TABLE 1

Reported cases of conjoined twins after assisted reproduction.

Case no.	Age	Gravity	Parity	Day of embryo transfer	Type of cycle	Treatment	Assisted hatching	No. of embryos transferred	No. of gestational sacs	No. of fetuses	Gestational age at disgnosis of CTs	Selective termination	No. of newborns	Result	Author
1	27	ND	0	ND	Fresh	IVF	–	2	2	3	10w	+	1	NVD	Boulot et al. (13)
2	35	ND	2	Day 3	Fresh	IVF	+	4	2	3	12w	+	1	Ongoing in the third trimester	Skupski et al. (18)
3	28	1	0	ND	Fresh	ICSI	–	3	2	3	8w4d	+	1	NVD (37w)	Goldberg et al. (15)
4	30	0	0	Day 3	FET	ICSI	+	2	2	3	10w	–	1	Abortion (12w), NVD (38w)	Sugawara et al. (19)
5	37	2	1	ND	FET	ICSI	–	4	3	4	11w3d	+	1	Elective C/S (38W)	Maymon et al. (16)
6	38	0	0	Day3	Fresh	ICSI	+	3	3	4	11w3d	+	2	Elective C/S (38w)	Allegra et al. (12)
7	36	0	0	Day 3 and 5	Fresh	IVF	–	3	1	2	10w	NA	0	Termination (11w)	Shimizu et al (17)
8	30	2	0	ND	Fresh	ICSI	–	2	1	2	28w	NA	1	C/S (30w, neonatal death)	Fujimori et al. (14)

Note: Cases 1–6 is in quadruplet or triplet pregnancy. Cases 7 and 8 were single gestational sac. C/S = cesarean section; FET = frozen-thawed embryo transfer; ICSI = intracytoplasmic sperm injection; NA = not applicable; ND = not documented; NVD = vaginal delivery.

Hirata. Conjoined twins after ART. Fertil Steril 2008.

To date, eight cases of CT after ART have been previously reported in the literature (Table 1) (12–19). A notable finding is that seven (including the present case) out of nine cases of CT involved ICSI or assisted hatching (AHA): four cases with ICSI, one case with AHA, and 2 cases with both ICSI and AHA. These techniques introduce small openings or thin areas in the zona pellucida that may cause herniation of the embryo through these defects. The embryo may also get pinched off and divide, forming identical twins and contributing to the development of CT. This has also been suggested to explain the higher rate of MZT that occurs after zona manipulation (20), such as ICSI (21, 22) and AHA (10, 23).

The zona pellucida may also be susceptible to in vitro culture conditions. Prolonged in vitro culture may harden the zona pellucida, contributing to the formation of MZT (10). Supporting evidence indicates that a high rate of MZT occurred after blastocyst stage transfer compared with day 3 embryo transfer (4–6). Regarding CT, to our knowledge there is only one earlier report in the literature documenting CT derived from blastocyst transfer (Table 1, case 7). However, in that case, it is unclear whether CT were derived from blastocyst or from day 3 embryos, because they report a unique technique in which two-step embryo transfer was performed. Therefore, we believe the present case is the first report of CT derived from blastocyst transfer.

The incidence of monozygotic splitting in pregnancies with two or more gestational sacs has been reported to be higher than that of pregnancies with a single gestational sac (5, 11). Similarly, the incidence of CT may increase in pregnancies with two or more gestational sacs than with a single gestational sac. Although we have very few published case reports available, supporting evidence indicates that two out of nine cases of CT were derived from a single gestational sac, whereas seven out of nine cases of CT were derived from two or three gestational sacs.

The present case was diagnosed by transvaginal sonography at 8.0 weeks' gestation, earlier than any other cases previously reported. The early diagnosis of CT by careful sonography permits the choice of selective termination in triplet or more pregnancy of different chorionicity. However, we did not know the best timing for selective termination, because the incidence of spontaneous abortion in conjoined twins is unknown. In this case, the conjoined twins had spontaneous cardiac arrest at 10 weeks 3 days of gestation. As shown in Table 1, it has been reported that irrespective of selective termination or spontaneous abortion of CTs, the subsequent course of residual viable fetus was uneventful.

In conclusion, to our knowledge, we report the first case of CT in a triplet pregnancy after ICSI and blastocyst transfer. Review of the literature and the present case suggests that ART might increase the incidence of CT, possibly owing to the manipulation of the zona pellucida. More research is needed to understand the mechanisms by which CT are formed with blastocyst transfer.

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Expression and possible implication of growth hormone–releasing hormone receptor splice variant 1 in endometriosis

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Objective: To determine possible involvement of splice variant 1 (SV1), a variant of the pituitary growth hormone-releasing hormone (GHRH) receptor, in the development of endometriosis.
Design: Comparative and laboratory study.
Setting: University teaching hospital reproductive endocrinology and infertility practice.
Patient(s): Eutopic and ectopic endometrial tissues, and peritoneal bone marrow-derived cells were collected from women with or without endometriosis. Normal ovarian tissues were collected from women without endometriosis.
Intervention(s): Ectopic endometrial stromal cells (ESC) were isolated and cultured with or without GHRH.
Main Outcome Measure(s): Gene expression of *GHRH* and *SV1* in the sample tissues was determined by reverse transcriptase (RT) nested polymerase chain reaction (PCR). Cyclic adenosine monophosphate (cAMP) production and 5-bromo-2'-deoxyuridine (BrdU) incorporation in ESC were measured using specific assay systems.
Result(s): We detected *SV1* messenger RNA (mRNA) in 17 out of 27 (63%) ectopic endometrial tissues, which was statistically significantly higher than that detected in eutopic endometrial tissues (2 out of 47, 4%) and normal ovarian tissues (0 out of 14). A relatively low rate of *GHRH* mRNA was detected in ectopic endometrial tissues (6 out of 27, 24%) and in eutopic endometrial tissues (12 out of 47, 26%). In contrast, relatively high rates were detected in normal ovarian tissues (14 out of 14, 100%) and peritoneal bone marrow-derived cells (13 out of 16, 81%). We found that GHRH stimulated the production of cAMP and the incorporation of BrdU in SV1-expressing ESC.
Conclusion(s): GHRH and SV1 may play a role in promoting the development of endometriosis. (Fertil Steril® 2008;■:■–■. ©2008 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, GHRH, SV1, proliferation

Endometriosis, defined as the presence of endometrium-like tissues outside the uterus, is one of the most common benign gynecologic disorders. The most widely accepted etiology of the disease is that endometrial tissues in retrograde menstrual flux implant and grow in the peritoneum. However, ectopic endometrium differs from eutopic endometrium, as demonstrated by gene expression analysis (1, 2). The specific characteristics of ectopic endometrium and the local environment surrounding it have been suggested to play important roles in the development of endometriosis.

Growth hormone-releasing hormone (GHRH), a peptide hormone of 42–44 amino acids, was originally thought to be secreted in the hypothalamus and to exert its actions on the pituitary gland. It is a well-established fact that GHRH stimulates the synthesis and secretion of growth hormone and regulates the proliferation and differentiation of pituitary somatotrophs (3). It is interesting that GHRH expression has also been demonstrated in normal human extrapituitary tissues and various tumors, suggesting a broader biological role for this peptide (4). An accumulating body of evidence indicates that GHRH functions as an autocrine growth factor for many neoplasms (5–7). The mitogenic effect of GHRH is mediated by a protein encoded by splice variant 1 (SV1), a splice variant of the GHRH receptor, which is highly similar to the pituitary GHRH receptor (8–14). Binding of GHRH to SV1 can stimulate adenylate cyclase to produce cyclic adenosine monophosphate (cAMP), a common secondary messenger of the pituitary GHRH receptor (8, 10, 13, 15).

