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Effects of 1,25-dihydroxy vitamin D_3 on endometriosis

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Context: Endometriosis is an estrogen-dependent, chronic inflammatory disease. Recent studies have shown that vitamin D (VD) is an effective modulator of the immune system and plays an important role in controlling many inflammatory diseases.

Objective: To clarify the *in vitro* effects of 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$) on human endometriotic stromal cells (ESCs) and to determine the serum levels of VD in endometriosis patients.

Design, Patients, and Main Outcome Measures: ESCs were isolated from ovarian endometrioma and cultured with $1,25(OH)_2D_3$. Gene expression of interleukin (IL)-8, cyclooxygenase-2, microsomal prostaglandin (PG) E synthase (mPGES)-1, mPGES-2, cytosolic PGES (cPGES), 15-hydroxyprostaglandin dehydrogenase (15-PGDH), matrix metalloproteinase (MMP)-2, and MMP-9 was examined using quantitative RT-PCR. The production of IL-8 and PGE_2 was measured using ELISA and EIA. Viable cell number was assessed using a cell counting assay, and DNA synthesis was assessed using the bromodeoxyuridine incorporation assay. Apoptosis was assessed using flow cytometry. The expression of IκBα protein was detected using Western blotting. The serum levels of 25-hydroxy vitamin D_3 ($25(OH)VD_3$) and $1,25(OH)_2D_3$ were measured by radioimmunoassay.

Results: *In vitro* studies showed that $1,25(OH)_2D_3$ significantly reduced IL-1β- or TNF-α-induced inflammatory responses, such as IL-8 expression and prostaglandin activity. $1,25(OH)_2D_3$ also reduced viable ESC numbers and DNA synthesis but did not affect apoptosis. MMP-2 and MMP-9 expressions were reduced by $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ inhibited NFκB activation. The serum $25(OH)VD_3$ levels were significantly lower in women with severe endometriosis than in the controls and women with mild endometriosis. Serum $1,25(OH)_2D_3$ levels were not different between groups.

Conclusions: VD modulates inflammation and proliferation in endometriotic cells, and a lower VD status is associated with endometriosis. Taken together, VD supplementation could be a novel therapeutic strategy for managing endometriosis.

Endometriosis is an estrogen-dependent, chronic inflammatory disease. It causes pain and infertility and remarkably deteriorates women's health (1). Therapeutic strategies for this disorder are limited to hormonal treatment; consequently, patients who desire to conceive cannot undertake these treatments (2).

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Vitamin D (VD) is a pleiotropic molecule and therefore has a broad range of biological activities (3). In addition to its roles in mineralizing the skeleton and in regulating plasma calcium levels, recent studies have shown that VD is an effective modulator of the immune system (4). For example, VD induces dendritic cell tolerance (5) and controls maturation (6), macrophage migration and adhesion (7), cytokine production (8), T lymphocyte proliferation (9), and cytokine production (10).

Recently, the anti-inflammatory, antiproliferative, and anti-invasive effects of VD have been documented as a result of in vitro studies of synovial fibroblasts in rheumatoid arthritis (RA) (11), dendritic cells in psoriasis (12), and cancer cells (13), whereas no such study has been conducted in endometriosis. Regarding the therapeutic effects of VD on endometriosis, a selective VD receptor agonist has been shown to reduce endometriosis development in a mouse model (14); however, the mechanism by which VD controls lesion development is unclear.

The aim of this study was to clarify the in vitro effects of 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$), a biologically active form of VD, on human endometriotic stromal cells (ESCs). We also determined the serum levels of VD in endometriosis patients.

Materials and Methods

Reagents and materials

Type I collagenase and deoxyribonuclease (DNase) I were purchased from Wako (Tokyo, Japan), and $1,25(OH)_2D_3$ was provided by Teijin Pharma (Tokyo, Japan). Antibiotics (a mixture of penicillin, streptomycin, and amphotericin B), interleukin (IL)-1β, and tumor necrosis factor (TNF)-α (TNF-α) were obtained from Sigma (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM)/F-12 medium, 2.5% trypsin, HEPES, and 0.25% trypsin–EDTA were obtained from Gibco (Grand Island, NY).

Patients and samples

The experimental procedures were approved by the Institutional Review Board of the University of Tokyo. Signed informed consent for the use of tissue or sera was obtained from each participant.

Isolation and culture of human endometriotic stromal cells (ESCs)

Endometriosis tissues for the in vitro study were obtained from patients with ovarian endometriomas (n = 35, women aged 36.7 ± 6.42 years, mean ± SD) who underwent laparoscopies or laparotomies. All patients had not received hormones or GnRH agonists for ≥ 3 months before surgery. Endometriosis tissue samples were obtained from the cyst wall of the ovarian endometrioma under sterile conditions and transported to the laboratory on ice in DMEM/F-12.

For isolation and culture, human ESCs were prepared as described previously (15, 16). Endometriosis tissue was minced into small pieces, incubated in DMEM/F-12 with 0.25% type I collagenase, 15 U/mL DNase I, 0.006% trypsin, and 0.02M HEPES for 1 to 2 hours at 37°C, and filtered through nylon cell strainers with apertures of 100 μm and then 70 μm. ESCs were cultured in DMEM/F-12 supplemented with 5% FBS and antibiotics. At the first passage, the cells were plated at a density of 2×10^5 cells per well into 6-, 12-, 24-, or 96-well culture plates at 1×10^4 cells per well and then incubated at 37°C in a humidified 5% CO_2/95% air environment.

Treatment of ESCs with $1,25(OH)_2D_3$

Each experiment was conducted using samples from different individuals (n = 4–7) since the amount of sample from one individual was insufficient to perform multiple sets of experiments. Unless otherwise indicated, ESCs were cultured in 2%–5% FBS medium and treated with 10^{-7} or 10^{-6} M $1,25(OH)_2D_3$ for 24 hours. To evaluate the effects of $1,25(OH)_2D_3$ on IL-1β- or TNF-α-induced IL-8 gene expression, the cells were treated with IL-1β (5 ng/mL) or TNF-α (10 ng/mL) in the absence or presence of $1,25(OH)_2D_3$ and incubated for 1, 3, 6, and 24 hours. To evaluate the effects of $1,25(OH)_2D_3$ on IL-1β-induced cyclooxygenase (COX)-2, microsomal prostaglandin (PG) E synthase (mPGES)-1, mPGES-2, cytosolic PGES (cPGES), and 5-hydroxyprostaglandin dehydrogenase (15-PGDH) gene expression, the cells were treated with IL-1β (5 ng/mL) in the absence or presence of $1,25(OH)_2D_3$, and the cells were incubated for 3 hours. This time point was chosen as the authors (17) and others (18) had found that 4 hours is late enough to detect the response of ESCs to IL-1β regarding mRNA expression of PGE_2-related enzymes. To evaluate the dose effects of $1,25(OH)_2D_3$ on IL-1β or TNF-α-induced IL-8 and PGE_2 secretion, the cells were cultured for 24 hours in the presence or absence of $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M) with IL-1β (5 ng/mL) or TNF-α (10 ng/mL). To evaluate the effects of $1,25(OH)_2D_3$ on inhibitor-κBα (IκBα) protein expression, ESCs were pretreated with $1,25(OH)_2D_3$ for 24 hours and then stimulated with TNF-α (10 ng/mL) for 5 minutes.

RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted, using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). One microgram of total RNA was reverse transcribed in a 20-μL volume using an RT-PCR kit (TOYOBO, Osaka, Japan). Real-time quantitative PCR was conducted using a LightCycler (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. Expression of each mRNA was normalized for RNA loading for each sample using human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA as the internal standard. Primer information and PCR conditions are listed in Supplemental Table 1. All PCR procedures were followed up with melting curve analysis.

Measurement of IL-8

After treatment, the conditioned media were collected, centrifuged, and stored at −80°C until assayed. IL-8 concentrations were measured using a specific ELISA kit (R&D Systems, Minneapolis, MN). The limit of sensitivity of ELISA was 31 pg/mL. There was no cross-reactivity or interference.

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Measurement of PGE_2

PGE_2 concentrations in the media were measured using a specific enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). The limit of sensitivity of the EIA was 7.8 pg/mL. The intra- and interassay coefficients of variation were 8.8 ± 3.2% and 15.6 ± 3.2% (mean ± SEM), respectively.

Viable cell number counting

The viable cell counting assay was performed using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. This kit measures the activity of dehydrogenase in cells by detecting the yellow formazan dye, which is reduced from tetrazolium by dehydrogenase. Since the activity of dehydrogenase is correlated with the number of living cells, the kit determines the number of living cells. ESCs were treated with or without $1,25(OH)_2D_3$ for 24 hours, after which 10 μL of the cell counting kit solution was added and cells incubated at 37°C for an additional 2 hours. The resultant color was read at 450 nm in an Epoch Microplate Spectrophotometer (BioTek, Winooski, VT).

Bromodeoxyuridine incorporation assay

To evaluate DNA synthesis, the bromodeoxyuridine (BrdU) incorporation assay was performed using the Biotrak cell proliferation ELISA system (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Briefly, ESCs were treated with $1,25(OH)_2D_3$ for 24 hours, and 10 μL BrdU solution was added and incubated at 37°C for an additional 2 hours. After removing the culture medium, the cells were fixed and the DNA denatured with the addition of 200 μL/well fixative. Immune complexes (peroxidase-labeled anti-BrdU bound to BrdU incorporated in newly synthesized, cellular DNA) were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm using an Epoch Microplate Spectrophotometer (BioTek).

