Tunicamycin enhances the apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand in endometriotic stromal cells

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BACKGROUND: The increase in concentration of osteoprotegerin, an antagonist of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), in the peritoneal fluid of women with endometriosis may interfere with TRAIL-induced apoptosis in endometriotic cells and promote the development of endometriosis. In the present study, the effect of tunicamycin, a possible apoptosis enhancer, on TRAIL-induced apoptosis in endometriotic stromal cells (ESC) was determined.

METHODS: ESC were isolated from cyst walls of ovarian endometrioma and cultured. ESC were incubated with or without tunicamycin (2 \( \mu \)g/ml) for the first 16 h, and then incubated with or without TRAIL (200 ng/ml) for the following 24 h. To examine whether caspases were involved in TRAIL-induced apoptosis, z-VAD-fmk (30 nM), a general caspase inhibitor, was added 1 h before TRAIL treatment. ESC were transfected with small interfering RNA (siRNA) for DR5, a receptor of TRAIL, before tunicamycin treatment to evaluate its role in ESC. DR5 mRNA level was determined by quantitative RT–PCR. Apoptosis in ESC was evaluated by flow cytometry.

RESULTS: Tunicamycin increases both DR5 mRNA (P < 0.005) and TRAIL-induced apoptosis (P < 0.0001) in ESC. The increase in TRAIL-induced apoptosis in ESC by tunicamycin was suppressed (P < 0.05) by z-VAD-fmk. Transfection with DR5 siRNA suppressed the tunicamycin-induced increase in DR5 mRNA and abrogated the up-regulation of TRAIL-induced apoptosis by tunicamycin.

CONCLUSIONS: The combined treatment with tunicamycin and TRAIL may have therapeutic potential in the treatment of endometriosis.

Key words: endometriosis / apoptosis / tunicamycin / tumor necrosis factor-related apoptosis-induced ligand

Introduction

Endometriosis, defined by the presence of viable endometriotic tissue outside the uterus, is an enigmatic disease. It deteriorates the health of women of reproductive age, and there is no ideal therapeutic treatment for the disease due to a lack of knowledge of its etiology (Momoeda et al., 2002; Osuga et al., 2002). Implantation and growth of endometrial cells from the overflow of menstrual blood...
into the peritoneal cavity is a widely accepted hypothesis for the pathogenesis of endometriosis. However, it is unclear why only a fraction of women develop endometriosis while retrograde menstruation is observed in most women.

Several lines of evidence indicate that a failure of apoptosis of ectopic and eutopic endometrial cells is a possible cause of endometriosis (Beliard et al., 2004; Harada et al., 2004b). Reduced apoptosis may be due to the decreased sensitivity of the endometrial and endometriotic cells to apoptotic stimuli, and/or impaired apoptotic stimuli to these cells in women with endometriosis. In this context, some studies have indicated that the concentration of osteoprotegerin (OPG) is elevated in the peritoneal fluid of women with endometriosis (Harada et al., 2004a; Bersinger et al., 2006). Since OPG has an antagonistic effect on tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), TRAIL-induced endometriotic cell apoptosis may be attenuated, thereby allowing endometriosis to develop in these women.

Recently, enhancement of TRAIL-induced apoptosis by tunicamycin, via induction of endoplasmic reticulum (ER) stress, has been reported in colon and prostate cancer cells, and melanoma cells (Jin et al., 2004; Shiraishi et al., 2005; Jiang et al., 2007). These studies suggest that tunicamycin-induced sensitization may be a promising strategy in cancer therapy. Meanwhile, the enhancement of TRAIL-induced apoptosis by tunicamycin might also be a unique therapy for endometriosis given that reduced apoptotic status of endometriotic cells contributes to the development of the disease. The current study investigates the effect of tunicamycin on endometriotic stromal cells (ESC), evaluating ER stress by mRNA expression of spliced XBP1 (sXBP1), a marker of ER stress (Ron and Walter, 2007), and apoptosis by flow cytometry analysis.