We had reported previously that gonadotropin-releasing hormones (GnRH) may play a role as local regulators in the development of endometriosis (16). Others have also shown that corticotrophin-releasing hormone may be involved locally in the progression of the disease (17). These findings imply that hypothalamic hormones are involved in the pathogenesis of endometriosis. It is interesting that the expression of *GHRH* mRNA has been detected in normal endometrial tissue

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(18, 19), although the implication of this finding remains to be elucidated. We therefore speculated that GHRH might stimulate the progression of endometriosis in a similar way to that observed in many tumors that express SV1. In this study, we first explored the messenger RNA (mRNA) expression patterns of *GHRH* and the different splice variants of the *GHRH* receptor in eutopic and ectopic endometrial tissues. Then we used a primary culture system to evaluate the effects of GHRH on cAMP production in ectopic endometrial stromal cells (ESC) and on the proliferation of ESC.

MATERIALS AND METHODS

Sample Collection

We collected ectopic endometrial tissues from the walls of ovarian endometriomas from 27 women (age range: 36.4 ± 7.0 years, mean ± SD) during laparoscopic surgery. Peritoneal fluid was aspirated from the pouch of Douglas immediately after insertion of the trocar to minimize contamination with blood. Eutopic endometrial tissues were also sampled during the operations. All of the patients had regular menstrual cycles. None had received hormone treatment in the 6 months preceding surgery or had undergone previous ovarian surgeries. According to the revised American Society for Reproductive Medicine classification of endometriosis, all of the women with endometriosis were classified as stages III/IV. For the control samples, normal endometrial tissues were collected from women (age range: 34.4 ± 7.9 years) with uterine fibroids without deformity of the endometrial cavity (n = 10), ovarian dermoid cysts (n = 8), or ovarian simple cyst (n = 2). Thirteen samples were obtained during the proliferative phase and 14 samples during the secretory phase in the endometriosis group, and 9 samples were obtained during the proliferative phase and 11 samples during the secretory phase in the control group. The days of menstrual cycle when the samples were taken were distributed similarly between the groups. Additional control samples were also collected and included normal ovarian tissues from 14 women (age range: 32.7 ± 8.9 years) undergoing laparoscopic ovarian cystectomy for dermoid cysts; after complete removal of the dermoid cysts, samples of normal ovarian tissue were obtained from the ovaries. Informed written consent for the use of the specimens was obtained from each patient, and consent forms and experimental protocols were approved by the University of Tokyo's institutional review board.

Ectopic endometrial tissues obtained for cell culture were placed in Dulbecco minimal essential medium (DMEM)/F12 medium (GIBCO-BRL/Invitrogen, Grand Island, NY) on ice and transported to the laboratory immediately. Eutopic and ectopic endometrial tissues and ovarian tissues obtained for reverse transcriptase polymerase chain reaction (RT-PCR) analysis were snap-frozen in liquid nitrogen immediately after sampling and stored at −80°C.

Isolation and Purification of Peritoneal Bone Marrow–Derived Cells

Grossly hemorrhagic peritoneal fluid specimens were discarded. Peritoneal bone marrow–derived cells were collected using a method previously described elsewhere (20), in which more than 90% of the cells stained positive for mouse monoclonal anti-human CD45 (leukocyte common antigen). Briefly, the collected peritoneal fluid was centrifuged at 200 × *g* for 5 minutes, and after the supernatant was removed, the cell pellet was resuspended in phosphate-buffered saline (PBS), layered onto Ficoll-Paque (Amersham Biosciences, Piscataway, NJ) and centrifuged at 150 × *g* for 30 minutes. Peritoneal bone marrow–derived cells were recovered from the interface.

Isolation and Culture of Human ESC

We purified ESC from ovarian endometriotic tissues and cultured them as described previously elsewhere (21, 22). The fresh endometriotic lesions were collected in sterile medium and dissected away from the underlying parenchyma. Approximately 1.0 to 1.5 g of the tissue was then minced into small pieces and incubated in DMEM/F12 (GIBCO-BRL) with 2.5 mg/mL type I collagenase (Sigma, St Louis, MO) and 15 U/mL deoxyribonuclease 1 (Takara, Tokyo, Japan) for 2 hours at 37°C with agitation. The resulting suspension was separated by serial filtration. Debris was removed with a 100-μm nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ), and some epithelial glands were eliminated with a 70-μm nylon cell strainer (Becton Dickinson). Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in DMEM/F12, plated onto 100-mm dishes (Iwaki, Chiba, Japan) and allowed to adhere at 37°C for 30 minutes, after which nonadhering epithelial cells and blood cells were removed with PBS rinses. The cells were cultured in DMEM/F12 supplemented with 10% charcoal-stripped fetal bovine serum (Hyclone, Logan, UT) and antibiotics (Sigma). The purity of the ESC population was greater than 95%, as confirmed by positive immunocytochemical staining for vimentin. When the cells reached confluence, they were used for experiments at the first passage.

RT-nested PCR for Detection of GHRH and Splice Variants (SVs) of the GHRH Receptor

Total RNA was extracted from tissues and cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). One microgram of total RNA in a total volume of 20 μL was reverse transcribed and then amplified using the ReverTra Dash RT-PCR kit (Toyobo, Osaka, Japan) following the manufacturer's protocol. Gene-specific primers were synthesized by Sigma Genosys, and the sequences are listed in Table 1. To investigate the existence of *GHRH* receptor SVs and to improve the specificity and sensitivity of amplification, nested PCR was carried out as described previously elsewhere (14). For *GHRH* receptor SVs, a primary amplification using primers I3-1 and E12 (0.3 μM each) was performed using the following cycling conditions: 94°C for 5 minutes followed by 30 cycles consisting of 94°C for 30 seconds, 60°C for 2 seconds, and 74°C for 70 seconds. Subsequently, 2 μL of the primary PCR product was were used as the DNA template for the secondary PCR, using the primers I3-2 and E8 (0.3 μM each) and

TABLE 1

Human *GHRH* receptor and *GHRH* gene-specific primers used in RT-nested PCR analysis.

mRNA	Primer name[a]	Direction	Location in cDNA	Sequence (5′–3′)	Product size (bp)
GHRH receptor	I3-1	Sense		CCT ACT GCC CTT AGG ATG CTG G	
	E12	Antisense	1156–1177	GCA GTA GAG GAT GGC AAC AAT G	
	I3-2	Sense		GCA CCT TTG AAG CCA GAG AAG G	[b]
	E8	Antisense	806–827	CAC GTG CCA GTG AAG AGC ACG G	
GHRH	E1	Sense	51–70	ATT TGA GCA GTG CCT CGG AG	
	E4-1	Antisense	352–371	TTT GTT CTG CCC ACA TGC TG	
	E3	Sense	207–228	ATG CAG ATG CCA TCT TCA CCA A	150
	E4-2	Antisense	336–356	TGC TGT CTA CCT GAC GAC CAA	

[a] The primers were named according to the location of their sequences in the respective gene (e.g., primer I3 is in intron 3, and E12 is in exon 12). Splice variants possess retained intronic sequence at their 5′ end.
[b] Expected product sizes of SV1, SV2, SV3, and SV4 were 720, 556, 390, and 335 bp, respectively.

Fu. GHRH receptor SV1 in endometriosis. Fertil Steril 2008.

the following cycling conditions: 94°C for 5 minutes followed by 30 cycles consisting of 94°C for 30 seconds, 63°C for 2 seconds, and 74°C for 20 seconds. This primer pair was designed to yield different product sizes for the *SV1*, *SV2*, *SV3*, and *SV4* mRNAs.

For *GHRH*, primers E1 and E4-1 (23) (0.25 μM each) were used for the primary amplification with the following cycling conditions: 94°C for 5 minutes followed by 30 cycles consisting of 94°C for 30 seconds, 64°C for 2 seconds, and 74°C for 10 seconds. We used 2 μL of the primary PCR product for the secondary PCR, using the primers E3 and E4-2 (24) (0.25 μM each) with the following cycling conditions: 94°C for 5 minutes followed by 25 cycles consisting of 94°C for 30 seconds, 62°C for 2 seconds, and 74°C for 5 seconds.