Assessment of cell death

Apoptosis of ESCs was assessed by double staining for annexin V and propidium iodide and using the Annexin V-EGFP Apoptosis detection kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. Briefly, ESCs were detached by 0.25% trypsin–EDTA, washed twice with PBS, and resuspended to 1×10^6 cells/mL in 1× binding buffer. Each sample solution was transferred to a 5-mL culture tube, after which 2 μL of annexin V-FITC and 2 μL of propidium iodide were added. Samples were then incubated for 10 minutes at 4°C in the dark, followed by filtration through a 40-μm nylon mesh (BD Biosciences, San Jose, CA) to remove cell clumps. After incubation, the samples were analyzed using flow cytometry (FACS Calibur and Cell Quest Pro; BD Biosciences). Annexin V-positive cells were regarded as apoptotic cells.

Western blotting

Cultured cells were homogenized in cell lysis buffer (Cell Signaling Technology, Beverly, MA,). Samples were resolved by 10% SDS-PAGE. Proteins were blotted onto a polyvinylidene difluoride membrane (Amersham Bioscience, Little Chalfont, UK) and incubated with rabbit anti-IκBα (1:1000; Cell Signaling Technology) as the primary antibody and then antirabbit horseradish peroxidase antibody (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immune complexes were visualized us an ECL western blotting system (Amersham).

Measurement of serum levels of 25-hydroxy vitamin D_3 ($25(OH)VD_3$) and $1,25(OH)_2D_3$

Serum samples for measuring VD were collected from another set of patients who underwent laparoscopy for treatment of either endometriosis or benign ovarian tumor at the University of Tokyo Hospital, Japan. Seventeen patients with stage 1 or 2 endometriosis (age, 35.4 ± 1.64 years), 22 patients with stage 3 or 4 endometriosis (age, 34.6 ± 1.53 years), and 37 controls who were negative for endometriosis confirmed by laparoscopy (age, 32.8 ± 1.05 years) were enrolled in the study. Endometriosis was staged according to the revised American Society for Reproductive Medicine classification. All patients and controls were negative for fibroids confirmed by laparoscopy. All patients and controls were Japanese residents and Mongoloid. All samples were taken from October to March (autumn to winter). All women were in the proliferative phase of their menstrual cycle. Validation samples (n = 73) were randomly selected from both patients and controls. All sera were collected the day before laparoscopy, and samples were stored at −80°C until the assay.

25-hydroxy vitamin D_3 ($25(OH)VD_3$) and $1,25(OH)_2D_3$ were measured by radioimmunoassay (RIA) (SRL, Inc, Tokyo, Japan). Briefly, after serum samples were mixed with antibody for VD, ^{125}I- labeled VD was added for competitive reactions, and the bound antigens were separated from the unbound ones and measured. Since RIA has been shown to have problems with specificity, accuracy and sensitivity for the measurement of $25(OH)VD_3$, and liquid chromatography coupled with tandem mass spectrometry (LS-MS/MS) has provided significant advances and is now considered the gold standard, the results of $25(OH)VD_3$ concentration measured by RIA were further verified by measuring validation samples using LS-MS/MS (LSI Medience Corporation, Tokyo, Japan) and RIA.

Statistical analysis

Data were evaluated using JMP software (version 10.0; SAS Institute Inc, Cary, NC). The differences between two samples were calculated using Student's *t* test. The differences among multiple samples were calculated using ANOVA, Tukey's test, and the Mann–Whitney *U*-test. The data are expressed as the mean ± SEM. A *P* value of < 0.05 was considered significant.

Results

The effect of $1,25(OH)_2D_3$ on IL-1β- or TNF-α-induced IL-8 mRNA and protein expression

$1,25(OH)_2D_3$ significantly reduced IL-1β-induced IL-8 mRNA expression at 6 and 24 hours after the treatments (67.4 ± 9.4% and 72.1 ± 1.7%, respectively, $P < .05$) (Figure 1a). $1,25(OH)_2D_3$ suppressed the secretion of IL-1β-induced IL-8 protein at a concentration of 10^{-9} to 10^{-7} M, with a dose dependency from 10^{-9} to 10^{-8} M and a flat response from 10^{-8} to 10^{-7} M (Figure 1*b*). $1,25(OH)_2D_3$ also significantly reduced TNF-α-induced IL-8 mRNA expression at 6 hours after the treatments (80.0 ± 6.0% of

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controls, $P < .05$) (Figure 1c). $1,25(OH)_2D_3$ significantly suppressed the secretion of TNF-α-induced IL-8 protein with a flat response from 10^{-9} to 10^{-7} M (Figure 1d).

The effect of $1,25(OH)_2D_3$ on IL-1β-induced PGE_2 secretion, PGE_2 synthesis, and degradative enzyme mRNA expression

$1,25(OH)_2D_3$ significantly suppressed the secretion of IL-1β-induced PGE_2 in a dose-dependent manner (Figure 2a). $1,25(OH)_2D_3$ significantly decreased the expression of IL-1β-induced COX-2, mPGES-1, and mPGES-2 mRNA and increased IL-1β-induced 15-PGDH mRNA expression by ESCs (57.9 ± 12.2%, 74.1 ± 7.4%, 84.1 ± 9.4%, and 224.9 ± 83.4% of controls, respectively, $P < .05$) (Figure 2, b–d, and f). The level of IL-1β-induced cPGES mRNA expression was not changed by $1,25(OH)_2D_3$ (Figure 2e).

Effect of $1,25(OH)_2D_3$ on viable ESC number, DNA synthesis, and apoptosis

$1,25(OH)_2D_3$ decreased the number of viable ESCs (90.0 ± 4.1% of controls, $P < .05$) (Figure 3a). $1,25(OH)_2D_3$ also decreased BrdU incorporation into ESC DNA (73.5 ± 4.6% of controls, $P < .05$) (Figure 3b). The percentage of apoptotic cells after $1,25(OH)_2D_3$ treatment was not significantly different from that of the controls (Figure 3c).

The effect of $1,25(OH)_2D_3$ on MMP-2 and MMP-9 mRNA expression

Treatment of ESCs with $1,25(OH)_2D_3$ significantly inhibited MMP-2 and MMP-9 mRNA expression (68.4 ± 3.7% and 65.6 ± 5.8% of controls; respectively; $P < .05$) (Figure 4).

The effect of $1,25(OH)_2D_3$ on TNF-α-induced IκBα expression

IκBα protein expression was decreased by TNF-α (34.0 ± 5.4% of controls, $P < .05$), but this effect was neutralized when ESCs were pretreated with $1,25(OH)_2D_3$ (10^{-7}M) for 24 hours (87.3 ± 28.6% of controls, $P < .05$) (Figure 5).

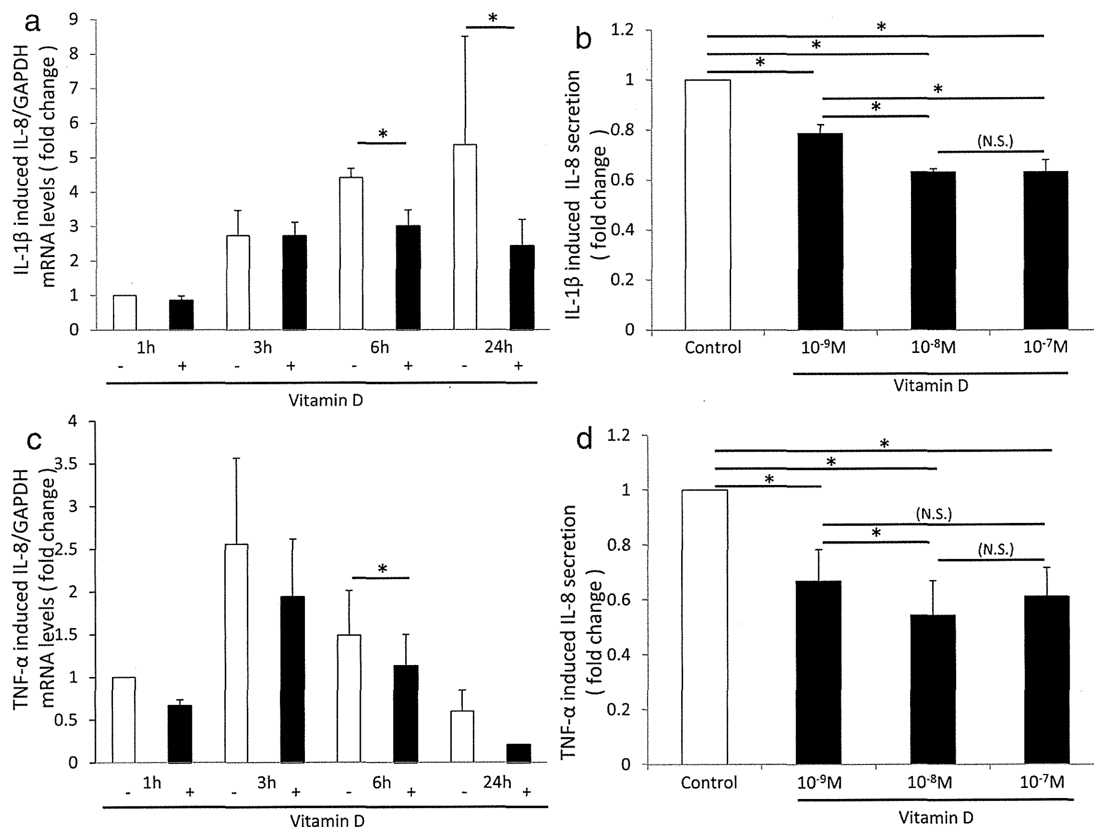

Figure 1. The effect of VD on IL-1β- and TNF-α-induced IL-8 expression. (a) IL-1β (5 ng/ml)-induced IL-8 mRNA expression in endometriotic stromal cells (ESCs) cultured in the absence or presence of $1,25(OH)_2D_3$ (10^{-7} M) at 1, 3, 6, and 24 hours and (b) IL-1β (5 ng/ml) -induced IL-8 protein in supernatant from ESCs cultured in the absence or presence of $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M) for 24 hours. * $P < .001$ vs control. (c) TNF-α (10 ng/ml)-induced IL-8 mRNA expression in ESCs cultured in the absence or presence of $1,25(OH)_2D_3$ (10^{-7} M) at 1, 3, 6, and 24 hours. (d) TNF-α (10 ng/ml) induced IL-8 protein in the supernatant from ESCs cultured in the absence or presence of $1,25(OH)_2D_3$ (10^{-9} to 10^{-7} M) for 24 hours. *$P < .05$ vs control.

