Endometriotic stromal cells lose the ability to regulate cell-survival signaling in endometrial epithelial cells in vitro

Hui Zhang, Mingjiang Li, Xiaojing Zheng, Ying Sun, Zeqing Wen, and Xingbo Zhao

Department of Obstetrics and Gynecology, Provincial Hospital Affiliated to Shandong University, 324 Jingwu Road, Jinan, Shandong 250021, People’s Republic of China

ABSTRACT: In normal endometrium, stromal factors regulate the growth of epithelial cells. However, epithelial cells in endometriotic lesions display increased proliferation and decreased apoptosis. This work tested the hypothesis that in endometriosis stromal cells lose the ability to regulate survival signaling and cell growth in epithelial cells. Primary normal, endometriotic eutopic and ectopic epithelial cells were cultured in the presence of medium conditioned by normal, eutopic and ectopic endometriotic endometrial stromal cells. Endometriotic epithelial cells showed higher Survivin expression than normal epithelial cells. Conditioned medium (CM) from normal or eutopic endometriotic stromal cells significantly inhibited the Survivin expression and AKt phosphorylation in normal or eutopic endometriotic epithelial cells. However, CM from ectopic endometriotic stromal cells did not have an inhibitory effect on normal or ectopic endometriotic epithelial cells. Inhibition of AKt phosphorylation and Survivin expression in normal or eutopic endometriotic epithelial cells in the presence of stromal factors from normal or eutopic endometriotic stromal cells was enhanced by progesterone, whereas progesterone had little effect in the presence of stromal factors from ectopic endometriotic stromal cells. The inability of ectopic endometriotic stromal cells to regulated PI3K/AKt/Survivin signaling and mediate the progesterone response in endometriotic epithelial cells may facilitate epithelial cell proliferation in endometriosis and promote the survival of endometriotic lesions.

Key words: endometriosis / PI3K / AKt / Survivin / progesterone resistance / stromal–epithelium communication

Introduction

Endometriosis is an estrogen-dependent chronic disease that is characterized by the presence of functional endometrium-like glands and stromal tissues in sites outside the uterine cavity. It affects 6–10% of women of reproductive age and the main clinical features are chronic pelvic pain and infertility (Giudice and Kao, 2004). Sampson (1927) has proposed that refluxed menstrual endometrium passed back to peritoneal surfaces via Fallopian tubes is the origination of endometriosis. Retrograde menstruation into the peritoneal cavity is present in most women while endometriosis occurs only in 6–10%, indicating that aberrant biologic behavior of the refluxed endometrium, such as molecular defects or survival enhancements, may decide their fate in ectopic foci (Lucidi et al., 2005).

In endometriosis, ectopic endometrium exhibits multiple molecular abnormalities involving cell apoptosis and survival (Dmowski et al., 2001; Bèläird et al., 2004), which can promote the dissemination and implantation of endometriotic cells at ectopic sites. Several cell apoptotic and survival signaling pathways were aberrantly expressed in endometriosis, including Bax/Bcl-2 (Bèläird et al., 2004; Johnson et al., 2005), RAS/RAF/mitogen-activated protein kinases (MAPK) (Matsuzaki et al., 2005; Honda et al., 2008) and phosphoinositide 3-kinase (PI3K)/AKt (PKB) (Matsuzaki et al., 2005; Honda et al., 2008; Grund et al., 2008). Recent tissular studies show that Survivin, a member of mammalian inhibitors of apoptosis (IAP) family, is up-regulated in endometriotic lesions (Tarkowski et al., 2001; Ueda et al., 2002; Goteri et al., 2005; Fujino et al., 2006).

Survivin is described to be a bifunctional protein capable of regulating cell proliferation and apoptotic cell death (Ambrosini et al., 1997). Its down-regulation is involved in cell death induction by various stimuli, including Fas, caspases, anti-cancer drugs (Ambrosini et al., 1998; Tamm et al., 1998; Grossman et al., 1999). Survivin expression was originally reported to be present in embryonic, fetal development and cancer (Ambrosini et al., 1997; Altieri and Marchisio, 1999; Adida et al., 1998; Guo and Hay, 1999) but later studies identified the presence of Survivin in normal tissues, including skin, endothelial cells and endometrium (Chiodino et al., 1999; Konno et al., 2000; O’Connor et al., 2000). Survivin expression in normal endometrium shows...
cyclic alterations dependent on the menstrual cycle (Konno et al., 2000; Tarkowski et al., 2000; Lehner et al., 2002), suggesting that steroid hormones regulate Survivin expression in endometrium. In addition, Survivin overexpression is shown to be present in hormone-dependent endometrial disorders, such as endometrial hyperplasia, carcinomas and endometriosis (Tarkowski et al., 2001; Takai et al., 2002, 2004; Pallares et al., 2005).

