pH 8.0, 25 $\mu\text{mol/l}$ EDTA, 100 $\mu\text{mol/l}$ NaCl, 0.1 mg/ml proteinase K, and 0.5% sodium dodecyl sulphate (SDS)] and cellular DNA were prepared according to the procedure described previously in detail (Izawa *et al.*, 1997). Extracted DNA were separated on a 1.5% agarose gel electrophoresis at a constant voltage of 100 V for 30 min, then visualized with ethidium bromide staining under UV light.

Statistical analysis

The data were analysed by one-way analysis of variance, followed by Fisher's protected least significant difference test. Values were expressed as means \pm SEM. $P < 0.05$ was considered statistically significant.

Results

Staurosporine-induced apoptosis in endometrial and endometriotic cells

Using staurosporine as an apoptosis-inducing agent (Izawa *et al.*, 1999), susceptibility to drug-induced apoptosis was examined in endometrial and endometriotic stromal cells (Figure 1). Normal skin fibroblastic cells were used in parallel as the control. Six hours after treatment with either 0.5 or 1.0 $\mu\text{mol/l}$ of staurosporine, phosphatidyserine, which is an early hallmark of apoptosis, appeared on the cell surface, and >60% of endometrial cells become Annexin V positive. This value is higher than that of fibroblasts. In contrast, the percentage of Annexin V-positive cells was <30% after 6 h treatment with 1.0 $\mu\text{mol/l}$ of staurosporine in endometriotic cells.

Apoptosis was also evaluated using detection of DNA fragmentation into a nucleosomal unit. In normal endometrium-derived cells, genomic DNA fragmentation into 180 bp units, a classical and late phase marker of apoptosis, was clearly demonstrated. In contrast, DNA fragmentation in response to staurosporine was markedly reduced in endometriotic cells (Figure 2).

Survival of endometrial and endometriotic cells by staurosporine exposure

The number of viable cells was estimated following exposure to staurosporine. Treatment of these cells with 1.0 $\mu\text{mol/l}$ of

