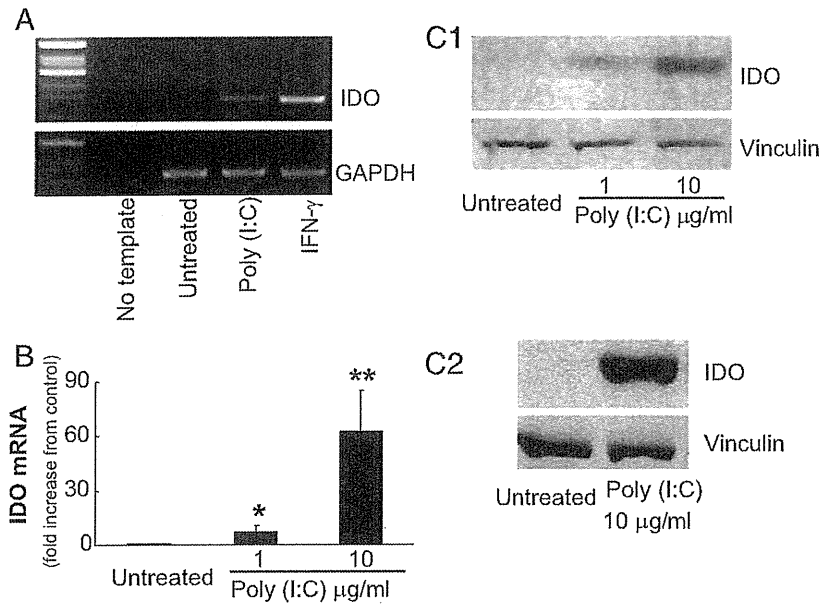

FIG. 1. IDO induction by poly(I:C) in human first-trimester trophoblasts. A, Primary first-trimester trophoblasts were cultured in the absence or presence of poly(I:C) (10 μg/ml) or IFN-γ (25 ng/ml, positive control) for 12 h. IDO mRNA expressions were evaluated by RT-PCR followed by electrophoresis. IDO mRNA expression was not detected in untreated trophoblasts but was induced by poly(I:C). B, Quantitative RT-PCR analysis indicated that poly(I:C) (1 or 10 μg/ml, 12 h) significantly increased the IDO mRNA level in a dose-dependent manner in primary trophoblasts ($n = 4$). Values are the mean ± SE. *, $P < 0.05$ (*vs.* control); **, $P < .01$ (*vs.* control). C1, Primary trophoblasts were cultured in the absence or presence of 1 or 10 μg/ml of poly(I:C) for 24 h. IDO protein expression was evaluated by Western blot analysis. Vinculin, a housekeeping protein, was used as a loading control. IDO protein expression was not detected in untreated trophoblasts but was induced by poly(I:C) in a dose-dependent manner. One of the representative results is shown. C2, Untreated Swan 71 trophoblast cells do not express IDO protein, whereas 10 μg/ml of poly(I:C) (24 h) induces IDO protein expression.

first-trimester trophoblasts do not express IDO protein constitutively, but expression was induced by poly(I:C) in a dose-dependent manner (Fig. 1, C1). Induction of IDO by poly(I:C) was also demonstrated in the Swan 71 cells (Fig. 1, C2).

Poly(I:C) induced IDO activity in the first-trimester trophoblasts

Given that poly(I:C) induced IDO mRNA and protein expression in trophoblasts, we next assessed the effect of poly(I:C) on the enzymatic activity of IDO in trophoblasts by measuring kynurenine concentrations in the conditioned media. As illustrated in Fig. 2, in comparison with conditioned media from untreated trophoblasts in which kynurenine levels did not change, the concentration of kynurenine in conditioned media from poly(I:C)-treated trophoblasts increased over time. The difference in kynurenine levels, between poly(I:C)-treated cells and untreated cells, peaked at 24 h but was significant at 8 h ($P < 0.01$) and remained significant for up to 36 h. This result indicated that poly(I:C) induces IDO enzymatic activity in trophoblasts and catabolizes tryptophan in the conditioned media.

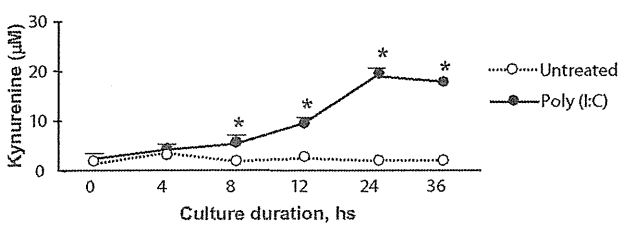

FIG. 2. IDO activity in the conditioned media of Swan 71 trophoblasts. Swan 71 cells were cultured with or without 10 μg/ml of poly(I:C). Conditioned media were collected at the indicated time points (0, 4, 8, 12, 24, and 36 h) and concentrations of kynurenine were determined. Data represent mean ± SD of quadruplicate determinations. *, $P < 0.001$ *vs.* untreated at the same indicated time points.

Conditioned media from poly(I:C)-treated trophoblasts suppressed T cell DNA synthesis

The effect of poly(I:C) treatment of trophoblasts on T cell response was then evaluated (Fig. 3). In comparison with T cells cultured in control conditioned media, DNA synthesis in T cells cultured in conditioned media taken from poly(I:C)-treated Swan 71 cells was significantly reduced. We confirmed that the residual poly(I:C) in the conditioned medium had no effect on T cells (data not shown). The DNA synthesis of T cells was significantly, although not completely, restored when L-tryptophan was added to the conditioned media from poly(I:C)-treated Swan 71 cells. This restoring effect of L-tryptophan indicates that the catabolism and depletion of tryptophan by IDO is responsible for the reduction of T cell DNA synthesis, although it is possible that other factors may also contribute to this reduction.

Poly(I:C) induced IFN-β and IFN-β induced IDO in the first-trimester primary trophoblasts

The mechanism by which TLR-3 ligation induces IDO in trophoblasts was then investigated by focusing on the possible involvement of IFN-β. IFN-β mRNA was not constitutively expressed in primary trophoblasts but was induced by poly(I:C) (Fig. 4A). This result is consistent with findings observed in HTR-8/SVneo cells, a trophoblast cell line (9). IFN-β induction of IDO was then investigated. As shown in Fig. 4, B and C, recombinant

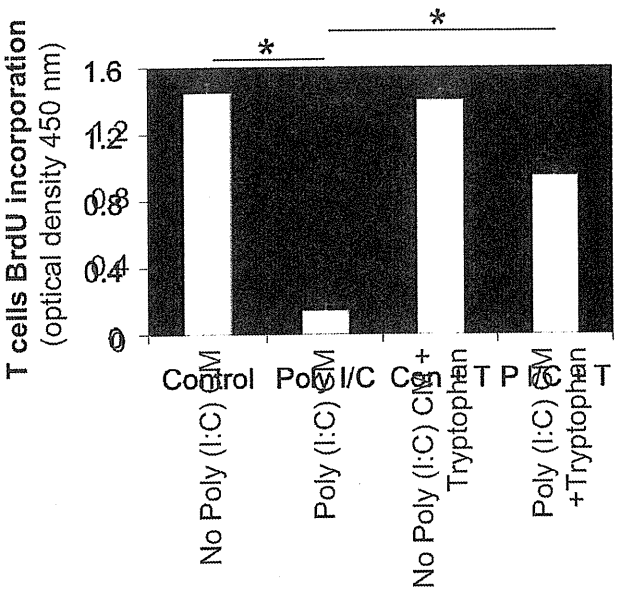

FIG. 3. Suppression of T cell DNA synthesis by conditioned media from poly(I:C)-treated Swan 71 trophoblasts. Conditioned media were prepared by culturing Swan 71 cells with or without 10 μg/ml of poly(I:C) for 24 h. T cells were cultured for 72 h in conditioned media with 5 μg/ml of phytohemagglutinin in the presence or absence of 100 μM of L-tryptophan. DNA synthesis of T cells was determined by BrdU incorporation. Conditioned media from poly(I:C)-treated Swan 71 cells suppressed the DNA synthesis of T cells. This suppression was restored when L-tryptophan was added to the culture. One of the representative data is shown (mean ± SEM of hexaplicate experiments). *, $P < 0.0001$.

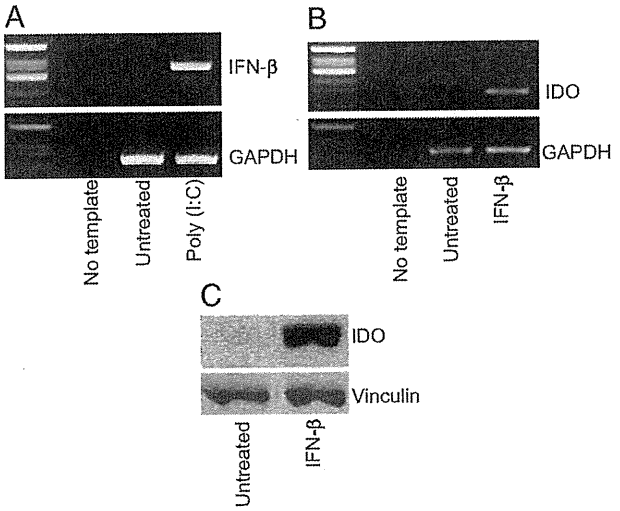

FIG. 4. Poly(I:C) induced IFN-β and IFN-β induced IDO expression in primary first-trimester trophoblasts. A, Primary trophoblasts were cultured with or without 10 μg/ml of poly(I:C) for 12 h. IFN-β mRNA expressions were evaluated by analysis of RT-PCR followed by electrophoresis. IFN-β was not constitutively expressed in first-trimester trophoblasts, but poly(I:C) induced IFN-β expression. B, Primary trophoblasts were cultured with or without 25 ng/ml of recombinant human IFN-β for 12 h. IDO mRNA expression was detected and analyzed by using RT-PCR. GAPDH was used as a loading control. C, Primary trophoblasts were cultured with or without 25 ng/ml of recombinant human IFN-β for 24 h. IDO protein expression was detected by using Western blot analysis. Vinculin was used as loading control. Both IDO mRNA and protein expression were induced by IFN-β.

IFN-β (25 ng/ml) induced IDO mRNA (Fig. 4B) and protein (Fig. 4C) in primary trophoblasts. Aggregated densitometry data showed a significant induction of IDO mRNA and protein by IFN-β ($n = 5$: $P < 0.01$, $n = 6$: $P < 0.01$, respectively).

The induction of IDO by poly(I:C) was preceded by that of IFN-β

The time-dependent nature of IFN-β and IDO induction by poly(I:C) was then investigated in Swan 71 cells. The induction of IDO mRNA was detected at 4 h after poly(I:C) treatment and peaked at 8 h (Fig. 5A). However, IFN-β mRNA levels peaked at 2 h after poly(I:C) treatment, which preceded the induction of IDO (Fig. 5B). Investigation of the time dependence of IDO induction by IFN-β revealed the earliest sign of induction at 2 h and peaked at 4 h (Fig. 5C).

Blocking IFN-β canceled the IDO induction by poly(I:C)

Given the possible involvement of IFN-β in IDO induction by poly(I:C), anti-IFN-β antibody was added to the culture system. Primary trophoblasts were pretreated with neutralizing antibody against human IFN-β (20 μg/ml) or control IgG for 30 min followed by treatment with poly(I:C) (10 μg/ml) for 8 h. Quantitative RT-PCR was performed to detect changes in IDO mRNA levels. Neutralizing antibody against IFN-β inhibited the induction of IDO by poly(I:C) in trophoblasts (Fig. 6) thereby confirming that IFN-β mediates the poly(I:C) stimulated induction of IDO.

Discussion

In the present study, we have demonstrated for the first time that IDO, which is involved in both antiviral activity and fetomaternal tolerance, is induced by the TLR-3 ligand poly(I:C) in human trophoblasts. This study has demonstrated that poly(I:C) induces the expression of IDO mRNA and protein in trophoblasts. Furthermore, our findings confirm that poly(I:C) induces IDO enzymatic activity and depletes tryptophan and consequently reduces DNA synthesis in T cells. In terms of elucidating the signaling pathway, IFN-β mediates the induction of IDO by poly(I:C) because blocking IFN-β specifically reduced this poly(I:C) effect.

