Altered apoptosis and proliferation in endometrial stromal cells of women with adenomyosis

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BACKGROUND: The eutopic endometrium in a woman suffering from adenomyosis is known to be biologically different from that of healthy women. The aim of this study was to examine the apoptosis and proliferation of eutopic endometrium from women with adenomyosis. METHODS: We enrolled 23 women with adenomyosis (study group) and 21 without (control group). Eutopic endometrium was obtained and separated into single endometrial stromal cells (ESCs). ESCs were treated in vitro with hydrogen peroxide (H2O2) to examine their apoptosis using a fluorescence-activated cell sorter. Cells were also treated with estradiol (E2), medroxyprogesterone acetate, interleukin (IL)-6, lipopolysaccharide and interferon-γ (IFN-γ) to test their proliferation using a non-radioactive cell proliferation assay. RESULTS: The percentage of annexin V (1)/7-amino-actinomycin D (1) ESCs was much lower in women with adenomyosis after 24 h culture with and without H2O2 treatment when compared with the control group. ESCs of adenomyosis proliferated more rapidly than those of the control group, whether they were cultured alone or were treated with E2, MPA, IL-6 or IFN-γ. The immunocytochemical Ki-67 labelling index was much more prominent in adenomyotic ESCs than that of the control group (7.7% versus 1.1%, P<0.001). CONCLUSIONS: Altered apoptosis and proliferation of eutopic endometrium possibly elucidate some aspects of the pathophysiology of adenomyosis. A high Ki-67 labelling index in immunocytochemistry might be a potential indicator in predicting the occurrence of adenomyosis.

Key words: adenomyosis/apoptosis/endometrial stromal cell/Ki-67/proliferation

Introduction

Adenomyosis is defined as the presence of ectopic endometrial glands and stroma within the myometrium. The pathogenic mechanism responsible for adenomyosis is not well known as yet. Our previous study revealed that the expression of killer cell immunoglobulin-like receptors on natural killer (NK) cells was decreased in eutopic endometrium in women with adenomyosis (Yang et al., 2004). It may be a compensatory effect in which the NK cytotoxicity is activated in order to eradicate the abnormal endometrial cells that might exit the eutopic site of endometrium. This finding suggested that abnormal endometrial cells, but not aberrant immunological phenomenon, account for the development of adenomyosis. Our following studies demonstrated that interleukin (IL)-6 was abnormally produced by endometrial stromal cells (ESCs) in women with adenomyosis, which was either overexpressive after in vitro co-culture with macrophage (Yang et al., 2006a) or resistant to the suppressive effect of medroxyprogesterone acetate (MPA) and danazol (Yang et al., 2006b). These studies strengthen the view that the eutopic endometrium of a woman with adenomyosis is biologically different from that of a healthy woman.

Apoptosis is a process of programmed cell death that is involved in a diverse group of processes including reproduction (Kokawa et al., 1996). Accumulated evidence suggests that apoptosis is directly involved in the regulation of the menstrual cycle through eliminating senescent cells from the functional layer of the uterine endometrium during the late-secretory and menstrual phases (Hopwood and Levison, 1976). In endometriosis, which is pathophysiologically similar to adenomyosis, reports demonstrated impaired apoptosis (Gebel et al., 1998; Dmowski et al., 2001) and accelerated proliferation (Meresman et al., 2002) of the eutopic endometrium. The resistance to apoptosis of endometrial cells and their increased sensitivity to proliferation suggest the possible pathogenesis of endometriosis.

There have been few studies investigating the apoptosis and proliferation of the eutopic endometrium of adenomyosis.
An immunohistochemical study found rare apoptosis in eutopic endometrium from women with adenomyosis as well as from the healthy controls (Jones et al., 1998). This report demonstrated that the B cell lymphoma/leukemia-2 (bcl-2) expression in stromal cells, which provided resistance to apoptosis, was less prominent in the eutopic endometrium of adenomyosis than the paired eutopic endometrium. Currently, there are no studies comparing the apoptosis and cell proliferation of eutopic endometrium between women with and without adenomyosis.