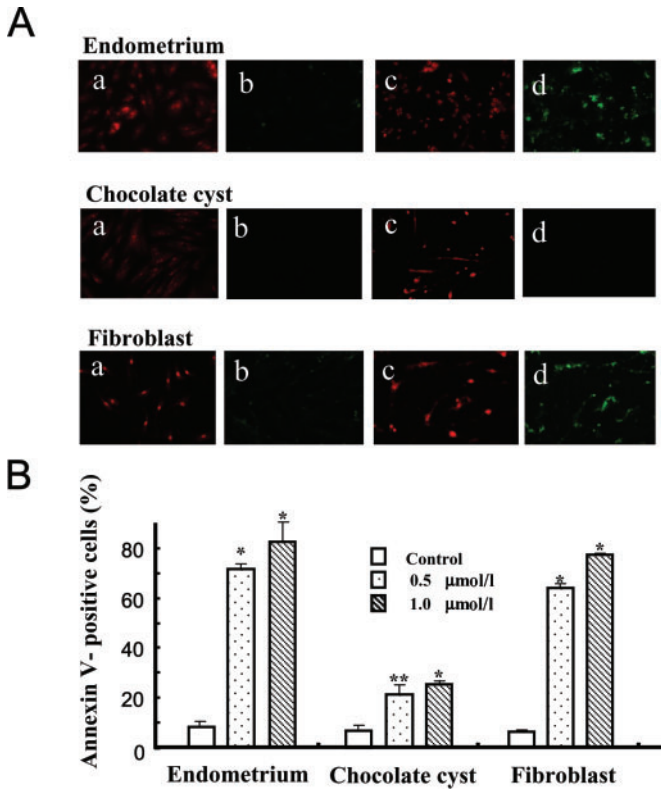


Figure 1. Exposure of phosphatidylserine on apoptotic cells from normal endometrium and chocolate cyst of endometriosis. Cells were treated with 0.5 or 1.0 $\mu\text{mol/l}$ staurosporine for 6 h, stained with Annexin V-EGFP and subsequently fixed with methanol for propidium iodide (PI) staining. The number of Annexin V-EGFP-positive cells was estimated as the percentage of PI-positive cells (total cell number) by fluorescence microscopy. Approximately 300 cells were counted in each cell population. (A) **a** and **c**, PI-positive cells. **b** and **d**, Annexin V-EGFP-positive cells. **a** and **b** are untreated control cells. **c** and **d** are cells treated with 1.0 $\mu\text{mol/l}$ staurosporine for 6 h. (B) Relative number of Annexin V-EGFP-positive cells was expressed as the percentage of total cell number. Results from cells from three patients (mean \pm SEM). * $P < 0.01$ (versus control), ** $P < 0.05$ (versus control).

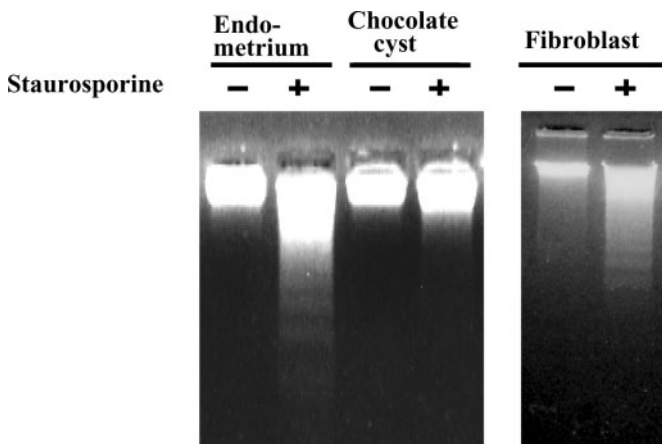


Figure 2. DNA fragmentation assay. Cells were treated with 1.0 $\mu\text{mol/l}$ staurosporine for 15 h, then cellular DNA was extracted and subjected to a 1.5% agarose gel electrophoresis as described in the text. DNA was visualized with ethidium bromide staining under UV light. A representative result from three independent experiments.

staurosporine for 15 h markedly reduced the number of viable cells (Figure 3). More than 80% of endometrial cells died in response to staurosporine, while in endometriotic cells, ~40% of cells remained alive. The cell death in response to staurosporine was partially but significantly rescued in the presence of a pan-caspase inhibitor Z-VAD (Figure 3).

The relative number of survival cells derived from the endometrium was <20% (Figure 4). Similar results were obtained in the cells derived from both proliferative and secretory phase. In contrast, in endometriosis cases, the relative number of survival cells was significantly higher than those of endometrial cells (Figure 4). The results clearly demonstrated that endometriotic cells have reduced susceptibility to drug-induced apoptosis.

Discussion

The present study demonstrated for the first time that endometriotic stromal cells derived from ovarian chocolate cysts show resistance to drug-induced apoptosis compared with their normal counterpart, eutopic endometrial cells. Apoptotic cell death was evaluated by staining with propidium iodide and Annexin V as well as by DNA fragmentation assay. We also confirmed that cell death induced by staurosporine was partially blocked by incubation with the caspase inhibitor, ZVAD-fmk, suggesting the role of a caspase cascade in the cell death process. Attenuated susceptibility to induced apoptosis in endometriotic stromal cells may be associated with abnormal survival in ectopic sites in an environment that is probably unfavourable for survival. These results indicate that apoptosis might be involved in the pathophysiology of endometriosis.

