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The Expression and Possible Roles of Chemokine CXCL11 and Its Receptor CXCR3 in the Human Endometrium

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IFN-γ secreted by a human embryo and trophoblast cells during implantation is suggested to play an important role in implantation and pregnancy. In the present study, we explored expression and possible functions of CXCL11, a CXC chemokine strongly induced by IFN-γ, and its receptor CXCR3 in the human endometrium. Secreted CXCL11 protein was not detected in cultured endometrial stromal cells (ESC) but was detected in cultured endometrial epithelial cells (EEC). IFN-γ stimulated the protein levels of CXCL11 in a dose-dependent manner in EEC and ESC. CXCL11 secreted from EEC with 100 ng/ml IFN-γ was 220-fold of the control, and 100-fold as compared with that secreted from ESC with the same dose of IFN-γ. CXCR3 was expressed in EEC, ESC, and trophoblast cells. Addition of IFN-γ to EEC increased the chemotactic activity of its culture medium to trophoblast cells and T cells, and the effect was suppressed by immunoneutralization with Abs of three CXCR3 ligands, including anti-CXCL11 Ab. CXCL11 significantly increased BrdU incorporation of ESC, which was inhibited by a p42/44 MAPK pathway inhibitor PD98059. In contrast, CXCL11 significantly decreased BrdU incorporation and increased the release of lactate dehydrogenase and the positive staining of annexin V in EEC. These findings suggest that IFN-γ promotes implantation by stimulating EEC to produce CXCL11, which induces migration of trophoblast cells and T cells, proliferation of ESC, and apoptosis of EEC. *The Journal of Immunology*, **2006, 177: 8813–8821.**

Implantation occurs on days 20–24 of a regular 28-day menstrual cycle which is compared to ~6 or 7 days after fertilization. Human implantation includes three stages which are called apposition, adhesion, and invasion. The initial unstable contact of the blastocyst to the uterine wall is apposition which occurs most commonly in the fundal wall of the uterus. The next stage, adhesion, is characterized by increased physical interaction between the blastocyst and the uterine epithelium. Subsequently, the third stage of implantation, invasion, begins and trophoblast cells infiltrate the uterine epithelium (1).

The endometrial epithelium is an important element where the molecular interactions between the embryo and the endometrium are initiated (2–4). A specific molecular cross-talk between embryo and endometrium has been reported during the human implantation process (5). Cytokines, such as IL-1, LIF, CSF-1, and IL-8, and their specific receptors, which are expressed in the endometrium and the embryo, are suggested to be involved therein (5–7).

IFN-γ secreted by the preimplantation embryo and endometrial leukocytes is suggested to play an important role in the process of implantation in humans (8–10). IFN-γ produced from the human embryo is highest when it develops to blastocyst and reaches a point of apposition in the uterus (8). This finding implies that IFN-γ may have some roles in the initial stages of implantation.

IFN-γ is known to strongly induce three CXC chemokines, CXCL9 (monokine induced by IFN-γ), CXCL10 (IFN-γ-inducible protein of 10 kDa), and CXCL11 (IFN-γ-inducible T cell α chemoattractant), in a range of cell types (11–14). These chemokines exert their effects through a shared receptor called CXCR3 (12, 13).

Recent studies demonstrated that these three chemokines were expressed in both pregnant and nonpregnant endometrium (15–18). In cultured endometrial stromal cells, IFN-γ, IL-1β, TNF-α, and LPS stimulated CXCL10 production (16) and progesterone induced secretions of CXCL9 and CXCL10 (17). Notably, a recent study showed that CXCL10 stimulates the migration and attachment of ovine trophoblast cells (19). In contrast, CXCL11 in the endometrium has been poorly studied despite its highest binding affinity to CXCR3 among the three chemokines (12).

Based on these findings, we speculated that IFN-γ could regulate implantation through CXCL11 production in the endometrium. In the present study, we first showed IFN-γ-induced production of CXCL11 in endometrial cells. We then examined the expression of CXCR3 in endometrial cells and trophoblast cells and effects of CXCL11 on these cells, aiming to assess the possible roles of CXCL11 and CXCR3 in implantation.

Materials and Methods

Reagents and materials

Type I collagenase, antibiotics, magnesium sulfate ($MgSO_4$), and streptokinase were purchased from Sigma-Aldrich. DMEM/Ham's F12 medium (DMEM/F12), RPMI 1640 medium, medium 199, 0.25% trypsin, and 0.25% trypsin/EDTA were obtained from Invitrogen Life Technologies. Charcoal-stripped FBS was obtained from HyClone. A specific inhibitor of ERK (MEK)-1, PD98059, was obtained from Calbiochem. Rabbit polyclonal Abs to human CXCL9 were obtained from PeproTech. Rabbit polyclonal Abs to human CXCL10 and human CXCL11 were obtained from BioVision. Rabbit polyclonal Abs to human total p42/44 MAPK and phospho-p42/44 MAPK were obtained from New England Biolabs. Anti-rabbit HRP secondary Ab and Ficoll-Paque Plus (1.077 g/ml) were obtained from Amersham Biosciences. Mouse monoclonal anti-human CXCR3 Ab, human

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recombinant IFN-γ, human recombinant CXCL11, and human recombinant IL-2 were obtained from R&D Systems. Isotype control mouse IgG1, isotype control rabbit IgG, and mouse mAbs to human vimentin, human cytokeratin, and human CD45 were obtained from DakoCytomation. Mouse mAbs to human cytokeratin type 7 were obtained from Immunologicals Direct. FITC-conjugated anti-mouse IgG (H+L) Ab was obtained from Beckman Coulter. DNase I was obtained from Takara.

Sources of tissues

Endometrial tissues were obtained from a total of 60 patients (aged 40.3 ± 4.9 years, mean ± SD) undergoing hysterectomy for benign gynecological conditions such as uterine fibroid without endometrial pathologies. Although the relatively high ages of the subjects in the reproductive age range and the pathologies of the myometrium may place some limitations on the present study, we used these samples due to the unavailability of endometrial tissue of healthy young women. All of them had regular menstrual cycles and had not received hormone therapy for at least 6 mo before surgery. The specimens were dated according to the patients' menstrual history and standard histological criteria by Noyes et al. (20). Placental tissues between 5 and 7 wk of gestation were obtained at elective termination of pregnancy. PBMC were obtained from normal volunteer donors. The Institutional Review Board of the University of Tokyo approved this study and written informed consent for use of the tissue was obtained from each woman. The tissues collected under sterile conditions were processed for primary cell cultures.

Isolation, purification, and culture of endometrial epithelial cells (EEC),[2] endometrial stromal cells (ESC), T cells, and trophoblast cells

Isolation and culture of human EEC and ESC was as described previously (21–24). 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 resultant dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer (BD Biosciences). Endometrial epithelial glands which remained intact were retained by the strainer, whereas dispersed ESC passed through the strainer into the filtrate. ESC in the filtrate were collected by centrifugation and resuspended in DMEM/F12 containing 10% FBS and antibiotics. ESC were plated 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 at a density of 2×10^5 cells/well into 12-well culture plates for the experiments of RT-PCR, Western blotting, and ELISA, or at the density of 1×10^4 cells/well into 96-well culture plates for the experiments of cell proliferation assay. 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 contaminated stromal cells to attach to the plate wall. The nonattached epithelial glands formed a monolayer of EEC after attachment with culture plates. EEC at a density of $\sim 2 \times 10^5$ cells/well in 12-well culture plates were used for the experiments of RT-PCR, Western blotting, ELISA, and assays of cytotoxicity and apoptosis, and EEC at the density of $\sim 1 \times 10^4$ cells/well in 96-well culture plates were used for the experiments of cell proliferation assay.

PBMC were separated by centrifugation of heparinized blood on Ficoll-Paque Plus. Ag-specific CD4-positive short-term T cell lines were generated from PBMC suspensions as previously described (25). Briefly, PBMC were stimulated in RPMI 1640 medium containing 5% autologous serum with streptokinase (100 U/ml) for 5 days. On day 6, activated T cells were expanded in the presence of human IL-2 (20 U/ml), and on day 15, they were used for migration assay. The expression of CXCR3 on the cells was identified by flow cytometry.

Trophoblast cells were prepared and maintained as previously described with some modifications (26–27). Briefly, the 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 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 Medium 199 with 10% FBS. The suspension was layered onto Ficoll-Paque Plus and centrifuged at $150 \times g$ for 15 min. Trophoblast cells recovered from the interface were washed with PBS and resuspended in Medium 199. The remaining leukocytes and syncytiotrophoblasts were removed by plating the cells for 30 min, followed by aspiration of the supernatant enriched with cytotrophoblasts. The cells were washed with PBS and the medium was changed to Medium 199 with 10% FBS and placed in a type IV collagen-coated 6-well plate (BD Biosciences) and kept at 37°C in a humidified 5% CO_2/95% air atmosphere. After incubation for 2 or 3 days, the cells were trypsinized and used for the experiments.