It has been previously demonstrated by our group that ligation of TLR-3 by the viral ligand, poly(I:C), causes

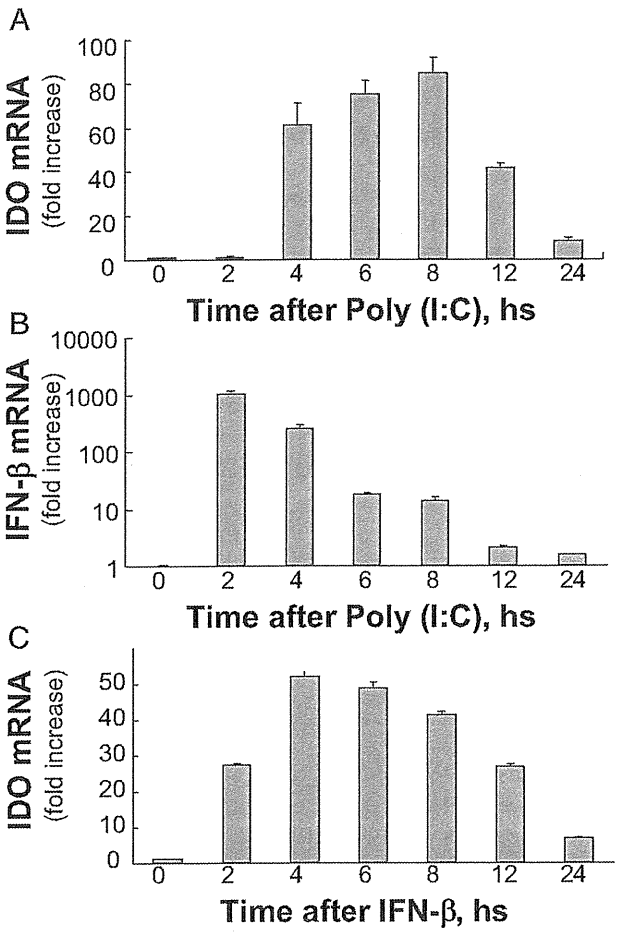

FIG. 5. Time-course studies of IDO induction by poly(I:C) (A), IFN-β induction by poly(I:C) (B), and IDO induction by IFN-β (C). Trophoblast Swan 71 cells were stimulated with 10 μg/ml of poly(I:C) or 25 ng/ml of IFN-β for the indicated time periods (0, 2, 4, 6, 8, 12, and 24 h). Quantitative RT-PCR was performed to detect changes in mRNA expression: the fold increases of IFN-β mRNA are indicated. Values are the mean ± SEM of triplicate cultures. Note that IDO induction by poly(I:C) was preceded by IFN-β induction by poly(I:C) (A *vs.* B) and IFN-β induced IDO more rapidly than poly(I:C) (C *vs.* A).

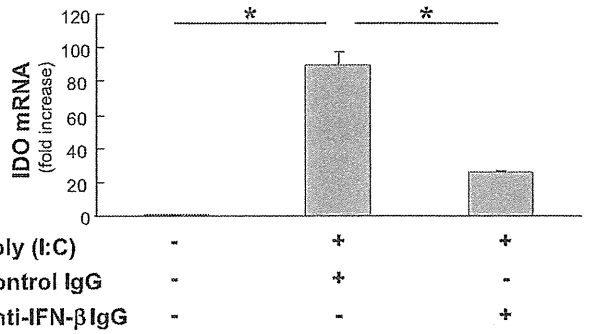

FIG. 6. Neutralizing antibody against IFN-β inhibited IDO induction by poly(I:C) in primary trophoblasts. Primary trophoblasts were pretreated with neutralizing antibody against human IFN-β (anti-IFN-β IgG) or nonimmune rabbit IgG (control IgG) for 30 min followed by treatment with 10 μg/ml of poly(I:C) for 8 h. The level of IDO mRNA was evaluated by the analysis of quantitative RT-PCR. Values are the mean ± SE of triplicate cultures. In comparison with controls, neutralizing antibody against IFN-β significantly inhibited poly(I:C)-induced IDO mRNA expression. *, $P < 0.001$.

trophoblasts to secrete chemokines such as regulated upon activation, normal T cell expressed, and secreted and antiviral factors such as IFN-β, secretory leukocyte protease inhibitor, 2′,5′-oligoadenylate synthetase, myxovirus-resistance A, and apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (9). These chemokines and antiviral factors are thought to modulate further immune responses and in turn contribute to the pathogenesis of pregnancy complications. As a result of the current study, IDO, which is a strong immune modulating factor, can now be added to the list of factors produced by trophoblasts upon TLR-3 ligation.

Previous studies have demonstrated that IDO has antiviral effects in various types of human cells. Bodaghi *et al.* (30) demonstrated that IDO controls human cytomegalovirus replication in human retinal pigment epithelial cells. Similar IDO effects were reported in human astrocytes for herpes simplex virus (31) and in epithelial, endothelial, and astroglial cells for measles virus (32). The mechanism by which IDO inhibits the replication of virus is the degradation of tryptophan, which is essential for virus replication (30, 31). In the case of a viral infection such as cytomegalovirus, the placenta and hence trophoblasts are targets for virus. In the current study, we found that TLR-3 ligation, which mimics viral infection, induces IDO expression and promotes tryptophan catabolism in first-trimester primary trophoblasts. This result further confirms our previous findings and other studies that suggest that trophoblasts or the placenta act as an active barrier to resolve infection and protect the fetus (1, 33–35).

On the other hand, IDO in the fetomaternal interface is also known to play an important role in maintaining tolerance for the establishment of a successful pregnancy. Munn *et al.* (21) proposed that the expression of IDO in the placenta is crucial to prevent immunological rejection of the fetus. They administered L-methyl-tryptophan, a pharmacological inhibitor of IDO, to pregnant mice and demonstrated rapid T cell-induced rejection of allogenic fetuses (21). In human pregnancy, IDO expression and activity have been demonstrated in the human placenta (36, 37), and reduced IDO expression in the human placenta and/or decidua has been linked to pathological pregnancy such as preeclampsia (38, 39), retarded intrauterine development (37), and miscarriage (40). In terms of the localization of IDO within the fetomaternal interface, the expression by human first-trimester trophoblasts is controversial (36, 37, 41). Some studies did not detect IDO in placental villi (37, 41), whereas Kudo *et al.* (36) demon-

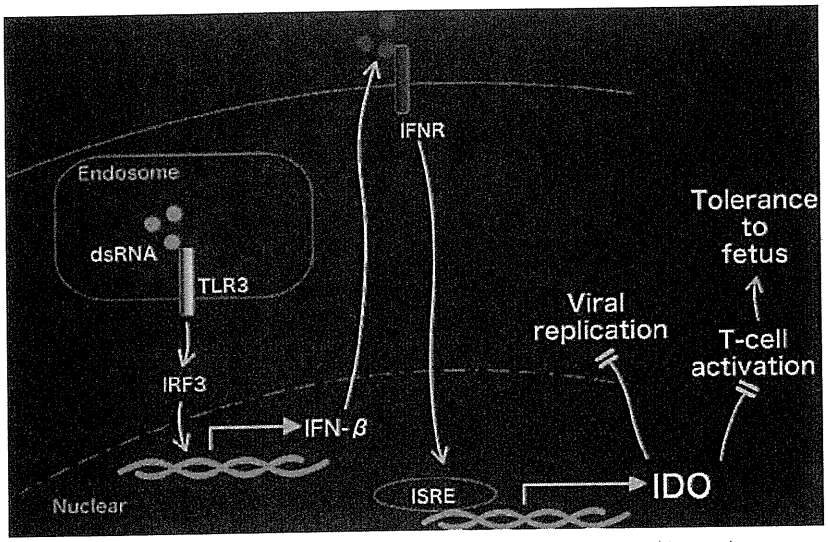

FIG. 7. Schematic representation of the TLR-3 signaling leading to antiviral immunity and tolerance to fetus in trophoblasts. TLR-3 is ligated by dsRNA such as poly(I:C). TLR-3 transduces signals to activate IRF3 kinases. IRF3 transactivates IFN-β (primary response gene). Secreted IFN-β binds to the type I IFN receptor (IFNR) in an autocrine manner and transactivates IFN-stimulated genes, which further stimulates the IFN-stimulated response element (ISRE). These findings identify IDO as one of the molecules induced by this transduction system in the human trophoblast. IDO subsequently inhibits viral replication and maintains the tolerance to the fetus.

strated strong expression of IDO in both the syncytiotrophoblast and invasive extravillous cytotrophoblast cell columns. On the other hand, decidua and uterine glandular epithelium have been shown to express IDO (36, 41). IDO expressions were also demonstrated in dendritic cells and macrophages in the fetomaternal interface (40). Therefore, it is plausible that IDO originated from a variety of types of cells that contribute to fetomaternal tolerance. In this study, quantitative real-time RT-PCR and Western blot analysis confirmed that IDO was not constitutively expressed in first-trimester trophoblasts; however, the activation of TLR-3 in trophoblasts dramatically induced IDO expression and reduced DNA synthesis in T cells. These findings suggest that trophoblasts induce production of IDO and contribute to the maintenance of tolerance in the case of viral infection, which can provoke hyperactive inflammation.

An important nature of IDO in this context is that this molecule can play a dual role, to provide antimicrobial action and maintain fetomaternal tolerance, in viral infection during pregnancy. IDO meets this seemingly paradoxical immunological task during pregnancy by strengthening defense against foreign pathogens without attacking the fetus, which is a semiforeign allograft. Therefore, inducing IDO upon viral infection is a very relevant response of trophoblasts in the fetomaternal interface and adds further evidence in support of the immunological role of the placenta.

Despite the significant roles of IDO in the placenta, factors that regulate IDO in the trophoblasts have not been well elucidated. By using a tissue culture system of term placenta, Kudo *et al.* (42) demonstrated that IFN-γ markedly stimulated IDO activity, whereas IFN-α and IFN-β were less potent. On the contrary, IFN-γ failed to induce IDO expression in the BeWo trophoblast cell line (43). In the current study, we used first-trimester primary cytotrophoblasts and identified TLR-3 ligation as a novel factor that significantly induces IDO expression in a dose- and time-dependent manner.

We further demonstrated that in primary trophoblasts, IFN-β modulates poly(I:C)-induced IDO expression by demonstrating that IFN-β directly induces IDO and that neutralizing antibody specific to IFN-β inhibits poly(I:C)-induced IDO expression (refer to the schematic diagram, Fig. 7). In general, TLR-3 ligation by dsRNA is known to activate IFN regulatory factor 3 (IRF3) kinase and stimulate the production of type I IFN (44, 45). In the current study, we have shown that IFN-β is not constitutively expressed but induced by poly(I:C) in primary trophoblasts. Secreted IFN-β may bind to the type I IFN receptor, which is expressed on trophoblasts (46), in an autocrine manner and transactivate IFN stimulated genes that further stimulate the IFN-stimulated response element. We have identified IDO here as one of the molecules induced by TLR-3 activation via this transduction system in human trophoblasts.

The expression and the function of IDO in the case of viral infection in *in vivo* settings remains to be elucidated. We and others have shown that administration of poly(I:C) to pregnant mice causes fetal resorption (47, 48) and preterm labor (49). Interestingly, however, we have also found that viral infection with murine gammaherpesvirus 68, which is able to activate TLR-3 (50), did not show the same outcome (51). In addition to the abovementioned IRF3 pathway, ligation of TLR-3 is known to activate the nuclear factor-κB pathway through a MyD88-dependent manner (44, 52). Activation of nuclear factor-κB signaling results in cytokine/chemokine production and consequently causes inflammatory responses (49), which could lead to pregnancy complications such as spontaneous abortion and preterm labor when it occurs at the fetomaternal interface. At the same time, activation of the

IRF3 pathway results in the production of type I IFN and inducible molecules such us IDO, which may function in an antiviral response and protect pregnancy. Therefore, we speculate that these two pathways, one harmful and one beneficial, finely tune the immune response at the fetomaternal interface and that the overall balance between these two pathways determines the clinical outcome of the infected pregnancy. Further studies are needed to elucidate the *in vivo* function of IDO in the case of viral infection during pregnancy.