Mesenchymal cells are essential in directing the growth, differentiation and function of epithelium (Donjacour and Cunha, 1991). In vitro studies show that in normal endometrium, stromal cells can regulate epithelial cells growth and differentiation (Arnold et al., 2001). Based on the studies showing the aberrant apoptosis and survival signaling in endometriosis (Béliard et al., 2004), we propose the hypothesis that endometriotic stromal cells may lose or partly lose the function to regulate the apoptotic and survival signaling in endometriotic epithelial cells that grow in ectopic foci.

Therefore, we aimed to investigate the role of stromal factors on epithelial survival signaling in normal and endometriotic endometrium. In the present studies, for the first time, we investigated the Survivin expression in normal and endometriotic endometrial epithelial cells through primary cell cultures as well as tissular studies. Then we examined the effects of normal and endometriotic stromal cells on the cell-surface hormone signaling in endometriotic epithelial cells.

**Materials and Methods**

**Materials**

Collagenase IA, trypsin, Matrigel, medroxyprogesterone and LY294002 were obtained from Sigma Corporation (St. Louis, MO, USA). Penicillin, DMEM/F12 (1:1) media without phenol red was obtained from HyClone Corporation (Beijing, China). Charcoal-stripped fetal bovine serum (FBS) from which the presence of hormones and some growth factors had already been excluded was obtained from GIBICO (Invitrogen Corporation, NY, USA). Rabbit anti-human Akt, p-Akt, Survivin and β-actin primary antibodies were obtained from Cell Signaling Technology, USA. Goat anti-rabbit horse radish peroxidase (HRP)-conjugated secondary antibodies were obtained from Jingmei, China. Diaminobenzidine (DAB) staining kits were purchased from Jingmei, China. Mammalian Cell Extraction Kit was purchased from BioVision, USA. ECL Plus Western Blotting Detection System was obtained from Amersham, UK. X-film was purchased from Kodak.

**Tissue collection and cells culture**

Normal biopsies were obtained from 14 women of reproductive age (proliferative phase: n = 7, mean age: 41.9 ± 2.4; secretory phase: n = 7, mean age: 41.4 ± 3.5) undergoing bilateral tubal ligation. Ectopic and ectopic endometriotic endometria were obtained from 11 patients with endometriosis (proliferative phase: n = 6, mean age: 40.2 ± 5.3; secretory phase: n = 5, mean age: 33.6 ± 6.7). Red lesions inside the ovary chocolate cysts were collected (Fig. 1). The phase of cycle was determined on the basis of the last menstruation, the ovarian findings during operation and histologic phase pattern of the endometrium (Noyes et al., 1950). Diagnosis was confirmed by histologic examination. None of the patients received any hormonal therapy during the 6 months before their operation. Informed consent was obtained from all participants prior to biopsy and the use of human tissues was approved by the institutional review board of Shandong University.

Methods for isolation and culture of endometrial cells were based on previously published data (Sugawara et al., 1997) with slight modifications. Briefly, specimens obtained during operation were placed immediately in a mixture of DMEM/F12 (1:1) supplemented with 100 U/ml of penicillin and 100 U/ml of streptomycin, and were transported to the laboratory. Tissues were then minced into small pieces and incubated in 0.25% collagenase type IA in a shaking water bath for 1 h at 37°C. The collagenase activity was stopped by adding two to three volumes of prewarmed medium containing 10% FBS. The cell suspension was firstly filtered through a 154-μm monofilament nylon mesh and then a 38.5-μm monofilament nylon mesh. To obtain stromal cells, the cell suspensions were collected and centrifuged at 110g for 10 min. The pellet was resuspended in medium and was incubated in cell culture dishes for 2 h at 37°C in 95% air and 5% CO₂. Then the medium was replaced with fresh medium and non-attached cells were discarded, whereas the attached stromal cells were cultured further. To obtain epithelial cells: the 35-μm monofilament mesh was washed thoroughly upside down with medium. The medium was collected and centrifuged at 110g for 10 min. The pellet was resuspended in medium and transferred into cell culture dishes. Culture media were changed every 2–3 days. Normal stromal cells (NSCs) and normal epithelial cells (NECs), endometriotic eutopic stromal cell (EuSCs) and epithelial cells (EuECs), endometriotic ectopic stromal cells (ESC) and epithelial cells (EECs) were isolated and cultured in DMEM/F12 (1:1) medium with 10% charcoal-stripped FBS and penicillin (100 U/ml) and streptomycin (100 U/ml) at 37°C in a humidified environment with 5% CO₂ in air. The cultured endometrial cells were characterized by immunohistochemistry with mouse anti-human vimentin and cytokeratin antibodies (Bruse et al., 2005). The purity for stromal cells was determined after the first and second passages and the purity was more than 98% (Fig. 1C). The purity for epithelial cells was determined before and after incubation with conditioned medium (CM) by stromal cells and the purity was more than 92% (Fig. 1D).