The secondary PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide. The RNA quality was tested by PCR amplification of human glyceraldehyde dehydrogenase (*GAPDH*) complementary DNA (cDNA) from the same RT reaction samples that were used for cDNA amplification of *GHRH* and *GHRH* SVs, as described earlier, using the control *GAPDH* primer set (Toyobo). An RT reaction without reverse transcriptase was used as a negative control.

Each PCR product was purified using the QIAEX II Gel Extraction Kit (Qiagen), and their sequence identities were confirmed using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Forster City, CA).

Treatment of ESC with hGHRH(1-29)NH2 to Study cAMP Response

To study the response of cAMP to exogenous GHRH, ESC were plated into a 24-well culture plate at 10^5 cells per well in 500 μL DMEM/F12 medium containing 10% FBS. When the cells had reached 80% confluence, they underwent serum starvation for 48 hours. Subsequently, the medium was replaced with 1% charcoal-stripped FBS medium containing 10^{-9} to 10^{-6} M hGHRH(1-29)NH_2 (Sigma) or vehicle with 50 μM isobutylmethylxanthine (IBMX), a cAMP phosphodiesterase inhibitor. We dissolved IBMX in 100% ethanol, and the final concentration of each vehicle in the medium was adjusted to 0.1%. Forskolin (10^{-7} M) was used as a positive control in each experiment.

After a 2-hour incubation (according to a preliminary experiment), conditioned media were collected, centrifuged, and stored at −80°C. Immediately after the conditioned media were collected, the wells were replenished with FBS-free medium so that the cell numbers could be counted using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Four separate experiments were done using tissues from four different women, and each experiment was performed in triplicate.

Measurement of cAMP Concentrations

The cAMP concentrations in aliquots of acetylated conditioned media were measured with a specific enzyme immunoassay (EIA) kit (Cayman Chemical Company, Ann Arbor,

MI), following the manufacturer's instructions. The cAMP concentrations were normalized to the cell counts in each corresponding well.

5-Bromo-2′-deoxyuridine (BrdU) Incorporation

The effect of GHRH on the proliferation of ESCs was evaluated by measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into cells. The incorporation of BrdU was detected using the Biotrak cell proliferation enzyme-linked immunosorbent assay (ELISA) system (Amersham Pharmacia Biotech), as reported previously elsewhere (25). Briefly, ESC were seeded into 96-multiwell Falcon plates at a density of 4000 cells per well in 100 μL of culture medium containing 10% FBS and were cultured for 48 hours, followed by a further 48 hours of serum starvation. After serum starvation, the medium was replaced with fresh medium supplemented with 1% charcoal-stripped FBS containing the control vehicle or hGHRH(1-29)NH_2 dissolved in dimethyl sulfoxide (DMSO) and diluted to 10^{-6} M with the medium. The final concentration of DMSO in the medium was 0.1%. Cells were incubated with 10 μL BrdU solution for the last 4 hours of the 24-hour and 48-hour culture periods. The culture medium was removed when the incubations had finished; the cells were fixed and the DNA was denatured by the addition of 200 μL fixative per well. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated into newly synthesized cellular DNA. The immune complexes were detected by the subsequent substrate reaction, and the resultant color was read at 450 nm using the DigiScan Microplate Reader (ASYS Hitech GmbH, Eugendorf, Austria).

Statistical Analysis

The data were expressed as mean ± standard deviation. Student's *t*-test was used for paired comparisons and one-way analysis of variance (ANOVA), and a post hoc test was used for multiple comparisons using StatView software (SAS Institute Inc., Cary, NC). $P<.05$ was considered statistically significant.

RESULTS

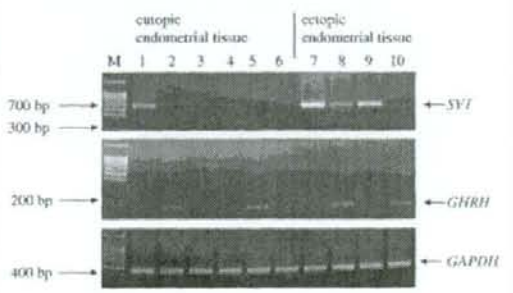
Expression of GHRH and GHRH Receptor Splice Variant mRNA in Eutopic and Ectopic Endometrial Tissues

We performed RT-nested PCR to examine the mRNA expression of *GHRH* and the splice variants of the *GHRH* receptor in eutopic and ectopic endometrial tissues from 27 women with endometriosis, and eutopic endometrial tissues from 20 women without endometriosis (Fig. 1). The number of *GHRH*-positive samples was similar between ectopic endometrial tissues (24%) and eutopic endometrial tissues in patients with (26%) or without (25%) endometriosis (Table 2). We detected *SV1* mRNA in 63% of ectopic endometrial tissue samples, whereas expression was statistically significantly lower in eutopic endometrial tissues in patients with (none detected) or without (10%) endometriosis. We detected *GHRH* in all of the normal ovarian tissue samples (100%) used as controls, whereas *SV1* was not detected in any of these samples. Bands representing *SV2*, *SV3*, and *SV4* were not detected in any of the samples. In peritoneal bone marrow–derived cells, *GHRH* mRNA was detected in 7 out of 10 (70%) women with endometriosis and 6 out of 6 (100%) women without endometriosis.

Each PCR product was sequenced and confirmed to be identical to the *SV1* and *GHRH* sequences (14, 24).

FIGURE 1

Reverse transcriptase polymerase chain reaction analysis of the splice variants of the growth hormone–releasing hormone (GHRH) receptor and *GHRH* gene expression in eutopic and ectopic endometrial tissues. Data represent eutopic and ectopic endometrial tissues from 27 women with endometriosis, and eutopic endometrial tissues from 20 women without endometriosis. Amplification of GAPDH was performed to ensure equal loading. Lane M: 100-bp DNA molecular marker. Lanes 1–3: eutopic endometrial tissues from different patients without endometriosis. Lanes 4–6: eutopic endometrial tissues from different patients with endometriosis. Lanes 7–10: ectopic endometrial tissues from different patients.



Fu. GHRH receptor SV1 in endometriosis. Fertil Steril 2008.

Effect of hGHRH(1-29)NH_2 on cAMP Production in ESC Expressing SV1

We first evaluated the expression of *SV1* mRNA in cultured ESC from 27 women. We detected *SV1* mRNA in 21 samples (77%), and the ESC that expressed *SV1* were used for the ensuing study. We evaluated the effect of hGHRH(1-29)NH_2 at concentrations between 10^{-9} M and 10^{-6} M on the production of cAMP. As shown in Figure 2, incubation with GHRH(1-29)NH_2 for 2 hours induced a dose-dependent increase in cAMP production in cultured ESC.

Effect of hGHRH(1-29)NH2 on DNA Synthesis in ESC

The effect of GHRH on DNA synthesis was examined in five independently cultured ESC preparations from five different

TABLE 2

Expression patterns of *GHRH* and *SV1* mRNA in three types of tissues.

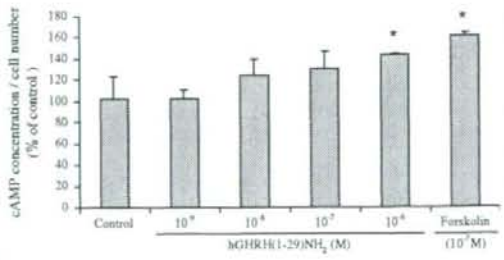
Tissue type	GHRH Rate	GHRH %	SV1 Rate	SV1 %
Endometriotic from ovarian endometrioma	6/27	24	17/27	63
Endometrial from women with endometriosis	7/27	26	0/27	0
Endometrial from women without endometriosis	5/20	25	2/20	10
Ovarian	14/14	100	0/14	0

Fu. GHRH receptor SV1 in endometriosis. Fertil Steril 2008.

women (Fig. 3). We found that hGHRH(1-29)NH_2 at a concentration of 10^{-7} M increased the incorporation of BrdU into DNA by 20% ($P<.05$) at 24 hours and 27% ($P<.05$) at 48 hours. At a concentration of 10^{-6} M, hGHRH(1-29)NH_2 increased BrdU incorporation by 23% ($P<.01$) at 24 hours and 33% ($P<.01$) at 48 hours. In contrast, hGHRH(1-29)NH_2 did not alter the amount of BrdU incorporated into the DNA of ESC that did not express *SV1* mRNA (data not shown, n = 3).