There are several limitations in our study. First, we used primary trophoblasts and a trophoblast cell line, Swan 71, rather than placental tissue, which has been preferably and widely used for these types of experiments (22, 53). Therefore, we cannot exclude possible contributions of cell populations other than trophoblasts on TLR-3 ligation in the fetomaternal interface. It is also possible that culture conditions such as FBS supplementation and using the Swan 71 cell line in experiments may have affected the results, given the possible effects of serum (54) or long-term trophoblast culture (55) on the immunosuppressive property of trophoblasts. The pure contribution of trophoblasts on the fetomaternal immune system, upon viral infection, in the *in vivo* setting remains to be determined.

In conclusion, our study demonstrates that TLR-3 ligation by poly(I:C) can induce IDO expression in human first-trimester trophoblasts via an IFN-β-dependent way. This discovery reveals a novel innate immune function of trophoblasts to meet the paradoxical immunological task during pregnancy: inducing antimicrobial activity and maintaining fetomaternal tolerance.

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Activin-A is induced by interleukin-1β and tumor necrosis factor-α and enhances the mRNA expression of interleukin-6 and protease-activated receptor-2 and proliferation of stromal cells from endometrioma

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Objective: To examine the regulation and the function of activin-A in stromal cells derived from endometrioma.
Design: Molecular studies.
Setting: University research laboratory.
Patient(s): Endometrioma stromal cells (EoSC) were obtained from 28 patients with ovarian endometrioma undergoing laparoscopy.
Intervention(s): EoSC were cultured with inflammatory stimuli or recombinant activin-A, followed by RNA extraction.
Main Outcome Measure(s): Activin mRNA expression was evaluated by real-time reverse transcription–polymerase chain reaction (RT-PCR), and activin-A concentration of supernatant of cultured EoSC was evaluated by ELISA. Also, the effect of activin-A on EoSC was evaluated with real-time RT-PCR and cell proliferation assay.
Result(s): Inflammatory stimuli, interleukin (IL) -1β, and tumor necrosis factor (TNF) -α induced inhibin/activin-βA subunit mRNA and activin-A protein expression in EoSC. Additionally, activin-A enhanced EoSC proliferation and increased the expression of IL-6 and protease-activated receptor (PAR)-2 mRNA.
Conclusion(s): An in vitro study revealed that activin-A, which is induced by IL-1β or TNF-α, might promote endometriosis by stimulating IL-6 and PAR-2 mRNA expression and increasing the proliferation of EoSC. (Fertil Steril® 2011;96:118–21. ©2011 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, activin-A, inflammatory cytokine, IL-6, PAR-2

Endometriosis is a gynecological condition in women of reproductive age, of which the primary symptoms are infertility and pain. Endometriosis is defined as the presence of viable endometrial glands and/or stroma outside of the uterine cavity. Although the etiology of endometriosis is unknown, multiple lines of evidence suggest that inflammation plays a pivotal role in the pathogenesis of the disease (1–4). We have shown that aberrant secretion of cytokines, such as interleukin (IL)-6, IL-8, and monocyte chemoattractant protein-1, in endometriotic tissue plays a role in modulating the inflammatory response. Moreover, we have demonstrated an association between protease-activated receptor (PAR)-2 and endometriosis.

PARs, which are members of the seven-transmembrane G-protein-coupled receptor family, are known to be important mediators of inflammation (5). We have reported that PAR-2 activation leads to the development of endometriosis by promoting endometrioma stromal cell (EoSC) growth and inflammation (6), while the formation of endometriotic lesions was suppressed in PAR-2 knockout mice (7).

Activins, which are a member of the TGF (transforming growth factor) -β superfamily, are homodimers of inhibin/activin-β subunits. Inhibins are heterodimers of inhibin-α and inhibin/activin-β subunits (8). Although activins are originally isolated as a factor that stimulates pituitary glands to secrete FSH (8), several studies provide evidence that activins also take part in inflammatory processes. For example, increased expression of activin-A has been shown in several inflammatory lesions, including ulcerative colitis, Crohn's disease, and interstitial pulmonary fibrosis (9, 10). Recent studies have demonstrated that activin-A and its receptors are expressed in endometriotic lesions (11, 12). Additionally, in vitro studies have demonstrated an increase in activin-A secretion from eutopic endometrium in women with endometriosis (12, 13).

These findings suggest that activin-A might be involved in the development and progression of endometriosis. However, the regulation of activin-A expression and its effect on the endometrium

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is unknown. In the present study, we examined the regulation of activin-A expression in EoSC, focusing on inflammatory stimuli. Additionally, we examined the effect of activin-A on cultured EoSC.

MATERIALS AND METHODS

Reagents and Materials

Dulbecco's minimum essential medium (DMEM)/Ham's F12 (F12) medium, 0.25% trypsin-ethylenediamineteraacetic acid (EDTA), and deoxyribonuclease I were from Invitrogen. Fetal bovine serum (FBS), type I collagenase, and antibiotics (mixture of penicillin, streptomycin, and amphotericin B) were purchased from Sigma. Recombinant human activin-A was from R&D Systems.

Patients and Samples

Tissue specimens were obtained from 28 patients with ovarian endometrioma undergoing laparoscopy after obtaining written informed consent under a study protocol approved by the Institutional Review Board of the University of Tokyo. All patients had regular menstrual cycles, and none had received hormone treatment for at least 3 months before surgery. Endometriotic tissue samples were obtained from the cyst wall of an ovarian endometrioma under sterile conditions and were histologically confirmed.

Isolation and Culture of Human EoSC

Primary EoSC culture was conducted as described elsewhere (14). Briefly, endometriotic tissue was dissected free of underlying parenchyma, minced into small pieces, incubated in DMEM/F12 with type I collagenase (2.5 mg/mL) and DNase I (15 U/mL) for 1–2 hours at 37°C, and separated using serial filtration. Debris were removed using a 100-μm nylon cell strainer (Becton Dickinson), and dispersed epithelial glands were eliminated with a 70-μm nylon cell strainer. Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in DMEM/F12 with 10% charcoal-stripped FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL), and plated onto 100-mm dishes (Iwaki). When the cells became confluent after 2 days, they were dissociated with 0.25% trypsin, harvested by centrifugation, and replanted in 6-well plates at 2×10^5 cells/well. They were kept at 37°C in a humidified 5% CO_2/95% air environment until they were grown to confluence over the course of 2 days. Purification of the stromal cell population was confirmed by immunocytochemical staining for the following antibodies: vimentin (stromal cells), cytokeratin (epithelial cells), and CD45 (monocytes and other leukocytes). The purity of the stromal cell was more than 98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin and CD45.

Treatment of Cultured EoSC

Cultured EoSC were treated with IL-1β (10 ng/mL; Genzyme) or tumor necrosis factor (TNF)-α (10 ng/mL; Genzyme) without FBS for 6 or up to 48 hours. In the experiment for mRNA expression, EoSC were cultured for 6 hours (n = 5). To examine the activin-A release into the medium, EoSC were plated to 12-well plates with 1 mL of DMEM/F12 with or without IL-1β or TNF-α. After incubation for 6, 24, and 48 hours, respectively, supernatants were collected and stored at −80°C until assay (n = 3). To examine the effect of activin-A on EoSC, cultured EoSC were stimulated with activin-A (0–300 ng/mL) without FBS for 6 hours to check mRNA levels. To examine the involvement of the activin receptor in activin-A-inducing responses in EoSC, SB431542 (10 μM), activin receptor–like kinase (ALK) -4, -5, and -7 inhibitor, or DMSO (control) was added 1 hour before activin-A stimulation (n = 3).

Reverse Transcription (RT) and Quantitative Real-Time Polymerase Chain Reaction (PCR) Analysis

Total RNA was extracted from EoSC, using the RNeasy minikit (Qiagen). RT was performed using Rever Tra Dash (TOYOBO). One microgram of total RNA was reverse transcribed in a 20-μL volume. For the quantification of various mRNA levels, real-time PCR was performed using LightCycler (Roche Diagnostic GmbH), according to the manufacturer's instructions. The PCR primers were selected from different exons of the corresponding genes to discriminate PCR products that might arise from possible chromosomal DNA contaminants. Especially, as activins are homodimers of inhibin/activin-β subunits and inhibins are heterodimers of inhibin-α and inhibin/activin-β subunits (8), the mRNA of the inhibin-α subunit and the inhibin/activin-βA and -βB subunits was examined, respectively.

The primer sequences were as follows: inhibin-α subunit (NM_002191: 369–388 and 602–582), inhibin/activin-βA subunit (NM_002192: 505–526 and 673–653), inhibin/activin-βB subunit (NM_002193: 1184–1204 and 1325–1305), PAR-2 (NM_005242: 369–389 and 549–530), and GAPDH (NM_002046: 628–648 and 1079–1060). PCR conditions were as follows: IL-6, 40 cycles of 95°C for 10 seconds, 66°C for 8 seconds, and 72°C for 12 seconds; PAR-2, 40 cycles of 95°C for 10 seconds, 64°C for 10 seconds, and 72°C for 8 seconds; GAPDH, 35 cycles of 95°C for 10 seconds, 64°C for 10 seconds, and 72°C for 18 seconds; inhibin-α subunit, inhibin/activin-βA subunit, and inhibin/activin- βB subunit, 40 cycles of 95°C for 10 seconds, 55°C for 10 seconds, and 72°C for 8 seconds. After amplification, melting curve analysis was performed. Relative expression of each mRNA was normalized by GAPDH mRNA.

Cell Proliferation Assay

To examine the effect of activin-A on the cell number of EoSC, preconfluent EoSCs in 96-well plates were treated with vehicle (phosphate-buffered saline) or activin-A (100 ng/mL) in DMEM/F12 without serum for 48 hours. After 48 hours of stimulation, cell numbers were measured with a cell counting kit (CCK-8; Dojindo), based on the method of colorimetric assay. Briefly, media were replaced with DMEM/F12 without serum and 10 μL of the CCK-8 solution was added to each well and incubated for 1 hour in the incubator. Absorbance was measured at 450 nm using a microplate reader. The data were shown as a ratio to controls (mean ± SD) from four different experiments.

ELISA

The concentration of activin-A in supernatant was measured in triplicate using specific ELISA kits (Quantikine; R&D Systems). The sensitivity of the assay was 3.6 pg/mL. The intra-assay and interassay coefficients of variation were <5% in the assays. The data obtained were shown as a relative ratio to the basal level of 6 hours of culture.

Statistical Analysis

Data were analyzed by Student's *t*-test for paired comparison and one-way ANOVA with post hoc test for multiple comparisons using Statview software (SAS Institute Inc.). $P<.05$ was considered statistically significant.

RESULTS

mRNA Expression of Inhibin/Activin Subunits in EoSC

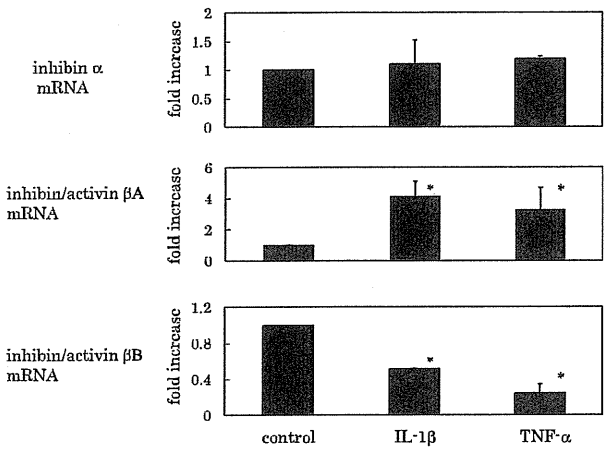
As shown in Figure 1, with IL-1β and TNF-α stimulation for 6 hours, the inhibin-α subunit mRNA of cultured EoSC was unchanged. There was an approximately 3- to 4-fold (n = 5; $P<.01$) increase in inhibin/activin βA subunit mRNA expression with IL-1β or TNF-α stimulation compared with each control sample, while expression of the inhibin/activin-βB subunit was reduced by more than 50% with these stimuli (n = 5, $P<.01$).