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Serum levels of VD

The serum levels of 25(OH)D_3 in samples from patients with severe endometriosis (17.2 ± 1.1 ng/mL) were significantly lower than the levels detected in samples from the controls (21.8 ± 1.3 ng/mL, $P < .05$) and the patients with mild endometriosis (21.5 ± 1.4 ng/mL, $P < .01$) (Figure 6a). In contrast, the serum 1,25$(OH)_2D_3$ levels were not different between the groups (Figure 6b). A strong correlation between the serum levels of 25(OH)D_3 measured using RIA and those measured using liquid chromatography coupled with tandem mass spectrometry was found ($R^2 = 0.6135$, $P < .05$, Figure 6c) in the validation sample, confirming the accuracy of the results using RIA.

Discussion

In this study, we found that 1,25$(OH)_2D_3$ significantly reduced IL-1β- or TNF-α-induced inflammatory responses, such as IL-8 mRNA expression, prostaglandin activity, and MMP mRNA expression. Further, 1,25$(OH)_2D_3$ regulates the viable ESC number and BrdU incorporation into ESC DNA but had no effect on apoptosis. 1,25$(OH)_2D_3$ inhibited NF-κB activation in ESCs. We also demonstrated that the 25(OH)D_3 levels were significantly lower in the sera from women with severe endometriosis than in that from the patients with mild endometriosis or controls. These results suggest that VD deficiency is associated with the pathogenesis of endometriosis, and VD supplementation may have therapeutic benefits in endometriosis management.

Treatment of ESCs with 1,25$(OH)_2D_3$ significantly reduced inflammatory responses. Inhibitory effects of VD on cytokine production have been demonstrated in various types of cells such as trophoblasts (19), placental cells (20), decidua cells (21) and immortalized myometrial smooth muscle cells (22) in the context of miscarriage,

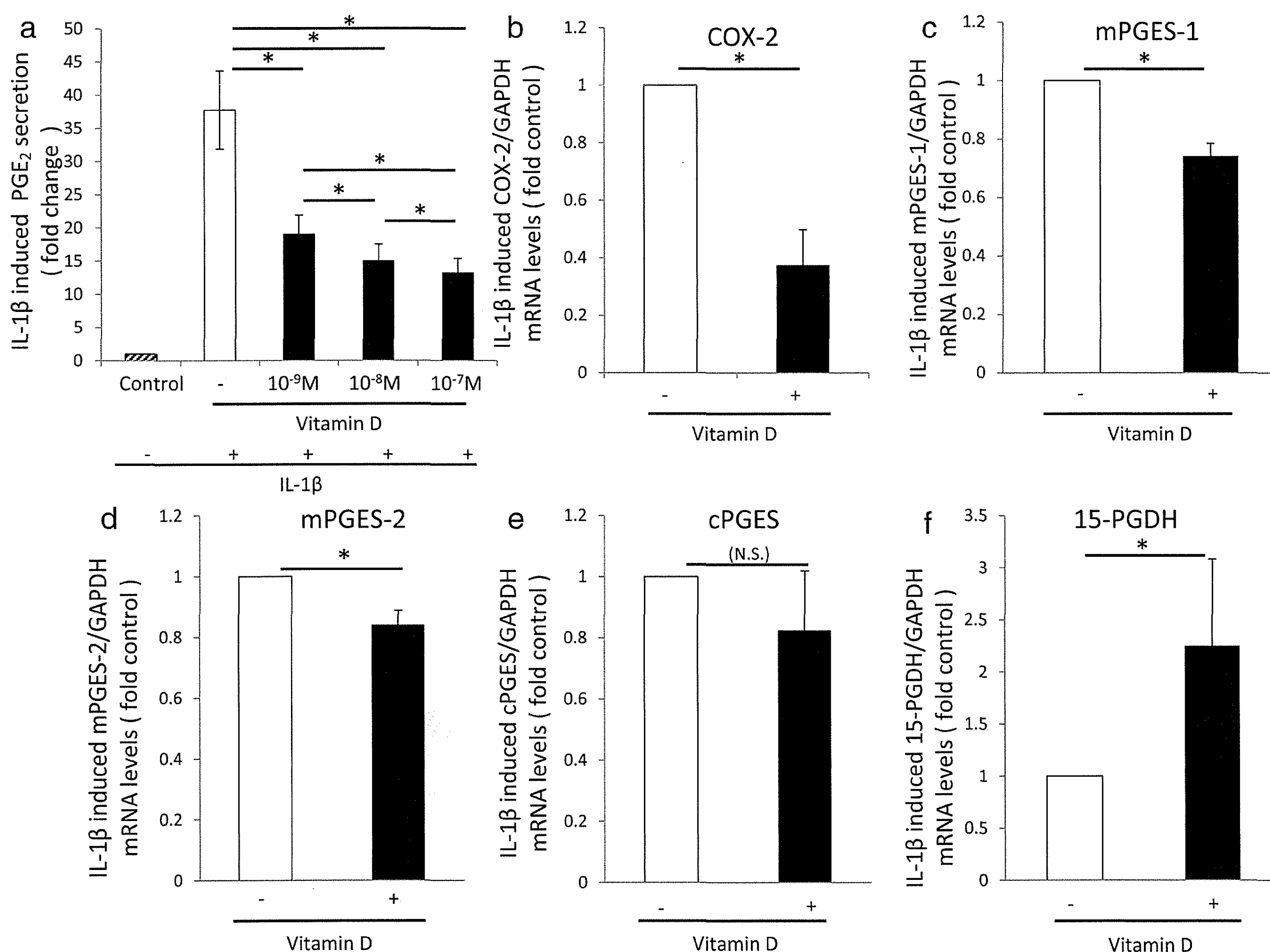

Figure 2. The effect of VD on IL-1β-induced prostaglandin E_2 (PGE_2) secretion and PGE_2 synthesis and degradative enzyme mRNA expression by endometriotic stromal cells (ESCs): (a) IL-1β (5 ng/ml) -induced PGE_2 levels in supernatant from ESCs cultured in the absence or presence of 1,25$(OH)_2D_3$ (10^{-9} to 10^{-7} M) for 24 hours. *$P < .05$ vs control. (b) IL-1β-induced cyclooxygenase (COX) –2 mRNA expression, (c) IL-1β-induced microsomal PGE synthase (mPGES) –1 mRNA expression, (d) IL-1β -induced mPGES-2 mRNA expression, (e) IL-1β-induced cytosolic PGE synthase (cPGES) mRNA expression, and (f) IL-1β-induced 15-hydroxyprostaglandin dehydrogenase (15-PGDH) mRNA expression. In each instance, ESCs were cultured with IL-1β (5 ng/ml) in the absence or presence of 1,25$(OH)_2D_3$ (10^{-7} M) for 3 hours. *$P < .05$ vs control.

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preterm labor, and preeclampsia. Indeed, in support of VD's potential role in the context of reproduction, we demonstrated that $1,25(OH)_2D_3$ significantly reduced IL-1β- or TNF-α-induced IL-8 mRNA expression and production in ESCs, suggesting that VD may exert in vivo regulatory effects on inflammation in endometriosis.