Materials and Methods

Reagents and materials

Type I collagenase, antibiotics (a mixture of penicillin, streptomycin and amphotericin B) and tunicamycin were purchased from Sigma (St Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium was obtained from Gibco (Grand Island, NY, USA). Charcoal-stripped fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). Deoxynuclease I (DNase I), 0.25% Trypsin–EDTA, Lipofectamine RNAi max and Opti-MEM I were from Invitrogen (Carlsbad, CA, USA). TRAIL was purchased from Peprotech (Rocky Hill, NJ, USA). General caspase inhibitor Z-Val-Ala-Asp(OMe)-CH2F (z-VAD-fmk) was purchased from Calbiochem (San Diego, CA, USA).

Sample collection

Endometriotic tissues were obtained from patients with ovarian endometriomas undergoing laparoscopy. Final diagnosis of ovarian endometrioma was confirmed by histopathological examination. Eutopic endometrial tissues were collected by curettage. Collected tissues were transported to the laboratory under sterile conditions and processed for the experiments. All women had regular menstrual cycles, and none had received hormonal treatment for at least 6 months before surgery. The institutional review board of the University of Tokyo approved the experimental procedures, and signed informed consent for the sample use was obtained from each patient.

Isolation and culture of ESC

The isolation and culturing of human ESC was performed as described previously (Hirotta et al., 2005a). Briefly, endometriotic tissues were minced into small pieces and incubated in DMEM/F12 containing type I collagenase (0.25%) and DNase I (15 IU/ml) for 2 h at 37°C. Dispersed endometriotic cells were separated by filtration through a 100-μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ, USA) and 70-μm nylon cell strainer. ESC in the filtrate were collected by centrifugation (250 g, 4 min, twice), resuspended in DMEM/F12 containing 5% FBS and antibiotics and plated on 100-mm culture dishes (Iwaki, Tokyo, Japan). Dishes were kept at 37°C in a humidified 5% CO2/95% air atmosphere for 1 or 2 days before the first passage. At the first passage, ESC were plated into 12-well plates at 1 × 105 cells/well for RT–PCR and small interfering RNA (siRNA) experiments, or 6-well plates at 4 × 105 cells/well for flow cytometry. The purity of ESC was more than 95%, according to positive cellular staining for vimentin (stromal cells) and negative cellular staining for cytokeratin (epithelial cells), CD45 (monocytes and other leukocytes) and von Willebrand factor (endothelial cells).

Small interfering RNA

ESC seeded at 1 × 105 cells per well in 12-well plates 1 day before transfection reached 70–80% confluence on the day of transfection. The siRNA constructs used were obtained as ON-TARGET plus SMARTpool DR5 (L-004448-00-0005) from Dharmacon. The non-targeting siRNA control, ON-TARGET plus siCONTROL non-targeting pool (D-001810-10-05) was also obtained from Dharmacon. Cells were transfected with 50 nmol/l siRNA for 24 h in Opti-MEM I with 5% FBS media using Lipofectamine RNAi max according to the manufacturer’s protocol. After transfection, media were removed and replaced with fresh media containing 1% charcoal-stripped FBS and antibiotics. After incubation for a further 12 h, cells were treated with tunicamycin and TRAIL as described above. For this experiment, samples from two women were used.