Activin-A Concentration in Supernatant of EoSC

In EoSC, the basal level of activin-A release into the medium was increased in a time-dependent manner (Fig. 2). In the presence of IL-1β or TNF-α, the concentration of activin-A was over 3- to 5-fold higher than that of each control sample throughout the 48-hour culture period (n = 3; $P<.01$). Compared with IL-1β- and TNF-α-stimulated EoSC, IL-1β rather than TNF-α induced activin-A in cultured supernatant significantly throughout 48 hours ($P<.01$).

FIGURE 1

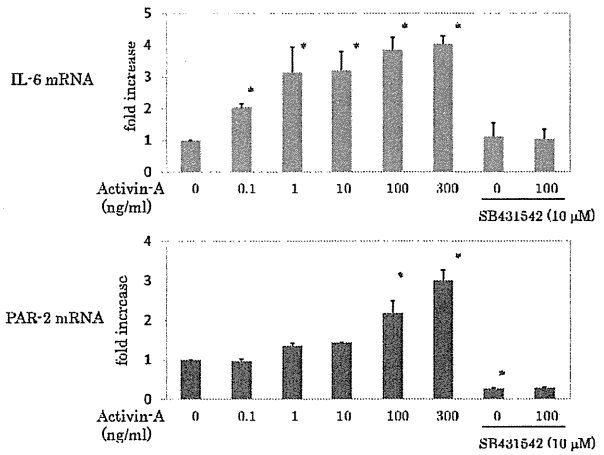
Inhibin/activin subunits mRNA in human EoSC. Cultured human EoSC were stimulated with IL-1β (10 ng/mL) or TNF-α (10 ng/mL) for 6 hours. Total RNA was extracted from the cells and subjected to real-time PCR to determine the inhibin/activin subunits mRNA levels. Data were normalized to GAPDH mRNA levels. Representative data from five different experiments were shown as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. *P<.01 (vs. control).



Yoshino. The role of activin-A in endometriosis. Fertil Steril 2011.

FIGURE 3

The role of activin-A in EoSC. Cultured human EoSC were stimulated with activin-A (0–300 ng/mL) for 6 hours. SB431542 (10 μM), ALK-4, -5, and -7 inhibitor, or DMSO (control) was added 1 hour before activin-A stimulation. Total RNA was extracted from the cells and subjected to real-time PCR to determine the IL-6 and PAR-2 mRNA levels. Data were normalized to GAPDH mRNA levels. Representative data from three different experiments were shown as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. *P<.01 (vs. control).



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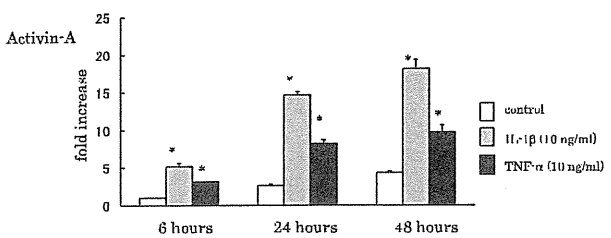
The Role of Activin-A in EoSC

As shown in Figure 3, activin-A significantly induced IL-6 and PAR-2 mRNA expression of EoSC in a dose-dependent manner. Although IL-6 mRNA expression was induced significantly with activin-A at the concentration of 0.1 ng/mL, upregulation of PAR-2 mRNA expression was observed at 100 ng/mL (n = 3; P<.01).

In the presence of SB431542 (10 μM), ALK-4, -5, and -7 inhibitor, activin-A-induced IL-6, and PAR-2 expression was suppressed. Moreover, SB431542 reduced the basal level of PAR-2 but not IL-6 mRNA expression (n = 3; P<.01).

FIGURE 2

Activin-A concentration in the supernatant of EoSC. Cultured human EoSC were stimulated with IL-1β (10 ng/mL) or TNF-α (10 ng/mL) for 6–48 hours. Activin-A concentration in the supernatant of cultured EoSC was measured in triplicate by ELISA, and data were shown as a relative ratio to the basal level of 6 hours of culture. One representative data point from three different experiments were shown. *P<.01 (vs. control).



Yoshino. The role of activin-A in endometriosis. Fertil Steril 2011.

In a cell proliferation assay, activin-A (100 ng/mL) stimulation for 48 hours exhibited a significant 1.3-fold increase in the number of EoSC (n = 4, mean ± SD, 1.3 ± 0.02; P<.0001).

DISCUSSION

In the present study, we found that inflammatory stimuli IL-1β and TNF-α induced activin-A expression in EoSC. Activin-A also enhanced EoSC proliferation and the expression of IL-6 and PAR-2 mRNA.

It is likely that an altered immunoinflammatory environment in the peritoneal cavity supports the survival and growth of endometriotic lesions. A variety of cytokines and growth factors derived from activated macrophages, endometriotic cells, and mesothelial cells in the abdominal cavities of women with endometriosis are thought to play a role in the development of the disease (1–4). IL-1β and TNF-α in particular may be key molecules involved in the disease process (1). These cytokines elicit pleiotropic effects, such as stimulation of cytokine secretion and cyclo-oxygenase (COX)-2 expression (14).

In the present study, we found that IL-1β and TNF-α increased the expression of the inhibin/activin-βA subunit in EoSC, without changing the inhibin-α subunit level. Consistent with the increase in mRNA expression, activin-A protein levels in the cultured supernatant were also upregulated with these stimuli. These results might explain the high concentration of activin-A protein that has been observed in the peritoneal fluid of patients with endometriosis (15).

Interestingly, mRNA expression levels of the inhibin/activin-βB subunit were suppressed with inflammatory stimuli. This result is consistent with observations by Reis et al., who showed that ovarian endometriotic cells fail to express the inhibin/activin-βB subunit (15), and Florio et al., who demonstrated that the concentration of inhibin-B in the supernatant of cultured EoSC is less than that of

inhibin-A, suggesting that the inhibin/activin βA subunit is predominant in endometriotic tissue (16). The lower expression levels of the inhibin/activin-βB subunit in ovarian endometriotic cells could also be the result of inflammation.

We also examined whether activin-A augmented the proliferation of endometriotic cells. The effect of activins on cell proliferation is known to be cell dependent. Activins stimulate proliferation of cells of various origins, including lung fibroblast, keratinocyte, thyroid cells, osteoblasts, and spermatogonial cells, but also exhibit an anti-proliferation effect on lymphocytes and prostate cancer cells (17). In the present study, activin-A induced proliferation of EoSC derived from endometrioma.

Furthermore, we found that activin-A induced expression of two known accelerators of endometriosis, IL-6 and PAR-2, in a dose-dependent manner. Dose-dependent studies revealed that IL-6 mRNA was induced at lower concentrations of activin-A compared with PAR-2 mRNA, suggesting the dose-dependent function of activin-A in the development of endometriosis. High IL-6 expression levels have previously been observed in the peritoneal fluid of women with endometriosis (18). Moreover, IL-6 secretion from endometriotic cells is a putative pathophysiological mechanism underlying the development of endometriosis (19). We also have shown that PAR-2 is involved in the pathogenesis of endometriosis (6, 7). Although the regulation of PAR-2 expression in EoSC has not been clarified, the present study revealed that activin-A could be an important factor in the regulation of PAR-2 expression. Notably, basal levels of PAR-2 but not IL-6 were reduced with the addition of SB431542, an inhibitor of several TGF-β family cytokines. This result suggests that cytokines of the TGF-β superfamily are likely to play a role in the regulation of the basal PAR-2 expression in EoSC.

In summary, we have demonstrated that activin-A is induced by inflammatory stimuli and then mediates the development of endometriosis by inducing cell proliferation and further increasing the inflammatory response.

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Interleukin-17F increases the secretion of interleukin-8 and the expression of cyclooxygenase 2 in endometriosis

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Objective: To examine the effects of interleukin (IL)-17F on the secretion of IL-8 and the gene expression of cyclooxygenase 2 (COX2) in endometriotic stromal cells.
Design: In vitro experimental study using human samples.
Setting: University hospital.
Patient(s): Endometriotic tissues were obtained from women with ovarian endometriomas undergoing laparoscopic surgery.
Intervention(s): Endometriotic stromal cells (ESCs) were cultured with IL-17F.
Main Outcome Measure(s): Concentrations of IL-8 were measured by a specific ELISA, and messenger RNA levels of IL-8 and COX2 were measured by real-time reverse transcription–polymerase chain reaction (PCR).
Result(s): IL-17F increased the secretion of IL-8 from ESCs, and the effect was inhibited by antibodies for IL-17 receptor A and IL-17 receptor C. Tumor necrosis factor α (TNF-α) synergistically enhanced IL-17F-induced increase in IL-8 secretion from ESCs. The IL-17F increased the gene expression of IL-8 and COX2 in ESCs.
Conclusion(s): These findings suggest that IL-17F may stimulate the development of endometriosis by up-regulation of IL-8 and COX2. (Fertil Steril® 2011;96:113–7. ©2011 by American Society for Reproductive Medicine.)

Key Words: Endometriosis, interleukin-17F, interleukin-8, cyclooxygenase 2

Endometriosis is an enigmatic disease, which is defined by the presence of endometriotic tissue outside the uterus. Although the precise etiology and pathogenesis of endometriosis remains unclear, it is widely believed that endometrial cells in retrograde menstruation implant and grow in the pelvic cavity. Given this theory, a puzzle emerges that only a fraction of women develop endometriosis, whereas retrograde menstruation is observed in most women. Multiple lines of evidence suggest that inflammation and immune responses play a pivotal role in the pathogenesis of endometriosis (1–3).

The Th17 cells are a new and distinct lineage of CD4+ helper T cells, which express interleukin (IL)-17A, but not IL-4 and interferon γ (IFN-γ). The Th17 cells play crucial roles in various autoimmune diseases and allergic diseases by promoting chronic inflammatory responses (4, 5). Previously, we demonstrated that Th17 cells were present in endometriotic tissues and that IL-17A stimulated IL-8 secretion, cyclooxygensase-2 (COX2) expression, and cell proliferation of endometriotic stromal cells (6, 7). Other investigators reported that IL-17A concentrations in peritoneal fluid (PF) correlated with the severity of endometriosis and infertility of this disorder (8). In addition, a recent study demonstrated that high expression of IL-17A was observed in the fluid of endometrioma positive for aromatase (9). These findings suggest that IL-17A and Th17 are involved in the pathogenesis of endometriosis.

Interleukin-17F is a member of the IL-17 family, which consists of six cytokines: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (10). Interleukin-17F consists of 153 amino acids approximately 50% homologous with IL-17A. It is expressed in Th17 cells (11), basophils, mast cells (12), and monocytes (13). Both IL-17A and IL-17F bind to the heteromeric receptor complex comprised of IL-17 receptor C (IL-17RC) and IL-17 receptor A (IL-17RA). However, each ligand has a different affinity for each receptor. Interleukin-17F binds with an extremely low affinity to IL-17RA, whereas it binds with higher affinity to IL-17RC compared with IL-17A (14). Interleukin-17A and IL-17F induce similar biological responses in many biological events, but also elicit markedly different responses in several inflammatory situations (15). It increases the production of various proinflammatory cytokines and chemokines in bronchial epithelial cells (12, 16), keratinocytes (17), colonic fibroblasts (18), and endothelial cells (13). Recent studies suggest the involvement of IL-17F in the pathophysiology of allergic asthma (19), inflammatory bowel disease (20), or psoriasis (17).