Numerous reports support the assertion that VD regulates the PG pathway in cancer and inflammatory diseases such as asthma. For example, $1,25(OH)_2D_3$ suppresses COX-2 expression in breast (13) and prostate (23) cancer cells and stimulates 15-PGDH in breast cancer cells (13). PG-inhibitory effects have also been shown in lung fibroblasts; for instance, $1,25(OH)_2D_3$ significantly reduced PGE_2 production and IL-1β-induced mPGES-1 and stimulated 15-PGDH (24). Given that the PG pathway plays important roles in the pathophysiology of endometriosis (25), we tested the effect of VD on ESCs. Similar to other cell type responses, $1,25(OH)_2D_3$ significantly re-

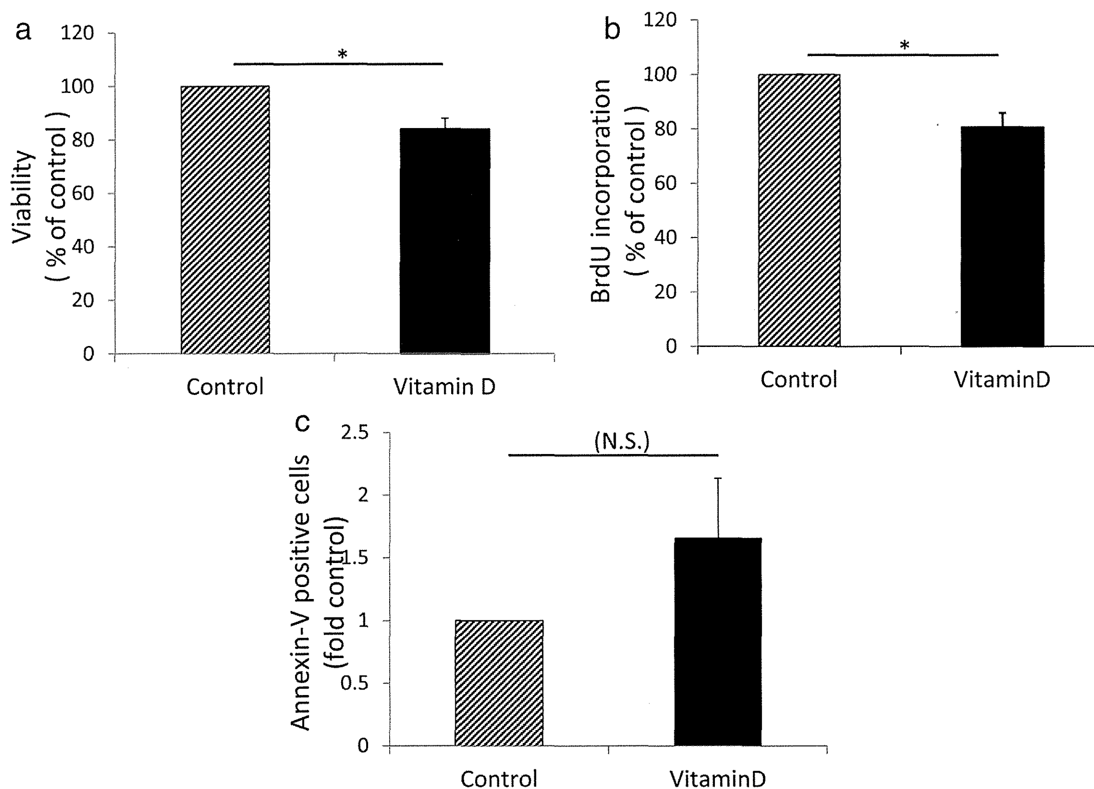

Figure 3. VD effects on endometriotic stromal cells (ESCs) number, DNA synthesis and apoptosis: (a) viable ESC numbers using the cell counting assay; (b) BrdU incorporation/DNA synthesis in ESCs; and (c) percentage of apoptotic/Annexin V positive ESCs. In each instance, ESCs were treated with $1,25(OH)_2D_3$ (10^{-6} M) for 24 hours, and $^*P < .05$ vs control.

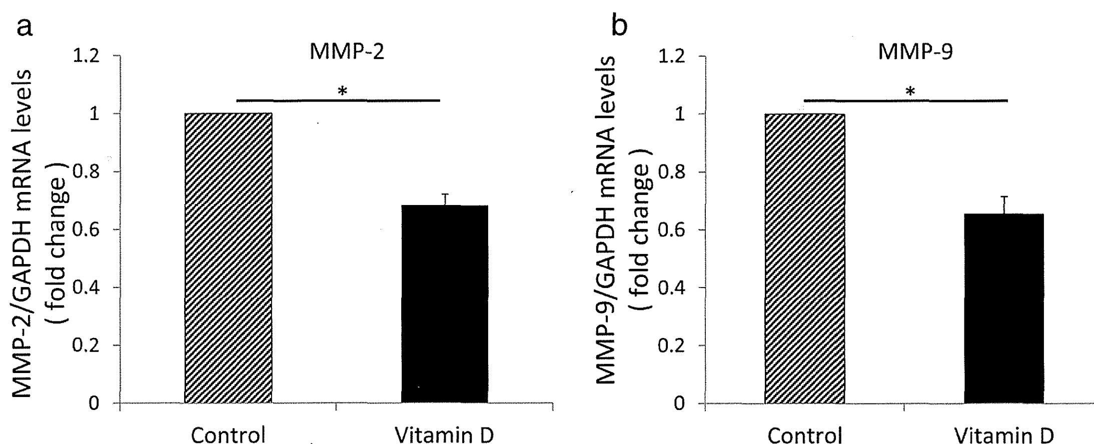

Figure 4. The effect of VD on matrix metalloproteinase (MMP) –2 and –9 mRNA expression by endometriotic stromal cells (ESCs): (a) MMP-2 mRNA expression; (b) MMP-9 mRNA expression. In each instance, ESCs were cultured in the absence or presence of $1,25(OH)_2D_3$ (10^{-7} M) for 24 hours. $^*P < .05$ vs control.

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duced PGE_2 production by ESCs. This may be owing to the reduction of PG-synthetic enzymes such as COX-2, mPGES-1, and mPGES-2 and the increase of the PG-degradative enzyme 15-PGDH and may reveal a mechanism by which VD affects endometriosis development.

We found that $1,25(OH)_2D_3$ significantly decreased viable ESC numbers as a result of reduced DNA synthesis rather than the induction of apoptosis. Previous reports have indicated that VD is proapoptotic in normal (26) or cancerous (13) cells, but this was not the case in ESCs. Endometriosis is resistant to apoptosis (27), and this may at least partially explain why VD did not induce apoptosis in ESCs.

VD can regulate MMP expression in endometrial cancer (28) and uterine fibroid cells (29). High MMP-2 and MMP-9 levels in serum or tissue (30) have been associated with the pathogenesis of endometriosis. Interestingly, the expression of MMP-9 in an endometriosis murine model was reduced by the administration of VD (31). In consideration of these findings, we examined the effect of $1,25(OH)_2D_3$ on MMP-2 and MMP-9 expression in ESCs. Given that $1,25(OH)_2D_3$ significantly reduced MMP mRNA expression, it is possible that VD exerts an anti-invasive effect by inhibiting MMP production. Further studies such as invasive assays or xenograft studies could be used to explore this mechanism.

We further demonstrated that $1,25(OH)_2D_3$ reduced activation of the NFκB pathway in ESCs by preserving IκBα. As NFκB mediates cytokine or prostaglandin production (32), MMP expression (33), and cell proliferation (34), our findings reveal the possible mechanism for the anti-inflammatory, antiproliferative, and anti-MMP effects of $1,25(OH)_2D_3$ reported in earlier studies. VD -induced inhibition of the NFκB pathway has also been reported in other cell types in various pathological conditions, such as uterine myometrial cells in preterm labor (35), peripheral blood mononuclear cells in Crohn's disease (36), and adipocytes in obesity (37); consequently, VD's therapeutic potential has been proposed.

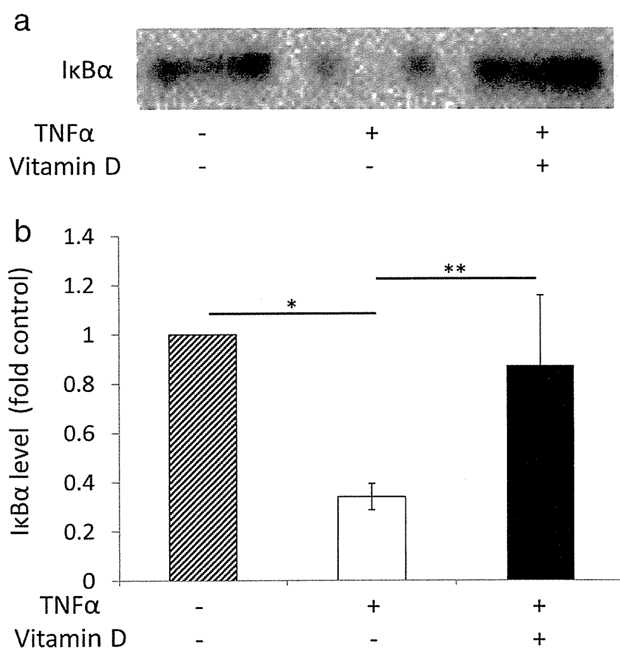

Figure 5. The effect of VD on TNF-α-induced IκBα protein expression by endometriotic stromal cells (ESCs). ESC were treated with TNF-α (10 ng/ml) for 5 minutes after pretreatment with $1,25(OH)_2D_3$ (10^{-7} M) for 24 hours. Western blotting was performed using antibody against IκBα. (a) Data shown are representative of four experiments. (b) Bars represent the average ± SEM. $*P < .05$ vs control. $**P < .05$ vs without $1,25(OH)_2D_3$.

There were several limitations in the current in vitro study. First, the ESCs were only from endometriomas and therefore cannot be considered as representative of all endometriosis. Second, assays of cell secretions such as cytokines are possibly affected by cell viability, although the observed differences in secretions were greater than those in viability.

Our findings, indicating an association between low serum levels of $25(OH)D_3$ and the severity of endometriosis, are consistent with a previous report (38), however, other groups have reported contrary results, ie, higher $25(OH)D_3$ levels with endometriosis (39) or no difference from that of healthy controls (40, 41). These discrepancies may be due to the variations in race and country of residence in these studies. Our study clarified, however, that serum $25(OH)D_3$ is reduced in Mongoloid and Japanese patients diagnosed with severe endometriosis and residing in Japan. It is also possible that seasonal influences (42), menstrual cycle, and disease stage influence $25(OH)D_3$ levels. Our study minimized these influences by sampling in autumn and winter during the proliferative phase of the menstrual cycle, and comparisons were made according to disease severity. Taken together, our results indicate that VD deficiency is associated with the progression of endometriosis.