Treatment of cell cultures

When EEC and ESC approached confluence, the complete medium was removed and replaced with fresh medium and antibiotics, and the cells were cultured for an additional 12–24 h. To evaluate the dose effects of IFN-γ, wells were replenished with serum-free medium with different concentrations of IFN-γ and the cells were incubated for 24 h. To assess the effect of IFN-γ on the expression of CXCL11 mRNA in EEC and ESC, the cells were incubated with IFN-γ at different concentrations in serum-free medium for 6 h. After the treatments, the conditioned medium were collected, centrifuged, and stored at −80°C for subsequent analysis.

Immunocytochemistry

In 48 h of culture, EEC, ESC, and trophoblast cells were fixed in cold methanol/acetone (1:1) at −20°C for 20 min and were washed twice in PBS. The fixed cells were treated with 3% hydrogen peroxide for 5 min to eliminate endogenous peroxidase. After blocking with 1.5% horse serum for 20 min, the cells were incubated with mouse mAbs to cytokeratin, vimentin, cytokeratin-7, and CD45 for 30 min at room temperature. Control cells were incubated with nonimmune murine IgG1, the concentration of which was adjusted to that of the primary Ab. The cells were then incubated with biotinylated goat anti-mouse IgG, followed by incubation with peroxidase-labeled streptavidin solution for 20 min at room temperature. The chromogenic reaction was conducted with diaminobenzidine. All cells were counterstained with hematoxylin. The experiments were repeated four times.

Western blotting

Cultured cells were homogenized in the lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.1% bromphenol blue and diluted to 1 mg of total protein/ml. The protein concentration in the homogenized cells was measured by a protein assay kit (Bio-Rad). Samples were resolved by 10% SDS-PAGE. Proteins were blotted onto a nitrocellulose membrane and incubated with rabbit polyclonal Abs to human CXCR9 (1/1000), human CXCL10 (1/1000), human CXCL11 (1/1000), total p42/44 MAPK (1/1000), and phosphospecific p42/44 MAPK (1/1000) as primary Abs and anti-rabbit HRP Ab (1/1000) as a secondary Ab. Immune complexes were visualized by use of ECL Western blotting system (Amersham Biosciences).

Measurement of CXCL11 in the supernatants of culture media

Concentrations of CXCL11 in conditioned culture media were measured using its specific ELISA kit (Quantikine; R&D Systems) according to the manufacturer's protocol. Absorbance was read at 450 nm with the DigiScan Microplate Reader (ASYS Hitech). Cultured cells were homogenized and the total protein amount in the homogenized cells was measured by a protein assay kit. Data were standardized by total protein of cell lysates.

RNA extraction, reverse transcription (RT), and real-time quantitative PCR of CXCL11, CXCR3, and its spliced variant CXCR3-B mRNA

RT and real-time quantitative PCR were performed as we have reported previously (21–24, 28–30). Total RNA was extracted from EEC and ESC, using the RNAeasy Mini kit (Qiagen). RT was performed using Rever Tra Ace-α (Toyobo); real-time quantitative PCR and data analysis were performed using LightCycler (Roche Diagnostic), according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed in a 20-μl total volume and cDNA was amplified using oligonucleotide primers based on human CXCL11, CXCR3, and the CXCR3-B sequence.

Recent studies indicate that in addition to the classic receptor CXCR3-A, alternatively spliced variant CXCR3-B is expressed in some cell types (31). CXCR3 primers amplified a common sequence to CXCR3-A and CXCR3-B. CXCR3-B primers amplified a unique sequence to CXCR3-B.

CXCL11 primers (sense, 5′-TTAAACAAACATGAGTGTGAAGGG-3′; antisense, 5′-CGTTGTCCTTTATTTTCTTTCAGG-3′) were chosen to amplify a 228-bp fragment. CXCR3 primers (sense, 5′-TGCCAATA CAACTTCCCACA-3′; antisense, 5′-CGGAACTTGACCCCTACAAA-3′) were chosen to amplify a 371-bp fragment. CXCR3-B primers (sense,

[2] Abbreviations used in this paper: EEC, endometrial epithelial cell; ESC, endometrial stromal cell; RT, reverse transcription; C_T, threshold cycle; LDH, lactate dehydrogenase; PI, propidium iodide.

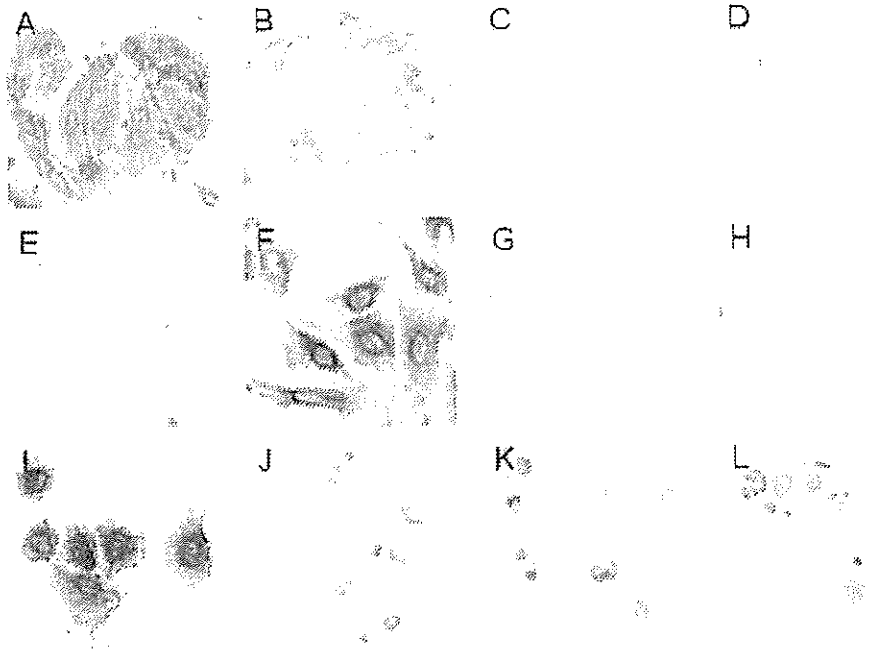

FIGURE 1. Immunocytochemical staining of primary cultured EEC, ESC, and trophoblast cells in early pregnancy. *A–D*, EEC were stained by anti-cytokeratin mAb (*A*), anti-vimentin mAb (*B*), anti-CD45 mAb (*C*), and isotype mIgG1 (*D*). EEC were positively stained with cytokeratin. *E–H*, ESC were stained by anti-cytokeratin mAb (*E*), anti-vimentin mAb (*F*), anti-CD45 mAb (*G*), and isotype mIgG1 (*H*). ESC were positively stained with vimentin. *I–L*, Trophoblast cells were stained by anti-cytokeratin-7 mAb (*I*), anti-vimentin mAb (*J*), anti-CD45 mAb (*K*), and isotype mIgG1 (*L*). Trophoblast cells were positively stained with cytokeratin-7. All cells were counterstained with hematoxylin. The result is representative of four separate experiments.

5′-TCACAAAAGAGTTCCTGCCA-3′; antisense, 5′-AAGAGGAGGCT GTAGAGGGC-3′) were chosen to amplify a 241-bp fragment. Expression of CXCL11, CXCR3, and CXCR3-B mRNA was normalized to RNA loading for each sample using GAPDH mRNA as an internal standard. Human GAPDH primers (Toyobo) were chosen to amplify a 452-bp fragment. The real-time PCR condition of CXCL11 was 40 cycles at 95°C for 15 s, 64°C for 10 s, 72°C for 16 s, followed by melting curve analysis. The PCR condition of CXCR3 was 40 cycles at 95°C for 15 s, 64°C for 10 s, 72°C for 15 s, followed by melting curve analysis. The PCR condition of CXCR3-B was 40 cycles at 95°C for 15 s, 64°C for 10 s, 72°C for 10 s, followed by melting curve analysis. Standardization of the data was performed by subtracting the signal threshold cycles (C_T) of the internal standard (GAPDH) from the C_T of CXCL11, CXCR3, and CXCR3-B. To quantify the expression of CXCR3-A, we subtracted the amount of CXCR3-B from that of CXCR3. Each PCR product was purified with a QIAEX II gel extraction kit (Qiagen) and their identities were confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems).

Flow cytometric analysis

Flow cytometric analysis was performed as we have reported previously (22). Adherent cells (EEC, ESC, and trophoblast cells) were detached by using 0.25% trypsin/EDTA. The cells (2×10^5 cells/sample) were washed twice with PBS containing 2% FBS and stained with the mouse anti-human CXCR3 mAb or isotype control mouse IgG1 for 30 min on ice. Then, the cells were washed twice and incubated with FITC-conjugated anti-mouse IgG Ab for 30 min on ice. Being washed twice, the cells were analyzed using EPICS XL flow cytometer (Beckman-Coulter) and EXPO 32 software (Beckman Coulter).