Although we have demonstrated the presence of Th17 cells and possible roles of IL-17A in endometriosis, the role of IL-17F in the disease has not been addressed. To study the possible roles of IL-17F in endometriosis, we examined the effects of IL-17F on

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the production of IL-8 and on the expression of COX2, possible key players in endometriosis, in endometriotic stromal cells (ESCs) (21–23). We also studied cooperative effect of IL-17F and tumor necrosis factor α (TNF-α), a typical cytokine implicated in the disease (24).

MATERIALS AND METHODS

Patients and Samples

Endometriotic tissues were obtained from patients with ovarian endometriomas undergoing laparoscopy. All patients had regular menstrual cycles, and none had received hormonal treatment (HT) for at least 6 months before surgery. The tissues, collected under sterile conditions, were processed for primary cell cultures. The experimental procedures were approved by the institutional review board of the University of Tokyo and signed informed consent for use of the endometriotic tissues was obtained from each patient.

Isolation and Culture of Mononuclear Cells From Endometriotic Lesions

Fresh endometriotic tissues collected in sterile medium were rinsed to remove red blood cells (RBC). The tissues were minced into small pieces and incubated in phenol red-free Dulbecco's minimum essential medium (DMEM)/F12 containing type I collagenase (0.25%; Wako Pure Chemical Industries) and deoxyribonuclease I (DNase; 15 IU/mL; Invitrogen) for 120 minutes at 37°C. The resulting dispersed cells were separated by filtration through 100-μm and 70-μm nylon cell strainers (Becton Dickinson). The filtrate was washed twice with phosphate-buffered saline (PBS). This pellet was resuspended in 40% Percoll (5 mL), layered gently onto 70% Percoll, and centrifuged at 1,800 rpm for 20 minutes. The interface was recovered and washed in PBS, resuspended in RPMI 1640 medium containing 10% charcoal-stripped fetal bovine serum (FBS; Hyclone), plated into 10-mm plates (Iwaki, Asahi Technology), and allowed to adhere at 37°C overnight. Nonadherent cells were collected and used for the experimental procedures as mononuclear cells from endometriotic lesions (EMMCs). The EMMCs were resuspended in 10% FBS in RPMI 1640 medium. The cells were stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 μg/mL of ionomycin (Sigma) for 48 hours. The conditioned media was harvested for assay.

Isolation and Culture of ESCs

Isolation and culture of human ESCs were processed as described previously (25, 26). Fresh endometriotic tissues collected in sterile medium were rinsed to remove blood cells. The tissues were minced into small pieces and incubated in phenol-red free DMEM/F12 containing type I collagenase (0.25%) and DNase I (15 IU/mL) for 120 minutes at 37°C. The resultant dispersed endometriotic cells were separated by filtration through a 100-μm nylon cell strainer and 70-μm nylon cell strainer. Stromal cells remaining in the filtrate were collected by centrifugation, resuspended in phenol-red free DMEM/F-12, and plated 100-mm dishes and allowed to adhere at 37°C for 12 hours. At the first passage, the cells were plated into 6-, 12-, or 48-well culture plates. The cells reached confluence in 2 or 3 days and then were used for the experiments. The purity of ESCs was more than 95%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin or CD45.

Treatment of ESCs

First, to examine the effect of IL-17F on IL-8 production, ESCs were incubated in 5% FBS DMEM/F12 medium with various doses of IL-17F for 24 hours. Second, for the time-course study of IL-8 and COX2 gene expression, ESCs were incubated with 5% FBS medium with IL-17F (10 ng/mL) for different periods up to 24 hours. Third, to examine the effect of a mouse anti-IL-17RA antibody and a goat anti-IL-17RC antibody (R&D Systems), ESCs were preincubated in 5% FBS DMEM/F-12 with each antibody or the control IgGs for 30 minutes and then stimulated with 50 ng/mL IL-17F for 24 hours. Finally, to evaluate the synergic effect of IL-17F and TNF-α on IL-8 secretion, the cells were stimulated with various doses of IL-17F (1–100 ng/mL) with or without TNF-α (1 ng/mL).

RNA Extraction, Reverse Transcription, and Real-Time Quantitative Polymerase Chain Reaction of IL-8 and COX2

We extracted total RNA from ESCs cultured in a 12-well plate using an RNeasy minikit (QIAGEN). One microgram of total RNA was reverse transcribed in a 20-μL volume using a reverse transcription–polymerase chain reaction (RT-PCR) kit (TOYOBO). Real-time quantitative PCR was performed as reported previously (27, 28). To assess IL-8 and COX2 messenger RNA (mRNA) expression, real-time quantitative PCR and data analysis were performed using Light Cycler (Roche Diagnostics GmbH). Expression of IL-8 and COX2 mRNA was normalized to RNA loading for each sample using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal standard. The primers for IL-8 and COX2 were the same as those used previously (7). The PCR conditions were as follows: for IL-8, 40 cycles at 95°C for 10 seconds, 66°C for 10 seconds, 72°C for 11 seconds; for COX2, 30 cycles at 95°C for 10 seconds, 66°C for 10 seconds, 72°C for 13 seconds; for GAPDH, 30 cycles at 95°C for 10 seconds, 64°C for 10 seconds, 72°C for 18 seconds. All PCR conditions were followed by melting curve analysis.

Immunocytochemistry

The ESCs were cultured in 16-well chamber slides (Nunc) in a humidified 5% CO_2–95% air environment and allowed to grow to about 50% confluence. The cells were fixed with cold methanol/acetone at −20°C for 20 minutes, washed twice with PBS. Endogeneous peroxidase was blocked by incubation for 20 minutes with a solution of 0.3% hydrogen peroxidase. Immunocytochemical cell labeling was performed using the avidin-biotin peroxidase method. After blocking with normal rabbit serum (Vector laboratories), the cells were incubated with 1 μg/mL anti IL-17RC antibody or goat IgG for 60 minutes at room temperature and incubated with avidin-biotin peroxidase complex (Vectastain Elite, Vector Laboratories), according to the manufacturer's instructions. Staining was detected with the diaminobenzidine chromogen after 3 minutes. All slides were counterstained with hematoxylin and evaluated under a light microscope.

Measurement of Cytokines

Concentrations of IL-17F or IL-8 in conditioned media were measured using specific ELISA kits (IL-17F, Pepro tech; IL-8, Genzyme/Techne). The intra-assay and interassay coefficients of variation (CV) were less than 5%.

Statistical Analysis

Data were evaluated using analysis of variance (ANOVA) with Scheffé's post hoc analysis for multiple comparisons and Student's t-test for two groups. $P<.05$ was accepted as statistically significant.

RESULTS

Expression of IL-17F in EMMCs and IL-RC in ESCs

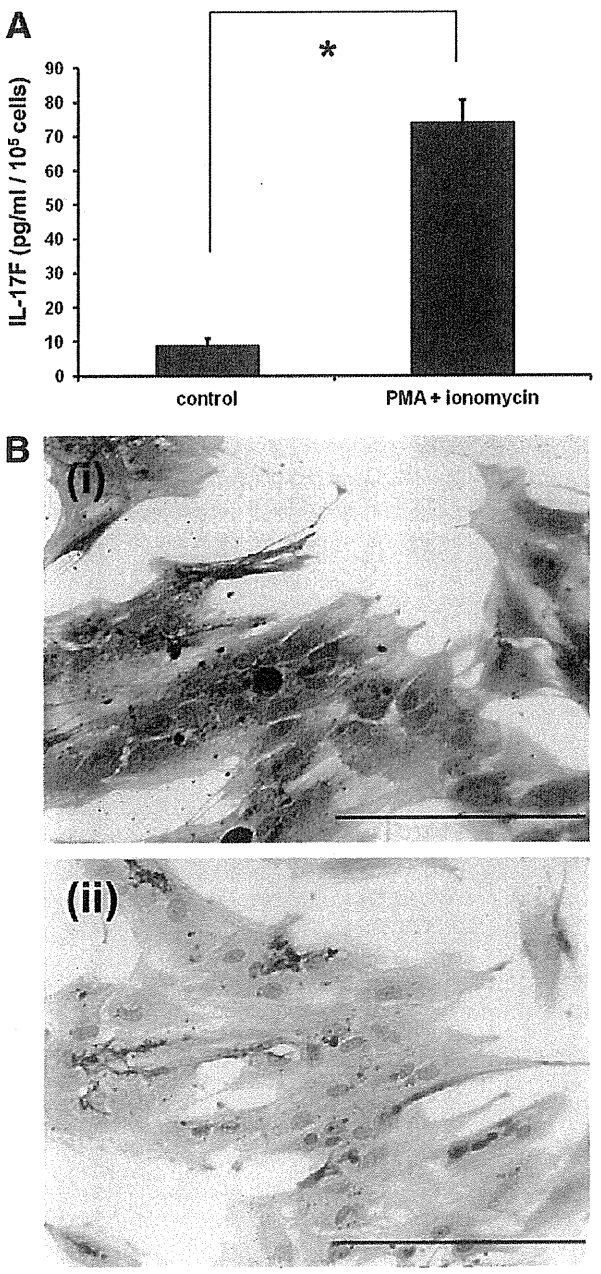
As shown in Figure 1A, EMMCs secreted IL-17F. The stimulation with PMA and ionomycin significantly enhanced the secretion of IL-17F from EMMCs. The presence of immunoreactive IL-17RC was demonstrated in ESCs (Fig. 1B). No staining was seen when goat IgG was used as a primary antibody.

Effect of IL-17F and Anti-IL-17RA or Anti-IL-17RC Antibodies on IL-8 Secretion From ESCs

As shown in Figure 2A, IL-17F at 10 ng/mL and a higher dose significantly increased the secretion of IL-8 from ESCs. The increase of IL-8 secretion by IL-17F at 100 ng/mL was 2.6-fold of the control. Treatment with the neutralizing antibodies for IL-17RA or IL-17RC significantly diminished IL-17F-induced increase in IL-8 secretion, whereas the control IgGs had no effect (Fig. 2B).

FIGURE 1

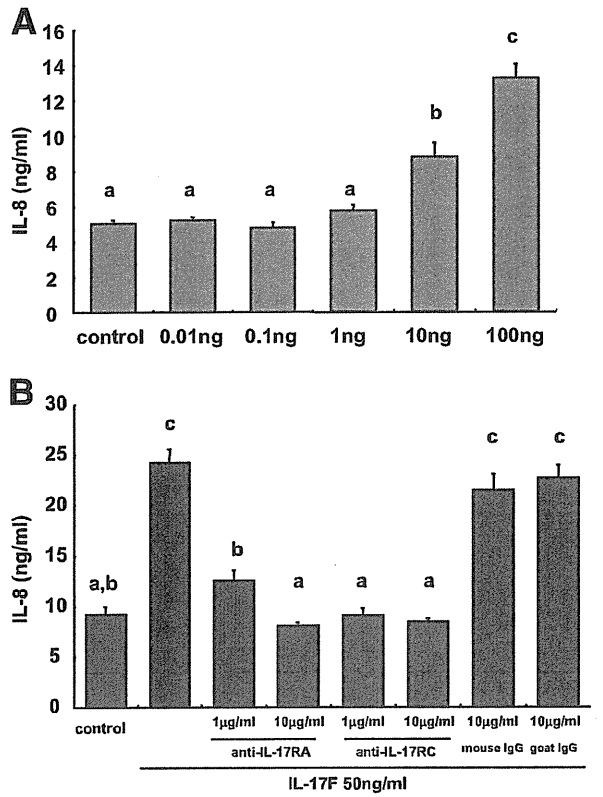
(A) Interleukin (IL)-17F secretion from mononuclear cells from endometriotic lesions (EMMCs). The EMMCs secreted IL-17F and the stimulation with phorbol ester (PMA; 50 ng/mL) and ionomycin (1 μg/mL) enhanced the secretion of IL-17F from EMMCs. Concentration of IL-17F in the conditioned medium was measured using a specific ELISA. Values are the means ± SEM of pentaplicate cultures. *$P<.0001$ versus control. The result is representative of three separate experiments using samples from different patients. (B) Immunocytochemistry of IL-17RC in endometriotic stromal cells. Cultured endometriotic stromal cells were immunostained with anti-IL-17RC antibody (i) or goat IgG isotype matched control (ii). Bars = 200 μm.