In contrast to $25(OH)D_3$, the levels of $1,25(OH)_2D_3$ did not differ between the groups. $1,25(OH)_2D_3$ is the biologically active form of VD and is, therefore, the relevant form for use in in vitro studies. Circulating levels, however, do not reflect the systemic VD status because of its short half-life (43). Therefore, a negligible difference in $1,25(OH)_2D_3$ levels between groups does not refute the association between VD status and endometriosis.

Having shown the association between low serum VD status and severe endometriosis, and the anti-inflammatory, antiproliferative, and anti-invasive properties of VD in ESCs, we propose that VD deficiency may promote the progression of endometriosis; therefore, VD supplementation may be used to manage the disease. Recently, VD supplementation trials have indicated that the vitamin is

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an effective therapeutic for many medical disorders, including Crohn's disease (44). Given that VD does not affect ovulation, VD supplementation may be an ideal therapy for those who wish to conceive. Clinical studies to evaluate the efficacy of VD on endometriosis symptoms and lesions are warranted in the future.

In summary, this study demonstrated that VD may prevent disease progression and that lower VD status was associated with endometriosis; therefore, VD supplements should be explored as a novel therapeutic strategy for managing the disease.

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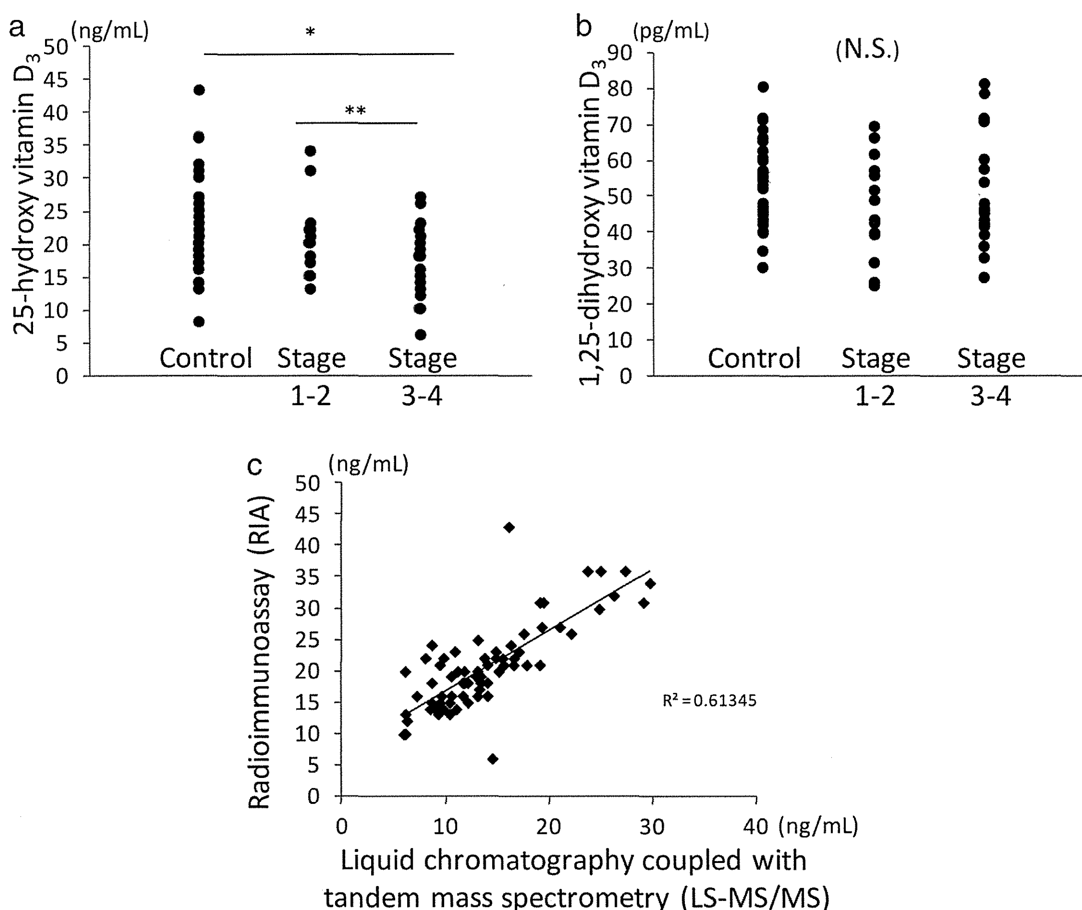

Figure 6. The serum levels of 25-hydroxy vitamin D_3 ($25(OH)D_3$, a)and 1,25-dihydroxy vitamin D_3 ($1,25(OH)_2D_3$, b) measured using RIA. Serum samples were collected from 37 control women, 17 patients with stage 1 or 2 endometriosis and 22 patients with stage 3 or 4 endometriosis. (c)The correlation between the serum concentrations of 25-hydroxy vitamin D_3 measured using RIA and those using liquid chromatography coupled with tandem mass spectrometry (LS-MS/MS) in validation samples collected from control and patients with endometriosis. (a) Serum levels of $25(OH)D_3$ from stage 3 or 4 endometriosis were significantly lower than levels detected in samples from controls and stage 1 or 2 endometriosis patients. * indicates $P < .05$, **indicates $P < .05$. (b) Serum $1,25(OH)_2D_3$ levels were not different between groups. (c) Positive correlation was observed with coefficient of correlation $R^2 = 0.61345$, $P < .05$.

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Original Article

Four Cases of Postoperative Pneumothorax Among 2814 Consecutive Laparoscopic Gynecologic Surgeries: A Possible Correlation Between Postoperative Pneumothorax and Endometriosis

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ABSTRACT **Study Objectives:** To evaluate the frequency of pneumothorax after laparoscopic surgery and to identify possible correlations to endometriosis.

Design: Retrospective review.

Setting: Tokyo University Hospital between 2006 and 2013.

Patients: Four patients among a total of 2814 patients with a postoperative pneumothorax.

Intervention: Laparoscopic surgery for gynecologic benign disease. The main outcome was the clinical frequency and characteristics of the patients with postoperative pneumothorax.

Measurements and Main Results: We observed 4 (0.14%) cases of postoperative pneumothorax after laparoscopic surgery, all of whom were diagnosed with endometriomas and developed a right-sided pneumothorax. The incidence of postoperative pneumothorax in 1097 patients with endometriomas was 0.36%, which was significantly higher than those without endometriomas.

Conclusion: The presence of endometrioma should be considered a risk factor for postoperative pneumothorax in gynecologic laparoscopic surgery. Journal of Minimally Invasive Gynecology (2015) 22, 980–984 © 2015 AAGL. All rights reserved.

Keywords: Endometriosis; Laparoscopic surgery; Postoperative pneumothorax

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Laparoscopic surgery is an acceptably safe and minimally invasive procedure for gynecologic lesions, because complications are reportedly rare, at an estimated rate of 0.36% to 0.72% [1–6]. The incidence of pneumothorax after laparoscopic surgery is especially rare, at a rate of 0.24%; however, this complication is often life-threatening [7]. Prolonged surgical duration (>200 min) has been reported to be a risk factor for postoperative pneumothorax [8], although the number of reported cases is quite low, and the mechanism of pneumothorax after laparoscopic gynecologic surgery remains unknown. Moreover, catamenial pneumothorax, which is caused by thoracic endometriosis, is reported to occur in approximately 25% of women with spontaneous pneumothorax [9] and is associated with a high incidence of pelvic endometriosis (58.7% [61%]) [10,11].

Here, we report 4 cases of postoperative pneumothorax after laparoscopic surgery for endometriomas over an

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8-year period through a retrospective review to estimate the frequency of pneumothorax and to elucidate correlations between postoperative pneumothorax and endometriosis.

Methods

We retrospectively reviewed the medical records of all patients who underwent laparoscopic surgery for gynecologic benign disease at the University of Tokyo Hospital (Tokyo, Japan) between January 2006 and December 2013. All the patients gave written informed consent before surgery.

In all cases, laparoscopy was performed with the patient in the lithotomy position under general anesthesia. Laparoscopic abdominal entry was performed using an open laparoscopic technique. Pneumoperitoneum was achieved by insufflation with carbon dioxide under an abdominal pressure of 8 to 10 mm Hg. The diaphragm was routinely examined, and the surgical procedure commenced after the patient was placed in the Trendelenburg position at approximately 20°.

All statistical analyses were performed using Statview software (SAS Institute Inc., Cary, NC). Data are presented as means ± SDs. Patient characteristics were compared using the Mann-Whitney U-test and Fisher's exact test. The χ^2 test was used to compare the incidence of a postoperative pneumothorax. A p value $<.05$ was considered statistically significant.

Results

A total of 2814 women underwent laparoscopic surgery for gynecologic benign diseases at the University of Tokyo Hospital from January 1, 2006 to December 31, 2013, which included laparoscopic hysterectomy or laparoscopic-assisted vaginal hysterectomy (311 cases), laparoscopic myomectomy (505 cases), laparoscopic oophorectomy (475 cases), and laparoscopic ovarian cystectomy (1319 cases) with overlaps. Among these patients, 4 (0.14%) developed postoperative pneumothorax. Patient characteristics and postoperative outcomes with or without postoperative pneumothorax are shown in Table 1. There were no significant differences in age, total surgical duration, or blood loss between patients with or without postoperative pneumothorax. All 4 patients with pneumothorax underwent laparoscopic surgery for ovarian endometriomas. The frequency of endometriomas was significantly higher in the cases with postoperative pneumothorax than those without endometriomas. We described 1 of the 4 pneumothorax cases after laparoscopic surgery as follows.

