

The role of survivin in the resistance of endometriotic stromal cells to drug-induced apoptosis

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BACKGROUND: Decreased susceptibility of endometrial tissue to apoptosis may contribute to the pathogenesis of endometriosis. We investigate the role of survivin in the pathophysiology of endometriosis through the ability of ectopic and eutopic endometrial stromal cells (ESCs) to resist apoptosis.

METHODS: Ectopic ESCs were obtained from ovarian chocolate cysts in patients undergoing laparoscopic surgery ($n = 22$). Eutopic ESCs were isolated from endometrial tissue of cyclic premenopausal women undergoing hysterectomy for fibroids ($n = 22$). Purified stromal cells were studied *in vitro*. The number of surviving cells and activation of caspases were assessed by WST-8 assay and immunoblotting. Expression of inhibitor of apoptosis proteins (IAP) family members: cIAP1 (*birc2*), cIAP2 (*birc3*), XIAP (*birc4*), survivin (*birc5*) were examined using cDNA array and real-time RT-PCR. Effects of gene silencing by small inhibitor RNAs (siRNA) were examined by WST-8-assay, Annexin-V staining and immunoblotting.

RESULTS: After staurosporine (SS) treatment, 55% of eutopic ESCs survived versus 70% of ectopic ESCs. Procaspase-3 or -7 was more intensely activated by SS treatment in eutopic than in ectopic ESCs ($P < 0.01$). mRNAs for IAP-family genes, such as cIAP-1, XIAP and survivin, were highly expressed in ectopic ESCs before SS treatment. The fold induction of survivin expression after SS treatment was higher in ectopic than eutopic ESCs (2.8 ± 0.27 versus 0.69 ± 0.07 , respectively). Survivin gene silencing in SS-treated ectopic ESCs led to an increase of apoptotic cells ($P < 0.05$, versus control siRNA).

CONCLUSIONS: We demonstrated that survivin plays a critical role in susceptibility of ESCs to apoptosis. Our results indicate that a survivin inhibitor may be effective as a novel treatment for endometriosis.

Key words: inhibitor of apoptosis proteins family / apoptosis / survivin / endometriosis

Introduction

Endometriosis, which occurs in approximately 10% of women of reproductive age, is defined as the presence of endometrial tissue outside the uterus (Harada *et al.*, 2001). Although endometriosis is a benign disease, it causes infertility and pelvic pain, compromising quality of life. Theories about the origin of endometriosis include metaplasia, Mullerian remnants and implantation and growth of endometrial tissues following retrograde menstrual reflux. Endometriotic cells may have a high potential for implantation and growth on peritoneal surfaces and may develop into endometriosis (Leyendecker *et al.*, 1998). Despite decades of clinical experience and research, endometriosis remains an enigma and its pathogenesis controversial.

Apoptosis, or programmed cell death, which plays a critical role in maintaining tissue homeostasis, is a normal function to eliminate excess or dysfunctional cells. Maintaining differentiated tissue by striking a balance between proliferation and cell death is essential for homeostasis. Evidence suggests that apoptosis may be directly involved in regulating the menstrual cycle by eliminating senescent cells from the functional layer of uterine endometrium (Kokawa *et al.*, 1996; Shikone *et al.*, 1996). In normal endometrium, apoptotic cells were identified in the glandular epithelium of late secretory and menstruating endometrium, whereas very little apoptosis was detected during the proliferative phase or at the beginning of the secretory phase (Tao *et al.*, 1997; Vaskivuo *et al.*, 2000). Eutopic endometrial cells and the ectopic endometrium seem to be fundamentally different in women with or without endometriosis and these

differences could contribute to the survival of endometrial tissues in the peritoneal cavity and the development of endometriosis. Impaired sensitivity of endometrial tissue to spontaneous apoptosis causes abnormal implantation and growth of endometrium at ectopic sites (Dmowski *et al.*, 2001), suggesting that decreased susceptibility of endometrial tissue to apoptosis may contribute to the pathogenesis of endometriosis. To gain further insight into the pathogenesis of endometriosis, we focused on the difference between eutopic and ectopic endometrial stromal cells (ESCs) in terms of the mechanism for regulating apoptosis.

We previously reported that ectopic ESCs have the distinct biological characteristic of resistance to drug-induced apoptosis compared with eutopic ESCs from women without endometriosis (Izawa *et al.*, 2006). In the present study, we used a staurosporine (SS)-induced apoptosis system to elucidate the nature and mechanism of the abnormal survival of endometriotic cells in ectopic sites. Our analysis focused on gene expression profiling, particularly in relation to resistance to apoptosis, in order to identify key factors that could be developed as strategies for prevention and treatment of endometriosis.