Hirata. IL-17F increases IL-8 in endometriosis. Fertil Steril 2011.

FIGURE 2

(A) Interleukin (IL)-17F stimulates IL-8 secretion from endometriotic stromal cells (ESCs). The ESCs were cultured in 5% fetal bovine serum with different doses of IL-17F for 24 hours. (B) Effect of a mouse anti-IL-17RA antibody or a goat anti-IL-17RC antibody on IL-17F-induced IL-8 secretion by ESCs. ESCs were preincubated in 5% fetal bovine serum medium with or without the antibodies or the control antibodies for 30 minutes and then stimulated with IL-17F (50 ng/mL) for 24 hours. (A, B) Concentration of IL-8 in the conditioned medium was measured using a specific ELISA. Values are the means ± SEM of pentaplicate cultures. Different letters denote significant differences between groups ($P<.05$). The result is representative of four separate experiments using samples from different patients.



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Synergistic Effect of IL-17F and TNF-α on IL-8 Secretion From ESCs

The TNF-α, together with IL-17F, triggered IL-8 secretion more than the combined levels generated by each stimulus alone (Fig. 3). This synergistic effect was apparent when TNF-α (1 ng/mL) was combined with 1 ng/mL IL-17F, and maximal synergy was obtained at the highest dose of IL-17F tested (100 ng/mL).

Effect of IL-17F on the Expression of IL-8 mRNA and COX2 mRNA in ESCs

We conducted time-course experiments to determine the effect of IL-17F on the expression of IL-8 mRNA (Fig. 4A) and COX2 mRNA (Fig. 4B). Real-time quantitative PCR analysis demonstrated

FIGURE 3

Effects of interleukin (IL)-17F on tumor necrosis factor α (TNF-α)-mediated IL-8 secretion from endometriotic stromal cells. Endometriotic stromal cells were treated with IL-17F or TNF-α or in combination for 24 hours. The conditioned medium was collected and assayed for IL-8 concentration using a specific ELISA. All values are expressed as the mean ± SEM of pentaplicate cultures. *$P<.0001$ versus each control. The data are representative of four independent experiments.

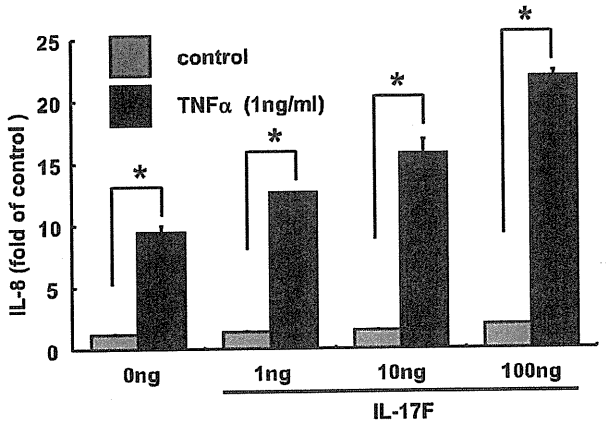

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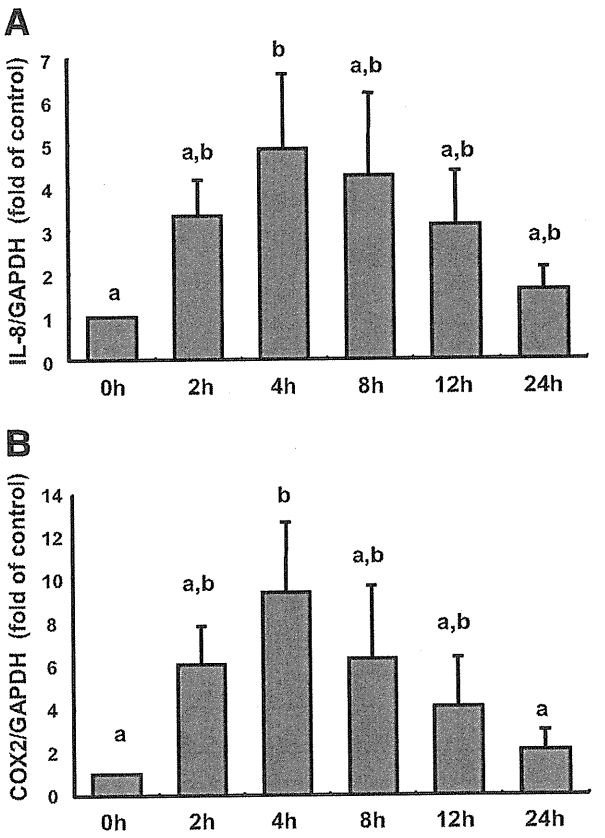
FIGURE 4

Effect of interleukin (IL)-17F on the expression of IL-8 (**A**) and cyclooxygenase 2 (COX2) (**B**) messenger RNA in endometriotic stromal cells (ESCs). The ESCs were incubated with IL-17F (10 ng/mL) for the indicated duration. Expression of IL-8 and COX2 messenger RNA in ESCs was examined by real-time quantitative polymerase chain reaction (PCR). The data shown are the relative ratio (**A**, IL-8 to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); **B**, COX2 to GAPDH) measured by real-time quantitative PCR. Data are the mean ± SEM of five independent experiments using samples from five different patients. Different letters denote significant differences between groups ($P<.05$).



Hirata. IL-17F increases IL-8 in endometriosis. Fertil Steril 2011.

that IL-17F up-regulated IL-8 and COX2 mRNA. Maximal increases in IL-8 and COX2 mRNA were observed at 4 hours, followed by a decrease with time up to 24 hours. The maximal increase of IL-8 mRNA was 4.9-fold of the control and that of COX2 mRNA was 9.4-fold of the control.

DISCUSSION

In the present study, we first demonstrated expression of IL-17F in EMMCs. We then showed that IL-17F stimulated IL-8 secretion from ESCs. The IL-17F increased IL-8 mRNA and COX mRNA to the maximal levels at 4 hours, followed by a decrease with time up to 24 hours. These response patterns appear to reflect self-protection of the cells from extreme inflammatory reactions that might damage the cells. We previously reported that ESCs expressed IL-17RA (7). In the present study, we showed that ESCs also expressed IL-17RC and that antibodies for IL-17RA and IL-17RC inhibited IL-17F-induced IL-8 secretion. The TNF-α synergistically enhanced IL-17F-induced IL-8 secretion. In addition, IL-17F stimulated COX2 expression in ESCs.

Pleiotropic functions of IL-8, such as chemoattraction and activation of neutrophils, angiogenesis, stimulation of proliferation, and survival of endometrial cells, are suggested to promote endometriosis (1, 21). The present study provided evidence that IL-17F stimulates IL-8 secretion in ESCs, suggesting that IL-17F participates in the development of endometriosis.

The present study also showed that IL-17F stimulates IL-8 secretion in ESCs through IL-17RA and IL-17RC. Our previous report demonstrated that IL-17A stimulates IL-8 secretion in ESCs through IL-17RA (7). It has been shown that IL-17RA and IL-17RC are necessary for the bioactivity of IL-17A and IL-17F (29). Furthermore, it has been demonstrated that a soluble form of IL-17RC could neutralize the activity of IL-17A and IL-17F (14). Accordingly, IL-17RA and IL-17RC could be a target for novel therapy of endometriosis.

The synergistic effect of IL-17F and TNF-α in stimulating secretion of IL-8 from ESCs was remarkable. Similar findings were reported for human bronchial epithelial cells (30) and colonic myofibroblasts (18). The TNF-α is a proinflammatory cytokine that plays multiple roles in the progression of endometriosis (2). During the inflammatory response, TNF-α is secreted from various cell types, such as peritoneal macrophage, endometrial epithelial, and stromal cells. The IL-17F may play a role as an accelerator of progression of endometriosis given that chronic pelvic inflammation entails increased TNF-α levels in the endometriotic tissues.

The COX2, a key enzyme in prostaglandin biosynthesis, is up-regulated in the lesions of endometriosis (31, 32). It plays an important role in the inflammatory responses by producing prostaglandins and is involved in the development of endometriosis (33). The present finding that IL-17F induced COX2 expression in ESCs provides further evidence for involvement of IL-17F in endometriosis.

Interleukin-17A and IL-17F induce similar inflammatory responses, inducing the proinflammatory cytokines and chemokines in many different cell types (34, 35). However, mice lacking either IL-17A or IL-17F exhibit distinct defects in experimental model of asthma and colitis (15). In addition, IL-17A and IL-17F are not always coexpressed or coregulated (36). Therefore, IL-17A and IL-17F are suggested to have distinct roles, although overlapping in many parts, in various physiological and pathological events. The present study indicated similar roles of IL-17A and IL-17F as a proinflammatory factor in the development of endometriosis. An additional study may reveal different roles, if any, of these molecules in endometriosis.

Our preliminary study showed that mononuclear cells from normal endometrial tissues appeared to secrete lower levels of IL-17F in the basal status, whereas higher levels of IL-17F under PMA and ionomycin stimulation compared with those in endometriotic tissues (data not shown). This finding may imply that IL-17F secreting cells might already be in the activated status in endometriotic tissues and relatively refractory to the stimulus compared with those in normal endometrial tissues.

In summary, we demonstrated that IL-17F stimulated the secretion of IL-8 and the expression of COX2 in endometriotic stromal cells. These findings indicate that IL-17F may promote endometriosis through these mechanisms.

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human reproduction

ORIGINAL ARTICLE *Reproductive biology*

TGF-β1 induces proteinase-activated receptor 2 (PAR2) expression in endometriotic stromal cells and stimulates PAR2 activation-induced secretion of IL-6

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BACKGROUND: Proteinase-activated receptor 2 (PAR2) is a G-protein-coupled receptor that is activated by several serine proteases. PAR2 activation in endometriotic stromal cells (ESCs) has been implicated in the development of endometriosis but the regulatory mechanism of PAR2 expression in ESC is unknown. Our objective was to study the mechanism by which PAR2 expression may be regulated in endometriotic lesions.

METHODS: Primary cultures of ESCs were treated with transforming growth factor-β (TGF-β) 1, tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and the expression of PAR2 was examined by real-time quantitative PCR. ESCs pretreated with or without TGF-β1 were treated with PAR2 agonist peptide (PAR2AP) and the secretion of the pro-endometriotic cytokine, IL-6, was measured using a specific enzyme-linked immunosorbent assay. Effects of TGF-β type I inhibitor, SB431542, and PAR2 small interfering RNA (siRNA) on the TGF-β1 stimulation of PAR2 gene expression and PAR2AP-induced IL-6 secretion were also evaluated. To study intracellular signaling, effects of inhibitors of mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K) and of Smad4 siRNA on the TGF-β1-induced PAR2 gene expression were studied.

RESULTS: Only TGF-β1, but neither TNF-α nor IL-1β, increased gene expression of PAR2. Activation of PAR2 with PAR2AP increased the secretion of IL-6 from ESCs. As expected, TGF-β1 pretreatment dose-dependently enhanced the PAR2AP-induced increase in IL-6 secretion from ESCs. Treatment of ESCs with the TGF-β type I inhibitor, SB431542, inhibited both TGF-β1-stimulation of PAR2 gene expression and PAR2AP-induced IL-6 secretion. Transfection of ESCs with PAR2 siRNA produced a similar inhibition of IL-6 secretion. The TGF-β1-induced increase in PAR2 gene expression was repressed by inhibition of p38 MAPK, p42/44 MAPK or PI3K, but not by knockdown of Smad4 expression.

CONCLUSIONS: In view of significant roles of PAR2 and IL-6 in endometriosis, the TGF-β1-induced increase in PAR2 expression may be an elaborate mechanism that augments the progression of the disease.