Table 1

Characteristics of patients with or without postoperative pneumothorax

	Pneumothorax (−)	Pneumothorax (+)	p value
No.	2810	4	
Age (yrs)	37.1 ± 8.1	41.5 ± 7.0	NS[a]
Parity	0.44 ± 0.81	0.50 ± 1.00	NS[a]
Operative time (min)	122.2 ± 58.4	112.8 ± 21.8	NS[a]
Cases with endometrioma	1093	4	p = .0123[b]
Total blood loss (mL)	122.2 ± 226.9	25 ± 50	NS[a]

Statistical analysis was done by [a]Mann-Whitney U test or [b]χ^2 test.

Case 1

A 33-year-old woman (gravida 0; weight, 46 kg; height, 158 cm; body mass index, 18.4 km/m^2) presented with dysmenorrhea and a 3-year history of primary infertility. Ultrasonography and magnetic resonance imaging revealed a 2.5-cm endometrioma in the right ovary. The serum level of cancer antigen-125 was 86 IU/mL. Laparoscopic cystectomy, adhesiolysis, and chromotubation were performed under an abdominal pressure of 8 mm Hg. The laparoscopic view during surgery confirmed an endometrioma in the right ovary and revealed complete obliteration of the Douglas pouch, whereas there was no endometriotic lesion of the diaphragm. The revised American Society for Reproductive Medicine score was 106, surgical duration was 105 min, and total blood loss was minimal. The operation was performed on the third day of the menstrual cycle. There was no sign of subcutaneous emphysema or pneumothorax during this procedure.

On postoperative day 1, the patient complained of right-sided chest pain and dyspnea. On examination, her oxygen saturation by pulse oximetry (SpO_2) level decreased to 90%, and a chest x-ray showed a right-sided pneumothorax. Pleural drainage of the right pleural cavity was performed, and the right lung was fully expanded, as observed by the chest x-ray on that day. The pleural fluid was blood tinged. On postoperative day 6, the right-sided chest drain was removed. On postoperative day 7, the patient was discharged. After that, infertility treatment was restarted. There was no recurrence of pneumothorax.

The patient characteristics and postoperative outcomes of all 4 cases with pneumothorax, including case 1, are reviewed in Table 2. All 4 patients had right-sided pneumothorax and underwent laparoscopic surgery for treatment of endometriomas. Although 3 cases showed severe endometriosis with cul-de-sac obliteration, they underwent adhesiolysis and not bowel surgery, because no bowel endometriosis-related symptoms were observed (e.g., dyspareunia or dyschezia). There was no incidence of subcutaneous emphysema or pneumothorax. An endometriotic lesion on the diaphragm was intraoperatively detected by laparoscopy only in case 4. On postoperative day 1 or 3, pneumothorax occurred with chest pain, dyspnea, or a decrease in SpO_2. Pleural drainage of the right pleural cavity was performed in all patients. In cases 1 and 2, the pleural fluid was blood tinged; however, no information on the pleural fluid was present in the medical records of cases 3 and 4. There was no recurrence of pneumothorax in any of the cases.

Table 2

Characteristics and operative outcome of patients with postoperative pneumothorax

Patient number	1	2	3	4
Characteristics of patients				
Age (years)	33	39	45	49
Gravida	0	0	1	2
Parity	0	0	0	2
BMI	18.4	18.6	23.4	19.3
Major complaint	Infertility	Chronic pelvic pain	Infertility	Increase in size of endometrioma
Smoking	—	—	—	—
CA-125 (U/mL)	86	59	117	15
Operative outcome				
Disease	Endometrioma	Endometrioma	Endometrioma	Endometrioma
Laterality of ov cyst	Right	Bilateral	Bilateral	Right
Size of ov cyst	2.5 cm	Right 6 cm, left 5 cm	Right 5 cm, left 2 cm	Right 4 cm, left 2 cm
Operative procedure	Right cystectomy adhesiolysis	Bilateral cystectomy adhesiolysis	Bilateral cystectomy adhesiolysis	Right salpingo-oophorectomy
Obliteration of cul de sac	Complete	Partial	Complete	None
Re-ASRM score	106	65	116	32
Endometriosis in the diaphragm	–	–	–	+
Operation time (min)	105	125	135	86
Operative blood loss (mL)	Minimal	100	Minimal	Minimal
Operation date (date of menstrual cycle)	3	15	15	11
Onset of pneumothorax (POD)	1	1	1	3
Primary symptom	Chest pain, dyspnea	Chest pain, dyspnea	Decrease in SpO_2	Chest pain, dyspnea
Laterality of pneumothorax	Right	Right	Right	Right

BMI = body mass index; ov = ovarian; POD = postoperative day; Re-ASRM = Revised American Society for Reproductive Medicine Classification of endometriosis; SpO_2 = oxygen saturation measured by pulse oximetry.

As shown in Table 3, there was a significant difference in the incidence of pneumothorax between patients with and without endometriomas (0.36% [4 of 1097] vs 0.00% [0 of 1717], respectively). Surgical duration was significantly shorter for cases with endometriomas than those without, whereas there was no significant difference in age or total blood loss.

Discussion

In this study, the incidence of postoperative pneumothorax among the patients who underwent laparoscopic surgery for benign gynecologic disease was 0.14%. Of all patients who developed a pneumothorax, all 4 had undergone surgery for endometriomas, and all 4 were affected only in the right lung. The incidence of postoperative pneumothorax was 0.36%, which was significantly higher in the cases with endometriomas than those without endometriomas. To the best of our knowledge, this is the first report to identify a correlation between postoperative pneumothorax and endometriomas.

Graybill et al [7] reported an incidence rate of pneumothorax after gynecologic laparoscopic surgery of 0.24%, whereas Solomon et al. [12] reported no instance of postoperative pneumothorax after 723 laparoscopic hysterectomies. Moreover, in a previous retrospective study, risk factors for pneumothorax during laparoscopy were reported as prolonged surgical duration (>200 min) and increased maximum end-tidal carbon dioxide [8]. However, in the present study, none of the 4 patients with pneumothorax exhibited these risk factors; rather, all shared the characteristics of endometriomas and a right-sided pneumothorax. Notably, these features shared a resemblance with the characteristics of a catamenial pneumothorax reported in the right lung, with thoracic endometriosis associated with the presence of pelvic endometriosis [10,13,14]. Furthermore, a high incidence of pouch of Douglas obliteration in catamenial pneumothorax has been reported [14–16], and 3 of the 4 cases in our series had pouch of Douglas obliteration.

Catamenial pneumothorax, which is caused by thoracic endometriosis, is reported to account for approximately 25% of women with pneumothorax [9]. Furthermore, a recent study reported that 98.9% of thoracic endometriosis–related pneumothorax cases occurred in the right lung [11]. This report also documented that thoracic endometriosis–related pneumothorax has several distinctive features from spontaneous pneumothorax, which include right-sided pneumothorax, history of pelvic endometriosis, age older than 31 years, and no history of smoking. Using these 4 factors, they established a predictive scoring system for

Table 3

Comparison of perioperative outcome with or without endometrioma

Variable	Endometrioma (+)	Endometrioma (−)	p value
No.	1097	1717	
Age (yrs)	36.8 ± 6.7	37.4 ± 8.9	NS[a]
Parity	0.36 ± 0.70	0.50 ± 0.87	p = .004[a]
Operation time (min)	122.1 ± 53.1	130.5 ± 59.2	p = .0002[a]
Total blood loss (mL)	124.4 ± 201.5	120.8 ± 241.4	NS[a]
Postoperative pneumothorax (+)	4	0	p = .0123[b]

Statistical analysis was done by [a]Mann-Whitney U test or [b]χ^2 test.

distinguishing thoracic endometriosis–related pneumothorax from primary spontaneous pneumothorax with a reported sensitivity of 93.5%, a specificity of 89.4%, and a high accuracy (0.9665 of area under the receiver-operating characteristic curve). Scored by this system, all of our 4 cases received the highest marks (full score). This finding led us to speculate that the incidence of postoperative pneumothorax among these cases was correlated to the occurrence of thoracic endometriosis.

Given that these cases with postoperative pneumothorax were associated with thoracic endometriosis, we suspected 3 pathogenic mechanisms. In the first scenario, pneumoperitoneum led to an increase in intra-abdominal pressure, which caused an influx of carbon dioxide into the pleural cavity through a diaphragmatic defect caused by diaphragmatic endometriosis. The second scenario implies that the pneumoperitoneum led to an increase in airway pressure, which caused membrane disruption, thereby weakening the alveolar and visceral pleura by occult thoracic endometriosis. Afterward, the air was allowed into the pleural cavity. In this scenario, ventilation may have caused a further increase in airway pressure and disruption of the alveolar or visceral pleural membrane. A similar mechanism was described in a previously reported case, which noted the occurrence of a postoperative right-sided hemothorax after laparoscopic surgery for endometrioma [17]. In this case report, the authors detected active bleeding from an endometriotic lesion of the right diaphragm by thoracoscopy and speculated that the observed elevation in abdominal and thoracic pressures by pneumoperitoneum provoked a disruption of the membranes or vessels of the diaphragmatic endometriotic lesion, which appears to be in line with our hypothesis. The third possibility is that women with endometriomas may have right-sided diaphragmatic defects more frequently than those without endometriomas. Such defects may not be associated with thoracic endometriosis, but they may be associated with the same embryonic development process that laid down the endometriosis. This embryonic process caused a right diaphragm with holes or weak points, which may allow the carboperitoneum to enter the pleural cavity.