FIGURE 2

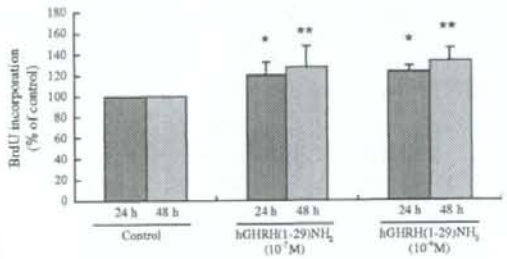
Effect of exogenous hGHRH(1-29)NH2 on cAMP production in cultured ectopic endometrial stromal cells (ESC). The cells were treated for 2 hours with hGHRH(1-29)NH2 (10^{-9} to 10^{-6} M) or forskolin (10^{-7} M). The cells were co-treated with isobutylmethylxanthine (IBMX) to suppress cAMP degradation. Conditioned media were assayed for cAMP concentration, and the cell numbers were determined using the Cell Counting Kit-8. Data represent the mean ± standard error of the mean of cAMP concentration per cell number of four separate experiments using different ESC preparations, expressed as a percentage of untreated controls. *$P<.05$ versus control.



Fu. GHRH receptor SV1 in endometriosis. Fertil Steril 2008.

FIGURE 3

Effect of hGHRH(1-29)NH_2 on the proliferation of ectopic endometrial stromal cells (ESC) determined by BrdU incorporation using a cell proliferation ELISA system. The ESC were treated with hGHRH(1-29)NH_2 (10^{-7} M and 10^{-6} M) for 24 hours and 48 hours. Data represent the mean percentage of the untreated control ± standard error of the mean of five independent experiments using hexaplicate wells. *$P<.05$. **$P<.01$ versus control.



Fu. GHRH receptor SV1 in endometriosis. Fertil Steril 2008.

DISCUSSION

Our study demonstrated that *GHRH* receptor *SV1* mRNA was expressed in ESC. We also detected *GHRH* mRNA in eutopic and ectopic endometrium and in peritoneal bone marrow–derived cells. In addition, our results showed that GHRH stimulated cAMP production and cell proliferation in ESC expressing *SV1*.

The receptors mediating the possible actions of GHRH in extrapituitary tissues have only been identified recently. In initial tumor studies, the pituitary GHRH receptor could not be detected in any of the cancers studied (26, 27). However, recent investigations demonstrated that various human cancers express SVs of the GHRH receptor (8–12, 14, 28–30), and of these, SV1 displays the greatest sequence similarity to the pituitary GHRH receptor. It is now believed that the gene product of SV1 is a functional receptor that relays mitogenic and other signals in response to GHRH in tumors (11, 26, 27, 31).

Positive immunostaining of the GHRH receptor in the human endometrium has been reported recently (32). In our study, we could not detect gene expression of the pituitary GHRH receptor in any endometrial samples. The reason for the discrepancy between our data and the immunohistochemical study is unknown. Nevertheless, we have provided a remarkable finding with the detection of *SV1* mRNA in 17 out of 27 (63%) endometriotic samples and in 2 out of 49 (4%) eutopic endometrial samples. It is well established that the gene expression profile of the ectopic endometrium is different from that of the eutopic endometrium (1, 2). The higher expression rate of *SV1* in the ectopic endometrium may therefore indicate a higher sensitivity to GHRH in the ectopic endometrium.

The protein sequence encoded by SV1 is similar to that of the pituitary GHRH receptor, preserving the transmembrane domains, intracellular loops, and the C-terminal end necessary for signal transduction. However, the large N-terminal extracellular tail characteristic of the pituitary GHRH receptor is truncated (14). Functional differences between the activity of SV1 and the pituitary GHRH receptor are not clear at the moment. Several studies have shown that stimulation of SV1-transfected cells and SV1-expressing cells by GHRH or GHRH analogs is followed by an increase in cAMP production (8, 10, 13, 15, 33). Our study has revealed that SV1-expressing ESC respond to GHRH with a dose-dependent increase in cAMP production. Together with the finding that the ESC did not express the pituitary GHRH receptor (data not shown), the observed cAMP response to GHRH appears to be mediated by SV1.

Our study demonstrated that hGHRH(1-29)NH_2 increased DNA synthesis in ESC, as determined by BrdU incorporation. The observed effects of GHRH make an interesting contrast with GnRH II, which decreases DNA synthesis in ESC (16). It is intriguing to speculate that several hypothalamic hormones play different roles in endometriotic tissues. In addition, given that GHRH acts as a growth factor in ESC, the antiproliferative action of GHRH antagonists in various cancer cell lines (6) may be extrapolated to therapeutic potential in endometriosis.

We determined the expression rate of *GHRH* mRNA in normal human endometrial tissues and ectopic endometrial tissues. The rate of expression was very similar in eutopic endometrial samples from women with and without endometriosis and in ectopic endometrial samples, at approximately 25%. These expression rates are relatively low compared with those reported in prostate cancer (86%), endometrial cancer (68%), and ovarian cancer (55%) (12, 34). Although it is not yet known why this rate is lower, it is possible that endometrial tissue–derived GHRH might not act alone on SV1 in ESC. When GHRH is derived from other sources such as ovarian tissues and bone marrow–derived cells in the peritoneal cavity, it might also contribute to activating SV1 in the endometriotic cells.

Our study has demonstrated that SV1 is expressed in endometriotic stromal cells and that the activation of SV1 by GHRH stimulates the proliferation of these cells. These findings suggest that GHRH and SV1 are involved in the development of endometriosis.

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ORIGINAL ARTICLE *Reproductive biology*

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 μg/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 μM), 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). Deoxyribonuclease 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)-CH_2F (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 (Hirota *et al.*, 2005a, c). 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 onto 100-mm culture dishes (Iwaki, Tokyo, Japan). Dishes were kept at 37°C in a humidified 5% CO_2/95% air atmosphere for 1 or 2 days before the first passage. At the first passage, ESC were plated into 12-well plates at 1×10^5 cells/well for RT–PCR and small interfering RNA (siRNA) experiments, or 6-well plates at 4×10^5 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).

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 the 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.

Small interfering RNA

ESC seeded at 1×10^5 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 three 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′-GAGTTAAGACAGCGCTTGGG-3′; antisense, 5′-ACTGGGCCTGCACCTGCTGCG-3′) amplify a 118 bp fragment; DR5 primers (sense, 5′-TGCAGCCGTAGTCTTGATTG-3′; antisense, 5′-GCACCAAGTCTGCAAAGTCA-3′) amplify a 389 bp fragment; GAPDH primers (sense, 5′-ACCACAGTCCATGCCATCAC-3′; antisense, 5′-TCCACCACCCTGTTGCTGTA-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. Each PCR product was purified with a Qiaex II gel extraction kit (Qiagen, Tokyo, Japan), and their identities were confirmed by DNA sequencing (ABI Prism 310 Genetic Analyzer; Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

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 FACS Calibur 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; Takemura 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

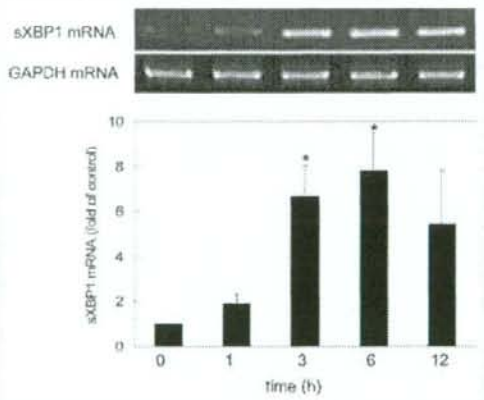

Figure 1 Tunicamycin-induced gene expression of sXBP1 in endometriotic stromal cells (ESC). ESC were cultured with tunicamycin for 0–12 h. Total RNA isolated from ESC was reverse transcribed, amplified by real-time PCR and representative amplified products are shown. The increase in levels of RNA was 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.