Key words: endometriosis / TGF-β1 / proteinase-activated receptor / interleukin-6

Introduction

Endometriosis is defined by the presence of viable endometriotic tissue outside the uterus and remains an incompletely understood disease. Endometriosis adversely affects the health of women of reproductive age, causing pain and infertility (Momoeda *et al.*, 2002; Osuga *et al.*, 2002). Although numerous studies have been conducted on the pathophysiology of the disease, its mechanism of progression is poorly understood. Multiple lines of evidence indicate that endometriosis is a chronic inflammatory disease and both immune and inflammatory responses contribute to the development of the disease. The immune and inflammatory responses are induced by interactions of

endometriosis-associated immune cells with endometriotic cells through various inflammatory substances, such as cytokines, chemokines, proteases, prostaglandins and growth factors. A number of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin (IL)-1, IL-6, IL-8 and IL-17, are suggested to play important roles, such as cell proliferation and angiogenesis, in promoting the disease (Lebovic *et al.*, 2001; Osuga, 2008; Osuga *et al.*, 2008, 2011).

Proteinase-activated receptor 2 (PAR2) is a G-protein-coupled receptor that is activated by cleavage within its extracellular N-terminal domain (Macfarlane *et al.*, 2001). We have previously reported that PAR2 activation stimulates the secretion of IL-6 and IL-8 in endometriotic stromal cells (ESCs) (Hirota *et al.*, 2005a). This response suggests that PAR2 has a functional role in endometriosis-associated inflammation. Moreover, PAR2 activation induces proliferation of ESCs, indicating that PAR2 activation may directly relate to the growth of the endometriotic lesion (Hirota *et al.*, 2005a). In a mouse model of endometriosis, both the number and the total weight of endometriotic lesions were significantly decreased in the PAR2-deficient mice compared with the wild-type mice. Interestingly, concentrations of IL-6 and monocyte chemotactic protein-1 were decreased in the peritoneal fluid and the serum of the PAR2-deficient mice, suggesting alleviated inflammation in the peritoneal cavity of the mice (Osuga *et al.*, 2008). These findings underscore the possible pivotal role of PAR2 in endometriosis. In the eutopic endometrium, PAR2 expression is increased during the menstrual phase (Hirota *et al.*, 2005b), which might contribute to the implantation of endometrial fragments in the retrograde menstruation to the peritoneum. PAR2 activation is induced by proteinases from neutrophils and mast cells, which are both observed in endometriotic tissues. PAR2 is also activated by the coagulation product, TF/VIIa, (Molino *et al.*, 1997; Uehara *et al.*, 2002, 2003), which could be formed by the local bleeding that is often observed in endometriosis.

Despite the observed effects of PAR2 in endometriosis, the regulation of PAR2 expression in endometriotic tissues remains unknown. TNF-α and IL-1β both increase PAR2 expression in neurons (Noorbakhsh *et al.*, 2005), whereas TGF-β increases PAR2 expression in fibroblasts (Materazzi *et al.*, 2007). Interestingly, TNF-α, IL-1β and TGF-β are all implicated in the development of endometriosis. In the present study, we found that TGF-β1, but neither IL-1β nor TNF-α, increased the gene expression of PAR2 in ESCs. This finding prompted us to investigate further the TGF-β1-induced expression of PAR2 in endometriosis. Therefore, we examined the effect of TGF-β1 on PAR2 activation-induced IL-6 secretion in ESCs. IL-6 is a representative pleiotropic cytokine involved in the development of endometriosis (Witz, 2000; Salmassi *et al.*, 2008). We also studied the possible intracellular mechanism by which TGF-β1 increases PAR2 expression in ESCs.

Materials and Methods

Patients and samples

Endometriotic tissues were obtained from patients with ovarian endometriomas undergoing laparoscopy. The diagnosis of endometriosis was confirmed by histopathological examination. Laparoscopic excision of ovarian endometriomas was performed as follows. After inspection of the pelvis, the ovary was freed from any adhesions. The endometrioma cyst wall was stripped away from the normal ovarian tissue gently and completely. Endometriotic tissue samples obtained from the excised endometrioma cyst wall were transported to the laboratory in DMEM/Ham's F12 medium (DMEM/F12; Invitrogen, Rockville, MD, USA) on ice under sterile conditions. All of the women had regular menstrual cycles, and none had received hormonal treatment for at least 6 months before surgery. This experimental procedure was approved by the Institutional Review Board of the University of Tokyo and signed informed consent for the use of the endometriotic tissues was obtained from each woman.

Isolation and culture of human ESCs

Isolation and culture of human ESCs were conducted as described previously (Hirota *et al.*, 2005a,c; Hirata *et al.*, 2008). Briefly, fresh endometriotic tissues collected in sterile medium were rinsed to remove blood cells and then were minced into small pieces and incubated in DMEM/F-12 containing type I collagenase (0.25%; Sigma, St Louis, MO, USA) and deoxyribonuclease I (15 U/ml; Takara, Tokyo, Japan) for 120 min at 37°C. The resulting dispersed endometriotic cells were separated by filtration through a 100 and 70 μm nylon cell strainers (BD, Franklin Lakes, NJ, USA). ESCs in the filtrate were collected by centrifugation and resuspended in phenol-red free DMEM/F-12 containing 5% charcoal-stripped fetal bovine serum (FBS), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B. ESCs were seeded in a 100 mm culture plate and kept at 37°C in a humidified 5% CO_2–95% air atmosphere. At the first passage, the cells were plated into 12- or 48-well culture plates (BD) at a density of 2×10^5 cells/ml. The purity of the ESC population was more than 95%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin, CD45 and von Willebrand factor.

Treatment of ESCs

When the ESC culture reached 70–80% confluence in 1 or 2 days, media were removed and replaced with fresh media containing 2% charcoal-stripped FBS and antibiotics. After culturing for an additional 12 h, the cells were ready for use in the experiments. To examine the effect of cytokines on PAR2 gene expression, ESCs were incubated with TGF-β1 (10 ng/ml), IL-1β (10 ng/ml) and TNF-α (10 ng/ml) (all cytokines were from R&D Systems, Minneapolis, MN, USA) for 6 h. We used PAR2 agonist peptide (PAR2AP; SLIGKV, BACHEM, Bubendorf, Switzerland) for the activation of PAR2 (Hirota *et al.*, 2005a). PAR2AP comprised the unmasked amino-terminal peptide of PAR2 cleaved by the activating proteinases. To examine the effect of TGF-β1 on IL-6 secretion induced by PAR2AP, ESCs were pretreated with TGF-β1 (10 ng/ml) for 24 h and then incubated with PAR2AP (30 μM) for 24 h. To examine the effect of inhibition of type I TGF-β receptor on gene expression of PAR2, ESCs were incubated with or without SB431542 (10 μM) (Calbiochem, La Jolla, CA, USA), and with TGF-β1 (10 μg/ml) for 6 h. To examine the effect of SB431542 on PAR2AP-induced IL-6 secretion, ESCs were pretreated with or without SB431542 (10 μM) and with TGF-β1 (10 ng/ml) for 24 h and then incubated with PAR2AP (30 μM) for 24 h. To see the effect of inhibitors of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) on TGF-β1-induced gene expression of PAR2, ESCs were pretreated with SB202190 (10 μM), PD98059 (25 μM), SP600125 (10 μM) or LY294002 (20 μM) [inhibitors of p38 MAPK, p42/44 MAPK, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and PI3K, respectively; Calbiochem] for 30 min before treatment with TGF- β for 6 h.

Small interfering RNA

The small interfering RNA (siRNA) constructs used were obtained as ON-TARGET plus SMART pool PAR2 (L-005095-00-0005) and Smad4 (L-003902-00-0005) from Dharmacon (Lafayette, CO, USA). 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 30 nmol/l siRNA for 24 h in Opti-MEM I using Lipofectamine RNAi max according to the manufacturer's protocol. After transfection, the medium was removed and replaced with fresh medium containing 5% charcoal-stripped FBS and antibiotics for 24 h. The cells were then treated with TGF-β1 and PAR2AP as described above.

RNA extraction, RT and real-time quantitative PCR

RNA extraction, RT and real-time quantitative PCR were performed as described previously (Takemura *et al.*, 2007; Hirata *et al.*, 2008). Total RNA was extracted from cultured ESCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Real-time quantitative PCR and data analysis were performed using a LightCycler (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions. Expression of PAR2 and Smad4 mRNA was normalized to RNA loading for each sample using human glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mRNA as an internal standard. The PAR2 primers chosen (sense, 5′-CTGCATCTGTCCTCACTGGA-3′; antisense, 5′-ACAGAG AGGAGGTCAGCCAA-3′) amplified a 181 bp fragment. The Smad4 primers chosen (sense, 5′-TGGCTGGTCGGAAAGGATTT-3′; antisense, 5′-ACTGGCAGGCTGACTTGTGG-3′), amplified a 431 bp fragment. The PCR conditions were as follow: for PAR2, 40 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 10 s; for Smad4, 40 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 18 s; for GAPDH, 30 cycles at 95°C for 10 s, 64°C for 10 s and 72°C for 18 s. All of the PCR experiments were followed by melting curve analysis.

Measurement of IL-6 protein

The concentration of IL-6 in the conditioned media was measured using a specific ELISA kit (R&D Systems). The sensitivity of the assay was 3.12 pg/ml, and the intra- and inter-assay coefficients of variation were less than 5%.

Statistical analysis

Data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons, or Student's *t*-test, appropriately. A value of $P < 0.05$ was considered significant.

Results

Effects of TGF-β1, TNF-α and IL-1β on gene expression of PAR2

TGF-β1 increased gene expression of PAR2, whereas neither TNF-α nor IL-1β affected PAR2 expression (Fig. 1A). The TGF-β1-induced PAR2 expression was dose-dependent between 1 and 10 ng/ml, the increase being significant from 1 ng/ml (Fig. 1B).

Effects of TGF-β1 on PAR2AP-induced IL-6 secretion

Although PAR2AP alone increased IL-6 secretion by 2.8-fold, TGF-β1 pretreatment dose-dependently enhanced PAR2AP-induced IL-6 secretion, with a total increase of 9.8-fold observed at 10 ng/ml TGF-β1 (Fig. 2).

Effects of SB431542 and PAR2 siRNA on TGF-β1-induced gene expression of PAR2 and on TGF-β1 stimulation of PAR2AP-induced IL-6 secretion

The TGF-β1 type I inhibitor, SB431542, suppressed the TGF-β1-induced expression of PAR2 (Fig. 3A). SB431542 also suppressed the TGF-β1 stimulation of PAR2AP-induced IL-6 secretion (Fig. 3B). Knockdown of PAR2 expression using PAR2 siRNA remarkably reduced PAR2 mRNA levels (Fig. 4A). Similar to the treatment with SB431542, PAR2 siRNA treatment inhibited the TGF-β1 stimulation of PAR2AP-induced IL-6 secretion (Fig. 4B).

Effects of MAP kinase inhibitors, PI3K inhibitor and Smad4 siRNA on TGF-β1-induced gene expression of PAR2

The Smad pathway, several MAPK pathways and PI3K pathway are typical intracellular signaling pathways activated by TGF-β1. To examine whether these pathways are involved in TGF-β1-induced gene expression of PAR2, ESCs were treated with MAPK inhibitors and Smad4 siRNA. The p38 MAPK, p42/44 MAPK and PI3K inhibitors (SB202190, PD98059 and LY294002 respectively) significantly

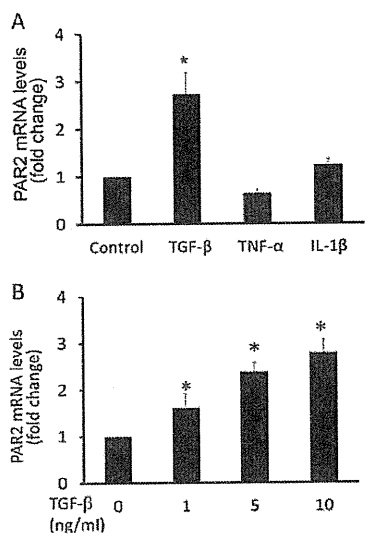

Figure 1 TGF-β1-induced increase in PAR2 mRNA expression in ESCs. (**A**) ESCs were cultured with TGF-β1 (10 ng/ml), TNF-α (10 ng/ml) and IL-1β (10 ng/ml) for 6 h. (**B**) ESCs were cultured with different concentrations of TGF-β1 for 6 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2. Data are the mean ± SEM of six (A) and five (B) independent experiments using different ESCs. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. *$P < 0.05$ versus control.