Treatment of ESC

When ESC reached 70–80% confluence in 1 or 2 days, media was removed and replaced with fresh media containing 1% charcoal-stripped FBS and antibiotics. After culturing for an additional 12 h, the cells were ready for use in the experiments. To see the effect of tunicamycin on mRNA levels of sXBP1, a marker of ER stress, ESC were incubated with 2 μg/ml tunicamycin for 0, 1, 3, 6 and 12 h. To examine the effect of tunicamycin on mRNA expression of DR5, a receptor of TRAIL, ESC were incubated with 2 μg/ml tunicamycin for 0, 1, 3, 6 and 12 h. For each experiment, samples from three women were used. DR4, another TRAIL receptor, was not studied because its expression had not been detected in ESC (Harada et al., 2004a). To see the effect of tunicamycin on TRAIL-induced apoptosis in ESC, ESC were incubated with or without tunicamycin (2 μg/ml) for the first 16 h, and then incubated with or without TRAIL (200 ng/ml) for the following 24 h. We selected the dose of TRAIL in reference to a recent study (Jiang et al., 2007), whereas a lower dose (25 ng/ml) is used in another study (Shiraishi et al., 2005). During the treatment, either the pan-caspase inhibitor z-VAD-fmk (30 μM), or a vehicle control was added 1 h before TRAIL treatment to examine whether caspases were involved in TRAIL-induced apoptosis in ESC. For this experiment, samples from two women were used.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from cultured ESC using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Total RNA was extracted from eight...
samples of eutopic endometrial tissues and eight samples of endometriotic tissues by the acid guanidinium–phenol–chloroform method using Isogen (Nippongene, Toyama, Japan). One microgram of total RNA was reverse transcribed in a 20-μl volume using Rever Tra Ace (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions. Real-time quantitative PCR was performed as previously reported (Hirata et al., 2008) to assess sXBP1 and DR5 mRNA, and data analyses were performed using a Light Cycler (Roche Applied Science, Mannheim, Germany). sXBP1 and DR5 mRNA levels were normalized to RNA loading for each sample using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. PCR primers were purchased from TOYOBO and are as follows: sXBP1 primers (sense, 5′-GAGTTAAAGACGCCGCTG-GG-3′; antisense, 5′-ACTGGCCTGACCTGTGGCG-3′) amplify a 118 bp fragment; DR5 primers (sense, 5′-TGCAGCCTAGTCTTTGATTG-3′; antisense, 5′-GCACCAAGTCGCAAAGCTCA-3′) amplify a 389 bp fragment; GAPDH primers (sense, 5′-ACCACAGTCCATGCACATGC-3′; antisense, 5′-TCCACCACCTGGTGTGTA-3′) amplify a 452 bp fragment. For real-time quantitative PCR, the conditions were as follows: for sXBP1, 28 cycles at 95°C for 10 s, 70°C for 10 s, 72°C for 5 s; for DR5, 28 cycles at 95°C for 10 s, 64°C for 10 s, 72°C for 16 s; for GAPDH, 25 cycles at 95°C for 10 s, 64°C for 10 s, 72°C for 18 s. All PCR experiments were followed by melting curve analysis.

Flow cytometry

Flow cytometric analysis was performed as reported previously (Hirota et al., 2006). Apoptosis of ESC was assessed by double staining [annexin V and propidium iodide (PI)] using the Annexin V-FITC Apoptosis detection kit I (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s instructions. Briefly, ESC were detached by 0.25% Trypsin–EDTA, washed twice with phosphate-buffered saline, and resuspended in 1× Binding Buffer at a concentration of 1 × 10^6 cells/ml. One hundred microliters of each sample solution were transferred to a 5 ml culture tube, 5 μl of annexin V-FITC and 2 μl of PI were added and the tubes incubated for 15 min at room temperature in the dark. After incubation, 400 μl of 1× Binding Buffer was added to each sample tube and the samples analyzed by FACSCalibur and Cell Quest Pro (BD Biosciences, San Jose, CA, USA). Annexin V-positive cells were regarded as apoptotic cells.

Isolation, culture and treatment of eutopic endometrial cells

The isolation and culture of eutopic endometrial cells of women with endometriosis (n = 4) or without endometriosis (n = 3) were performed according to the method that we have been using (Harada et al., 2005; Hirota et al., 2005b; Takeamura et al., 2006). In the same way as the treatment of ESC, eutopic endometrial cells were incubated with or without tunicamycin (2 μg/ml) for the first 16 h, and then incubated with or without TRAIL (200 ng/ml) for the following 24 h in order to see the effect of tunicamycin on TRAIL-induced apoptosis in eutopic endometrial cells.

Statistical analysis

Data were evaluated using analysis of variance with post hoc analysis (Fisher’s protected least significance) for multiple comparisons. P < 0.05 were considered statistically significant.

Results

As shown in Fig. 1, treatment of ESC with tunicamycin increased mRNA levels of sXBP1, with a maximal increase of 7.8-fold compared with the basal level observed at 6 h. Tunicamycin also increased the gene expression of DR5, which reached 3.7-fold higher levels compared with the basal level at 12 h (Fig. 2).