To confirm the specificity of stromal cell effects, a human embryonic lung fibroblast cell line (HELF) was also employed in the experiment. HELF cells were cultured in DMEM/F12 (1:1) medium supplemented with 10% charcoal-stripped FBS and penicillin (100 U/ml) and...
streptomycin (100U/ml) at 37°C in a humidified atmosphere of 5% CO₂. Culture media were changed every 2–3 days.

Preparation of conditioned culture medium by stromal cells

Stromal cells of passage 1–2 were used in the experiments. 2 x 10⁵ stromal cells were plated on top of a thin Matrigel layer (0.5 mm). Cells were cultured with phenol red-free DMEM/F12 (1:1, v/v) supplemented with 2% charcoal-stripped serum, which was called basic medium (BM), was added to each well. For hormonal treatment, specified dosage of medroxyprogesterone (P, 10⁻⁸ M) was used in cell cultures. Ethanol concentrations were not higher than 0.1% in culture medium. Cell cultures were maintained for 5 days at 37°C, 5% CO₂ in a humidified atmosphere. Wells containing BM without cells was used to generate a negative control for the effects of the 5-day incubation. At day 5 the supernatants were collected in centrifuge tubes and the cell debris were removed by centrifugation (1851 g, 10 min).

HELFCells of 2 x 10⁵ were also plated on top of a thin Matrigel layer (0.5 mm) and cultured with phenol red-free DMEM/F12 (1:1, v/v) in 24-well plates. To get CM, 2 ml phenol red-free DMEM/F12 (1:1, v/v) supplemented with 2% charcoal-stripped serum, which was called basic medium (BM), was added to each well. Cell cultures were maintained for 5 days at 37°C, 5% CO₂ in a humidified atmosphere. At day 5 the supernatants were collected in centrifuge tubes and the cell debris were removed by centrifugation (1851 g, 10 min).

Epithelial cells of 5 x 10⁴ were plated in 6-well culture plates with phenol red-free DMEM/F12 (1:1, v/v) supplemented with 10% charcoal-stripped FBS and 100 U/ml of penicillin, 100 U/ml of streptomycin. After cell adherence, culture medium was removed and co-culture medium was added to each dishes. Co-culture-medium contained CM and BM at a ratio of 2.5:1 plus 1% charcoal-stripped FBS. Epithelial cells were treated with CM from the stromal cells from the same patients. Cultures were maintained for 5 days, followed by MTT test and western blot. Specimens used for immunocytochemistry analysis were obtained by placing sterile coverslips in culture dishes before planting the cells.

Tissue samples used for immunohistochemistry were normal endometria, and endometriotic eutopic endometria were also used for cell isolation and culture. Fresh tissues were washed with PBS twice to clean blood and then fixed in 4% parafomaldehyde (pH 7.0) for 24 h and embedded in paraffin. Samples were cut into 4-µm sections and mounted onto glass slides. Deparaffinated, rehydrated sections were incubated with 3% H₂O₂ in for 10 min to block endogenous peroxidase activity. Antigen retrieval was performed by microwave heating three times for 8 min in citrate buffer at pH 7.6. The sections were rinsed in PBS, blocked with 10% normal goat serum for 30 min and then incubated with the primary rabbit anti-human Survivin antibody (diluted 1:500 in PBS; CST, USA) overnight in a wet chamber at 4°C. A HRP-conjugated goat anti-rabbit IgG was used as secondary antibody. HRP activity was detected using DAB tetrahydrochloride as substrate for 2 min in accordance with the manufacturer’s instructions. Sections were counterstained with hematoxylin before mounting. Sections incubated with PBS instead of primary antibody were used as negative control. Immunostaining with rabbit anti-human β-actin antibody of the same dilution was used as a negative control for nuclear staining.

The isolated endometrial epithelial cells cultured on coverslips (Weiyi, Beijing, China) were used for the following immunocytochemistry analysis. After treatment, epithelial cells on slips were fixed with 4% paraformaldehyde (pH 7.0) for half an hour following with Triton X-100 for 20 min at room temperature. Endogenous peroxidase was blocked by incubation in 0.3% H₂O₂ for 10 min at room temperature. The slips were incubated with confining liquid for 10 min and then with rabbit anti-human Survivin (diluted 1:500 in PBS; CST, USA), mouse anti-human vimentin and cytokeratin (diluted 1:500 in PBS; Zhongshan, China) as primary antibodies for 2 h at 37°C, followed by rinsing with PBS and incubation with an HRP-conjugated goat anti-rabbit or rabbit anti-mouse IgG as the second antibody. HRP activity was detected using DAB tetrahydrochloride as substrate for 2 min in accordance with the manufacturer’s instructions. Sections and coverslips incubated with PBS instead of primary antibodies were used as negative control. A human endometrium adenocarcinoma cell line, Ishikawa cells, cultured on coverslips, which were reported to be positive of Survivin expression (Chen et al., 2009), were used as a positive control.