Fluorescence-activated cell sorter (FACS) unlike immunohistochemistry, is useful in assessing changes in the intensity of expression of surface markers and, therefore, can be used to quantify their up- or down-regulation. There has been a study comparing immunohistochemistry and FACS results in gastric carcinoma using a monoclonal antibody against the carcinoembryonic antigen. Comparable results were achieved with these two methods. However, quantitative evaluation of the immunoreactive cell populations was available only with the use of FACS (Tamai et al., 1993). In addition, FACS facilitates the evaluation of larger numbers of cells than tissue sections in small specimens. Studies have proven the efficacy of FACS in the apoptotic analysis of endometrial cells (Stackievicz et al., 2001; Wang et al., 2005).

In this study, ESCs were obtained from women with and without adenomyosis. We investigated the apoptosis of ESCs with FACS and determined the proliferation of ESCs using non-radioactive assay kits and immunocytochemistry, in an attempt to find possible deviations in apoptosis and proliferation in the eutopic endometrium of adenomyosis.

**Materials and methods**

**Subjects and specimens**

This study consisted of 23 women who suffered from adenomyosis (study group) and 21 women in whom cervical intraepithelial neoplasia or leiomyoma was found (control group). These women underwent hysterectomy, either via abdominal or vaginal route, at our hospital. All the participating women were at premenopausal status. As apoptosis and cell proliferation might vary in different phases of the menstrual cycle (Kokawa et al., 1996; Jones et al., 1998; Matsumoto et al., 1999), only women in the early- to mid-secretory phase were included in this study. The histology of the early- to mid-secretory phase endometrium was characterized by tortuous glands, subnuclear vacuoles in the glandular epithelium, prominent glandular secretion, stromal edema and inconspicuous spiral arteries. The diagnosis of adenomyosis was made by histopathologic examination when there is ingrowth of endometrium below the endometrial–myometrial interface more than 2.5 mm. Informed consent was obtained from each woman before surgery, and this study protocol was approved by the institutional review board at our hospital.

**Purification of ESCs**

Endometrium was obtained immediately after the uterus was removed from the women, and was placed in an ice-cold 1 : 1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F-12 for transport to the laboratory. The tissue was gently dissected into small pieces (1–2 mm³) and washed by centrifugation (400 x g) in a fresh medium to remove any debris or excess blood cells. The tissue was then incubated for 2 h at 37°C in a shaking water bath in DMEM/F-12 containing 0.5% collagenase and 0.02% DNase. 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, 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 air with 5% CO₂ for 30 min. After sedimentation, the top 8 ml of medium was collected. The medium containing stromal cells was filtered through a 40-μm nylon mesh. Final purification was achieved by allowing stromal cells to adhere selectively to culture dishes for 30 min at 37°C in 5% CO₂ in air. Non-adhering epithelial cells were removed. Cell purity was assessed by immunocytochemistry using antibodies against cytokeratin (Dako Corp., Carpenteria, CA, USA), vimentin (Dako) and Factor VIII (Dako). The purity of the stromal cell preparations used in this study was more than 98%.

**Fluorescence-activated cell sorter**

The ESCs were divided into three portions. The first portion was processed immediately for FACS analysis. The second portion was cultured for 24 h in T25 flasks containing DMEM/F-12 supplemented with 100 IU ml⁻¹ penicillin G, 50 mg ml⁻¹ of streptomycin and 2.5 μg ml⁻¹ of amphotericin B at 37°C in air with 5% CO₂. The third portion was also cultured for 24 h as was done in the second portion, except that 200 μM of hydrogen peroxide (H₂O₂) was added to the media. The second and third portions were processed for FACS analysis after culture.

The method of FACS analysis has been described in detail previously (Yang et al., 2000, 2004). Briefly, after washing twice with cold phosphate-buffered saline (PBS), the ESCs were resuspended in 1× binding buffer at a concentration of 1×10⁶ cells ml⁻¹. About 100 μl of ESCs were incubated with 5 μl of phycoerythrin (PE)-conjugated annexin V (BD Biosciences, San Jose, CA, USA) and 5 μl of 7-amino-actinomycin D (7-AAD, BD Biosciences) for 15 min at room temperature (RT) in the dark. After that, 400 μl of 1×binding buffer was added to the ESCs.