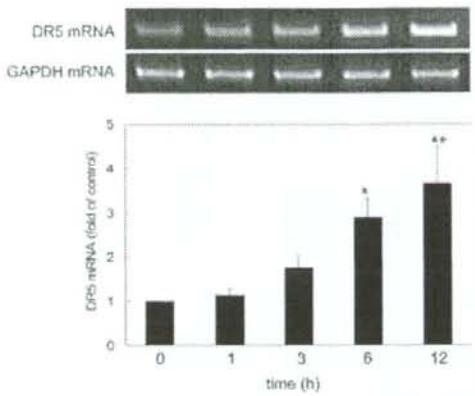

Figure 2 Tunicamycin-induced gene expression of DR5 in ESC. ESC were cultured with tunicamycin for 0–12 h and the same procedures as described in Fig. 1 were used to measure DR5 mRNA. Values are the mean ± SEM of three independent experiments using samples from three different women. $^{*}P < 0.05$; $^{**}P < 0.005$ (both 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 hypoestrogen state in the patient, and alternative treatment that does not affect the hormonal status of the patient is required.

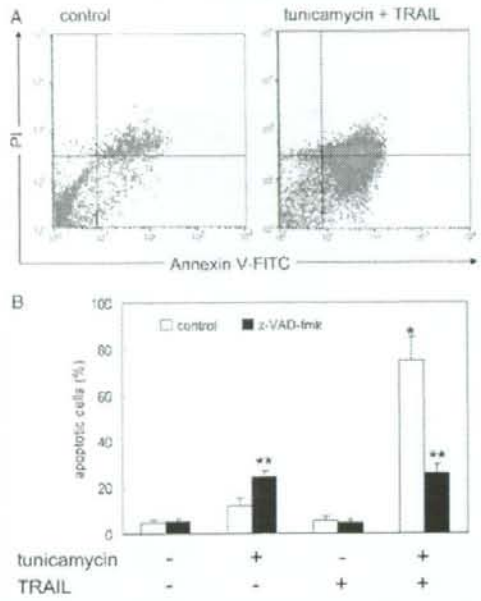

Figure 3 Effect of tumor necrosis factor-related apoptosis-induced ligand (TRAIL) on apoptosis of ESC with or without tunicamycin pretreatment, and effect of a general caspase inhibitor, z-VAD-fmk, on the TRAIL-induced apoptosis. ESC were cultured with or without tunicamycin (2 μg/ml) for 16 h, then treated with or without TRAIL (200 ng/ml) for 24 h. One hour before TRAIL treatment, z-VAD-fmk or the control vehicle was added to the culture. Apoptosis was analyzed by flow cytometry on 5×10^4 ESC that were double stained [annexin V and propidium iodide (PI)]. Annexin V-positive cells, both dead and live, were regarded as apoptotic cells. (**A**) The representative flow cytometry data. (**B**) Percentage of apoptotic cells in each treatment. Values are the mean ± SEM of two independent experiments using samples from two different women. $^{*}P < 0.0001$ versus all others; $^{**}P < 0.05$ versus all tunicamycin (−) groups. FITC, fluorescein isothiocyanate.

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

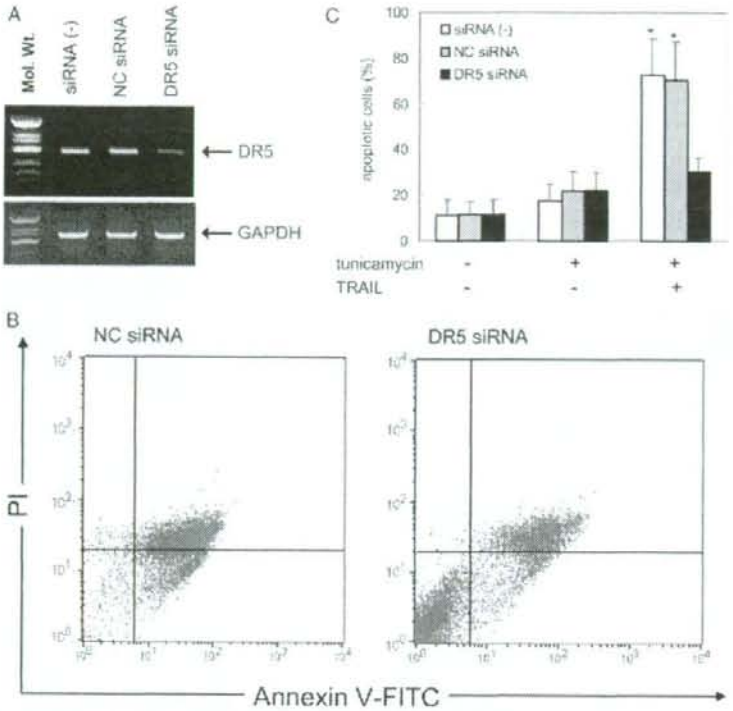

Figure 4 Effect of DR5 small interfering RNA (siRNA) on TRAIL-induced apoptosis of ESC pretreated with tunicamycin. First, ESC were transfected with the control, or DR5 siRNA for 24 h [siRNA (−), mock transfection; NC siRNA, negative control siRNA transfection; DR siRNA, DR siRNA transfection]. Subsequently, 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 5×10^4 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.

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 proapoptotic genes are down-regulated in endometriotic tissues as compared with eutopic endometrial tissues (Arimoto *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.

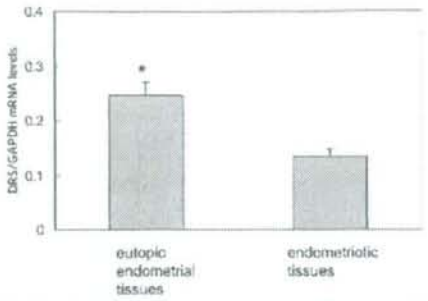

Figure 5 DR5 mRNA levels in eutopic endometrial tissues and endometriotic tissues of women with endometriosis. Both tissues were collected from eight women with endometriosis. Total RNA isolated from the tissues was reverse transcribed and amplified by real-time PCR. The levels of RNA were calculated by subtracting the signal threshold cycles of the internal standard (GAPDH) from the threshold cycles of DR5. Values are the mean ± SEM. *$P < 0.005$ versus endometriotic tissues.

Table I Percentage of apoptotic cells in eutopic endometrial cells from women with ($n = 4$) or without ($n = 3$) endometriosis.

	Without endometriosis	With endometriosis
control	10.9 ± 0.8[a]	11.5 ± 3.7
Tunicamycin	27.1 ± 4.4[b]	18.3 ± 4.5
TRAIL	13.8 ± 1.3[a]	12.9 ± 3.7
Tunicamycin + TRAIL	29.5 ± 4.4[b]	27.0 ± 4.7

Endometrial stromal cells of women with or without endometriosis were cultured with or without tunicamycin (2 μg/ml) for 16 h, then treated with or without tumor necrosis factor-related apoptosis-induced ligand (TRAIL, 200 ng/ml) for 24 h. Values are mean ± SEM. $P < 0.05$, a versus b.

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 DR5 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 DR5 mRNA.