Immunohistochemistry

Human endometrial and placental tissues were fixed overnight in 10% neutral-buffered formalin and embedded in paraffin, and 5-μm sections were prepared. Sections were treated with 0.03% hydrogen peroxide for 30 min to eliminate endogenous peroxidase. After blocking with 1.5% BSA, the sections were incubated with anti-human CXCR3 mAb (1/400) for 30 min at room temperature. Control slides were incubated with isotype control mouse IgG1, the concentration of which was adjusted to that of the primary Ab. The sections were then incubated with biotinylated goat anti-mouse IgG, followed by incubation with peroxidase-labeled streptavidin solution for 20 min at room temperature. The chromogenic reaction was conducted with diaminobenzidine. All sections were counterstained with hematoxylin. Assessments of immunostaining were based on agreement among three independent observers who were blind to the phases of the menstrual cycle at which specimens were collected.

In vitro migration assay

Migration assay was performed in 24-well plates (Costar) carrying Transwell permeable supports with 3-μm polycarbonate membrane for T cells and with 8-μm polycarbonate membrane for trophoblast cells as previously reported (32, 33). Supernatants of EEC were either stimulated or not by IFN-γ (100 ng/ml) for 24 h, were preincubated for 1 h with 10 μg/ml anti-CXCL9/10/11 Ab or isotype control rabbit IgG, and were plated on the lower chambers. Cells were plated on the upper wells of Transwell membranes containing 100 μl of serum-free DMEM/F12. A total of 5×10^6 T cells were incubated for 2 h at 37°C and 5% CO_2 atmosphere, and 2×10^5 trophoblast cells were for 24 h. T cells and trophoblast cells on the upper surface of membranes were completely removed; migrated cells were fixed with acetone/methanol. The number of T cells, resuspended in 10 ml PBS, was determined using a Coulter Counter Z1 (Beckman Coulter). Migration indices of trophoblast cells were determined by counting the number of trophoblast cells stained with H&E in 10 randomly selected nonoverlapping fields of the wells under light microscope.

Cell proliferation assay

Cell proliferation assay was performed as we have reported previously (21, 30, 34, 35). The effect of CXCL11 on the proliferation of ESC and EEC was examined by measuring BrdU incorporation into DNA by using the Biotrak Cell Proliferation ELISA System (Amersham Biosciences) according to the manufacturer's instructions. ESC and EEC were seeded into Falcon 96-multiwell plates (BD Biosciences) at a density of 1×10^4 cells/well in 100 μl of the culture medium. To assess the effect of CXCL11 on cell proliferation, the cells were incubated with CXCL11 at different concentrations in serum-free medium. To evaluate the effects of a MAPK inhibitor, the cells were preincubated with MEK inhibitor PD98059 for 1 h before CXCL11 treatment. After 24 h, 100 μl of BrdU solutions were added and incubated at 37°C for an additional 2 h. After removing the culture medium, the cells were fixed and the DNA was denatured by the addition of 200 μl/well fixative. The peroxidase-labeled anti-BrdU bound to the BrdU incorporated in newly synthesized cellular DNA. The immune complexes were detected by the subsequent substrate reaction and the resultant color was read at 450 nm in the DigiScan Microplate Reader.

Assessment of cell death

Cytotoxicity of EEC was assessed by the measurement of lactate dehydrogenase (LDH) activity released from the cytosol of damaged cells into supernatant using the Cytotoxicity Detection kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Absorbance was read at 492 nm with the DigiScan Microplate Reader. Values were expressed relative to measurement from control LDH.

Table I. *IFN-γ-stimulated CXCL11 mRNA expression in EEC and ESC*[a]

	IFN-γ (ng/ml)			
	0 (=control)	10	100	1000
EEC	0.0028 ± 0.0020	0.21 ± 0.15[b]	0.74 ± 0.41[b]	0.96 ± 0.72[b]
ESC	0.00039 ± 0.00029	0.23 ± 0.21[c]	0.37 ± 0.30[c]	0.41 ± 0.32[c]

[a] The values of CXCL11 mRNA represent relative ratios compared with GAPDH mRNA level. Values are the mean ± SEM of three separate experiments using different EEC and ESC preparations. Logarithm of the values was used in statistical analysis.
[b] $p < 0.0001$, each vs. control of EEC.
[c] $p < 0.0001$, each vs. control of ESC.

Apoptosis of EEC was assessed by double staining of annexin V and propidium iodide (PI) using an Annexin V^{FITC} kit (Beckman Coulter) according to the manufacturer's instructions. Annexin V is a phosphatidylserine-binding protein used to detect phosphatidylserine translocation from the inner to the outer plasma membrane leaflet which is assumed to be a feature of apoptosis. Cell death, including necrosis and late phase of apoptosis, was detected by PI, a marker for cell membrane permeability. Briefly, EEC were detached by using 0.25% trypsin/EDTA, washed twice with PBS, and pelleted in annexin V-binding buffer containing FITC-conjugated annexin V. PI was then added and samples were incubated for 10 min on ice and analyzed by EPICS XL flow cytometer and EXPO 32 software.

Statistical analysis

Data were evaluated using ANOVA with posthoc analysis (Fisher's protected least significance) for multiple comparisons and the Student *t* test for paired comparisons. A value of $p < 0.05$ was accepted as significant.

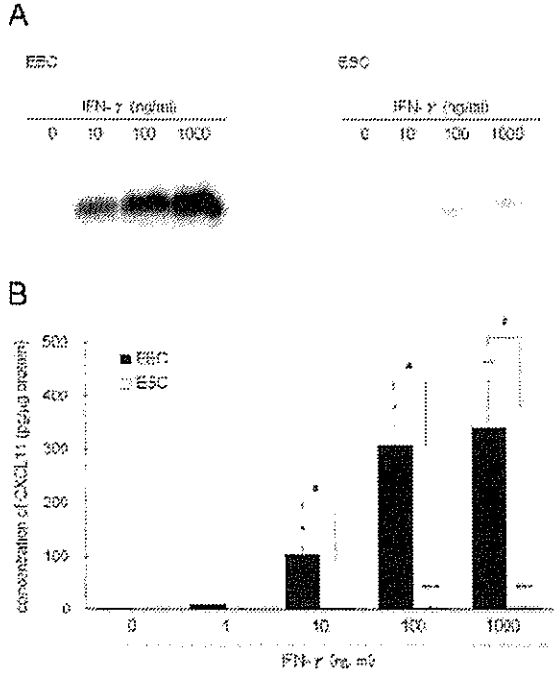

FIGURE 2. IFN-γ-stimulated CXCL11 protein production in EEC and ESC. *A*, EEC and ESC were cultured in serum-free medium with different doses of IFN-γ for 24 h. Cell extracts were prepared and assayed for CXCL11 by Western blotting. The result is representative of three separate experiments. *B*, EEC and ESC were cultured in serum-free medium with different doses of IFN-γ for 24 h. The conditioned medium were collected and assayed for CXCL11 concentrations by ELISA. The values were normalized with total protein of cell extracts. Values are the mean ± SEM of the combined data of five separate experiments using different EEC and ESC preparations. #, $p < 0.0001$, between EEC and ESC with IFN-γ at 10, 100, 1000 ng/ml. *, $p < 0.01$; **, $p < 0.0001$, both vs control of EEC; ***, $p < 0.0005$, both vs control of ESC.

Results

Verification of the purity of EEC, ESC, and trophoblast cells

We confirmed the purity of EEC, ESC, and trophoblast cells with immunocytochemistry. The purity of EEC preparations was >95%, as judged by positive cellular staining for cytokeratin and negative cellular staining for vimentin and CD45 (Fig. 1, *A–D*). The purity of ESC preparations was >98%, as judged by positive cellular staining for vimentin and negative cellular staining for cytokeratin and CD45 (Fig. 1, *E–H*). The purity of trophoblast cell preparations was >90%, as judged by positive cellular staining for cytokeratin-7 and negative staining for vimentin and CD45 (Fig. 1, *I–L*).

IFN-γ-induced protein and mRNA expression of CXCL11 in EEC and ESC

IFN-γ stimulated the mRNA expression and the cellular and secreted protein levels of CXCL11 in a dose-dependent manner in EEC and ESC (Table I and Fig. 2). CXCL11 protein levels both in the cells and in the medium were remarkably higher in EEC than in ESC. The secreted protein levels of CXCL11 in the control were 1.41 ± 0.26 pg/μg protein in EEC, but undetectable in ESC. IFN-γ at 100 ng/ml increased secreted CXCL11 protein levels up to 308 ± 35 pg/μg protein in EEC and 3.16 ± 1.69 pg/μg protein in ESC.

IFN-γ-induced protein expression of three CXCR3 ligands CXCL9, CXCL10, and CXCL11 in EEC

As illustrated in Fig. 3, IFN-γ dose-dependently induced CXCL9, CXCL10, and CXCL11 in EEC.