For staining with caspase-3, ESCs were resuspended in 0.5 ml of Cytotox/Cytperm (BD Biosciences) solution for 20 min. After being washed twice with Perm/Wash buffer (BD Biosciences), the ESCs were incubated with PE-conjugated anti-caspase-3 (BD Biosciences) for 30 min in the dark at RT. For staining with bcl-2, ESCs were treated with 0.5 ml of lysing solution for 10 min, and were cultured with 0.5 ml of permeablizating solution for 10 min. The ESCs were washed with 0.5% bovine serum albumin (BSA) in 1× PBS and 0.1% sodium azide, and then incubated with 20 μl of PE-conjugated anti-bcl-2 (BD Biosciences) for 30 min in the dark at RT.

FACS analysis was done within 1 h using a FACSScan cytometer (BD) with computer interface to software (Hewlett-Packard Consor 32, BD) for full-list mode data storage, recovery and analysis. Samples of ESCs were subjected to FACS, and background noise in the clinical samples was excluded by establishing a window in which only the signals derived from ESCs appeared. Electrical compensation was performed to eliminate the interference of fluorescence during simultaneous two-colour measurement. The compensation procedure was performed before each measurement. In each cell suspension, a minimum of 30 000 events acquired for gated ESCs were measured (Figure 1).

**Proliferation assay**

ESC proliferation was determined by CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA). This kit is a colorimetric assay for determining the number of
viable cells as a surrogate measure of proliferation. It is composed of solutions of a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS)] and an electron coupling reagent phenazine methosulfate (PMS). MTS is bioreduced by cells to a formazan product that is soluble in tissue culture medium. The absorbance of formazan at 490 nm can be measured directly in 96-well assay plates without additional processing. The conversion of MTS into aqueous, soluble formazan was accomplished by dehydrogenase enzymes found in metabolically active cells. The quantity of formazan product, as measured by the amount of 490 nm absorbance, was directly proportional to the number of living cells in culture. Experiments have demonstrated a high correlation between this assay and [3H]thymidine incorporation assay (Bounous et al., 1992; Wagner et al., 1999). Its advantages over the [3H]thymidine assay are that it does not deal with radioactivity, it is less costly and not very labour intensive (Ahmed et al., 1994).

ESCs (2 × 10^4 per well) were seeded in 96-well culture plates. The first column of each 96-well plate did not contain any cells and was used as a blank. The remaining columns were ESCs without treatment, followed by ESCs treated with estradiol (E2) 10^{-8} M, E2 10^{-7} M, MPA 10^{-8} M, MPA 10^{-7} M, E2 10^{-8} M + MPA 10^{-7} M, E2 10^{-7} M + MPA 10^{-7} M, IL-6 10 ng ml^{-1}, IL-6 50 ng ml^{-1}, lipopolysaccharide (LPS) 10 ng ml^{-1} and interferon-γ (IFN-γ) 100 ng ml^{-1}. Cell proliferation was evaluated after 48 h of treatment when 20 μl of MTS/PMS solution was added to all wells, and plates were incubated at 37°C for 4 h. At the end of the incubation period, plates were read with an enzyme-linked immunosorbent assay plate reader (SpectraMax, Molecular Devices, Menlo Park, CA, USA). Data were expressed in optical density (OD) units. Experiments were conducted in triplicate.