It is believed that the eutopic endometrium of women with endometriosis shares changes with ectopic tissue, and that these changes are not found in the eutopic endometrium of disease-free

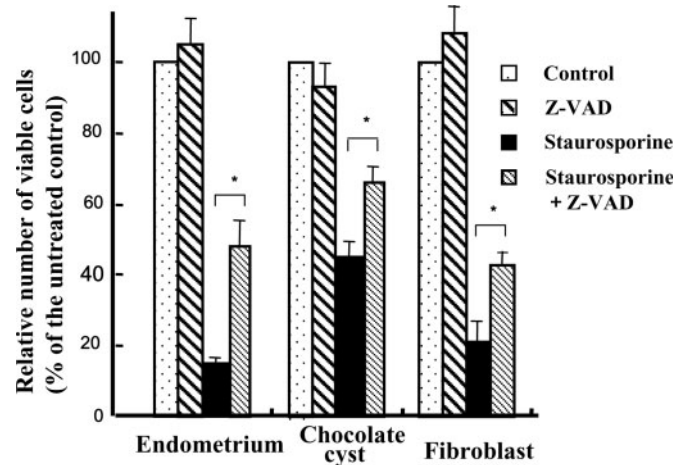


Figure 3. Effect of Z-VAD-fmk on the staurosporine-induced apoptosis. Cells were pretreated with 30 $\mu\text{mol/l}$ Z-VAD-fmk for 1 h, then treated with 1.0 $\mu\text{mol/l}$ staurosporine for 15 h in the presence or absence of Z-VAD-fmk. The relative number of viable cells was estimated by WST-8 assay and expressed as the percentage of untreated control as described in the text. Each value represents the mean of at least four determinations (\pm SEM). * $P < 0.05$ (versus control). A representative result from three independent experiments.

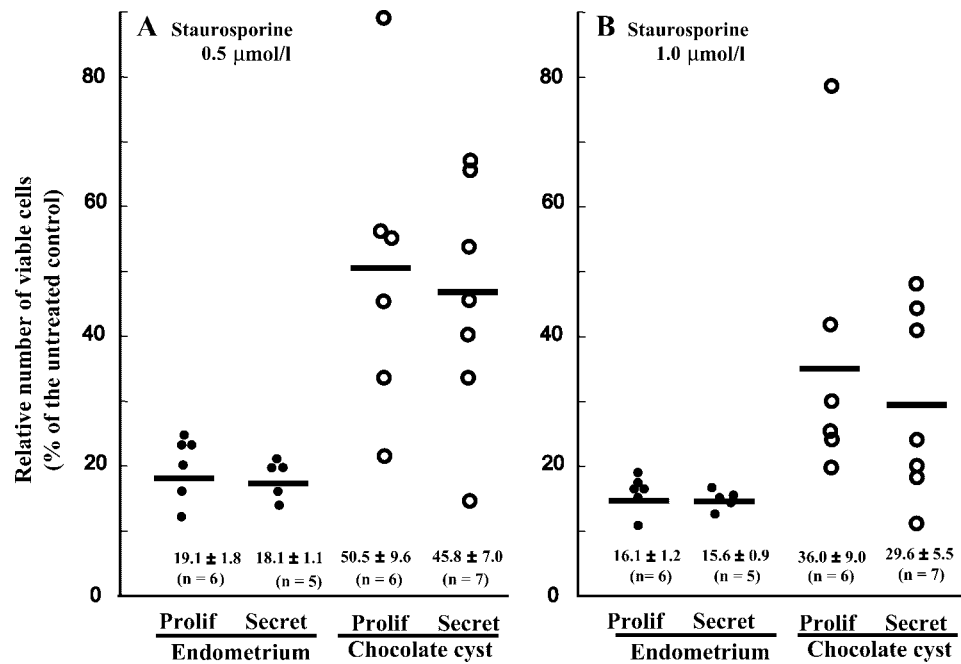


Figure 4. Staurosporine-induced cell death in endometrium- and chocolate cyst-derived cells. Cells were treated with 0.5 or 1.0 μmol/l staurosporine for 15 h. The relative number of viable cells was estimated by WST-8 assay and expressed as percentage of the untreated control. Each value represents the mean of at least four determinations in each patient. Results are expressed as the mean (horizontal bar in the figure) ± SEM. The numbers of patients are indicated in parentheses. $P < 0.01$ (proliferative phase endometrium versus chocolate cyst, secretory phase endometrium versus chocolate cyst). Prolif = cells in the proliferative phase; Secret = cells in the secretory phase.

women. The primary defect in endometriosis is to be found in the eutopic endometrium (Sharpe-Timms, 2001). Apoptotic process is one of the endometrial alterations appearing in eutopic and ectopic endometrium of women with endometriosis (Harada *et al.*, 2004).