Expression of CXCR3 in EEC, ESC, and trophoblast cells

We examined the expression of CXCR3 in cultured EEC, ESC, and trophoblast cells. As a positive control, activated T cells showing a Th1-polarized profile of cytokine production were used

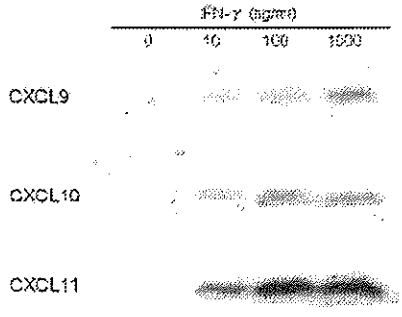

FIGURE 3. IFN-γ-induced protein expression of three CXCR3 ligands CXCL9, CXCL10, and CXCL11 in EEC. EEC and ESC were cultured in serum-free medium with different doses of IFN-γ for 24 h. Cell extracts were prepared and assayed for CXCL9, CXCL10, and CXCL11 by Western blotting. All the chemokines were expressed in dose-dependent manners. The result is representative of three separate experiments.

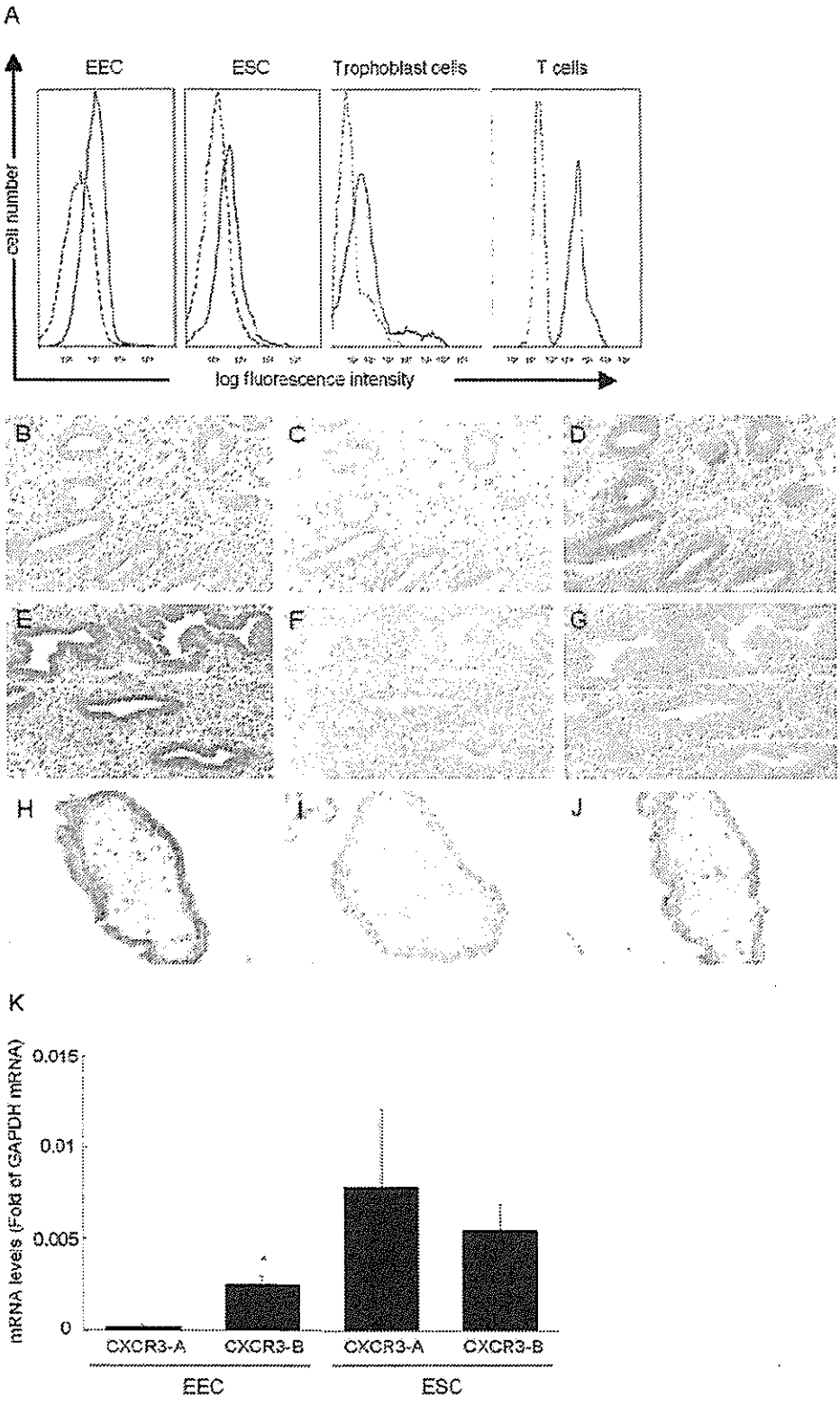

FIGURE 4. Expression of CXCR3 in human EEC, ESC, and trophoblast cells. *A*, Cultured ESC, EEC, and trophoblast cells were collected and stained with anti-CXCR3 Ab (solid line) or isotype control mouse IgG1 (dotted line). T cells were used to be positive control cells for CXCR3. *B–G*, Immunohistochemistry of CXCR3 in human endometrium. Endometrial sections of proliferative phase (*B–D*) and secretory phase (*E–G*) were immunostained with anti-human CXCR3 mouse Ab (*B* and *E*) or isotype mouse IgG1 (*C* and *F*) and stained with H&E (*D* and *G*). Magnification, ×200. *H–J*, Immunohistochemistry of CXCR3 in human villi. Sections of villi were immunostained with anti-human CXCR3 mouse Ab (*H*) or isotype mouse IgG1 (*I*) and stained with H&E (*J*). Magnification, ×200. *K*, Expression of CXCR3-A and -B mRNA in EEC and ESC. Total RNA isolated from EEC and ESC of 13 women was reverse transcribed and amplified by PCR using primers of CXCR3 and CXCR3-B. CXCR3 primers amplify common sequence to CXCR3-A and -B. The data were calculated by subtracting the signal C_T of the internal standard (GAPDH) from the C_T of CXCR3 and CXCR3-B. To quantify the expression of CXCR3-A, we subtracted the amount of CXCR3-B from that of CXCR3. The values of CXCR3-A and -B mRNA represent relative ratios compared with GAPDH mRNA level. Values are the mean ± SEM of samples from 13 women. *, $p < 0.0001$, CXCR3-A mRNA in EEC vs CXCR3-B mRNA in EEC.

(36, 37). As shown in Fig. 4*A*, flow cytometry using an anti-CXCR3 Ab demonstrated that all the four types of cells, i.e., EEC, ESC, trophoblast cells, and Th1 cells, expressed CXCR3 on the cell surface.

As shown in Fig. 4, *B–G*, the presence of CXCR3 in human endometrium was demonstrated in both proliferative and secretory phases. Both stromal and epithelial cells were stained. The intensity of staining in EEC appeared to be stronger than that in ESC in the same section, regardless of the phases of the menstrual cycle. The intensity of the staining seems relatively weak during the proliferative phase and was enhanced during the secretory phase. The presence of CXCR3 in human villi was shown in Fig. 4, *H–J*.

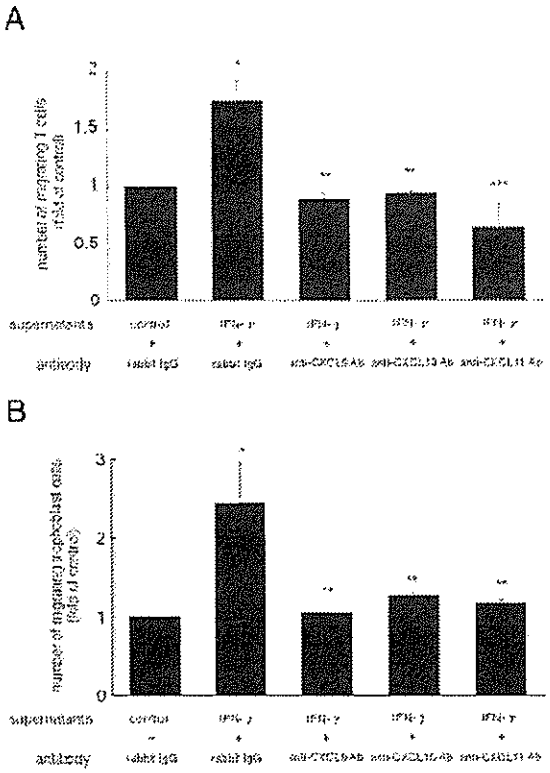

FIGURE 5. Effects of conditioned medium of IFN-γ-stimulated EEC on the migration of T cells and trophoblast cells. Migration assay was performed to study whether the migration of T cells and trophoblast cells was affected by endometrial CXCL11 expression. Supernatants of EEC either stimulated or not by IFN-γ (100 ng/ml) for 24 h were preincubated for 1 h with 10 μg/ml anti-CXCL9 Ab, anti-CXCL10 Ab, anti-CXCL11 Ab, or isotype control rabbit IgG, and plated on the lower chambers. Cells were plated on the upper wells of Transwell membranes containing 100 μl of serum-free DMEM/F12. 5×10^4 T cells (*A*) were incubated for 2 h, and 2×10^5 trophoblast cells (*B*) were for 24 h. After the incubation, T cells and trophoblast cells on the upper surface of membranes were completely removed and migrated cells were fixed with acetone/methanol. Migration indices were determined by counting the cell number. The values represent relative ratios of the cell number compared with those in using the control supernatants of EEC with rabbit IgG. *A*, Values are the mean ± SEM of the combined data from three independent experiments using different T cell preparations. *, $p < 0.05$, control with rabbit IgG vs IFN-γ with rabbit IgG. **, $p < 0.001$, each vs IFN-γ with rabbit IgG. *B*, Values are the mean ± SEM of the combined data from three independent experiments using different trophoblast cell preparations. *, $p < 0.05$, control plus rabbit IgG vs IFN-γ plus rabbit IgG. **, $p < 0.05$, each vs IFN-γ plus rabbit IgG.