The immunohistochemical score was composed of two elements: the percentage of positively staining glandular epithelial cells (i.e. quantity score (no staining: 0, 1–10% positively staining cells: 1, 11–50% positively staining cells: 2, 51–80% positively staining cells: 3, 81–100% positively staining cells: 4) and the recorded staining intensity (0: negative, 1: weakly staining, 2: moderately staining and 3: strongly staining). The immunoreactivity was shown as quantity score x staining intensity. Two sections per sample were assessed by two observers. All slides were evaluated blind for immunostaining without any knowledge of the clinical or pathologic data.

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Stromal cells of passage 1–2 were used in the experiments. 2 x 10⁵ stromal cells were plated on top of a thin Matrigel layer (0.5 mm). Cells were cultured with phenol red-free DMEM/F12 (1:1, v/v) in 24-well plates. To get CM, 2 ml phenol red-free DMEM/F12 (1:1, v/v) supplemented with 2% charcoal-stripped serum, which was called basic medium (BM), was added to each well. For hormonal treatment, specified dosage of medroxyprogesterone (P, 10⁻⁸ M) was used in cell cultures. Ethanol concentrations were not higher than 0.1% in culture medium. Cell cultures were maintained for 5 days at 37°C, 5% CO₂ in a humidified atmosphere. Wells containing BM without cells was used to generate a negative control for the effects of the 5-day incubation. At day 5 the supernatants were collected in centrifuge tubes and the cell debris were removed by centrifugation (1851 g, 10 min).

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Treatment with LY294002

LY294002 (PI3K inhibitor, Sigma Aldrich) was dissolved in dimethylsulfoxide (DMSO) at a stock concentration of 50 mmol/l and stored at 4°C. A final concentration of 0.5 μmol/l was used in cell cultures. 5 x 10⁵ of epithelial cells were plated in 6-well culture plates with phenol red-free DMEM/F12 (1:1, v/v) supplemented with 10% charcoal-stripped FBS and 100 U/ml of penicillin, 100 U/ml of streptomycin. After adhesion, cells were treated with LY294002 (0.5 μmol/l) in red-free DMEM/F12 (1:1, v/v) supplemented with 1% charcoal-stripped FBS. Mock treatment with an identical volume of DMSO was used as a control.

Cell proliferation and methyl thiazolyl tetrazolium assay

The proliferation of epithelial cells was assessed by methyl thiazolyl tetra-zolium (MTT) test (Chakraborty and Guha, 2007). Cells were seeded in 96-well culture plates at a concentration of 5 x 10⁴ cells/well. After different treatments, 20 μl of MTT working solution (5 mg/ml, Sigma) was added to each well and incubated for 4 h at 37°C. Then the medium was carefully discarded and the formazan crystals were dissolved in 150-μl DMSO. The absorbance of each well was measured with a microculture plate reader at 570 nm.

Immunohistochemistry on paraffin sections of tissue and immunocytochemistry on coverslips of cells

The proliferation of epithelial cells was assessed by methyl thiazolyl tetra-zolium (MTT) test (Chakraborty and Guha, 2007). Cells were seeded in 96-well culture plates at a concentration of 5 x 10⁴ cells/well. After different treatments, 20 μl of MTT working solution (5 mg/ml, Sigma) was added to each well and incubated for 4 h at 37°C. Then the medium was carefully discarded and the formazan crystals were dissolved in 150-μl DMSO. The absorbance of each well was measured with a microculture plate reader at 570 nm.

Total protein extraction and western blot analysis

Cells were harvested by trypsinization using 0.25% trypsin (Sigma Aldrich) and centrifugation (172g, 8 min). Total protein was extracted using the Mammalian Cell Extraction Kit (BioVision) according to the manufacturer’s instructions. Fifty micrograms of total protein extracted from cells was diluted in 5 x loading buffer, applied to a 10% polyacrylamide gel and subjected to electrophoresis, using PageRuler Prestained Protein Ladder (Fermentas) as size markers. The proteins were transferred to
Hybond-P polyvinylidene difluoride membranes (Amersham) in trans-
buffer containing 25-mM Tris and 185-mM glycine, pH 8.3, together
with 20% methanol. After transfer, the membranes were blocked
for 1 h in blocking buffer (TBS containing 0.1% Tween 20 (TBST), sup-
plemented with 5% non-fat dry milk) and then the membranes were incu-
bated overnight at 4°C with rabbit anti-human AKt, p-AKt, Survivin and
β-actin monoclonal antibodies in blocking buffer (diluted 1:10000). After
being washed three times with TBST, the membranes were incubated
with HRP-conjugated goat anti-rabbit secondary antibodies (diluted
1:10 000), and then the immunoblots were developed with the ECL
Plus western bloting agent (Amersham) and exposed to X-ray film
(Kodak). Western blot densitometric analysis was performed using an
Alphalmager 2200 gel documentation system with image analysis software.
The band densities for AKt, p-AKt and Survivin were normalized to the
densities of β-actin.