The inability of endometriotic cells to transmit a 'death' signal and their ability to avoid cell death is associated with increased expression of anti-apoptotic factors and decreased expression of pre-apoptotic factors. An inhibitor of apoptosis proteins (IAPs) interacts with multiple cellular partners and inhibits apoptosis induced by a variety of stimuli (Salvesen and Duckett, 2002). Human IAP family members include neuronal apoptosis inhibitory protein (*birc1*), cellular IAP1 (*cIAP1*, *birc2*), *cIAP2* (*birc3*), X chromosome-linked IAP (*XIAP*, *birc4*), Survivin (*birc5*), Apollon (*birc6*), melanoma IAP (*birc7*) and IAP-like protein 2 (*birc8*). Among the regulators of cell death, IAPs have recently emerged as modulators in an evolutionarily conserved step in apoptosis, which may potentially involve the direct inhibition of the terminal effectors, caspase-3 and -7, in the signaling pathway of apoptosis. Overexpression of IAPs confers protection against a number of proapoptotic stimuli in malignant diseases (Wright and Duckett, 2005; Schimmer, 2004).

On the basis of previous data, we focused on enhanced survivin expression in ESCs, being representative of the IAP family. Survivin expression has a prominent cancer bias because it is undetectable in most adult tissue but is expressed at high levels in a majority of human tumors. The mechanisms for survival and for regulating the inhibition of apoptosis are still not understood. In the present study, we investigated the role of survivin in the pathophysiology of endometriosis using a drug-induced apoptosis system in human ectopic and eutopic ESCs in culture. Our results indicate that a survivin inhibitor may be effective as a novel treatment for endometriosis.

Materials and Methods

Isolation of endometriotic and ESCs

With their informed consent, we recruited 22 women with endometriomas who had regular ovulatory cycles, had not received hormone therapy for at least 6 months before surgery, and underwent laparoscopy for ovarian endometriomas at Tottori University Hospital. The Institutional Review Boards of Tottori University School of Medicine approved this project. The chocolate cyst linings of the ovaries of patients with endometriosis were the source of endometriotic tissues. Eutopic endometrial tissues ($n = 22$) were obtained from the uteri of cyclic premenopausal

women who underwent hysterectomy for fibroids. All endometrial samples were grouped either in the proliferative ($n = 11$) or secretory phase ($n = 11$). Because we previously confirmed that the apoptosis rates in the proliferative and secretory phase endometrium were similar (Izawa *et al.*, 2006), the samples derived from endometriomas were not classified according to menstrual phase for analysis.

Stromal cells were isolated from endometriotic and endometrial tissues according to the method of Osteen *et al.* (2003), described in detail previously. The tissues were minced into small pieces in Dulbecco's modified Eagle's medium/Ham's F-12 medium (DMEM/F-12) and digested with 0.5% collagenase in DMEM/F-12 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 cells clumps by differential sedimentation at unit gravity. The medium containing stromal cells was filtered through a 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% CO₂ in air. We used the stromal cells in a monolayer culture after the first passage. To confirm the purification of stromal cells, immunohistochemical staining of the isolated ectopic and eutopic ESCs was performed using cytokeratin (DAKO, Kyoto, Japan) as a marker of epithelial cells, vimentin (DAKO) as a marker of stromal cells, CD14 (Nichirei, Tokyo, Japan) as a marker of activated macrophages, and factor VIII (DAKO) as a marker of endothelial cells. The results showed that the purity of stromal cells were more than 98%.

Induction and assessment of apoptosis

Ectopic and eutopic ESCs were seeded in DMEM/F-12 (1 : 1 vol/vol) with 10% fetal bovine serum (FBS), and maintained in each well of a 96-well plate (5×10^4 cells per well) for 24 h, then treated with SS (0.5 μM), which has the biological ability to induce apoptosis (Izawa *et al.*, 2006). After SS treatment for 8 h, apoptotic cells were measured from the number of viable cells, as 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 an early event of apoptosis, we examined exposure of phosphatidylserine on the cell surface. Briefly, ectopic ESCs were plated on a cover slip in a 35-mm dish and incubated for 48 h post-transfection with small interfering RNAs (siRNAs). Ectopic ESCs were then treated with SS for 3 h to induce apoptosis as described above, and stained with Annexin V-tagged enhanced green fluorescent protein, EGFP (MBL, Nagoya, Japan). The relative number of Annexin V-EGFP positive cells was expressed as the percentage of cells stained with propidium iodide (Sigma, St Louis, MO, USA) under UV microscopy. On the basis of our previous data (Izawa *et al.*, 2006), we chose exposure to 0.5 μM SS for 3 or 8 h to detect the early and active phase of apoptosis for annexin V staining or WST-8 assay.