Trophoblast cells were strongly stained. No staining was seen when the primary Ab was replaced with nonimmune mouse IgG1.

Expression of CXCR3-A and CXCR3-B mRNAs in EEC and ESC

We examined the mRNA expression of CXCR3-spliced variants CXCR3-A and CXCR3-B in cultured EEC and ESC. In EEC, expression level of CXCR3-B mRNA was 12 times as high as that of CXCR3-A, whereas, in ESC, there was no significant difference between expression levels of CXCR3-A and CXCR3-B (Fig. 4*K*).

Stimulation of IFN-γ on the migration of T cells and trophoblast cells through secretion of CXCR3 ligands

To study chemotactic activity of CXCR3 ligands secreted from EEC including CXCL11 on the migration of T cells and trophoblast cells, in vitro migration assay was performed. As illustrated

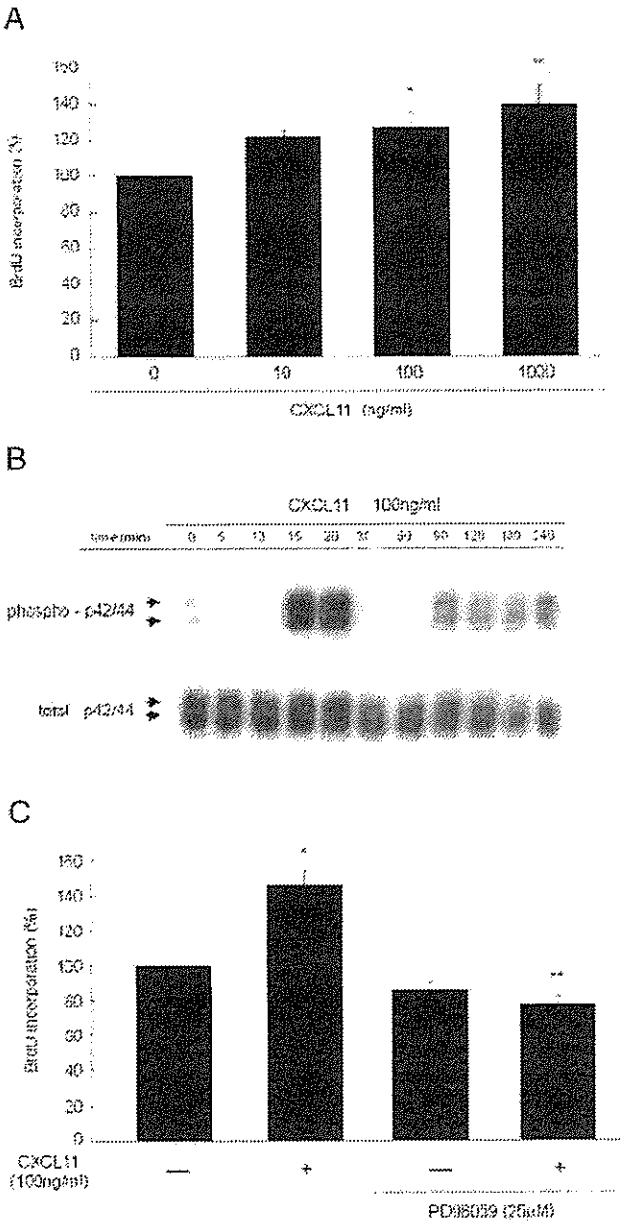

FIGURE 6. CXCL11-induced ESC proliferation via p42/44 MAPK activation. *A*, The effect of CXCL11 on the proliferation of ESC was examined by measuring BrdU incorporation into DNA by using the cell proliferation ELISA. ESC were treated with CXCL11 at different concentrations for 24 h. The values represent relative ratios compared with those in untreated cells. Values are the mean ± SEM of the combined data from five independent experiments using different ESC preparations. *, $p < 0.005$; **, $p < 0.0005$, both vs control. *B*, ESC were incubated with 100 ng/ml CXCL11 for the indicated times (0–240 min). Cell extracts were prepared and assayed for phosphorylated p42/44 MAPK (phospho-p42/44) or total p42/44 MAPK (total-p42/44) by Western blotting. The result is representative of three separate experiments. *C*, Effects of MEK inhibitor PD98059 on CXCL11-induced cell proliferation of ESC was examined by measuring BrdU incorporation into DNA by using the cell proliferation ELISA. ESC were treated with or without PD98059 (25 μM), for 1 h, and then stimulated with CXCL11 (100 ng/ml). After 24 h incubation, BrdU incorporation into DNA in ESC was measured using the cell proliferation ELISA. The values represent relative ratios compared with those in untreated cells. Values are the mean ± SEM of the combined data from four independent experiments using different ESC preparations. *, $p < 0.0001$, control vs CXCL11. **, $p < 0.0001$ CXCL11 vs CXCL11 with PD98059.

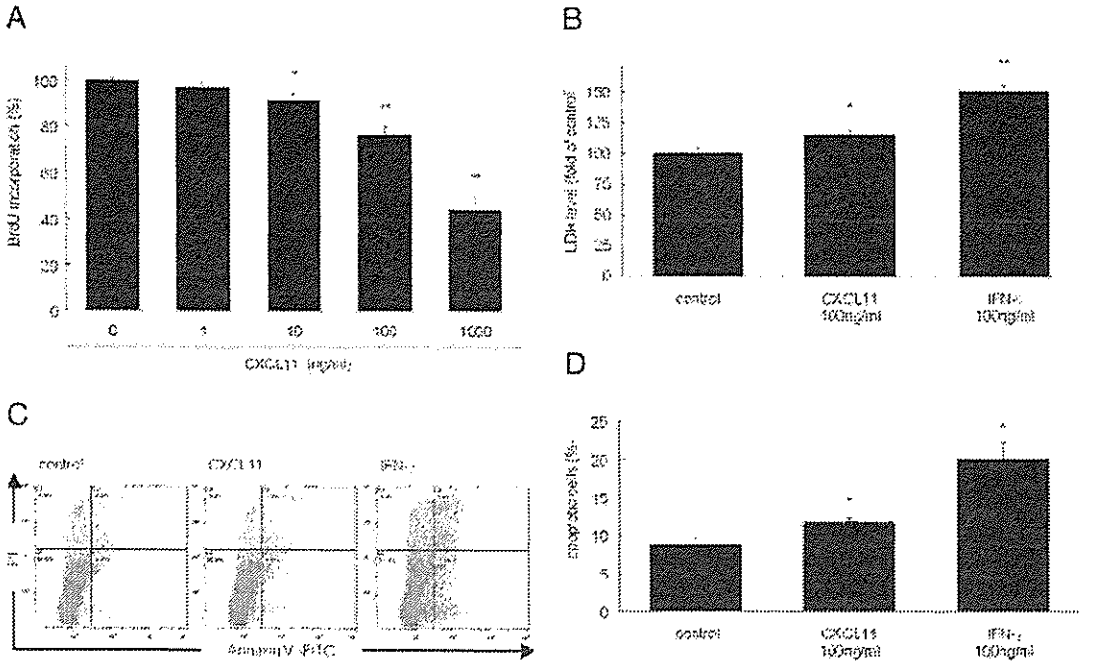

FIGURE 7. CXCL11-induced inhibition of proliferation and stimulation of apoptosis in EEC. *A*, The effect of CXCL11 on the proliferation of EEC was examined by measuring BrdU incorporation into DNA by using the cell proliferation ELISA. EEC were treated with CXCL11 at different concentrations for 24 h. The values represent relative ratios compared with those in untreated cells. Values are the mean ± SEM of the combined data from four independent experiments using different EEC preparations. *, $p < 0.05$; **, $p < 0.0001$, both vs control. *B*, The effect of CXCL11 on cell death of EEC was determined by the measurement of LDH in CXCL11-treated EEC supernatant. EEC were treated with CXCL11 at 100 ng/ml or with IFN-γ at 100 ng/ml for 24 h. The values represent relative ratios compared with those in untreated cells. Values are the mean ± SEM of the combined data from three independent experiments using different EEC preparations. *, $p < 0.05$; **, $p < 0.0001$, both vs control. *C*, The effect of CXCL11 on apoptosis of EEC was determined by double staining of annexin V and PI. EEC were treated with CXCL11 at 100 ng/ml or with IFN-γ at 100 ng/ml for 48 h. The cells were stained with Annexin V^{FITC} and PI. Apoptosis was analyzed by flow cytometry on 5×10^4 EEC. The result is representative of four separate experiments. *D*, The percentage of apoptotic EEC treated with CXCL11 and IFN-γ was significantly higher than that of the control. Annexin V-positive cells were regarded as apoptotic cells. Values are the mean ± SEM of the combined data from four independent experiments using different EEC preparations. *, $p < 0.05$, both vs control.

in Fig. 5, supernatants of IFN-γ-stimulated EEC up-regulated the migration of T cells and trophoblast cells as compared with those of nonstimulated EEC. Moreover, immunoneutralization with Abs of three CXCR3 ligands, CXCL9, CXCL10, and CXCL11, reduced the chemotactic activity of IFN-γ-stimulated supernatants.