**Immunocytochemistry for Ki-67**

ESCs were plated in four-well chamber slides (Nunc, Naperville, IL, USA). After 24 h of incubation, the medium was removed and cells were fixed for 5 min with 4% paraformaldehyde in PBS. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at RT. ESCs were blocked by incubating at RT in 1% BSA in PBS for 1 h, followed by 3% H_2O_2 for 5 min. Cells were then incubated with a 1:50 dilution of mouse monoclonal anti-human Ki-67 antibody (BD, Franklin Lakes, NJ, USA) as a primary antibody for 40 min at RT. Next, cells were washed in PBS, incubated with biotinylated mouse anti-human antibody (BD) for 40 min, and washed again in PBS. Cells were then incubated with 3,3’ diaminobenzidine chromogen (Dako) for 10 min, and were...
washed again in PBS. Finally, cells were counterstained with hematoxylin and slides were mounted with water-soluble mounting medium. Experiments with the omission of primary antibodies were used as negative controls.

Ki-67 protein is a nuclear and nucleolar protein that is strictly associated with cell proliferation. The Ki-67 labelling index was calculated manually by counting the number of Ki-67 positive cells per 1000 total cells by two trained observers blinded to the experiment. Two consecutive, randomly selected 100× microscopic fields were selected for each specimen processed.

**Statistical analysis**

All values are expressed as mean ± SE. The one-way analysis of variance (ANOVA) followed by Tukey’s post hoc test was employed to compare between treated and untreated ESCs, and between the study and control groups in cell proliferation. The independent samples’ t-test was used to compare the difference between the study and control groups in cell apoptosis and immunocytochemistry. A P-value < 0.05 was considered statistically significant.

### Results

The mean age was 44.4 ± 0.8 years for women with adenomyosis and 44.6 ± 1.2 years for those without adenomyosis. The body mass indices were similar between the two groups (mean, 24.8 versus 23.6). All the participating women were in the early- to mid-secretory phase which was confirmed by histopathologic examination.

### ESC apoptosis

In Figure 1B, viable ESCs are negative for both annexin V and 7-AAD. ESCs in early apoptosis are annexin V positive and 7-AAD negative, and those in late apoptosis or already dead are positive for both annexin V and 7-AAD. In ESC suspensions tested on the day of specimen collection (day 0), both the percentages of annexin V (+)/7-AAD (+) and annexin V (+)/7-AAD (−) were similar between women with and without adenomyosis. After 24 h culture, however, the percentage of annexin V (+)/7-AAD (+) ESCs was much lower in women with adenomyosis than in those without (4.9 ± 1.2% versus 14 ± 3.5%, respectively, P = 0.021), but the percentages of annexin V (+)/7-AAD (−) ESCs remained similar between the two groups. In ESCs treated with H2O2 for 24 h, the percentages of annexin V (+)/7-AAD (+) ESCs increased even further. These percentages were also lower in women with adenomyosis than in women without (8 ± 2.3% versus 20.5 ± 4.6%, respectively, P = 0.019) (Figure 2).

The percentages of ESCs positive for bcl-2 staining were similar between women with and without adenomyosis on day 0 (9 ± 2.7% versus 6.6 ± 1.2%) as well as after 24 h culture with H2O2 treatment (7.6 ± 2.7% versus 5.6 ± 0.9%) or without H2O2 treatment (7.5 ± 2.5% versus 5.2 ± 1.2%). The percentages of ESCs positive for caspase-3 staining were also not different between these two groups (Figure 3).

### ESC proliferation

With regard to the effects of various agents on ESC proliferation, the OD units were not different (P > 0.98 by ANOVA) between ESCs cultured alone and ESCs treated with various agents including E2, MPA, E2 + MPA, IL-6, LPS and IFN-γ, either in women with or without adenomyosis. However, the OD unit of ESCs cultured alone for 48 h in women with adenomyosis was significantly higher than that in the control group (0.42 ± 0.05 versus 0.24 ± 0.03 respectively, P < 0.05). After treatment with E2 10⁻⁸ M (P < 0.05), E2 10⁻⁷ M (P < 0.01), MPA 10⁻⁸ M (P < 0.05), MPA 10⁻⁷ M (P < 0.05), E2 10⁻⁸ M + MPA 10⁻⁷ M (P < 0.05), E2 10⁻⁷ M + MPA 10⁻⁷ M (P < 0.05), IL-6 10 ng ml⁻¹ (P < 0.05), IL-6 50 ng ml⁻¹ (P < 0.05) and IFN-γ 100 ng ml⁻¹ (P < 0.05) for 48 h, the OD units were also much higher in women with adenomyosis than those in women without (Figure 4).