Fig. 3A depicts representative flow cytometry data of ESC that were untreated (control) or treated with tunicamycin followed by TRAIL, clearly indicating increased apoptosis in the treated ESC. As shown in Fig. 3B, the percentage of apoptotic cells in control ESC was 4.7%, and TRAIL alone did not increase apoptosis in ESC. In contrast, pretreatment of ESC with tunicamycin followed by TRAIL treatment significantly increased apoptosis to 74.6%, while the addition of z-VAD-fmk reduced it to 26.1%. When control ESC were treated with tunicamycin alone, the percentage of apoptotic cells appeared slightly increased (11.8%).

In view of these findings, it was speculated that the increase in apoptosis following treatment of ESC by tunicamycin and TRAIL was a result of a tunicamycin-induced increase in DR5 expression. Knockdown of DR5 expression using DR5 siRNA dramatically reduced DR5 mRNA levels in tunicamycin-stimulated ESC (Fig. 4A). Importantly, DR5 siRNA also inhibited the increase in TRAIL-induced apoptosis in tunicamycin-treated ESC, whereas negative control siRNA did not affect the TRAIL-induced increase in apoptosis (Fig. 4B and C).

Fig. 5 shows DR5 mRNA levels in eutopic endometrial tissues and endometriotic tissues of women with endometriosis. DR5 mRNA was

![Figure 1](https://example.com/f1.png)

**Figure 1** Tunicamycin-induced gene expression of sXBP1 in endometriotic stromal cells (ESC). ESC were cultured with tunicamycin for 0–12 h. Total RNA isolated from ESC was reverse transcribed, amplified by real-time PCR and representative amplified products are shown. The increase in levels of RNA was calculated by subtracting the signal threshold cycles of the internal standard (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) from the threshold cycles of sXBP1. Values are the mean ± SEM of three independent experiments using samples from three different women. *P < 0.05 versus 0 h.
at significantly higher levels in eutopic endometrial tissues than in endometriotic tissues.

In order to supplement the present study, we examined apoptosis induced by tunicamycin and TRAIL in eutopic endometrial cells of women with and without endometriosis (Table I). In eutopic endometrial cells of women without endometriosis, tunicamycin in combination with TRAIL showed no additive effect on tunicamycin alone, which induced an increase of apoptosis compared with the control. In eutopic endometrial cells of women with endometriosis, tunicamycin tended to increase TRAIL-induced apoptosis, though the increased levels seem to be quite low compared with those observed in ESC.

Discussion

The present study has demonstrated that the addition of tunicamycin to ESC increases the production of sXBP1 mRNA, a marker of ER stress, and the mRNA levels of DR5, a typical proapoptotic receptor of TRAIL. A substantial increase in apoptosis was observed upon the addition of TRAIL to ESC pretreated with tunicamycin, and apoptosis was significantly suppressed by the treatment of ESC with the general caspase inhibitor, as well as DR5 siRNA.

Reduced apoptosis of endometrial and/or endometriotic cells has been noted as a possible mechanism of the development of endometriosis. This implies that the induction of apoptosis in these cells may suppress the progress of the disease. GnRH analogues are currently widely used to treat endometriosis, and GnRH analogue-induced apoptosis of endometriotic cells has been observed in vivo and in vitro (Imai et al., 2000; Meresman et al., 2003). However, GnRH analogue treatment has various serious side effects, such as inducing a hypostrogen state in the patient, and alternative treatment that does not affect the hormonal status of the patient is required.

Recent studies have identified compounds that regulate apoptosis in ESC, and several of these increase apoptosis of endometriotic cells. Their usefulness for endometriosis therapy is currently being evaluated (Nasu et al., 2005, 2007; Wang et al., 2005).