cDNA array analysis

The human apoptosis gene oligo GE array™ kit, containing 96 marker genes involved in the regulation and mediation of apoptosis (e.g. IAP-, Bcl-2-, tumor necrosis factor (TNF) ligand-, TNF receptor-, Caspase-, TRAF-, Death domain-family, p53 and ATM pathway), was obtained from SABioscience Corp. (Frederick, MD, USA). After SS treatment for 2 h, total cellular RNA was extracted from proliferative phase eutopic ($n = 3$) and ectopic ESCs ($n = 3$) using RNeasy Minikit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNAs (3 μg) from respective samples were used as a template to generate ³²P labeled cDNA probes using the GE array ampolabelling kit. The cDNA probes corresponding to the mRNA population were then denatured

and hybridization carried out in GEHyb solution to nylon membranes spotted with gene-specific fragments. Membranes were then washed in $2\times$ standard saline citrate (SSC), 1% sodium dodecyl sulfate (SDS) twice for 15 min each at 60°C , followed by $0.1\times$ SSC, 0.5% SDS twice for 15 min each at 60°C . To detect radioactivity, the phosphor-imager (FLA-8000, Fuji-Film, Tokyo, Japan) was used to visualize the expression levels of each transcript, and results were quantified with GEArray Analyzer software. The relative expression level of each gene was determined by comparing the signal intensity of each gene in the array after correction for background and normalizing the signal intensities against signals derived from glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Genes were considered to be differentially expressed in the untreated and SS-treated samples if the change was greater than 2-fold.

Transfection of siRNA

siRNAs (Silencer[®] predesigned siRNAs for human XIAP-, survivin- and control-siRNA) were purchased from Ambion, Inc. (Austin, TX, USA). ESCs were plated in media containing 10% FBS to give 30–50% confluency, and transfection of the siRNA oligonucleotides was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) to result in a final RNA concentration of 50 nM prior to SS exposure.

Real-time RT-PCR

Total RNA was extracted from ectopic or eutopic ESCs using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription of RNA (1 μg) was performed using a Gene Amp RNA PCR Core Kit (Perkin Elmer, Branchburg, NJ, USA). The mRNA levels were quantified in triplicate using the Applied Biosystems 7900 HT real-time PCR system. The specific ABI Taqman probes for human *BIRC2* (cIAP1), *BIRC3* (cIAP2), *BIRC4* (XIAP), *BIRC5* (survivin) and Taqman Human GAPDH control Reagents (Applied Biosystems, Tokyo, Japan) were used. The absolute values for gene expression were normalized to that for GAPDH, and the relative values compared with the respective control (i.e. ectopic or eutopic ESCs without SS exposure) were calculated. All samples were tested in triplicate, and each run included no-template and no-reverse transcriptase controls.

Immunoblot analysis

Ectopic and eutopic ESCs were cultured in 100-mm dishes until subconfluent. After 0.5 μM SS treatment for 8 h, whole cell lysates were prepared with the lysis buffer (50 mM Tris-HCl, 125 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA, 50 mM NaF, 0.1% phenylmethylsulphonyl fluoride and protease inhibitors). The suspension was centrifuged at 15 000g for 30 min at 4°C , and supernatant was collected. The total protein concentration of supernatant lysate was quantified using Bradford assay (Bio-rad protein assay, Bio-Rad Laboratories, Hercules, CA, USA). Equal quantities of cell lysates (50 μg) were applied on the 4–20% gradient polyacrylamide gel for electrophoresis and transferred to polyvinylidene difluoride membrane. Transferred proteins were reacted with antibodies of caspase-3 and -7 proteins (Cell Signaling Technology, Beverly, MA, USA). These antibodies recognize both the full length and cleaved fragments of their respective proteins. The bound antibodies were visualized with horseradish peroxidase-conjugated antibodies using a chemiluminescence kit.

Statistical analysis

All experiments were repeated a minimum of three times. Results are expressed as means \pm SE. For cDNA array data, values were quantified with GEArray Analyzer software (SABioscience). All data were assessed for statistically significant differences via a one-way analysis of variance followed by the Fisher's protected least-significant differences *post hoc* test.

All statistical analyses were carried out using Statview 5.0 Software (SAS Institute, Inc., Cary, NC, USA).

Results

Resistance to SS-induced apoptosis in ectopic ESCs

SS treatment reduced the number of viable cells in eutopic ESCs by approximately 50%, whereas 70% of ectopic ESCs survived (Fig. 1A). Interestingly, SS treatment of eutopic ESCs in the proliferative phase induced the cleavage of pro-caspase-3 and -7. In contrast, those cleaved bands were faint in ectopic ESCs (Fig. 1B and C). These findings support the notion that ectopic ESCs are resistant to apoptosis.

Profiling of apoptosis-related gene expression in ectopic ESCs

We employed a targeted cDNA array procedure to investigate how SS exposure impacts gene expression involved in regulating apoptosis in ectopic ESCs. After exposure to SS, which can induce apoptosis by inhibiting various kinase proteins, genes susceptible to SS-induced apoptosis were examined. The change in a given transcript from one experiment was estimated by comparing with paired specimens: SS-treatment versus no treatment (Fig. 2A and Tables I and II).