Statistical analysis
Statistical analyses were performed with ANOVA using SPSS 11.5 (SPSS
Inc., Chicago, IL). Values are expressed as mean ± SD. Differences
between two and multiple groups were determined by analysis of variance
(ANOVA). A P-value < 0.05 was considered statistically significant.

Results
Aberrant Survivin expression in endometriosis
Immunohistochemical analysis showed that the staining pattern of Sur-
vivin was different between endometrium of the proliferative and
secretory phases. Stronger Survivin immunostaining was observed in
normal and endometriotic eutopic endometria of the proliferative
phase compared with those of the secretory phase (P < 0.05; Figs 2, 3A). During the proliferative phase, the glands and stratum
were positive for Survivin immunostaining and the staining was
restricted to the nuclei (Fig. 2A–F). In addition, endometriotic
eutopic glands (Fig. 2D–F) exhibited higher total and nuclear Survivin
expression than normal glands (Figs 2A–C and 3; P < 0.01), in which
the Survivin staining was negative or weak and heterogeneous. During
the secretory phase, the Survivin immunostaining was mostly cyto-
plasmic in pattern in glands whereas negative in stroma (Fig. 2H–
M). Nuclear Survivin-positive glandular cells were mostly localized in
the functional layer (Fig. 2M). In this phase, no significant difference
of Survivin staining was noted between endometriotic eutopic glands
(Fig. 2H–J) and normal glands (Figs 2K–M and 3; P > 0.05). In addi-
tion, in normal and endometriotic endometria, the percent of Survivin-null glands was much higher during the secretory
phase than during the proliferative phase (P < 0.01).

Primarily cultured endometrial epithelial cells isolated from differ-
ent endometrium showed different Survivin expression in vitro. As
expected, in normal and EuECs, immunocytochemical analysis showed
that the staining pattern of Survivin was different between the proliferative and secretory phases. Stronger Survivin immuno-
 staining was noted in eutopic epithelial cells of the proliferative
phase compared with those of the secretory phase (Fig. 4A,B). How-
ever, no significant difference was observed in EECs throughout
the menstrual cycle (Fig. A,B). During the proliferative phase, the
Survivin staining was strong and localized in both the nuclei and

Paracrine factors from stromal cells regulates cell-survival signaling in epithelial cells
in normal and endometriotic eutopic endometrium
To examine the effect of normal stromal factors on survival signaling in
NECs in vitro, NECs were treated with CM from NSCs cultured on
Matrigel. Survivin expression and AKt phosphorylation in NECs
were determined using western blot analysis. CM from NSCs cultured
on Matrigel caused a reduction of Survivin expression to 37% of the
control in NECs (Fig. 6A, P < 0.01) and a reduction of AKt phos-
phorylation to 38% of the control (P < 0.001). To explore the
ability of NSCs on mediating progesterone response, NECs were cul-
tured with CM from NSCs treated with progesterone (10−8 M).
When NECs were treated with P in the absence of stromal CM, no
significant hormonal response was noted in cell-survival signaling in
NECs. When NECs were cultured with CM from NSCs treated with
P plus the same dose of fresh P, a 55% reduction in Survivin
expression and a 61% reduction in AKt phosphorylation were noted in
NECs compared with controls (P < 0.001).

To explore the stromal–epithelial communication in endometriotic
eutopic endometrium, we examined the effect of endometriotic eutopic
stromal factors on survival signaling in EuECs in vitro. EuECs were
treated with CM from EuSCs cultured on Matrigel. Survivin expression
and AKt phosphorylation in EuECs were also determined using
western blot analysis. CM from EuSCs cultured on Matrigel caused a
reduction of Survivin expression by 45% in EuECs (Fig. 7A, P < 0.05)
and a reduction of AKt phosphorylation by 37% (P < 0.001). As expected, when EuECs were treated with P in the absence of
stromal CM, no significant hormonal response was found in cell-
survival signaling in EuECs either (Fig. 7A). When EuECs were cultured
with CM from EuSCs treated with P plus same dose of fresh P, 57%
reductions in Survivin expression and AKt phosphorylation were
noted in EuECs (P < 0.001).
Ectopic stromal cells lose the ability to regulate cell-survival signaling in epithelial cells

To explore the stromal–epithelial communication in endometriotic ectopic endometrium, we examined the effect of ectopic stromal factors on survival signaling in EECs in vitro. EECs were treated with CM from ESCs cultured on Matrigel. Survivin expression and AKt phosphorylation in EECs were determined using western blot analysis. CM from ESCs cultured on Matrigel caused no significant reduction of Survivin expression or AKt phosphorylation in EECs (Fig. 8A, B). However, EECs showed a 41% reduction in Survivin expression and a 37% reduction in AKt phosphorylation compared with controls when treated with CM from NSCs grown on Matrigel (P < 0.001), suggesting that EECs do not lose the responsiveness to normal stromal factors.