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Interleukin (IL)-1β Stimulates Migration and Survival of First-Trimester Villous Cytotrophoblast Cells through Endometrial Epithelial Cell-Derived IL-8

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IL-1, secreted by human embryos and trophoblast cells, is important for successful implantation and pregnancy. We previously reported that IL-1β induced IL-8 production in human endometrial stromal cells (ESCs) and that induction was regulated by substances implicated in implantation. In the present study using human primary cells in culture, we measured IL-1β-induced production of IL-8 from endometrial epithelial cells (EECs) and ESCs and examined effects of the endometrium-derived IL-8 on migration and number of first-trimester villous cytotrophoblast cells (vCTs). Both basal and IL-1β-induced IL-8 levels of cell supernatants were much higher in EECs than ESCs. Addition of IL-1β to EECs increased the chemotactic activity of the supernatants to vCTs, and this effect was suppressed by immunoneutralization with anti-IL-8 antibody. Supernatants of IL-1β-stimulated EECs yielded significantly higher number of vCTs compared with those of untreated EECs, and the effect was inhibited by IL-8 antibody. These findings suggest that IL-1 promotes implantation by stimulating EECs to produce IL-8, which subsequently induces migration of vCTs and contributes to survival of vCTs. (*Endocrinology* 150: 350–356, 2009)

Migration and survival of trophoblast cells are essential for establishing a pregnancy. These phenomena are controlled elaborately by various substances that are secreted from the embryo, the endometrium, or both during the implantation process (1–5). In particular, molecular cross talk between the embryo and endometrium is indispensable for their coordinated development, which is required for successful implantation (6, 7).

IL-1 is a typical cytokine that affects the implantation process at the interface between the embryo and endometrium (8–10). In view of the findings that IL-1 increases secretion of prostaglandin E2 and leukemia inhibitory factor (LIF) and expression of the integrin β3-subunit in human endometrium (11–13), IL-1 may be one of the first signals that the blastocyst exerts on the endometrium. The notion is also supported by evidence of IL-1 expression in human embryo and trophoblast as described below. It has been shown that successful implantation after *in vitro* fertilization is correlated positively with high concentrations of IL-1α and IL-1β, two active ligands of IL-1, in the embryos' culture media (14, 15). Immunohistochemical studies have demonstrated the localization of IL-1β in first-trimester villous cytotrophoblast cells (vCTs) (16, 17). In addition, an *in vivo* study in mice has shown that administration of IL-1 receptor antagonist significantly reduces the number of implanted embryos (18).

With the aim of gaining a better understanding of the interaction between the human endometrium and trophoblast, a recent study identified the gene expression profile of endometrial stromal cells (ESCs) cocultured with first-trimester trophoblast explants (19). The study revealed that one of the most up-regulated genes was chemokine IL-8, which was up-regulated more than 300-fold. In another study, IL-8 from decidual natural killer cells promoted invasion of first-trimester extravillous trophoblast cells (20). These findings imply that endometrial IL-8 is involved in the process of implantation under the stimulus of trophoblast.

Endometrial IL-8 acts as not only a chemoattractant of leukocytes but also an autocrine growth factor (21). IL-8 acts on cells through IL-8 chemokine receptors CXCR-1 and CXCR2, which are expressed in endometrium (22). In contrast to the roles

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Abbreviations: CCK-8, Cell Counting Kit-8; CXCR, chemokine receptor; DNase I, deoxyribonuclease I; EEC, endometrial epithelial cell; EEC-SN, supernatant of EEC; ESC, endometrial stromal cell; ESC-SN, supernatant of ESC; F12, Ham's F12 medium; FBS, fetal bovine serum; HPF, high-power field; IL-8Ab, IL-8 antibody; LIF, leukemia inhibitory factor; mIgG, mouse IgG1; PF, peritoneal fluid; PL, peritoneal leukocyte; vCT, villous cytotrophoblast cell.

of IL-8 on endometrium, its chemotactic and proliferative effects on first-trimester vCTs still remain obscure. As for expression of the receptors, a study has shown that CXCR2 is expressed in primary first-trimester trophoblast cells and a trophoblast cell line BeWo cells but has not examined the CXCR1 expression (23).

We reported previously that IL-1 induces IL-8 production in human ESCs and the induction is modulated by substances that are implicated in implantation (24, 25). Combined with the above findings, we hypothesized that the development of trophoblast in early pregnancy is affected by endometrial IL-8, which is up-regulated by embryo-derived IL-1. To address this issue, we first examined IL-1β-induced production of IL-8 from human endometrial epithelial cells (EECs) and ESCs. We then assessed effects of the culture media from EECs stimulated by IL-1β on migration and number of human first-trimester vCTs.

Materials and Methods

Reagents and materials

Type I collagenase, antibiotics, and magnesium sulfate ($MgSO_4$) were purchased from Sigma (St. Louis, MO). DMEM and Ham's F12 medium, DMEM/F12, 0.25% trypsin and 0.25% trypsin/EDTA, were from Life Technologies (Rockville, MD). Charcoal-stripped fetal bovine serum (FBS) was from HyClone (Logan, UT). Ficoll-Paque Plus (1.077 g/ml) was from Amersham Biosciences (Piscataway, NJ). Mouse monoclonal antihuman IL-8 antibody (IL-8Ab), human recombinant IL-1β and human recombinant IL-8 were from R&D Systems (Minneapolis, MN). Isotype control mouse IgG1 (mIgG) and mouse monoclonal antibodies to human pan-cytokeratin (AE1/AE3), human vimentin, and human CD45 were from Dako (Glostrup, Denmark). Mouse monoclonal antibody to human cytokeratin 7 was from AbD Serotec (Oxford, UK). Deoxyribonuclease I (DNase I) was from Takara (Tokyo, Japan).

Tissue sources

Endometrial tissues were obtained from a total of 20 women (43.7 ± 4.2 yr, mean ± SD) undergoing hysterectomy for benign gynecological conditions such as uterine fibroids without endometrial pathologies. Although the relatively high reproductive age range of the subjects and the myometrium pathology results may place some limitations on the present study, we used these samples due to the unavailability of endometrial tissue in healthy young women. All subjects had regular menstrual cycles and had not received hormone therapy for at least 6 months before surgery. Placental tissues between 5 and 8 wk of gestation (7.4 ± 6.7 wk) were obtained from a total of 25 women (29.6 ± 6.7 yr) undergoing elective terminations of pregnancy for isolation of first-trimester vCTs. Peritoneal leukocytes (PLs) were used as a positive control in the experiment of RT-PCR. To obtain PL, peritoneal fluid (PF) with PL was obtained from a woman with endometriosis undergoing laparoscopy. PF was collected via a laparoscopic cannula introduced into the cul-de-sac before starting any manipulative procedures. The Institutional Review Board of the University of Tokyo approved this study, and written informed consent for use of the tissue samples was obtained from each woman. The tissues were collected under sterile conditions and were processed for primary cell cultures.

Isolation, purification, and culture of EECs, ESCs, first-trimester vCTs, and PLs

The isolation and culture of human EECs and ESCs was carried out as described previously (2, 26). Endometrial tissues were minced and incubated in DMEM/F12 containing 0.25% type I collagenase and 15 U/ml DNase I for 60 min at 37 C. The resulting dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer (Becton Dickinson, Lincoln Park, NJ). Intact endometrial epithelial glands were retained by the strainer, whereas dispersed ESCs passed through the strainer into the filtrate. ESCs in the filtrate were collected by centrifugation and resuspended in DMEM/F12 containing 5% FBS and antibiotics. ESCs were then plated in a 100-mm culture plate and incubated at 37 C in a humidified 5% CO_2-95% air atmosphere. At the first passage, ESCs were plated at a density of 2×10^5 cells/well into 12-well culture plates for experimental use. Cells enriched with endometrial epithelial glands were collected by backwashing the strainer with DMEM/F12, plated in a 100-mm plate, and incubated at 37 C for 60 min to allow any contaminating stromal cells to attach to the plate wall. The nonattached epithelial glands formed a monolayer of EEC after attachment to culture plates. EECs at a density of 2×10^5 cells/well in 12-well culture plates were used for the experiments.