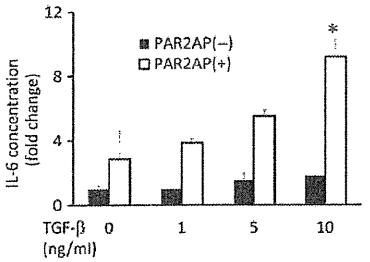

Figure 2 TGF-β1-induced increase in PAR2AP-induced ESC secretion of IL-6. ESCs were pretreated with TGF-β1 (0, 1, 5 and 10 ng/ml) for 24 h and subsequently incubated with or without 30 μM PAR2AP for 24 h. At the end of the incubation period, the conditioned medium was collected and assayed for IL-6 by ELISA. The values are presented as the mean ± SEM of four separate cultures. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. *$P < 0.05$ versus ESCs stimulated with PAR2AP but without TGF-β1 pretreatment. The result is representative of three repeated experiments using samples from three different women.

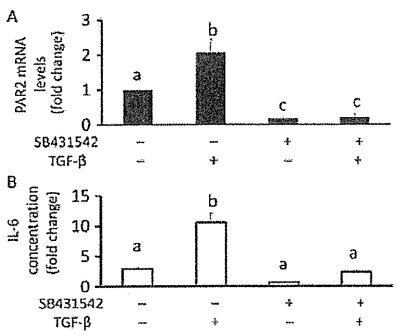

Figure 3 Effects of SB431542 on the TGF-β1-induced increase in PAR2 mRNA expression and in PAR2AP-induced IL-6 secretion from ESCs. (**A**) ESCs were cultured with or without SB431542 (10 μM) and TGF-β1 (10 ng/ml) for 24 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2. The values are presented as the mean ± SEM of three independent experiments. The data were analyzed by Student's *t*-test. (**B**) ESCs were pretreated with or without SB431542 (10 μM) and TGF-β1 (10 ng/ml) for 24 h and subsequently incubated with PAR2AP (30 μM) for 24 h. At the end of the incubation period, the conditioned media were collected and assayed for IL-6 by ELISA. The values are presented as the mean ± SEM of four separate cultures. Data are shown as the fold change in IL-6 concentrations in ESCs not pretreated with SB431542 and TGF-β1 and not stimulated with PAR2AP. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. The result is representative of three repeated experiments using samples from three different women. (A and B) Different letters denote significant differences between groups ($P < 0.05$).

diminished TGF-β1-induced PAR2 gene expression (Fig. 5A). In contrast, neither the SAPK/JNK inhibitor (SP600125) nor treatment with Smad4 siRNA had any effect on TGF-β1-induced PAR2 gene expression, although Smad4 siRNA markedly decreased gene expression of Smad4 (Fig. 5B).

Discussion

In the present study, we demonstrated that TGF-β1, but neither TNF-α nor IL-1β, increased gene expression of PAR2. TGF-β1 dose-dependently increased the secretion of IL-6 in PAR2AP-stimulated ESCs. SB431542, an inhibitor of the TGF-β receptor, inhibited the TGF-β-induced increase in gene expression of PAR2 in ESCs and the TGF-β1-augmented IL-6 secretion from PAR2AP-stimulated ESCs. Likewise, PAR2 siRNA inhibited the TGF-β1-induced increase in gene expression of PAR2 in ESCs and the TGF-β1-augmented

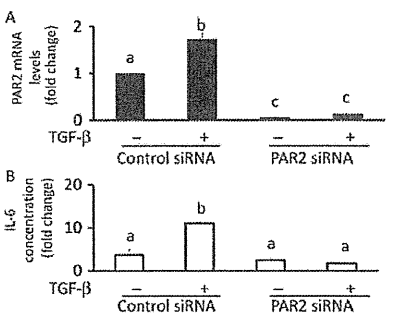

Figure 4 Effects of PAR2 siRNA on TGF-β1-induced increase in PAR2 mRNA expression and in PAR2AP-induced ESC secretion of IL-6. (**A**) ESCs were transfected with 30 nmol/l PAR2 siRNA or negative control siRNA for 24 h. After transfection, the medium was removed and replaced with medium containing 5% charcoal-stripped FBS and antibiotics for 24 h. Thereafter, ESCs were cultured with or without TGF-β1 (10 ng/ml) for 24 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2. The values are presented as the mean ± SEM of three independent experiments using different ESCs. The data were analyzed by Student's *t*-test. (**B**) ESCs were transfected with 30 nmol/l PAR2 siRNA or negative control siRNA for 24 h. After transfection, the medium was removed and replaced with the medium containing 5% charcoal-stripped FBS and antibiotics for 24 h. Thereafter, ESCs were pretreated with or without TGF-β1 (10 ng/ml) for 24 h and subsequently incubated with PAR2AP (30 μM) for 24 h. At the end of the incubation period, the conditioned medium was collected and assayed for IL-6 by ELISA. The values are presented as the mean ± SEM of four separate cultures. Data are shown as fold changes in IL-6 concentrations in ESCs pretreated with control siRNA but without TGF-β1, and not stimulated with PAR2AP. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. The result is representative of three repeated experiments using samples from three different women. (A and B) Different letters denote significant differences between groups ($P < 0.05$).

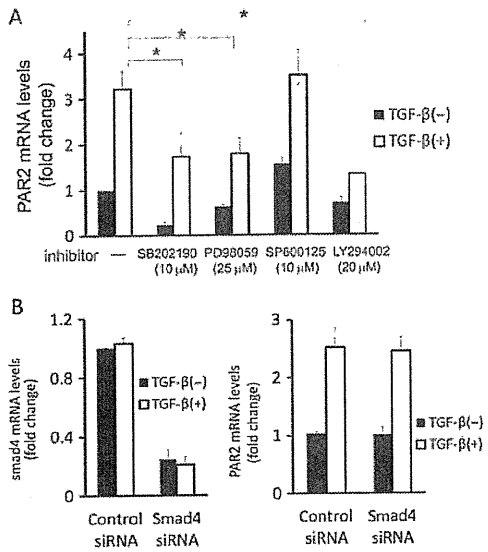

Figure 5 TGF-β1-induced expression of PAR2 mRNA in ESCs treated with inhibitors of MAPKs or PI3K, or Smad4 siRNA. (**A**) ESCs were treated with inhibitors of p38 MAPK (SB202190), p42/44 MAPK (PD98059), SAPK/JNK (SP600125) or PI3K (LY294002) for 30 min and stimulated with TGF-β1 (10 ng/ml) for 6 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2. Data are the mean ± SEM of nine independent experiments using different ESCs from nine patients. The data were analyzed by ANOVA, followed by *post hoc* analysis for multiple comparisons. $^*P < 0.05$ versus ESC treated without the inhibitors but with TGF-β1. (**B**) ESCs were transfected with 30 nmol/l Smad4 siRNA for 24 h. After transfection, media were removed and treated with or without TGF-β1 (10 ng/ml) for 6 h. Total RNA isolated from ESCs was reverse transcribed and amplified by real-time PCR using primers for Smad4 and PAR2. Values were calculated by subtracting data for signal threshold cycles (C_t) of the internal standard (GAPDH) from C_t values for PAR2 and Smad4. Data are the mean ± SEM of three independent experiments using different ESCs from three women. The data were analyzed by Student's *t*-test.

IL-6 secretion from PAR2AP-stimulated ESCs. SB202190, a p38 MAPK inhibitor, PD98059, a p42/44 MAPK inhibitor, and LY294002, a PI3K inhibitor, suppressed the TGF-β1-induced gene expression of PAR2. Suppression of Smad4 expression by the siRNA had no effect on TGF-β1-induced gene expression of PAR2.

It is interesting that neither IL-1β nor TNF-α increased gene expression of PAR2 in ESCs, compared with the stimulation by both molecules of PAR2 expression in neurons, osteoarthritis chondrocytes and osteoblasts (Noorbakhsh *et al.*, 2005, Xiang *et al.*, 2006; Boileau *et al.*, 2007; Amiable *et al.*, 2009). We observed that TGF-β1 increased PAR2 expression in ESCs, which is consistent with TGF-β1 stimulation of PAR2 expression in human dermal fibroblasts (Materazzi *et al.*, 2007). Therefore, PAR2 expression appears to be differentially regulated in different cell types.

TGF-β may play multiple roles in different stages of the progression of endometriosis (Omwandho *et al.*, 2010). The present study demonstrates a new role of TGF-β1 in the development of endometriosis, in the induction of PAR2 expression in ESCs. The increase in PAR2 expression consequently enhanced IL-6 secretion from PAR2AP-stimulated ESCs. Given that the proteinases and the coagulation product that activate PAR2 are present in endometriotic tissues, this sequence may partly explain the increased expression of IL-6 in both endometriotic tissue and in peritoneal fluid of women with endometriosis (Salmassi *et al.*, 2008; Velasco *et al.*, 2010). The elevation in TGF-β levels in endometriotic tissues is therefore likely to contribute to this sequence of events (Tamura *et al.*, 1999; Komiyama *et al.*, 2007).

IL-6 is a multifunctional cytokine that is involved in numerous immunological and proliferative responses in endometriosis (Witz, 2000). In particular, IL-6 increases aromatase activity, haptoglobin production and hepatocyte growth factor production in endometriotic cells and/or endometriotic cells (Piva *et al.*, 2001; Khan *et al.*, 2005; Velasco *et al.*, 2006; Sharpe-Timms *et al.*, 2010). IL-6 is also known to stimulate the proliferation of ESCs (Khan *et al.*, 2005). These findings indicate that IL-6 stimulates the progression of endometriosis via various events such as cell proliferation, angiogenesis and immunomodulation. Therefore, the TGF-β1-stimulated increase in PAR2 expression and the resulting increase in IL-6 is a possible mechanism by which TGF-β1 can amplify PAR2-mediated disease progression. This hypothesis is also consistent with the previous finding that PAR2 activation stimulates the proliferation of ESCs (Hirota *et al.*, 2005a,b,c). In addition, increased IL-6 production via a TGF-β1-stimulated increase in PAR2 expression might contribute to endometriosis-associated infertility, because IL-6 is suggested to be a causative factor for infertility in endometriosis (Gomez-Torres *et al.*, 2002, Yoshida *et al.*, 2004; Deura *et al.*, 2005).

The effect of TGF-β1 on PAR2 expression might provide a novel insight in the pathogenesis of endometriosis. Immune cells are an important component of endometriotic tissues and are involved in the development of the disease. In particular, our recent studies suggest that Th2 cells and Th17 cells contribute to disease progression by inducing inflammation and cell proliferation (Hirata *et al.*, 2008; OuYang *et al.*, 2008, 2010). The differentiation of Th cells is under the strict control of cytokines, with Th17 cells being induced from naïve Th cells by TGF-β1 in combination with IL-6. Without IL-6, naïve Th cells will differentiate into regulatory T cells under the influence of TGF-β1 (Miossec *et al.*, 2009). Therefore, we hypothesize that TGF-β1 stimulates the environment to aid the development of Th17 cell by increasing PAR2 activation-induced secretion of IL-6 in endometriotic tissues. This process could be potentiated by enhancement of PAR2 activation by the proteolytic enzymes produced by neutrophils activated by Th17 cells (Miossec *et al.*, 2009). In this way, TGF-β1 may co-operate with Th17 cells to stimulate disease progression. Further studies are warranted to corroborate the notion.

TGF-β utilizes multiple signaling pathways to stimulate different cells. Smad4 is essential for TGF-β signal transduction (Prud'homme, 2007), but suppression of Smad4 expression by siRNA did not inhibit the TGF-β1-induced increase in PAR2 expression in ESCs. In contrast, a p38 MAPK inhibitor and a p42/44 MAPK inhibitor suppressed the effect of TGF-β1. The present findings indicate that the activation of