Figure 2 Different Survivin expressions in normal endometrium and eutopic endometrium from patients with endometriosis. A–F: Survivin expression in normal (A–C) and endometriotic eutopic (D–F) endometrium during the proliferative phase by immunohistochemistry. In normal endometrial epithelium, Survivin expression was heterogeneous and weaker (A–C). Survivin-null glands (A) coexisted with Survivin-positive glands (B,C). Immunostaining was localized in both the cytoplasm and the nuclei. In endometriotic eutopic endometrial epithelium, Survivin expression was also heterogeneous but stronger than normal endometrium (D–F). No Survivin-null glands were noted. Immunostaining was localized in both the cytoplasm and the nuclei. H–M: Survivin expression in normal (H–J) and endometriotic eutopic (K–M) endometrium during secretory phase by immunohistochemistry. In both the normal and the endometriotic eutopic endometrial epithelium, Survivin expression was heterogeneous and weaker than proliferative endometrium. Survivin-null glands (j) coexisted with Survivin-positive glands (H,I). Immunostaining was mostly localized in the cytoplasm. Nuclear-Survivin-positive gland cells were mostly found in the surface layer (M). (N) Negative control using PBS instead of primary Survivin antibody. (O,P) Nuclear-negative controls using primary actin antibody at the same dilution as Survivin antibody.
When EECs were treated with P in the absence of stromal CM, no significant hormonal response was found in cell-survival signaling in EECs (Fig. 8A). To determine whether ESCs had the ability to mediate progesterone response, EECs were cultured with CM by ESCs treated with progesterone (10^{-6}M). When EECs were cultured with CM from ESCs treated with progesterone plus the same dose of fresh progesterone, only a 17% reduction in Survivin expression and a 13% reduction in AKt phosphorylation were noted in EECs (P < 0.05) while a 54% reduction in Survivin expression and a 61% reduction in AKt phosphorylation were noted in EECs when EEC were cultured in the presence of CM from NSCs treated with progesterone (P < 0.001). To demonstrate whether the loss-of-function on regulating survival signaling of ectopic stromal factors was specific to EECs, NECs were also treated with CM from ESCs cultured on Matrigel. Survivin expression and AKt phosphorylation in EECs were determined using western blot analysis. Interestingly, CM from ESCs cultured on Matrigel caused no significant reduction of Survivin expression or AKt phosphorylation in NECs, neither in the presence of progesterone (Fig. 8A, B). These data suggested that the aberrant stromal—epithelial communication in ectopic endometrium is due to the loss-of-function of ESCs.

To confirm the specificity of stromal cell effects, CM was also collected from a parallel culture of HELF cells. NECs (n = 4) were cultured in the presence of CM conditioned by HELF cells. As shown in Fig. 9, instead of down-regulating Survivin expression and AKt phosphorylation, CM from the HELF cells exhibited a 20% increase effect compared with unconditioned medium controls.

**Survivin expression regulation is mediated by PI3K/AKt pathway in normal and endometriotic endometrium**

To investigate whether the Survivin expression regulation in epithelial cells was mediated by PI3K/AKt pathway, a specific PI3K inhibitor, LY294002 was employed in cell cultures. Western blot analysis showed that PI3K inhibition significantly inhibited AKt phosphorylation and Survivin expression in all epithelial cells, including NECs, EuECs and NECs (Figs 6–8A,B; P < 0.001). The correlation coefficients between AKt phosphorylation and Survivin expression under different treatments in the three epithelial cells were: NECs r = 0.880, P < 0.001; EuECs: r = 0.916, P < 0.001; EECs: r = 0.899, P < 0.001, suggesting the positive relationship between the PI3K/AKt pathway activity and the Survivin expression.

**Ectopic stromal factors lose the ability to regulate ectopic epithelial cells growth in vitro**

To evaluate the role of different stromal factors in epithelial cells proliferation, MTT test was performed. As shown in Fig. 10, CM by ESCs showed no significant growth regulation on NECs (P > 0.05) and EECs (P > 0.05), neither of progesterone alone nor progesterone plus CM by ESCs (P > 0.05; Fig. 10C). In contrast, both the CM from NSCs and the progesterone plus CM from NSCs significantly decreased the NECs and EECs growth (P < 0.001; Fig. 10A). As expected, CM from EuSCs also exhibited growth regulation on EuECs (P < 0.001; Fig. 10B). PI3K inhibition by LY294002 inhibited growth by more than 90% in all epithelial cells, suggesting that the PI3K/AKt/survivin signaling pathway plays an essential role in growth of normal and endometriotic endometrial epithelial cells.