Among the presented cases with postoperative pneumothorax, we detected a diaphragmatic endometriotic lesion in only 1 of 4 patients. However, some diaphragmatic endometriotic lesions go undetected by the rigidity of conventional laparoscopy, which renders this modality sufficient to investigate only a part of the diaphragm [18]. Therefore, we could not exclude the possibility of diaphragmatic endometriosis in the other 3 cases.

Video-assisted thoracoscopy is very useful for the diagnosis and treatment of thoracic endometriosis because it can detect small lesions by magnification [19]. Nezhat et al reported the use of a combined approach of laparoscopy and thoracoscopy for pelvic and diaphragmatic endometriosis [15,20]. Fortunately, pneumothorax did not recur in our cases to this point. However, given that endometriosis can be a progressive condition and requires multiple surgeries during women's lifetime, subsequent treatment may be necessary when they develop pneumothorax or thoracic endometriosis.

A literature review retrieved 6 reports of pneumothorax during or after gynecologic laparoscopy [21–26]. Among these, 2 cases occurred in the bilateral lungs [21,22], and 4 cases occurred in the right lung [23–26]. Of these, 2 occurred during laparoscopic surgery for bilateral endometrioma or pelvic endometriosis [22,23], 2 for ovarian cysts with pelvic pain, although the endometrioma was not addressed [21,24], 1 during diagnostic laparoscopy for infertility [25], and 1 during laparoscopic-assisted vaginal hysterectomy for myoma [26]. According to the findings of these reports, some postoperative instances of pneumothorax may occur without endometriosis. However, these cases may have been associated with postoperative pneumothorax from other causes.

Our study had some limitations. First, this study was retrospective. Second, the incidence of postoperative pneumothorax was extremely low, which might have resulted in inadequate power to determine the incidence of postoperative pneumothorax and accurately identify the characteristics of patients. Therefore, future studies are required to further investigate other correlations.

In conclusion, we found that the incidence of postoperative pneumothorax in patients who underwent laparoscopic

surgery for benign gynecologic disease was quite low (0.14%). Furthermore, all of our 4 patients were diagnosed with endometriomas and developed right-sided pneumothorax postoperatively. Hence, the frequency of postoperative pneumothorax was significantly higher among cases with endometriomas than those without endometriomas. Thus, endometrioma should be recognized as a risk factor for postoperative pneumothorax in gynecologic laparoscopic surgery.

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ORIGINAL ARTICLE

Resveratrol Enhances Apoptosis in Endometriotic Stromal Cells

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Keywords
Anti-apoptosis, endometriosis, survivin, Therapy, TNF-α-related-apoptosis-inducing ligand

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Problem

Resistance to apoptosis, together with inflammatory and invasive activity, contributes to the pathogenesis of endometriosis; therefore, approaches that can safely enhance apoptosis in endometriotic tissue are highly sought after as a means of managing the disease. Although resveratrol (RVT) is known to induce apoptosis or increase sensitivity to apoptotic stimuli in various cancer cell types, its effect on human endometriosis has remained uncertain. This study aimed to investigate whether RVT induces or enhances apoptosis in human endometriotic stromal cells (ESCs).

Method of study

Endometriotic tissues were collected, during laparoscopies, from women affected by ovarian endometriosis. ESCs were prepared, cultured, and treated with RVT. Apoptosis was assessed by annexin V–PI staining. Survivin mRNA expression in ESCs was examined using RT-PCR. ESCs were pre-treated with or without RVT and then incubated with TNF-α-related-apoptosis-inducing ligand (TRAIL), which is a known pro-apoptotic molecule.

Results

RVT alone did not induce apoptosis in ESCs. RVT significantly reduced survivin mRNA expression ($P < 0.05$). Pre-treatment with RVT significantly enhanced TRAIL-induced apoptosis ($8.13 \pm 0.83\%$ (control) versus $29.19 \pm 7.39\%$ (pre-treated with RVT), $P < 0.05$).

Conclusion

This study indicates that RVT suppresses survivin expression and enhances TRAIL-induced apoptosis in ESCs.

Introduction

Endometriosis, defined as the presence of endometrial tissue exterior to the uterus, is a common gynecological condition that is associated with symptoms such as pelvic pain and infertility.[1,2] Endometriosis is estrogen-dependent, and it can afflict women during their reproductive years. To date, therapeutics for endometriosis are limited to hormone analogs, and therapeutics that do not interfere with ovulation are preferred to alternatives.

The pathogenesis of endometriosis[3,4] is known to be associated with inflammation, invasion, and resistance to apoptosis. Endometriosis escapes apoptosis despite the presence of apoptotic stimulants, such as TNF-α,[5,6] TNF-α-related-apoptosis-inducing ligand

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(TRAIL),[4,7] and FAS[8,9] in the lesion. Further, apoptosis-inducing agents such as TRAIL[4] and staurosporine[10] fail to have a marked effect on the survival of endometriotic stromal cells (ESCs). A promising strategy for controlling endometriosis could include enhancing endogenous or exogenous apoptotic stimulants in the lesion.

Resveratrol (RVT) is a phytoalexin polyphenol naturally produced by several plant species in response to bacterial and fungal infection.[11] The therapeutic effect of RVT on endometriosis was firstly described by Bruner-Tran et al. in 2011.[12] Many studies since have been conducted to verify the effect of RVT on endometriosis and to elucidate the mechanism of action. It has been shown that RVT controls the development of endometriosis by suppressing proliferation,[13,14] vascularization,[13–15] invasiveness,[12] and cell survival,[14] and by increasing apoptosis[14] in rodent models. Further, RVT has been shown to reduce invasiveness[12] in *in vitro* studies using ESCs. Our group previously demonstrated that RVT suppressed the TNF-α-induced inflammatory response in ESCs[16]; however, whether RVT induces apoptosis in endometriosis *in vitro*, to our knowledge, has not been confirmed.

RVT has been shown to stimulate apoptosis in various types of cells such as ovarian cancer cells.[17,18] Recently, reports indicate that RVT has the potential to increase sensitivity to apoptotic stimuli by suppressing the expression of anti-apoptotic molecules[19,20] in various cancer cell types. These findings encouraged us to investigate whether RVT induces or enhances apoptosis in ESCs.

Materials and methods

Patients and Samples

Endometriosis tissues were obtained during laparoscopies conducted on patients with ovarian endometriosis. Diagnosis of endometriosis was confirmed by histopathological examination. All patients reported regular menstrual cycles prior to laparoscopies, and they did not receive hormonal treatment for ≥6 months before surgery. The tissues were collected under sterile conditions and processed for primary cell culture. All experimental procedures used for this study were approved by the institutional review board at the University of Tokyo. Signed informed consent for the use of tissue was obtained from each patient.

Isolation and Culture of Endometriotic Stromal Cells

Isolation and culture of stromal cells from ovarian endometriosis tissues were processed as described previously.[16,21] Fresh tissues collected in sterile medium were rinsed to remove blood cells. The tissues were minced into small pieces and incubated in DMEM/F-12 (Gibco, Grand Island, NY, USA) with type I collagenase (0.25%; Sigma-Aldrich, St Louis, MO, USA) and deoxynuclease I (15 U/mL; TaKaRa, Tokyo, Japan) for 120 min at 37°C. The tissue was separated by filtration through nylon cell strainers (70 μm; BD, Franklin Lakes, NJ, USA), and cells passing through the strainer were collected and used as endometriotic stromal cells (ESCs). ESCs were resuspended in phenol red-free DMEM/F12 containing 5% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/L amphotericin B. At the first passage, ESCs were plated onto 6- or 12-well culture plates at a density of 2×10^5 cells/mL. TRAIL and RVT were purchased from Sigma-Aldrich. For apoptosis assays, ESCs were plated onto 6-well plates and incubated with RVT for 24 h followed by stimulation with TRAIL for 18–24 h. For mRNA analysis, ESCs were plated onto 12-well plates and incubated with RVT for 24 h. RVT was used at 40–120 μM and TRAIL at 100 ng/mL in this study.

Annexin V Flow Cytometry

Flow cytometric analysis was performed as reported previously.[4] ESCs were stained with annexin V and propidium iodide (PI) using the Annexin V-FITC Apoptosis Detection Kit I (Abcam, Cambridge, UK) according to the manufacturer's instructions. In brief, ESCs were resuspended in 300 μL of 1× binding buffer and incubated with 3 μL of annexin V-FITC and 3 μL of PI for 15 min at room temperature in darkness. ESC viability was analyzed using FACSCalibur (BD Biosciences, San Jose, CA, USA). Annexin V-positive cells were regarded as apoptotic cells.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA from ESCs was extracted using an RNeasy minikit (QIAGEN, Hilden, Germany). RNA was reverse transcribed using an RT-PCR kit (TOYOBO, Osaka, Japan) according to the manufacturer's

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instructions. qRT-PCR was performed using a LightCycler 480 (Roche Diagnostics GmbH, Mannheim, Germany). The expression of survivin mRNA was normalized against GAPDH mRNA (the internal standard). Primer pairs and the PCR conditions were as follows: for GAPDH, 5′- ACCACAGTCCATGCCAT CAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, 95°C for 10 s, 64°C for 10 s, and 72°C for 18 s; for survivin, 5′-GGACCACCGCATCTCTACAT-3′ and 5′- GC ACTTTCTTCGCAGTTTCC-3′, 40 cycles at 95°C for 10 s, 57°C for 10 s, and 72°C for 11 s. All PCRs were followed up with melting curve analysis.