CXCL11-induced proliferation of ESC via the p42/44 MAPK pathway

Cell proliferative effects of CXCL11 on EEC were studied by BrdU incorporation assay. As shown in Fig. 6*A*, BrdU incorporation into DNA was significantly increased by CXCL11 at 100–1000 ng/ml. At the concentration of 100 ng/ml, the level of BrdU incorporation was 127% of the control.

It has been reported that CXCR3 ligands induce activation of p42/44 MAPK and cell proliferation in vascular pericytes, glomerular mesangial cells, and that the mitogenic response is mediated by p42/44 MAPK signaling (38). We therefore tested whether activation of p42/44 MAPK was required for cell proliferation induced by CXCL11 in ESC. As depicted in Fig. 6*B*, CXCL11 at 100 ng/ml stimulated a biphasic phosphorylation of p42/44 MAPK in ESC. The phosphorylation levels were reached maximal at 15 and 20 min, followed by decrease to basal levels in 30–60 min and reincrease over 90 min. A p42/44 MAPK pathway inhibitor (MEK inhibitor) PD98059 significantly abrogated the CXCL11-induced BrdU incorporation of ESC (Fig. 6*C*).

CXCL11 inhibited proliferation and stimulated apoptosis in EEC

In contrast to ESC, EEC showed significantly decreased BrdU incorporation into DNA by addition of CXCL11 at 10–1000 ng/ml (Fig. 7*A*). At the concentration of 100 ng/ml, the level of BrdU incorporation was down to 77% of the control.

Effects of CXCL11 on cell death of EEC were determined by measurement of LDH in the supernatants. As shown in Fig. 7*B*, the addition of CXCL11 increased the release of LDH from EEC significantly. Additions of 100 ng/ml CXCL11 and 100 ng/ml IFN-γ enhanced the levels of LDH up to 115 and 150% of the control, respectively.

Apoptotic effects of CXCL11 on EEC were evaluated by staining of annexin V. Fig. 7*C* shows a representative data. Cells expressing annexin V (*lower* and *upper right quadrants* combined) were defined as apoptotic cells. The percentage of apoptotic cells in CXCL11- and IFN-γ-stimulated cells was higher than that in the control cells. As shown in Fig. 7*D*, the combined data from four independent experiments demonstrated that apoptotic cells were significantly increased by CXCL11 at 100 ng/ml.

Discussion

In the present study, we demonstrated that IFN-γ induced production of the chemokine CXCL11 in EEC and that the receptor of CXCL11, CXCR3, is expressed in EEC, ESC, and trophoblast cells. CXCL11 secreted from EEC stimulated migration of trophoblast cells

and T cells. In addition, CXCL11 stimulated proliferation of ESC and apoptosis of EEC.

The present finding that IFN-γ induced the production of CXCL11 in EEC in a markedly larger amount than in ESC implies an endometrial response to the adjacent embryo in apposition and attachment, an initial phase of implantation. IFN-γ production from the human embryo is highest when it develops a blastocyst and reaches a point of apposition in the uterus (8), suggesting that IFN-γ have regulatory roles at the beginning of implantation of the embryo. The endometrial epithelium is an important element where the molecular interactions between the embryo and the endometrium commence (2–4). Therefore, it is feasible that embryo-derived IFN-γ play important roles for implantation partly by provoking CXCL11 production in EEC.

CXCL11 had a positive proliferative effect on ESC and a negative effect on EEC, while both cells have CXCR3, a receptor of CXCL11. It has been reported that CXCR3 ligands, CXCL9, CXCL10, and CXCL11, up- or down-regulate cell proliferation in a cell type-dependent manner. For example, they stimulate proliferation of human vascular pericytes, including glomerular mesangial cells (38, 39) and smooth muscle cells (40), whereas they inhibit growth of vascular endothelial cells. It is interesting to note that the reverse response pattern demonstrated in ESC and EEC mimics that observed in vascular pericytes and endothelial cells. In a recent study, CXCR3-B, when activated by its ligands, induces apoptosis and inhibits cell proliferation (31). In contrast, activated CXCR3-A induces cell proliferation (31). The present study demonstrated that CXCR3-B was mainly expressed in EEC and both CXCR3-A and -B were expressed in ESC. The opposite functions of CXCL11 on cell proliferation might be explained by the interaction of CXCL11 with CXCR3 variants.

The present study showed that CXCL11 induced a biphasic activation of p42/44 MAPK. Interaction between CXCR3 and its ligands leads to p42/44 MAPK activation, whose pattern is either monophasic or biphasic (38). A biphasic activation of p42/44 MAPK has been indicated to stimulate progression of the cell cycles (41, 42). It is thus speculated that CXCL11 promotes ESC proliferation through a biphasic activation of p42/44 MAPK.

Cell death induced by CXCL11 may play a physiological role in the process of implantation. Cell death, especially apoptosis, of endometrial epithelial cells occurs in implantation sites not only in mice (43), rats (44), and hamsters (45), but also in humans (3, 46). Embryo-induced apoptosis of epithelial cells is an important mechanism for invading the luminal epithelium and breaching the epithelial barrier; the immediate consequence is that the trophoectoderm come in direct contact with the basement membrane and, then, stromal invasion can proceed (3). The apoptotic mechanism in endometrial epithelial cells is triggered by a direct contact between blastocysts and epithelial cells (3). In view of the present finding that IFN-γ and CXCL11 induced apoptosis of EEC, we speculate that embryo-derived IFN-γ kills EEC for implantation and that the apoptotic effect is partially indebted to IFN-γ-induced CXCL11 in EEC.

A chemotactic activity of CXCR3 ligands, including CXCL11, on the trophoblast may subserve spreading and invasion of trophoblast cells during the implantation period. Multiple factors such as insulin-like growth factor II, insulin-like growth factor-binding protein-1, endothelin-1, and heparin-binding epidermal growth factor have been shown to promote migration of trophoblast cells into the endometrium (47). In an ovine study, CXCL10 expressed in the endometrium was suggested to stimulate the migration and attachment of trophoblast cells (19). The present study demonstrated that CXCR3 ligands, CXCL9, CXCL10, and CXCL11, which derived from EEC stimulated by IFN-γ, increased migration of trophoblast cells as well as T cells. CXCR3 expressed on the trophoblast cells, which was demonstrated by immunohistochemistry and flow cytometry, may be involved in the chemotactic effect of CXCR3 ligands. Taken together, CXCR3 ligands including CXCL11 could be added to the list of chemotactic factors of trophoblast cells.

Several chemokines expressed in the fetal and maternal annexes during the early pregnancy are thought to regulate cellular movement and positioning of leukocytes, which infiltrated into the subepithelial stromal regions of the uterus. CXCL9 and CXCL10 are suspected to modulate the distribution of leukocytes in the endometrium, contributing to the establishment of immunological environments suitable for implantation and subsequent development (17). Chemotactic activity of three CXCR3 ligands on T cells may also tune the immune environments of the endometrium for implantation.

In summary, we have shown that IFN-γ induced expression of CXCL11 in the endometrial epithelial cells and that CXCR3, the receptor of CXCL11, is expressed on the endometrial cells and trophoblast cells. The demonstrated pleiotropic functions of CXCL11 on trophoblast cells and endometrial cells are suggested to regulate the implantation process of the embryo.

Disclosures

The authors have no financial conflict of interest.

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Recurrence of ovarian endometrioma after laparoscopic excision

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BACKGROUND: To analyse risk factors that influence the recurrence of endometrioma after laparoscopic excision. METHODS: A total of 224 patients who had a minimum of 2 years of post-operative follow-up after laparoscopic ovarian endometrioma excision were studied retrospectively. Recurrence was defined as the presence of endometrioma more than 2 cm in size, detected by ultrasonography within 2 years of surgery. Fourteen variables (age, presence of infertility, pain, uterine myoma, adenomyosis, previous medical treatment of endometriosis, previous surgery for ovarian endometriosis, single or multiple cysts, the size of the largest cyst at laparoscopy, unilateral or bilateral involvement, co-existence of deep endometriosis, revised American Society for Reproductive Medicine (ASRM) score, post-operative medical treatment and post-operative pregnancy) were evaluated to assess their independent effects on the recurrence using logistic regression analysis. RESULTS: The overall rate of recurrence was 30.4% (68/224). Significant factors that were independently associated with higher recurrence were previous medical treatment of endometriosis [odds ratio (OR) = 2.324, 95% confidence interval (95% CI) = 1.232–4.383, $P = 0.0092$) and larger diameter of the largest cyst (OR = 1.182, 95% CI = 1.004–1.391, $P = 0.0442$). Post-operative pregnancy was associated with lower recurrence (OR = 0.292, 95% CI = 0.028–0.317, $P = 0.0181$). CONCLUSIONS: Previous medical treatment of endometriosis or large cyst size was a significant factor that was associated with higher recurrence of the disease. Post-operative pregnancy is a favourable prognostic factor.