**Discussion**

Impaired spontaneous apoptosis and decreased apoptosis susceptibility in endometriotic tissue (Gebel et al., 1998; Béliard et al., 2004) as well as eutopic endometrium (Garcia-Velasco and Arici, 2003) from women with endometriosis, favors the survival and development of this disease. Multiple molecular abnormalities in endometriosis including the activation of cell-survival signaling have been reported in previous studies (Hornung et al., 1997; Kao et al., 2003; Wu et al., 2005). Survivin, a member of inhibitor of apoptosis (IAP) proteins, is one of the regulators of cell death (Ambrosini et al., 1997; Kao et al., 2003; Wu et al., 2005). Survivin was aberrantly expressed in endometriotic endometrium and that Survivin gene expression might facilitate the apoptosis reduction and invasive phenotype in endometriosis. In the current study, we analyzed the Survivin expression and regulation in normal and endometriotic eutopic,
ectopic endometrium by both tissular and in vitro cell-culture studies. Previous researches on the Survivin expression have been performed mainly using tissular studies (Ueda et al., 2002; Fujino et al., 2006), to our knowledge, this is the first analysis of Survivin expression in normal and endometriotic endometrium using in vitro primary cell cultures. We also proved here that the regulation of Survivin expressions in normal and endometriotic endometrium was mediated by PI3K/Akt signaling. In addition, we presented data supporting our hypothesis that the aberrant Survivin expression in endometriotic epithelial cells was due to the loss-of-function of endometriotic stromal cells on regulating survival signaling and progesterone responsibility in epithelial cells.

Previous reports show controversial conclusions on the correlation between Survivin expression and menstrual cycles (Konno et al., 2000; Tarkowski et al., 2000; Lehner et al., 2002; Takai et al., 2002; Ueda et al., 2002). Data from Ueda et al. showed that no cyclic variation for Survivin expression was present in normal endometrium (Fujino et al., 2006). However, our studies suggested that the Survivin

**Figure 4** Survivin expression in primarily cultured normal (A: a,b) and endometriotic ectopic (A: c) endometrial epithelial cells by immunocytochemistry. Survivin expression was negative or weak in NECs during secretory phase (a) and became positive or stronger during proliferative phase (b). No significant difference was noted between ectopic epithelial cells of proliferative phase and secretory phase (c). (d) Ishikawa cells were used as a positive control of Survivin immunostaining. (B,C) Immunostaining score of immunocytochemical analysis.
expression in normal endometrium alters during menstrual cycles. Immunohistochemical analysis on tissues showed that endometrium of the proliferative phase had a much higher Survivin expression and the Survivin staining was mainly localized in the nuclei, whereas the Survivin expression was lower or negative in the endometrium of the secretory phase with the Survivin staining mainly in the cytoplasm. These data suggest that the Survivin expression and subcellular location are responsible to hormonal alteration and the subcellular location of Survivin may play an important role in hormonal growth regulation in endometrium, which needs further investigations.

Cyclic variation of Survivin expression was also noted in endometriotic eutopic endometrium. However, EuECs showed higher Survivin expression compared with normal endometrial epithelium during the proliferative phase by tissue immunohistochemical studies and cellular western blot analysis. All these data suggest that the Survivin expression and subcellular location are responsible to hormonal alteration and the subcellular location of Survivin may play an important role in hormonal growth regulation in endometrium, which needs further investigations.

Expression in normal endometrium was menstrual cycle-independent in ectopic endometrial epithelial cells. Higher Survivin expression was noted in ectopic epithelial cells from patients with endometriosis throughout the menstrual cycle. The cyclic expression of Survivin in normal endometrial epithelial cells suggests that the Survivin gene expression may

Figure 5 Survivin expression in primarily cultured NECs, EuECs and EECs by western blot analysis. (A) Representative results were shown. (B) Densitometric results were expressed as Survivin protein expression in epithelial cells after normalization to actin (means ± SD). Survivin expression in NECs and EuECs exhibited cyclic variation (P < 0.001) during proliferative phase and secretory phase, which was lost in EECs (P > 0.05).

Figure 6 Survivin expression in primarily cultured NECs under different treatments analyzed by western blot. (A) Representative results were shown. O = control; P = progesterone; CM-ESCs = conditioned medium from eutopic stromal cells; CM-ESCs (P) = conditioned medium from eutopic stromal cells treated with progesterone; CM-NSCs = conditioned medium from normal stromal cells; CM-NSCs (P) = conditioned medium from normal stromal cells treated with progesterone; LY294002 = specific PI3K inhibitor. (B) Densitometric results were expressed as percentage of control (O) (means ± SD). Actin was used as a loading control.
be regulated by steroid hormones and the increase of progesterone in the secretory phase may lead to the down-regulation of Survivin expression. The loss-of-cyclic variation of Survivin expression in ectopic endometrium implies the presence of progesterone resistance in EECs.