In untreated ectopic ESCs, the signals of *bad*, *bax*, *bcl-x*, *birc2* (cIAP1), *birc4* (XIAP), *birc5* (survivin), *mch3* (caspase 7), *nol3* (Nop30), *tnfrsf1a* (TNFR-1), *tnfrsf5* (CD40) and *tnfrsf14* (HVEM-L), were innately intense in the panel of the Apoptosis cDNA array (Fig. 2A and Table I). After SS treatment, *bad*, *bax*, *birc5*, *tnfrsf10b* (DR5), *tnfrsf1a* and *tnfrsf5* expression in ectopic ESCs showed more than a 2-fold increase versus untreated cells (Fig. 2A and Table II), whereas these spots in eutopic ESCs were faint regardless of SS treatment (data not shown). Interestingly, the degree of survivin expression before and after SS treatment was remarkably different between ectopic and eutopic ESCs (2.8 ± 0.27 versus 0.69 ± 0.07) (Table II).

Enhanced survivin expression with SS exposure in ectopic ESCs

To elucidate the role of survivin in apoptosis resistance, we analyzed the SS-inducible changes of mRNA levels using real-time RT-PCR. Survivin mRNA was detectable in all 22 samples of ectopic ESCs and 19/22 samples of eutopic ESCs. In the survivin-positive samples, the change of survivin mRNA expression levels in ectopic ESCs after SS treatment was approximately 2.5-fold higher than in proliferative and secretory eutopic ESCs. In contrast, the SS-induced changes of cIAP-1, cIAP-2, and XIAP expression levels in ectopic ESCs were not significantly different from those in eutopic ESCs (Fig. 2B).

SS-induced apoptosis by survivin silencing in ectopic ESCs

Silencing of the survivin gene led to a significant decrease in the number of viable ectopic ESCs following SS treatment (approximately 75% versus control-siRNA), whereas XIAP gene silencing in ectopic ESCs had no such effect (Fig. 3A). Three hours after SS treatment,