Key words: endometriosis/laparoscopy/ovary/recurrence/risk factors

Introduction

Ovarian endometrioma is a common disease lesion among women with endometriosis. Regardless of its symptoms, surgery is most frequently chosen for its treatment because medical treatment alone is inadequate (Jones and Sutton, 2000). In addition, a likelihood of malignant change in this disease is not negligible (Nishida *et al.*, 2000), and European Society of Human Reproduction and Embryology (ESHRE) guidelines recommend that histology should be obtained to exclude malignancy in cases of endometrioma of more than 3 cm in diameter (Kennedy *et al.*, 2005).

Because this disorder is commonly diagnosed in women of reproductive age (Giudice and Kao, 2004), laparoscopic excision of endometrioma, instead of oophorectomy, is applied for most cases. When it is done in infertile woman, laparoscopic excision is also known to improve fertility (Beretta *et al.*, 1998).

One of the most frustrating aspects of treating endometrioma with laparoscopic excision is disease recurrence after surgery (Busacca *et al.*, 1999). When planning a laparoscopy, gynaecologists should be aware of each individual's expected likelihood of recurrence as well as her symptoms and desire for current or future fertility. By having information about factors that may be related to a recurrence of ovarian endometrioma, gynaecologists will be able to distinguish patients at risk, optimize the timing of laparoscopy and plan pre- and post-operative management properly. However, little study has been done to analyse various variants that may have impacts on a recurrence of endometrioma after laparoscopic excision.

To date, recurrence of ovarian endometrioma after laparoscopy has always been discussed focusing on a single factor, such as the effect of post-operative (Muzii *et al.*, 2000) or pre-operative (Muzii *et al.*, 1996) medication, the method of laparoscopic treatment (Saleh and Tulandi, 1999) and the anatomical location (Ghezzi *et al.*, 2001). There is only one multivariate analysis that analysed six variables on the recurrence of

endometrioma by Busacca *et al.* (1999). To analyse risk factors that might influence the recurrence of endometrioma after laparoscopic excision, we retrospectively evaluated 14 variables to assess their independent effects on the recurrence.

Materials and methods

Subjects

A total of 224 patients who had a minimum of 2 years of post-operative follow-up after laparoscopic ovarian endometrioma excision performed at University of Tokyo Hospital between 1995 and 2002 were studied retrospectively. Patient characteristics are summarized in Table I. Institutional Review Board approval was not requested because laparoscopic excision of endometrioma is the standard treatment used in our department. All the procedures followed were in accordance with the revised Declaration of Helsinki, and patients gave informed consent before surgery.

We did not routinely administer pre- or post-operative medical therapy, however, some of the patients were given medical therapy according to their specific needs, e.g. relief of pain. One hundred and two patients had undergone medical treatment previously. Among them, 65 had continued their medication until the operation. The average duration of pre-operative medical therapy was 9.7 months. Post-operative medical therapy was given in 32 cases. The average duration of post-operative medical therapy was 9.5 months. More detailed information about the medication is summarized in Table II.

Surgery

Laparoscopic excision of ovarian endometrioma was performed as follows. After inspection of the pelvis, the ovary was freed from any adhesions. A sharp cortical incision was made, and a cleavage plane was identified. The capsule of the cyst was stripped away from the normal ovarian tissue completely, using bilateral traction and sharp dissection. Other endometriotic peritoneal implants were excised with scissors or coagulated with bipolar electrocoagulation completely, whereas a part of deep endometriosis might be left untreated. Haemostasis was accurately achieved with bipolar electrocoagulation.

The recurrence of ovarian endometrioma was defined as the presence of cysts with a typical aspect detected by transvaginal ultrasonography (Exacoustos *et al.*, 2003) more than 2 cm in diameter within 2 years of surgery. When the cyst was indistinguishable from a transient corpus luteum cyst or an intraovarian haematoma, the diagnosis of recurrence was made only when the cyst had not disappeared after several successive menstrual cycles. Fourteen variables [age, presence of infertility, pain, uterine myoma, adenomyosis, previous medical treatment of endometriosis, previous surgery for ovarian endometriosis, single or multiple cysts, the size of the largest cyst (see abstract) at laparoscopy, unilateral or bilateral involvement, co-existence of deep endometriosis, revised american society for reproductive medicine (ASRM) score, post-operative medical treatment and post-operative pregnancy] were evaluated to assess their effects on the recurrence of ovarian endometrioma. The pain was defined as requiring analgesia at least once a month for dysmenorrhea or chronic pelvic pain. Univariate analysis of the possible risk factors for recurrence followed by a forward step-wise variable selection and logistic regression analysis were performed to eliminate confounding factors. A P value of less than 0.05 was considered statistically significant.

Table I. Characteristics of patients

Factors	Number of cases (%)
Age (years)	32.2 ± 5.4[a]
Infertility	76 (33.9)
Pain	131 (58.5)
Presence of uterine myoma	18 (8.1)
Presence of adenomyosis	60 (26.9)
Previous medical treatment of endometriosis	102 (45.5)
Previous surgery of ovarian endometrioma	30 (13.4)
Multiple cysts	98 (43.8)
Largest cyst diameter (cm)	5.2 ± 1.9[a]
Bilateral involvement	85 (37.9)
Co-existence of deep endometriosis	63 (28.1)
Revised ASRM score	58.1 ± 32.2[a]
Post-operative medical treatment	32 (14.2)
Post-operative pregnancy	39 (17.4)

ASRM, American Society for Reproductive Medicine
[a]Mean ± SD.

Table II. Number of patients who underwent medical treatment before and after the operation

Treatment	Number of patients who underwent medical treatment	
	Before operation	After operation
GnRH agonist	87	15
Danazol	21	5
Oral contraceptives	5	15

Results

The overall rate of recurrence was 30.4% (68/224). Table III presents P values, odds ratio (OR) and 95% confidence interval (95% CI) of univariate and logistic regression analysis.

Using univariate analysis, age, presence of infertility, pain, uterine myoma, adenomyosis, previous surgery for ovarian endometrioma, single or multiple cysts, unilateral or bilateral involvement, co-existence of deep endometriosis and post-operative medical treatment did not significantly influence recurrence. Previous medical treatment of endometriosis, larger diameter of the largest cyst and higher revised ASRM score appeared to be associated with higher recurrence, whereas post-operative pregnancy was associated with lower disease recurrence.

According to a forward step-wise variable selection, five variables (previous medical treatment of endometriosis, the size of the largest cyst at laparoscopy, co-existence of deep endometriosis, revised ASRM score and post-operative pregnancy) were selected for logistic regression analysis. Significant factors that were independently associated with higher recurrence were previous medical treatment of endometriosis [rate of recurrence was 25.5% (29/112) versus 38.2% (39/102) in untreated versus treated patients, respectively, OR = 2.324, 95% CI = 1.232–4.383, $P = 0.0092$] and larger diameter of the largest cyst (OR = 1.182, 95% CI = 1.004–1.391, $P = 0.0442$). Neither co-existence of deep endometriosis nor higher revised ASRM score was significantly associated with recurrence. Post-operative pregnancy was significantly associated with lower recurrence [rate of recurrence was 34.1% (63/185) versus 12.8% (5/39) in no pregnancy versus pregnancy group, respectively, OR = 0.292, 95% CI = 0.028–0.317, $P = 0.0181$].

Table III. Univariate and logistic regression analysis of factors related to the recurrence of ovarian endometrioma

Factors	Univariate analysis	Logistic regression analysis	
	P values	*P* values	Odds ratio (95% confidence interval)
Age (years)	NS		
Infertility	NS		
Pain	NS		
Presence of uterine myoma	NS		
Presence of adenomyosis	NS		
Previous medical treatment of endometriosis	<0.05	<0.01	2.324 (1.232–4.383)
Previous surgery of ovarian endometrioma	NS		
Multiple cysts	NS		
Largest cyst diameter (cm)	<0.05	<0.05	1.182 (1.004–1.391)
Bilateral involvement	NS		
Co-existence of deep endometriosis	NS	NS	0.456 (0.198–1.052)
Revised score	NS	NS	1.010 (1.000–1.021)
Post-operative medical treatment	NS		
Post-operative pregnancy	<0.05	<0.05	0.292 (0.028–0.317)

ASRM, American Society for Reproductive Medicine

Discussion

Many previous studies discussed the recurrence of ovarian endometrioma after laparoscopic excision, in view of requirements of reoperation (Busacca *et al.*, 1999; Saleh and Tulandi, 1999; Abbott *et al.*, 2003) or pain recurrence (Busacca *et al.*, 1999; Abbott *et al.*, 2003). In this study, we focused on the mechanism of ovarian endometrioma recurrence *per se* and used a definition of the recurrence as the presence of cysts more than 2 cm in diameter by ultrasonography, which might be rather objective and cover minimum lesions. Under this definition, we observed a recurrence rate of 30.4%.