Mesenchymal cells are essential in directing the growth and functioning of overlying epithelium (Donjacour and Cunha, 1991), and appropriate stromal factors play a crucial role in regulating the proliferation and differentiation of epithelium in normal endometrium (Arnold et al., 2001). In the current study, we found that normal stromal factors regulated cell-survival and apoptosis signaling in endometrial epithelial cells. Here we used Matrigel as extracellular matrix in endometrial stromal cells cultures to mimic in vivo stromal microenvironment. Medium conditioned by NSCs cultured on Matrigel exerted a significant inhibition on AKt phosphorylation and Survivin expression in NECs. Unexpectedly, progesterone could only inhibit AKt phosphorylation and Survivin expression in epithelial cells in the presence of normal stromal factors, indicating that endometrial epithelial cells cultured in vitro may lose the original hormonal responsiveness, or epithelial cells can only show hormonal response in the presence of stromal factors, or it is stromal cells that mediate appropriate hormonal response in epithelial cells, which need further investigations. Taken together, these data suggest that in normal endometrium, stromal cells may produce paracrine factors that are involved in regulating survival and apoptosis signaling in epithelial cells. In normal hormonal cycles, endometrial epithelial cells exhibit increased proliferation in the estrogen-dominant proliferative phases and decreased proliferation in the progesterone-secreting phases. Progesterone may suppress cell proliferation via inhibiting AKt phosphorylation and Survivin expression in epithelial cells and the presence of normal stromal factors are essential in this effect of progesterone.

We found that higher Survivin expression existed in endometriotic eutopic endometrium and primarily cultured EuECs. These data lead to the hypothesis that impaired stromal–epithelial cross-talk may be present in eutopic endometrium. However, CM by endometriotic eutopic stromal cells decreased Survivin expression and AKt phosphorylation in EuECs in vitro, indicating that eutopic stromal cells still have the ability to regulate survival and apoptosis signaling in eutopic epithelial cells. In addition, paracrine factors from eutopic stromal cells mediated the inhibition effect by progesterone on survival signaling in epithelial cells. These data suggest that the overactivity of cell-survival signaling present in eutopic epithelial cells is not due to the aberrant stromal–epithelial communication. Considering the survival overactivity is only observed during the proliferative phase, we estimate that steroid hormones may play a role in the Survivin overexpression in eutopic endometrium, which needs further investigations. However, the presence of stromal factors contributes to
the maintenance of the cyclic variation of cell-survival and apoptosis signaling in eutopic epithelial cells during menstrual cycle.

Higher and menstrual cycle-independent Survivin expression was observed in ectopic epithelial cells. As expected, paracrine factors from endometriotic ectopic stromal cells cultured on Matrigel exerted no inhibition on Survivin expression and Akt phosphorylation in ectopic or NECs, whereas paracrine factors from NSCs significantly inhibited the Survivin expression and Akt phosphorylation in both epithelial cells, suggesting that ectopic stromal cells lose the ability, which is present in NSCs, to regulate survival or apoptosis signaling in epithelial cells. Progesterone showed little inhibition on Survivin expression and Akt phosphorylation in ectopic epithelial cells even in the presence of CM from ectopic stromal cells, which may explain the absence of cyclic variation of Survivin expression and Akt phosphorylation in ectopic epithelial cells during menstruation.

We speculate that the loss-of-function of ectopic stromal cells on regulating survival signaling and mediating progesterone function may facilitate the maintaining and development of ectopic lesions.

PI3K/Akt signaling, a cell-survival signaling, is aberrantly activated in endometriosis (Matsuzaki et al., 2005; Honda et al., 2008; Grund et al., 2008). Here we found that PI3K inhibition decreased the Survivin expression in normal, eutopic and ectopic endometrial epithelial cells and Akt phosphorylation was positively correlated with Survivin expression in these cells under different treatments, indicating that Survivin expression in normal, eutopic or ectopic endometrial epithelial cells can be regulated via PI3K/Akt pathway.

Another important point needs to be demonstrated was that the growth regulation by stromal factors was specific to endometrial stromal cells. Interestingly but as expected, no survival signaling regulation or growth inhibition was noted in ectopic epithelial cells when treated with CM by human embryonic lung fibroblast cell line HELF cultured on Matrigel. Using HELF cells as a control also confirmed that the epithelial cells survival inhibition by CM was not due to the lack of nutrients.

In conclusion, for the first time, the possible mechanism of aberrant Survivin expression in endometriotic eutopic and ectopic epithelial cells were investigated via in vitro cell cultures. Higher Survivin expression was present in eutopic and ectopic epithelial cells compared with NECs but only ectopic epithelial cells lost the cyclic variation of Survivin expression throughout menstruation cycles. Ectopic stromal cells lost their regulating functions on survival signaling, PI3K/Akt/Survivin signaling, in epithelial cells and also lost the ability to mediate the inhibition effect of progesterone on survival signaling in epithelial cells. Altogether, these findings suggest that the loss-of-function or impaired function of ectopic stromal cells may favor part of the impaired molecular changes, aberrant biologic behaviors of ectopic epithelial cells, which needs more investigation in future studies.

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References


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