The present study has demonstrated that tunicamycin has distinctive characteristics among the drugs that induce apoptosis of endometriotic cells. Given that the peritoneal environment of endometriotic women interferes with the proapoptotic function of TRAIL, as shown by the increase in OPG concentrations (Harada et al. 2004a; Bersinger et al., 2006) and the resultant decrease in TRAIL/OPG ratio in the peritoneal fluid of endometriotic women, an agent that sensitizes endometriotic cells to TRAIL-induced apoptosis could be highly effective for the treatment of endometriosis. Tunicamycin, in combination with TRAIL, may be that agent since it substantially increased apoptosis in ESC. The combined treatment with...
Tunicamycin and TRAIL may have therapeutic potential in the treatment of endometriosis. However, careful evaluation of low doses of tunicamycin in combination with TRAIL is required before in vivo investigations are carried out because tunicamycin induces varying degrees of apoptosis in normal cells, such as melanocytes, fibroblasts and human umbilical venous endothelial cells (Jiang et al., 2007). Endometriotic tissues are in a state of low apoptosis (Harada et al., 2004b) and are generally resistant to drug-induced apoptosis (Izawa et al., 2006). In light of a genome-wide study which showed that pro-apoptotic genes are down-regulated in endometriotic tissues as compared with eutopic endometrial tissues (Animoto et al., 2003), genetic differences between endometriotic cells and eutopic endometrial cells may contribute to the resistance of the former to apoptosis. In this context, decreased levels of DR5 mRNA in endometriotic tissues compared with eutopic endometrial tissues is a notable finding, and up-regulation of DR5 by tunicamycin may be a reasonable approach to sensitizing ESC to apoptotic stimulation by TRAIL.

Tunicamycin is a typical inducer of ER stress, a cellular stress response to perturbations in the protein folding functionality of the ER (Xu et al., 2005). ER stress activates the unfolded protein response, which is mediated by the activation of three signal transduction cascades originating from the three ER transmembrane proteins PERK, ATF6 and IRE1. Activated IRE1 splices XBP1 to produce sXBP1. In this study, the tunicamycin-induced increase of sXBP1 mRNA in ESC indicates enhanced ER stress and activation of IRE1. A recent study has shown that suppression of the IRE1 signal transduction pathway inhibited tunicamycin-induced up-regulation of DR5 in one melanoma cell line but not in another melanoma cell line, implying a diversity of pathways regulating tunicamycin-induced DR5 expression (Jiang et al., 2007). We have also shown that knockdown of IRE1 using siRNA inhibited the increase of sXBP1, but did not suppress the increased expression of DR5 and the increased apoptosis in ESC (data not shown). Tunicamycin-induced up-regulation of DR5 in ESC appears to be mediated by a pathway other than the IRE1 pathway.

**Figure 4** Effect of DR5 small interfering RNA (siRNA) on TRAIL-induced apoptosis of ESC pretreated with tunicamycin. First, ESC were transfected with the control, or DR5 siRNA for 24 h [siRNA (--), mock transfection; NC siRNA, negative control siRNA transfection; DR siRNA, DR siRNA transfection]. Subsequently, ESC were cultured with or without tunicamycin (2 μg/ml) for 16 h, followed by treatment with or without TRAIL (200 ng/ml) for 24 h. Apoptosis of ESC was analyzed by flow cytometry on 5 × 10⁴ ESC that were double stained (annexin V and PI). Annexin V-positive cells, both dead and live, were regarded as apoptotic cells. (A) DR5 mRNA in ESC after tunicamycin treatment for 16 h. Amplification of GAPDH was used as a reference for determining RNA quality and amounts. (B) Representative flow cytometry data of ESC after treatment with TRAIL for 24 h. (C) Percentage of apoptotic cells after each treatment. Values are the mean ± SEM of three independent experiments using samples from three different women. *P < 0.005 versus all others.
In women with endometriosis, tunicamycin in combination with TRAIL appeared to enhance the apoptosis of eutopic endometrial cells. However, the levels of apoptosis induced by tunicamycin with TRAIL in the eutopic endometrial cells were low, about a third of that observed in ESC. These findings suggest that ESC are more sensitive to the tunicamycin-enhanced TRAIL-induced apoptosis than eutopic endometrial cells. Whether this characteristic might have any relevance to the pathophysiology of endometriosis would be a matter of future study.

In summary, tunicamycin induces ER stress and increases levels of DRS mRNA, a death receptor for TRAIL, in ESC. Tunicamycin also substantially increases TRAIL-induced apoptosis in ESC in a caspase-dependent manner, and the effect is mediated by increasing DRS mRNA.

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