Apoptosis, or programmed cell death, is a distinctive form of cell death defined by characteristics of morphological and biochemical events that result in the efficient elimination of cells from tissue without eliciting an inflammatory response (Kerr *et al.*, 1972). Accumulating evidence suggests that apoptosis is also critically involved in the regulation of menstrual cycles and the development of endometriosis (Harada *et al.*, 2004).

Gebel *et al.* (1998) reported that the percentage of apoptosis in sloughed endometrial cells was greatly reduced among women with endometriosis, implying that the number of surviving cells that enter the peritoneal cavity is greater in women who develop endometriosis. Dmowski *et al.* (2001) demonstrated that the apoptotic index in glandular epithelium was significantly lower in women with endometriosis than in controls. This difference was caused primarily by a significant decrease in apoptosis during the late secretory/menstrual and early proliferative phases in women with endometriosis. The cyclic variability of apoptosis was lost in these women. Although we did not compare the susceptibility with drug-induced apoptosis of eutopic endometrial cells between women with and without endometriosis, this may be an intriguing issue for future study.

One can speculate that if the decrease in apoptosis facilitates ectopic survival and implantation of the endometrial cells, there must be an inverse correlation between the level of apoptosis

and the severity of the disease. To test this hypothesis, Dmowski *et al.* (2001) analysed the apoptotic index according to the stage of endometriosis. They found that there was a trend toward decreased apoptosis with increasing stage of the disease, but the difference lacked statistical significance (Dmowski *et al.*, 2001).

The susceptibility to drug-induced apoptosis in endometriotic cells varies widely among patients (Figure 4). Apoptosis was markedly induced in two patients. The responses of the cells derived from these patients were similar to the eutopic endometrial cells. No distinctive clinical feature, such as disease stage, age and absence of drug treatment history, was observed in these patients. Endometriotic cells from different patients may differ in their ability to survive. For example, red peritoneal endometriotic implants are known to have a higher proliferative activity than those of black lesions (Nisolle *et al.*, 1997; Matsuzaki *et al.*, 2001).

Imai *et al.* (2000) reported that exposure to GnRH resulted in changes of the sensitivity of endometrial cells of patients with endometriosis to spontaneous apoptosis. These changes in sensitivity may, in turn, release endometrial cells from resistance to apoptosis and result in reduced survival and growth (Imai *et al.*, 2000). A recent study also showed that treatment using 30 days of combination oral contraceptives suppresses cell proliferation and enhances apoptosis of eutopic endometrial tissues from patients with endometriosis (Meresman *et al.*, 2002). Treatment modalities of under-use may have potential beneficial effects on cell growth and apoptosis in eutopic endometrium. The effects of drug treatments on staurosporine-induced apoptosis

in endometriotic cells may be a useful *in vitro* model system of future drug investigation.

It is important to note that staurosporine is now recognized as kinase inhibitor for a number of kinases including protein kinase C (PKC). In our preliminary experiments to evaluate the ability of several drugs which had been well recognized as apoptosis-inducing agents, such as PKC-specific inhibitors, bisindolylmaleimide and H-7, were far less effective than staurosporine in terms of apoptosis induction. Etoposide exhibited no caspase-dependent cell death (data not shown).

Our present findings about attenuation in drug-induced apoptosis in endometriotic stromal cells may reveal the distinct biological characters of endometriotic cells. Using our newly established system, further study on the molecular basis of attenuated apoptotic machinery in endometriotic cells may expose the fundamental characteristics of endometriosis, and lead to unveiling the pathophysiology of the disease.

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