The isolation and culture of first-trimester vCTs were performed as described previously (2). Briefly, the first-trimester placental tissues were washed in PBS, and the soft villous material was cut away from connective tissue and vessels. The washed tissue was incubated in sterile PBS containing 1 mM $MgSO_4$, 0.125% trypsin, and 30 U/ml DNase I for 30 min at 37 C with mild stirring. The suspension was then filtered through a 100-μm nylon cell strainer, and the cells were centrifuged at $200 \times g$ for 5 min to obtain a cell pellet, which was resuspended in DMEM/F12 with 5% FBS. The cell suspension was layered onto Ficoll-Paque Plus and centrifuged at $150 \times g$ for 15 min. The cells recovered from the interface were washed with PBS and resuspended in DMEM/F12. The remaining leukocytes were removed by plating the cells for 30 min, followed by aspiration of the supernatant enriched with cytotrophoblast cells. The cells were washed with PBS, the medium was changed to DMEM/F12 with 10% FBS, and the cells were placed in type IV collagen-coated plates (BD Biosciences, Bedford, MA) and incubated at 37 C in a humidified 5% CO_2-95% air atmosphere. The cells were plated at a density of 2×10^4 cells/well in 96-well plates for cell number assays, and at a density of 4×10^5 cells/well in 6-well plates for RT-PCR and *in vitro* migration assay. After incubation for 24 h, the cells were ready for experimental use.

PLs were collected as previously described (27). PF containing peritoneal leukocytes was centrifuged at $200 \times g$ for 5 min, and the supernatant removed. The cell pellet was resuspended in PBS, layered onto Ficoll-Paque Plus and centrifuged at $150 \times g$ for 30 min. PLs were recovered from the interface.

We confirmed the purity of EECs, ESCs, vCTs, and PLs by immunocytochemistry as we described previously (2). The purity of EEC preparations was greater than 95%, as judged by positive cellular staining for pan-cytokeratin (a marker for epithelial cells), negative cellular staining for vimentin (a marker for stromal cells), and CD45 (a marker for leukocytes). The purity of ESC preparations was greater than 98%, as judged by positive cellular staining for vimentin and negative cellular staining for pan-cytokeratin and CD45. The purity of vCT preparations was greater than 90%, as judged by positive cellular staining for cytokeratin 7 (a marker for trophoblast cells) and negative staining for vimentin and CD45. The purity of PLs was greater than 90%, as judged by positive cellular staining for CD45.

Treatment of endometrial cell cultures

When ESCs and EECs approached confluence, the complete media were removed and replaced with fresh media and antibiotics, and the cells were cultured for 24 h. The wells were then replenished with FBS-free media containing different concentrations of IL-1β (0, 0.1, 1, and 10 ng/ml) and incubated for an additional 24 h. After IL-1β treatment, the cell supernatants of ESCs and EECs (ESC-SN and EEC-SN, respectively) were collected, centrifuged and stored at − 80 C for subsequent analysis.

Measurement of IL-8 in endometrial cell supernatants

Concentrations of IL-8 in endometrial cell culture media were measured using human IL-8 ELISA kit (R&D Systems) following the manufacturer's protocol. Absorbance was read at 450 nm with the DigiScan microplate reader (ASYS Hitech GmbH, Eugendorf, Austria). The total amount of IL-8 in the culture medium was calculated from the liquid

volume and the concentration of IL-8. After collection of the culture media, the cultured cells were homogenized, and the amount of total protein in the homogenized cells was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Data of IL-8 content in the culture media were normalized against the total protein content of the cell lysates.

In vitro migration assay

In vitro migration assay was performed in 24-well plates containing Transwell permeable supports with an 8-μm polycarbonate membrane (Costar, Cambridge, MA) for vCTs as previously described (2). EEC-SNs that had or had not been stimulated by 0.1, 1, and 10 ng/ml IL-1β for 24 h [IL-1β (0.1, 1, 10 ng/ml) EEC-SN and control EEC-SN, respectively] or FBS-free medium with or without IL-8 (100 pg/ml) was added to the lower chambers. In the experiments using the neutralizing antibody, control EEC-SN and EEC-SN that had been stimulated by 1 ng/ml IL-1β for 24 h [IL-1β (1 ng/ml) EEC-SN] were preincubated for 1 h with 1 μg/ml of IL-8Ab or isotype control mIgG and plated in the lower chambers. Cultured vCTs were plated at a density of 2×10^5 cells/well in the upper chamber of the Transwell membranes, which contained 100 μl FBS-free DMEM/F12, and were incubated for 72 h at 37 C in a 5% CO_2 atmosphere. After the incubation, the upper surface of the membranes was gently cleansed with a cotton swab. Then the cells that had migrated through the pores were fixed with acetone/methanol and stained with hematoxylin and eosin. The filter was gently cut from the chamber, and the migrated cells were counted from the underside of the filter. The number of vCTs that migrated across the filters was counted in 10 randomly selected high-power fields (HPFs) per filter under the light microscope.

Measurement of vCT number

To measure the cell number, we used the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) as we previously described (28).

The cultured medium was removed from vCTs and replaced with fresh medium supplemented with antibiotics 24 h before treatment. Cultured vCTs were treated with control and IL-1β (0.1, 1, 10 ng/ml) EEC-SN or FBS-free medium with or without 100 pg/ml IL-8. In the experiments using neutralizing antibody, control and IL-1β (1 ng/ml) EEC-SN were preincubated for 1 h with 1 μg/ml IL-8Ab or isotype control mIgG before the treatment of vCTs was started. After 72 h incubation, CCK-8 assay was performed.

In the CCK-8 assay, CCK-8 solutions containing the tetrazolium salt WST-8 were added and incubated at 37 C for an additional 2 h. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product in culture medium. The amount of formazan, which is directly proportional to the number of living cells, was evaluated by measuring the OD at 450 nm in the DigiScan microplate reader (ASYS Hitech).

RT-PCR of *CXCR1* and *CXCR2* mRNA

RT-PCR was performed as reported previously (29). Total RNA was extracted from vCTs and PLs using the RNAeasy minikit (QIAGEN, Hilden, Germany). Reverse transcription was performed using ReverTra Ace-α (Toyobo, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a total volume of 20 μl, and cDNA was amplified using oligonucleotide primers based on the human *CXCR1* and *CXCR2* sequences. PCR was performed using the ReverTra Dash kit (Toyobo) according to the manufacturer's instructions. *CXCR1* primers (sense, 5′-TACTGTTGGACACACCTGGC-3′; antisense, 5′-TAGACATCAGTGACGGAGCG-3′) were designed to amplify a 294-bp fragment. *CXCR2* primers (sense, 5′-CACAGTGAAGACATCGGTGG-3′; antisense, 5′-AGGGATTCTGGTTCACATGG-3′) were designed to amplify a 246-bp fragment. The expression levels of *CXCR1* and *CXCR2* mRNA were normalized to *GAPDH* mRNA, which was used as an internal control and as a loading control. Human *GAPDH* primers (Toyobo) were designed to amplify a 452-bp fragment. The PCR conditions for *CXCR1* consisted of 35 cycles at 98 C for 10 sec, 60 C for 4 sec, and 74 C for 12 sec. The PCR conditions for *CXCR2* consisted of 35 cycles at 98 C for 10 sec, 60 C for 4 sec, and 74 C for 12 sec. PCR products were purified using the QIAEX II gel extraction kit (QIAGEN), and their sequence identities confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Statistical analysis

Data were evaluated using ANOVA with *post hoc* analysis using Fisher's protected least-significance difference test. $P < 0.05$ were accepted as significant.