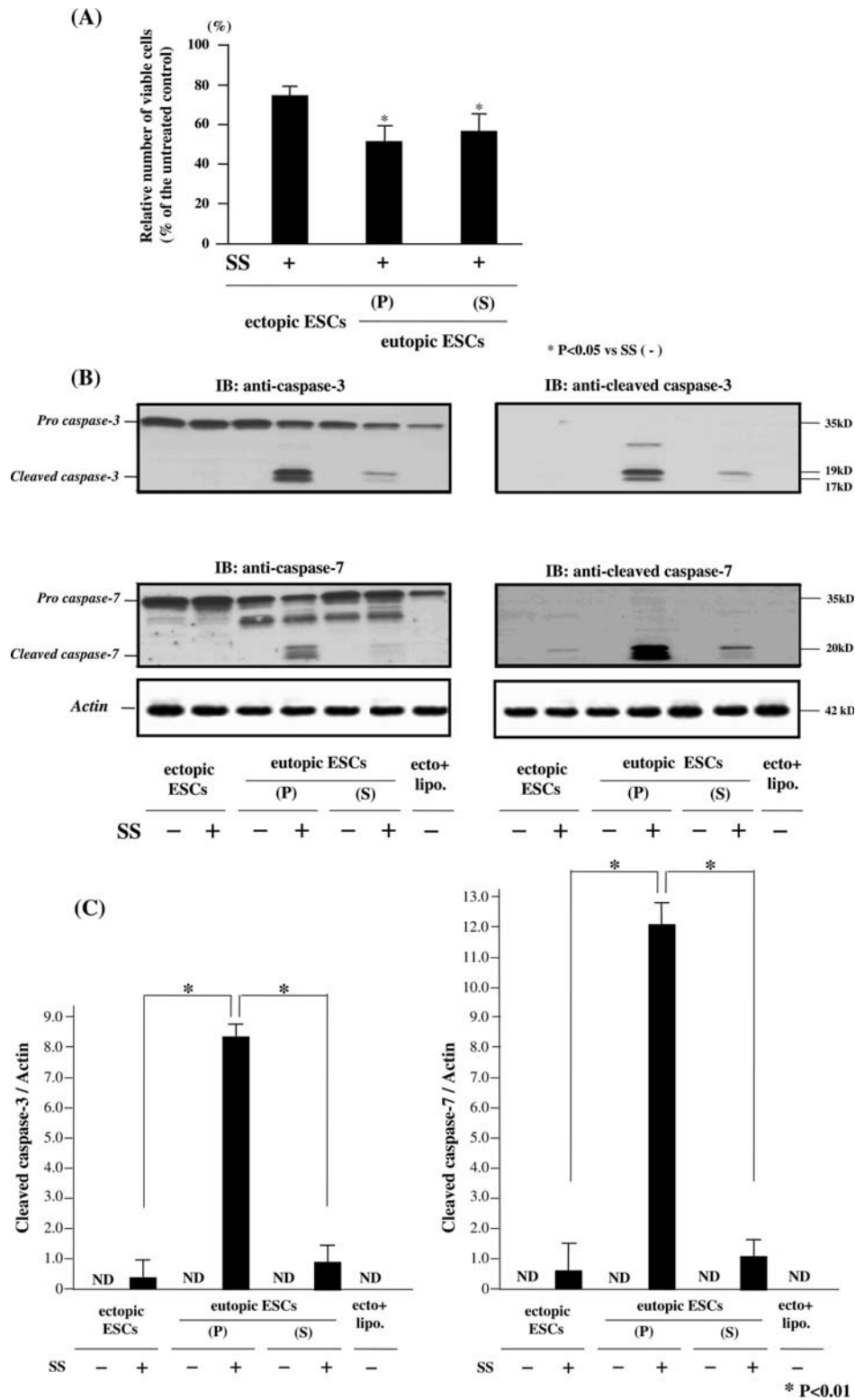


Figure 1 (A) Cell viability in eutopic and ectopic ESCs following treatment with SS. Eight hours after SS exposure ($0.5 \mu\text{M}$), cell viability was assessed using the WST-8 assay. Data are expressed as mean \pm SE of three independent experiments. * $P < 0.05$ versus SS(-), assigned a value of 100%. (B) Cleavage of caspase-3 and -7 in response to SS in ectopic and eutopic ESCs. Immunoblot analysis was performed on whole cell lysates prepared from ectopic and eutopic ESCs using caspase-specific antibodies (caspase-3 or -7) and actin. Antibodies recognizing procaspases (Left) and cleaved caspases (Right) were used. IB: immunoblotting; P: proliferative phase; S: secretory phase; ecto+lipo.: ectopic ESCs treated only with the transfection agent (Lipofectamine 2000). (C) Relative densitometric units of the bands with the density of the eutopic ESCs of the secretory phase with SS exposure set arbitrarily at 1.0. ND: not detectable. * $P < 0.01$.