### Ki-67 labelling index of ESCs

The immunocytochemical staining of ESCs was shown in Figure 5. A high density of immunopositive nuclei is a feature of proliferating ESCs. Ki-67 positive cells were observed in 7.7 ± 1.2% of ESCs in women with adenomyosis,
which was significantly higher than that in the control group 
(1.1 ± 0.2%, P < 0.001) (Figure 6).

Discussion

There have been several reports describing the cyclic apoptosis of endometrial cells in the regulation of menstrual cycles (Kokawa et al., 1996; Tao et al., 1997). A decreased sensitivity of endometrial cells to apoptosis was found in the ectopic locations (Beliard et al., 2004; Nishida et al., 2005, Izawa et al., 2006) as well as in the eutopic endometrium (Dmowski et al., 1998; Gebel et al., 1998) of endometriosis when compared with the fertile controls. For the first time, the present study demonstrates reduced apoptosis and increased proliferation of the eutopic endometrium in women with adenomyosis.

Our results revealed that ESCs of adenomyosis were resistant to late apoptosis (both annexin V-PE and 7-AAD positive) after 24 h incubation when compared with the control group. This differential susceptibility of ESCs to apoptosis between women with and without adenomyosis was also found when ESCs were treated with H₂O₂. H₂O₂ is an apoptosis inducing agent that injures various kinds of cells by both apoptosis and necrosis (Wagner et al., 2000; Nakamura et al., 2006). Our findings suggest that the apoptosis-resistant ESCs might escape from programmed cell death and lead to implantation in the ectopic sites and the development of adenomyosis.

Bcl-2 is a regulator of the apoptotic process. Elevated levels of bcl-2 can provide resistance to cell death (DiGiuseppe et al., 1996; Reed 1997). Bcl-2 has been considered to inhibit apoptosis in the human endometrium during the proliferative phase (Otsuki et al., 1994). Meresman et al. (2000) reported an increased expression of bcl-2 protein in the proliferative eutopic endometrium from women with endometriosis when compared with the normal controls. In this study, however,
be expressed on ESCs during in vitro culture (Zarmakoupis et al., 1995), and that the expression level of IL-6 receptor did not differ between the proliferative and the secretory phases (Yoshioka et al., 1999). However, our results did not demonstrate increased endometrial cell proliferation after stimulation with IL-6.

LPS was reported to promote (Iba et al., 2004), and IFN-γ to suppress (Clayton et al., 2004; Nishida et al., 2005) endometrial cell proliferation. The action of LPS on ESCs was mediated through toll-like receptor 4 (Hirata et al., 2005), and that of IFN-γ on ESCs was through IFN-γ receptor 1 (Nishida et al., 2005). However, we did not achieve similar results, in which neither the ESCs with adenomyosis nor those of the control group proliferated differently after treatment with LPS and IFN-γ when compared with untreated ESCs. Our method of purifying ESCs from uterine endometrium is consistent with what has been used previously (Osteen et al., 1989) and yields cells of very good purity, >98% based on vimentin, cytokeratin and Factor VIII staining. However, the isolation procedure that relies on a combination of enzymatic digestion, unit gravity sedimentation, filtration and adherence is not likely to remove macrophages from the ESC cultures. As we attempted to stimulate ESCs with various agents including those that are expected to activate macrophages (e.g. LPS, IFN-γ, E2), but did not find any effect of LPS or IFN-γ on proliferation of ESCs, this would suggest that our ESC cultures are not contaminated with macrophages, which is in contrast to those seen by others who have suggested stimulation of proliferation by LPS (Iba et al., 2004) and suppression by IFN-γ (Clayton et al., 2004; Nishida et al., 2005). In addition, previous reports studied ESCs from women with endometriosis, whereas we investigated ESCs from women with adenomyosis. ESCs between these two disease entities might respond differently to the cytokine stimulation. Furthermore, cells that proliferated prominently after the stimulation with LPS are ESCs (Iba et al., 2004) which might be biologically different from the eutopic ESCs in this study.