Results

Both basal and IL-1β-induced IL-8 levels of culture media are higher in EECs than ESCs

Both ESCs and EECs have been reported to produce IL-8 in response to IL-1 (24, 25, 30). IL-1 has two bioactive ligands, IL-1α and IL-1β, possessed of similar biological effects (31). Therefore, in the present study, we used IL-1β as a representative of IL-1 ligands, and compared IL-8 production in response to IL-1β between ESCs and EECs. As shown in Fig. 1, both the basal IL-8 levels and the IL-1β-induced IL-8 levels were higher in EEC-SN than in ESC-SN. The basal IL-8 level of EEC-SN was 72-fold higher than that of ESC-SN. The IL-8 level of media in EECs stimulated by 1 ng/ml IL-1β was 5 times higher than that of ESCs. Based on these results, we chose to use the EEC-SN in all subsequent experiments to evaluate the effects of endometrial IL-8 on first trimester vCT.

Migration of first-trimester vCTs is stimulated by EEC-SN treated with IL-1β via IL-8

To study the chemotactic effects of EEC-derived IL-8 on first-trimester vCTs, *in vitro* migration assay was performed. As illustrated in Fig. 2A, more vCTs migrated to IL-1β (0.1, 1, 10 ng/ml) EEC-SN than control EEC-SN. The effect appeared to be maximal at 1 ng/ml. Recombinant IL-8 also promoted the chemotaxis of

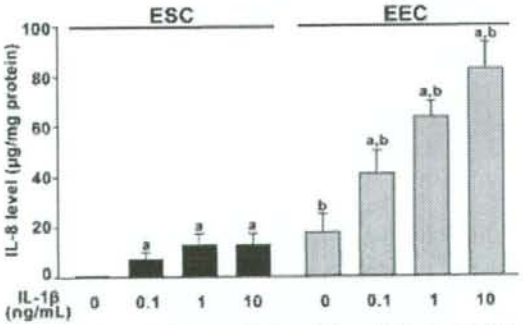

FIG. 1. IL-1β-stimulated IL-8 secretion from ESCs and EECs. Human ESCs (n = 10) and EECs (n = 10) were cultured in FBS-free media with different doses of IL-1β for 24 h. The supernatants were collected and assayed for IL-8 by ELISA. Values are normalized to the total protein content of the cell lysate. The values (total IL-8 content of the supernatants/total protein content of the cell extract, micrograms per milligram protein) are presented as the mean ± SEM of the combined data of separate experiments using different ESC and EEC preparations. a, $P < 0.05$ when compared with the same cell type without IL-1β stimulation; b, $P < 0.05$ when compared with ESCs stimulated by the same dose of IL-1β.

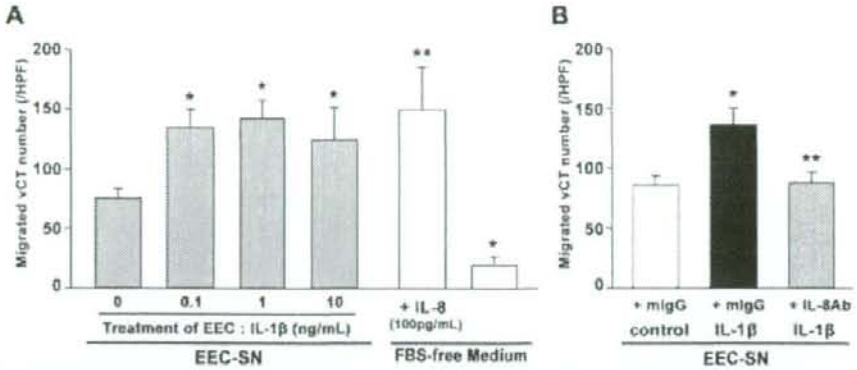

FIG. 2. Stimulatory effect of EEC-SN treated with IL-1β on migration of first-trimester vCTs through IL-8. *In vitro* migration assay was performed to determine whether migration of human first-trimester vCTs was affected by endometrial IL-8 expression. Cultured vCTs were plated at a density of 2×10^5 cells in the upper chambers of Transwell membranes for 72 h. After incubation, vCTs that had migrated through the pores were fixed and counted. The number of vCTs that had migrated across the filters was counted in 10 randomly selected HPFs per filter under the light microscope. Values represent the cell number per HPF. A, EEC-SN that had or had not been treated with IL-1β for 24 h (IL-1β EEC-SN and control EEC-SN, respectively) or FBS-free media with or without IL-8 were plated in the lower chambers. Values are the mean ± SEM of the combined data from six independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN; **, $P < 0.05$ when compared with FBS-free media without IL-8. B, Control EEC-SN and EEC-SN that had been treated with 1 ng/ml of IL-1β for 24 h [IL-1β (1 ng/ml) EEC-SN] were preincubated for 1 h with 1 μg/ml of anti-IL-8Ab or isotype control mIgG and plated in the lower chambers. Values are the mean ± SEM of the combined data from 10 independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN + mIgG; **, $P < 0.05$ when compared with IL-1β (1 ng/ml) EEC-SN + mIgG.

vCTs. In contrast, immunoneutralization with IL-8Ab abolished the chemotactic activity of IL-1β (1ng/ml) EEC-SN (Fig. 2B).

EEC-SN treated with IL-1β yielded higher number of first-trimester vCTs via IL-8

To study the effects exerted by EEC-derived IL-8 on number of first-trimester vCTs, we used CCK-8 assay. As illustrated in Fig. 3A, when compared with control EEC-SN, IL-1β (0.1, 1, 10 ng/ml) EEC-SN up-regulated the vCT number. Recombinant IL-8 also increased in the vCT number. The effects of IL-1β (1ng/ml) EEC-SN were eliminated by immunoneutralization with IL-8Ab (Fig. 3B).

CXCR1 and *CXCR2* mRNA are expressed in first-trimester vCTs

To examine the expression of IL-8 receptors in first-trimester vCTs, RT-PCR was performed using primers specific to *CXCR1* and *CXCR2* (receptors for IL-8). A sample of PLs was used as a positive control for IL-8 receptor-expressing cells (32–35). As demonstrated in Fig. 4, mRNAs of IL-8 re-

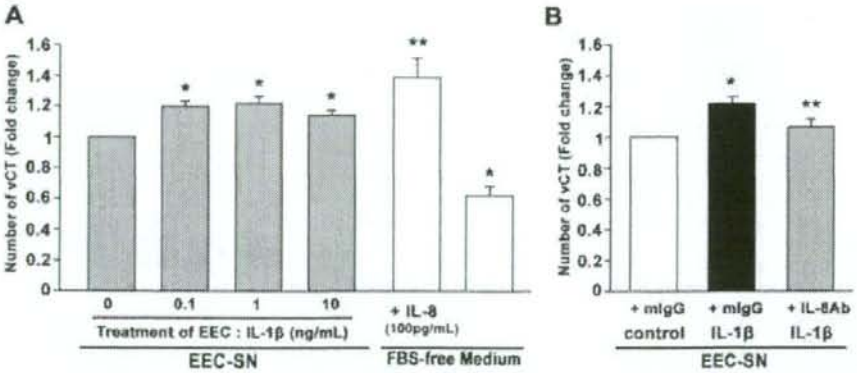

FIG. 3. Stimulatory effect of EEC-SN treated with IL-1β on increase in number of first-trimester vCTs through IL-8. The CCK-8 assay was performed to determine whether number of human first-trimester vCTs was affected by endometrial IL-8 expression. Before the assay, vCTs were treated for 72 h. A, vCTs were treated with control EEC-SN and IL-1β (0.1, 1, and 10 ng/ml) EEC-SN or FBS-free media with or without 100 pg/ml of IL-8. The values represent the ratio of the cell number relative to control EEC-SN. Values are the mean ± SEM of the combined data from seven independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN; **, $P < 0.05$ when compared with FBS-free media without IL-8. B, Control EEC-SN and IL-1β (1 ng/ml) EEC-SN were preincubated for 1 h with 1 μg/ml of IL-8Ab or mIgG. The values represent the ratio of the cell number relative to control EEC-SN with mIgG. Values are the mean ± SEM of the combined data from nine independent experiments using different vCT preparations. *, $P < 0.05$ when compared with control EEC-SN + mIgG; **, $P < 0.05$ when compared with IL-1β (1 ng/ml) EEC-SN + mIgG.