Statistical Analysis

The data are presented as mean ± SEM. Statistical analyses were carried out using Student's *t*-test. A P value of <0.05 was considered as statistically significant.

Results

RVT did not Induce Apoptosis in ESCs

RVT induces apoptosis in various cancer cells, but it has been reported that ESCs are resistant to apoptotic stimuli.[4,10] Our examination of RVT effects on cell survival indicated a similar result. As shown in Fig. 1a, RVT did not change the proportion of annexin V-positive cells. The proportion of apoptotic cells to total cells was 5.03 percent (±0.52%) in the control group and 6.25 percent (±1.55%) in the RVT-treated cells, but the difference was not significant (Fig. 1b).

RVT Decreased Survivin mRNA Expression in ESCs

Survivin is known to be involved in resistance to apoptosis in endometriosis. To investigate the effect of RVT on survivin expression, ESCs were treated with RVT for 24 h and survivin mRNA levels were measured by qRT-PCR. Survivin mRNA levels were significantly reduced to 10.0 percent (±5.7%, $P < 0.05$) by RVT treatment (Fig. 2).

RVT Enhanced TRAIL-Induced Apoptosis

Several agents can accentuate the effect of pro-apoptotic molecules such as TRAIL.[22,23] ESC survival is not markedly affected by TRAIL.[4] To test whether

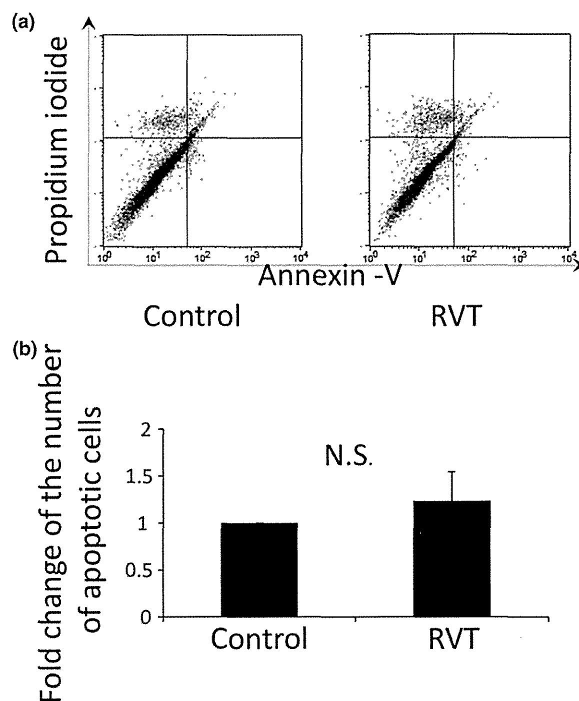

Fig. 1 Resveratrol (RVT) does not induce apoptosis in endometriotic stromal cells (ESCs). ESCs were cultured with or without (control) RVT for 24 hr. ESCs were stained with annexin V-FITC and propidium iodide (PI). Annexin V-positive cells were regarded as apoptotic cells. (a) A representative flow cytometry result is shown. (b) The bar graph indicates the fold change of the proportion of apoptotic cells to total cells. Data are mean ± SEM of three independent experiments using different ESC preparations. N.S. indicates 'no significant' difference.

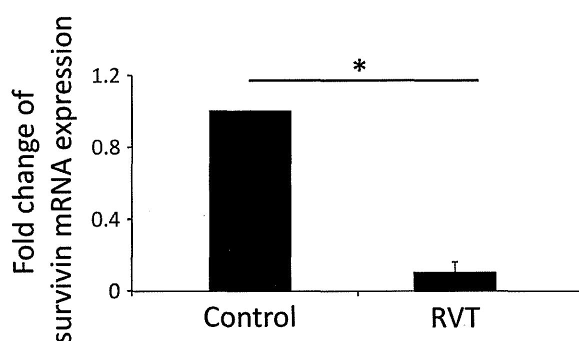

Fig. 2 Resveratrol (RVT) decreases survivin mRNA expression in endometriotic stromal cells (ESCs). ESCs were cultured with or without RVT for 24 hr. Survivin mRNA expression was measured using qRT-PCR and normalized against GAPDH expression. The bar graph indicates the fold change of survivin mRNA expression. Data are mean ± SEM of three independent experiments using different ESC preparations. *Indicates $P < 0.05$.

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RVT enhances the effect of TRAIL, ESCs were cultured with TRAIL for 18–24 hr, after pre-treating the cells with (or without) RVT for 24 hr. Pre-treatment with RVT enhanced apoptosis induced by TRAIL (Fig. 3a). Statistically, the proportion of apoptotic cells to total cells in the control cells was 5.32 percent (±0.67%) and 8.13 percent (±0.83%, $P < 0.05$ versus control) in the TRAIL-only cells, and 29.19 percent (±7.39%, $P < 0.05$ versus TRAIL only) in the RVT-pre-treated cells.

Discussion

This study shows that RVT alone does not induce apoptosis in ESCs; however, the results indicate that RVT reduces the expression of survivin in ESCs. In addition, this study clearly demonstrates that RVT enhances TRAIL-induced apoptosis in ESCs.

RVT did not induce apoptosis in ESCs, which is in contrast to findings reported in endometriosis mouse model.[14] The reason why RVT by itself induces apoptosis in this endometriosis mouse model,[14] whereas RVT by itself did not induce apoptosis in the current *in vitro* experiment may be explained by several reasons. Firstly, the apoptosis observed in endometriosis mouse model was not the direct result of apoptosis-inducing effects of RVT, but secondary results of RVT such as suppressing of vascularization[13–15] and invasiveness.[12] It is also possible that RVT induces apoptosis by enhancing putative endogenous pro-apoptotic factors in *in vivo* setting.

The absence of apoptosis-inducing effect of RVT in ESCs is also distinguished from findings reported in cancer cells.[17,24–26] Regarding the mechanism, RVT exerts its apoptosis-inducing effects by inhibiting various survival pathways such as PI3-kinase[27] and STAT3[28] and suppressing the expression of anti-apoptotic molecules such as survivin,[19,29] bcl-2,[30] and bcl-x.[20] The absent of apoptosis-inducing effects of RVT in ESCs may possibly related to the inherent anti-apoptotic property in endometriosis.

Having known that RVT by itself does not induce apoptosis in ESCs, we then asked whether RVT can reduce survivin. Survivin has been shown to be a key molecule in resistance to apoptosis in endometriosis. Watanabe et al[10] conducted a comprehensive gene-profile analysis and found that

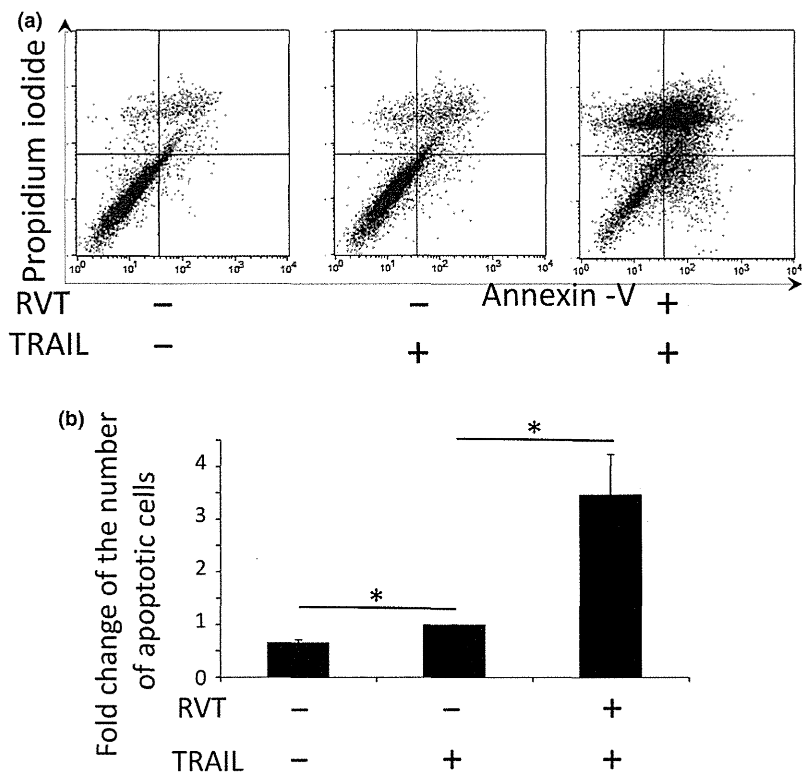

Fig. 3 Resveratrol (RVT) enhances TRAIL-induced apoptosis in endometriotic stromal cells (ESCs). ESCs were pre-treated with or without RVT for 24 hr and then cultured with TRAIL for 18–24 hr. ESCs were stained with annexin V-FITC and propidium iodide (PI). Annexin V-positive cells were regarded as apoptotic cells. (a) A representative flow cytometry result is shown. (b) The bar graph indicates the fold change of the proportion of apoptotic cells in total cells. Data are mean ± SEM of six independent experiments using different ESC preparations. *Indicates $P < 0.05$.

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