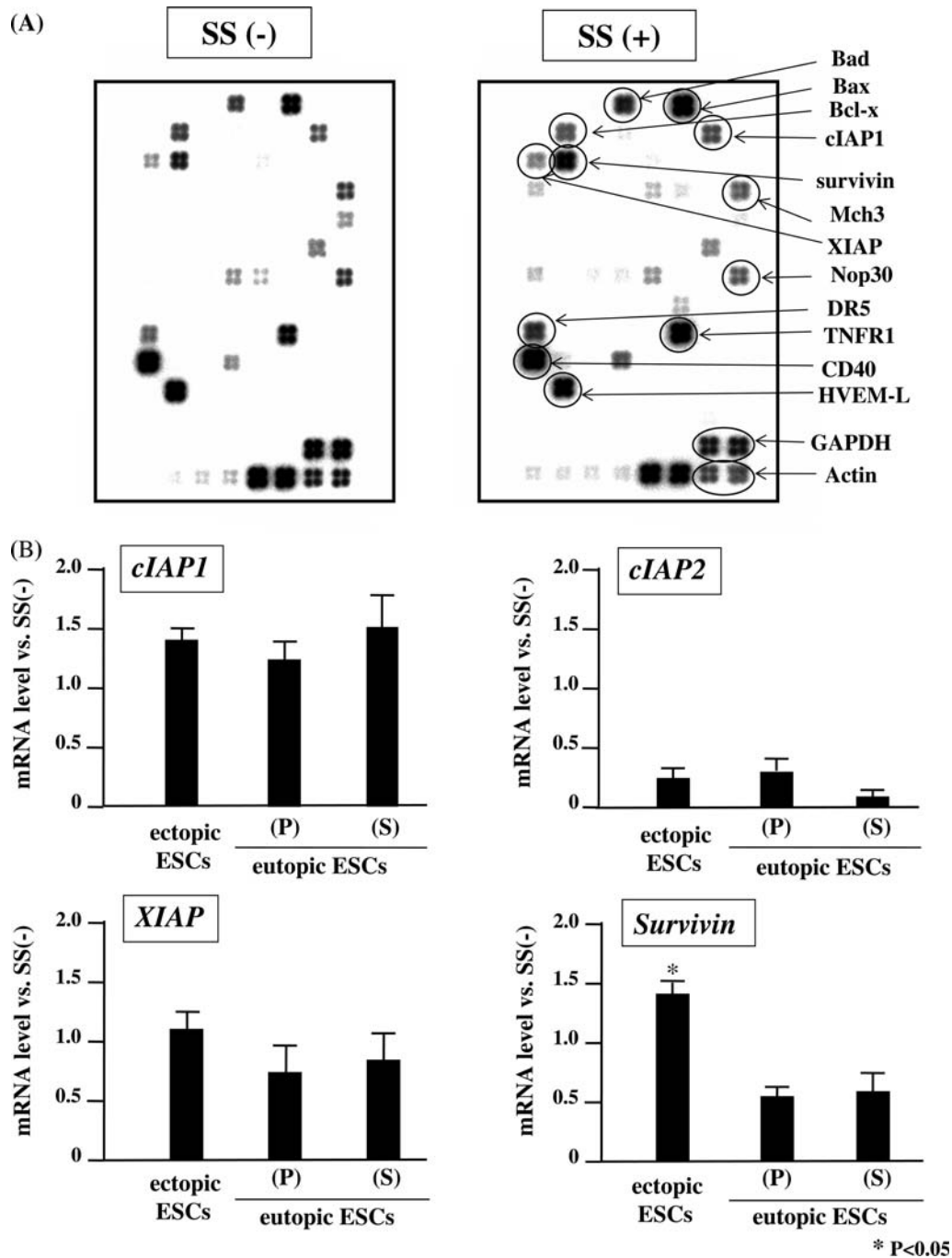


Figure 2 (A) Profiling of gene expression in ectopic ESCs before and after SS exposure. Ectopic ESCs are left untreated, or treated with SS for 2 h. The relative expression level was determined using GE array analyzer software (average values are shown in Tables I and II). Data shown here are representative of three experiments. Relative expression levels of selected mRNAs were normalized by adjusting for the values derived from glyceraldehyde-3-phosphate dehydrogenase (GAPDH), denoted as 1.0. (B) Quantitative analysis for gene expression of the inhibitor of apoptosis proteins (IAP) family in ectopic and eutopic ESCs. Gene expression of *birc2* (*cIAP1*), *birc3* (*cIAP2*), *birc4* (*XIAP*), *birc5* (*survivin*) in response to SS exposure in ectopic and eutopic ESCs was compared by real-time RT-PCR. Each value was assigned after comparison with other genes and correcting for background and GAPDH. The data are mean \pm SE of three independent experiments. * $P < 0.05$ versus SS(-).

the percentage of the Annexin-V positive cells (an early event marker of apoptosis) was <15% in ectopic ESC. In contrast, 25% of ectopic ESCs were positively stained by the pretreatment with survivin-siRNA (Fig. 3B). The arrows in the inset of panels 'g' and 'h' show the

phosphatidylserine exposed on the cell surface and chromatin condensation, respectively (Fig. 3B). After SS treatment, silencing of the survivin gene in ectopic ESCs markedly potentiated the cleavage of caspase-3 and -7 (Fig. 3C and D).

Table I Highly-expressed genes in untreated ectopic ESCs

Gene	Group	Relative mRNA expression
<i>bad</i>	Bcl-2 Family	0.32 ± 0.07
<i>bax</i>	Bcl-2 Family	0.35 ± 0.15
<i>bcl-x</i>	Bcl-2 Family	0.33 ± 0.09
<i>birc2</i> (<i>clAPI1</i>)	IAP Family	0.27 ± 0.07
<i>birc4</i> (<i>XIAP</i>)	IAP Family	0.14 ± 0.02
<i>birc5</i> (<i>survivin</i>)	IAP Family	0.47 ± 0.09
<i>mch3</i> (<i>caspase 7</i>)	Caspase Family	0.31 ± 0.00
<i>nol3</i> (<i>Nop30</i>)	CARD Family	0.33 ± 0.03
<i>tnfrsf1a</i> (<i>TNFR1</i>)	TNF Receptor Family	0.65 ± 0.12
<i>tnfrsf5</i> (<i>CD40</i>)	TNF Receptor Family	1.5 ± 0.46
<i>tnfrsf14</i> (<i>HVEM-L</i>)	TNF Ligand Family	1.2 ± 0.16

Representative transcripts highly expressed in ectopic ESCs are listed. Relative mRNA levels (mean ± SE, *n* = 3) were expressed as arbitrary units normalized by the mRNA level of glyceraldehyde-3-phosphate dehydrogenase.

IAP: inhibitor of apoptosis proteins; TNF: tumor necrosis factor; CARD: caspase recruitment domain.

Table II Differential gene expression in response to staurosporin (SS) in ectopic (*n* = 3) and eutopic (*n* = 3) ESCs

Gene	Fold-induction	
	Ectopic ESCs	Eutopic ESCs
<i>bad</i>	2.7 ± 0.60	1.1 ± 0.11
<i>bax</i>	2.2 ± 0.54	0.75 ± 0.07
<i>birc2</i>	1.9 ± 0.55	1.4 ± 0.33
<i>birc4</i>	1.9 ± 0.51	1.1 ± 0.27
<i>birc5</i>	2.8 ± 0.27	0.69 ± 0.07
<i>tnfrsf10b</i> (<i>DR5</i>)	3.4 ± 0.15	2.0 ± 0.45
<i>tnfrsf1a</i>	3.1 ± 0.84	0.71 ± 0.04
<i>tnfrsf5</i>	2.3 ± 0.17	0.60 ± 0.06

Values (mean ± SE) are the fold induction of gene expression in response to SS.

Discussion

The underlying mechanisms by which endometrial tissues survive and grow in ectopic sites are an enigma. Alteration of the apoptotic process is one of the physiological changes occurring in endometriotic cells. A relationship may exist between the altered apoptotic machinery and enhanced survival of endometriotic cells in ectopic sites. As the present study shows, ectopic ESCs constitutively exhibit a high expression of *clAPI1*, *XIAP* and *survivin*, supporting the notion that ectopic ESCs may potentially possess innate anti-apoptotic characteristics. We focused on the action of *survivin* during the early stage of apoptosis because *survivin* was highly expressed in ectopic ESCs

with SS exposure and have demonstrated that *survivin* plays a critical role in ESC's susceptibility to apoptosis.