Using immunocytochemistry, we demonstrated a higher Ki-67 labelling index in ESCs with adenomyosis than in the control group. Ki-67 protein is a nuclear and nucleolar protein that is strictly associated with cell proliferation (Verheijen et al., 1989). A detailed cell cycle analysis revealed that the Ki-67 antigen was present in the nuclei of cells in the G₁, S and G₂ phases of the cell division cycle as well as in mitosis. Quiescent or resting cells in the G₀ phase did not express the Ki-67 antigen (Gerdes et al., 1984).

The identification of an individual indicator that predicts the occurrence of adenomyosis is still several years away. Our results revealed a mean immunocytochemical Ki-67 index of 7.7% in adenomyosis, whereas the fraction of Ki-67-positive cells in the control group is generally <2%. Although it is unclear whether the Ki-67 protein expression remains constant before and after the development of adenomyosis, the much higher Ki-67 protein expression of ESCs in adenomyosis might be a potential indicator of adenomyosis. Further studies are required to establish which values of Ki-67 labelling index might be used to predict the occurrence of adenomyosis.

Figure 6. Ki-67 labelling index in women with adenomyosis (●, n = 23) and without (○, n = 21) adenomyosis. This index was calculated by counting the number of Ki-67 positive cells per 1000 total ESCs. Two consecutive, randomly selected 100× microscopic fields were selected for each specimen processed. The mean value of the Ki-67 labelling index was 7.7% in women with adenomyosis and 1.1% in women without adenomyosis. The P-value was <0.001 by independent-samples’ t test.

The bcl-2 expression on ESCs was not different between women with and without adenomyosis. This result implies that the decreased apoptosis of ESCs in adenomyosis is regulated by mediators other than bcl-2. It might also be due to the fact that we studied endometrium in the early- to mid-secretory phase, whereas Meresman et al. (2000) investigated endometrium in the proliferative phase.

Caspase-3 is a key protease that is activated during the early stage of apoptosis. In this study, we did not demonstrate different expression of caspase-3 in ESCs between women with and without adenomyosis. The reason may be that the membrane phospholipid phosphatidyserine externalization, which has a high affinity for annexin V, may not be directly mediated by caspase-3 (Johnson et al., 2000). It is compatible with a previous report that caspase-independent pathways of apoptosis are also involved in the programmed cell death (Bouscary et al., 2000).

Proliferative disorders (hyperplasia and cancer) of the human endometrium have been linked to hyperestrogenic states and to unopposed estrogen replacement therapy (Rose, 1996). In this study, there was a trend that the ESCs treated with E₂ proliferated more rapidly than the untreated ESCs did, and the proliferation was limited after the addition of MPA to E₂. This is compatible with previous reports that E₂ augments endometrial cell proliferation, but MPA arrests their growth (Harada et al., 2004; Blauer et al., 2005).

IL-6 has been reported to promote endometrial cell proliferation (Khan et al., 2005). IL-6 achieves its effects through the ligand-specific IL-6 receptor. IL-6 receptor has been found to
In this study, we used uterine samples from patients with cervical intraepithelial neoplasia and leiomyoma as controls. Although it is certainly true that they do not have adenomyosis, they would not be considered normal in the strictest sense of the word. Cervical intraepithelial neoplasia, in particular, is a dysplastic condition that precedes an invasive neoplastic process in which substantial field cancerization can be present in the surrounding tissues. The expression of bcl-2 in affected tissues would be predicted to be higher when compared with truly normal cells.

In conclusion, we report for the first time the differential expression of ESC apoptosis and proliferation between women with and without adenomyosis. The altered apoptosis and proliferation of the eutopic endometrium possibly reveal some aspects of the pathophysiology of adenomyosis. A high Ki-67 labelling index in immunocytochemistry might be a potential indicator in predicting the occurrence of adenomyosis.

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