Survivin, which is not usually seen in terminal differentiated tissues, can be regarded as an oncogene. In previous studies, *survivin* expression was limited to the epithelial cells of the endometrium (Konno *et al.*, 2000). Ueda *et al.* (2002) reported positive *survivin* immunostaining in both stromal cells and glandular epithelial cells of ovarian endometriomas, although very few specimens with positive stromal cells were included in that study. We showed that *survivin* mRNA was highly expressed in all samples derived from ectopic ESCs (Fig. 2A and B). Furthermore, we confirmed *survivin* protein synthesis in all samples of ectopic ESCs using enzyme-linked immunosorbent assay (75.1 ± 7.4 pg/mg protein; Assay Designs, Ann Arbor, MI, USA; data not shown): these studies and our present data suggest that enhanced *survivin* expression in endometriotic tissues are related to an escape from apoptosis and may facilitate cell viability.

Recently, the concept of intrinsic and extrinsic apoptotic pathways was reported (Vucic and Fairbrother, 2007). The intrinsic pathway is initiated by stimuli such as irradiation and treatment with chemotherapeutic agents. The extrinsic pathway is triggered when death receptors, such as DR5 (TNF receptor family) which is highly expressed in ectopic ESCs (Table II), are engaged by their responsive ligands, leading to activation of caspase-3 and -7. Once activated, these caspases cleave and activate the downstream effector caspases, which in turn cleave nuclear proteins and induce apoptosis. IAPs are believed to represent the ultimate line of defense against cellular suicide because they inhibit caspase-3 and -7. *Survivin* regulates the G2/M phase of the cell cycle by associating with the mitotic spindle microtubules and directly inhibits caspase-3 and -7 (Olie *et al.*, 2000). Figure 1 shows the differences in cell viability and activation of caspase-3 and -7 between eutopic and ectopic ESCs in response to SS exposure. We and other researchers also demonstrated that susceptibility to drug-induced (e.g. SS or interferon- γ) apoptosis in ectopic ESCs was attenuated compared with eutopic ESCs (Nishida *et al.*, 2005; Izawa *et al.*, 2006), implying that endometriotic cells have the potential to survive and develop in ectopic sites. In addition, it is important to note that SS is a pan-kinase inhibitor, including protein kinase C, and we have to assume that the activities of many kinases are inhibited by SS treatment in this experimental model.

Fibroids and endometriosis, which are estrogen-dependent, represent abnormal proliferation of a particular kind of cell from the female reproductive tract. Obtaining endometrial tissue from cycling women who do not have fibroids is difficult: thus, an overlap may exist in the biochemical and inflammatory changes associated with endometriosis and fibroids. For this study, we compared eutopic ESCs with fibroids as a control. In the future, we need to study the eutopic endometrium of disease free women. As in previous studies, our study consistently used cultured ectopic ESCs obtained from the internal layer of ovarian endometriomas. We performed no experiments using specimens from deep or peritoneal endometriotic lesions because it is difficult to purify and culture cells from these tissues.

We did not determine the levels of IAPs expression in ectopic ESCs in different phases of the menstrual cycle. However, Goumenou and coworkers reported that the apoptotic rates as well as Bcl-2 and Bax expression in ovarian endometriotic cells are not affected by stage of endometriosis or phase of menstrual cycle (Goumenou *et al.*, 2004). Although Bcl-2 expression in the eutopic endometrium

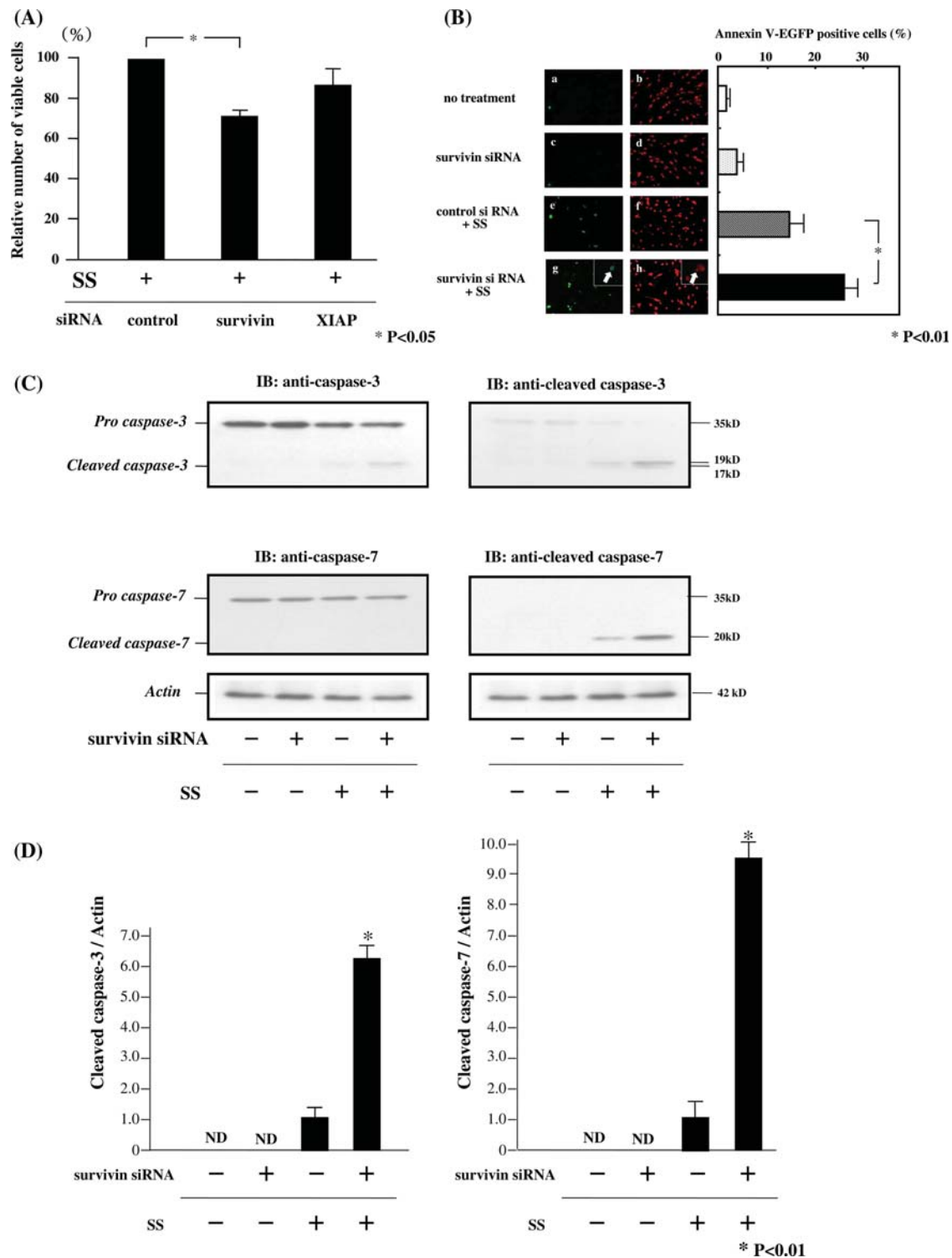


Figure 3 (A) Effect of silencing the survivin gene on ectopic ESCs viability in presence of 0.5 μ M SS. After transfection of control-, survivin- or XIAP-small interfering RNA (siRNA) for 48 h, ectopic ESCs were treated with SS for 8 h. Cell viability was assessed by WST-8 assay. Values are the mean \pm SE of three independent experiments. (B) Annexin-V staining assay. Ectopic ESCs were treated with SS for 3 h. The relative number of Annexin-V-tagged enhanced green fluorescent protein (EGFP)-positive cells was expressed as the percentage of total cell number, estimated by propidium iodide staining of fixed cells. Approximately 300 cells were counted in each cell population. The data are representative of three independent experiments. a, c, e, g: Annexin V-positive cells; b, d, f, h: PI positive cells; *P < 0.01. (C) Cleaved caspase-3 or -7 as shown by immunoblotting (described in legend to Fig. 1). After transfection with survivin siRNA for 48 h, ectopic ESCs were treated with SS (0.5 μ M) for 8 h. (D) Relative densitometric units of the immunoblot bands are shown, with density of ectopic ESCs + SS set arbitrarily at 1.0. *P < 0.01 versus no survivin siRNA.

of patients with endometriosis has a cyclic pattern, these cyclic changes may not be apparent in peritoneal and ovarian endometriotic tissues (Watanabe *et al.*, 1997). Hence, cyclic variability of apoptosis-related factors may be lost in ovarian endometriotic cells.

As our understanding of endometriosis improves, newer treatment modalities are being developed that target specific aspects of its pathophysiology. The use of siRNA has great potential as human therapy because it can target and silence expression of a distinct gene. Effective therapies can also exploit the large change of survivin expression with SS exposure in ectopic ESCs, as well as the difference in apoptotic reaction between ectopic and eutopic ESCs. Changing the expression of particular IAPs has resulted in an increase in spontaneous cell death induction or increased sensitivity to death stimuli (Tamm *et al.*, 2000). YMI55, a novel small-molecule survivin suppressant, induces regression of hormone-refractory prostate cancer (HRPC) (Nakahara *et al.*, 2007) and phase II clinical studies of YMI55 for HRPC, melanoma and non-small cell lung cancer are in progress. Further studies of YMI55 seem to be worthwhile to develop this novel therapeutic approach for treating endometriosis. However, the YMI55 survivin inhibitor might cause adverse effects in the whole body, and extensive tests will need to be carried out before it could be considered for use in benign disease. In conclusion, we provide new data suggesting that survivin expression may play a crucial role in the pathogenesis and progression of endometriosis.

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