The patient's age, presence of infertility and pain did not significantly influence the recurrence. The presence of neither uterine myoma nor adenomyosis was significant. As for the characteristics of endometrioma, single or multiple cysts and unilateral or bilateral ovarian involvement were not significant, whereas patients with larger endometrioma had higher probability of recurrence, which agrees with the finding of earlier studies (Busacca *et al.*, 1999; Saleh and Tulandi, 1999). Because most ovarian endometrioma are associated with extra ovarian endometriosis (Redwine, 1999), we evaluated revised ASRM score and co-existence of deep endometriosis. Revised ASRM score did not independently correlate the recurrence. Co-existence of deep endometriosis did not influence the recurrence either.

A new observation demonstrated in this study was that previous medical treatment of endometriosis was a significant factor that was associated with higher recurrence, whereas previous surgery of ovarian endometrioma was not. The less-favourable prognosis for women who have already had medical treatment may be explained by two possible reasons. The first is that the medication may mask endometriotic lesions and allow them to escape from removal at operations. Because more than half of the women who were categorized into previous medical treatment group had continued their medication until the time of operation, it may be possible that the medication might yield latent lesions that remain and recur after the operation. Our findings may also support the study of Muzii *et al.* (1996), which suggests that pre-operative GnRH agonist treatment does not seem to offer any advantage in terms of surgical performance based on various parameters including recurrence rates.

The second possible reason for negative impact of medical treatment on endometrioma recurrence is that hormonal suppressive therapy may alter some genomic characteristics of endometriotic lesions. As for malignant transformation of endometriosis, it is proposed that hormonal ablative treatments may cause negative selection, suppress the normal, eukaryotic cells more than aneuploid cells bearing chromosomal aberrations and increase the rate of dyskaryotic cells in the endometriotic implants (Blumenfeld, 2004). We suppose that the 'negative selection' may also contribute to the recurrence of disease, making the lesion more active, progressive and prone to recurrence.

Patient with post-operative pregnancy had a much lower rate of recurrence, which indicates that subsequent pregnancy may have a protective effect on endometrioma recurrence. On the contrary, laparoscopic excision of endometrioma is known to improve fertility, when it is done in infertile women (Beretta *et al.*, 1998). Taken together, gynaecologists should optimize the timing of laparoscopy according to the patient's desire for current and future pregnancy.

Our study was in line with previous observations that post-operative medical treatment did not significantly influence disease recurrence (Bianchi *et al.*, 1999; Muzii *et al.*, 2000; Busacca *et al.*, 2001). Three-month GnRH analogue (Busacca *et al.*, 2001) or danazol (Bianchi *et al.*, 1999) therapy after laparoscopy was demonstrated to provide no significant advantage in preventing disease recurrence. Post-operative administration of low-dose cyclic oral contraceptives for 6 months had also no significant effect on the long-term recurrence rate of endometrioma (Muzii *et al.*, 2000). However, the treatment period of these studies, and also ours, was less than 1 year, and there is no information about the effect of longer period of treatment. It is therefore possible that medical treatments longer than 1 year may have an effect to prevent endometrioma recurrence. Further studies, e.g. randomized controlled trials, are needed to determine the effectiveness of these therapies.

In summary, this study demonstrated significant factors that were independently associated with a higher or lower recurrence of endometrioma after laparoscopic excision.

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Expression of Adiponectin Receptors and Its Possible Implication in the Human Endometrium

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Adiponectin, a pleiotropic cytokine, exerts its effects via the specific receptors AdipoR1 and AdipoR2. Whereas circulating adiponectin concentrations decrease in women with endometriosis and endometrial cancer, possible effects of adiponectin and the presence of the receptors in the endometrium have not been determined. In this study, we examined the expression of adiponectin receptors AdipoR1 and AdipoR2 in the human endometrium and assessed effects of adiponectin in endometrial cells. Expression of AdipoR1 and AdipoR2 in endometrial tissues was evaluated by real-time quantitative PCR, *in situ* hybridization, and Western blotting. The effects of adiponectin on phosphorylation of AMP-activated protein kinase, a regulator of energy homeostasis, in cultured endometrial stromal cells (ESCs) and epithelial cells (EECs) were studied by Western blotting. The effects of adiponectin on IL-1β-induced secretion of IL-6, IL-8, and monocyte chemoattractant protein 1 from cultured ESCs were determined using specific ELISAs. The expression of AdipoR1 and AdipoR2 was detected in the endometrium. The expression of both genes was increased in the midluteal phase, the period of embryo implantation. *In situ* hybridization revealed that both AdipoR1 and AdipoR2 appeared to be equally expressed in the epithelial cells and in the stromal cells. Adiponectin increased phosphorylation of AMP-activated protein kinase in ESCs and EECs. Adiponectin decreased IL-1β-induced secretion of IL-6, IL-8, and monocyte chemoattractant protein 1 from ESCs. These findings suggest that adiponectin exerts energy-homeostatic and antiinflammatory effects in the endometrium, and these effects might be relevant to pathological and physiological endometrium-related events such as implantation and endometriosis. (*Endocrinology* 147: 3203–3210, 2006)

ADIPONECTIN IS A hormone that structurally belongs to the complement 1q family (1). It is highly expressed in differentiated adipocytes and circulates at high levels in the bloodstream (2–5). Adiponectin levels both in adipose tissue and in circulation are reduced in obesity (2, 3). An increasing body of evidence indicates that adiponectin plays an important role in regulating energy metabolism and insulin sensitivity (3, 6). In addition to its antidiabetic effects, adiponectin has been shown to have pleiotropic activities such as antiinflammatory, antiangiogenic, and antiatherosclerotic effects (7–9).

Two adiponectin receptors (AdipoR1 and AdipoR2) have recently been identified (10). The receptors contain seven transmembrane domains but are structurally and functionally distinct from G protein-coupled receptors. Activation of these receptors phosphorylates AMP-activated protein kinase (AMPK), a regulator of energy homeostasis of the cell, and stimulates fatty acid oxidation and glucose uptake. In mice, AdipoR1 is abundantly expressed in the skeletal muscle, whereas AdipoR2 is predominant in the liver (10). In humans, although the expression of the receptors has been reported in a few tissues and cells (11–15), the expression in reproductive organs has been poorly understood.

A growing body of evidence indicates that many adipokines, including adiponectin, have biological implications for female fertility (16). Interestingly, we and others have recently demonstrated that serum adiponectin levels are decreased in women with endometriosis (17) and endometrial cancer (18, 19). Given the diverse effects of adiponectin, these findings imply that adiponectin exerts some effects on the endometrium.

With these backgrounds, we surmised that the adiponectin receptors are expressed in the human endometrium and that adiponectin has possible effects therein. To address this thesis, we studied the presence of the adiponectin receptors in endometrial tissues, and adiponectin-induced activation of the receptors was examined by measuring AMPK phosphorylation of endometrial cells. In addition, effects of adiponectin on IL-1β-induced secretion of IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 from the endometrial cells was determined, considering that these proinflammatory cytokines are important in pathology and physiology of the endometrium (20–22).

Materials and Methods

Reagents and materials

Type I collagenase and antibiotics (a mixture of penicillin, streptomycin, and amphotericin B) were purchased from Sigma Chemical Co. (St. Louis, MO). DMEM/Ham's F12 (F-12) medium was from Life Tech-

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Abbreviations: AICAR, 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; DIG, digoxigenin; EEC, endometrial epithelial cell; ESC, endometrial stromal cell; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MCP, monocyte chemoattractant protein; SSC, standard saline citrate.

nologies, Inc. (Grand Island, NY). Human recombinant adiponectin was obtained from R&D Systems (Minneapolis, MN) and generated in our laboratory (6). 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was obtained from Toronto Research Chemicals (Toronto, Canada). Antihuman rabbit antibodies of AMPK-α (no. 2532) and phospho-AMPK-α (no. 2535) were from Cell Signaling (Beverly, MA). Rabbit antibodies for AdipoR1 (ADIPOR12-A) and AdipoR2 (ADIPOR22-A) were purchased from Alpha Diagnostic International (San Antonio, TX). Antirabbit horseradish peroxidase secondary antibody was from Amersham Biosciences (Little Chalfont, UK). Recombinant IL-1β was purchased from Genzyme/Techne (Minneapolis, MN). Charcoal-stripped fetal bovine serum (FBS) was from HyClone (Logan, UT). Deoxyribonuclease I was from Invitrogen (Carlsbad, CA).

Collection of samples

Endometrial tissues were obtained from women undergoing hysterectomy for benign gynecological conditions. In total, 77 women aged 32–45 yr were recruited to the present study. All women had regular menstrual cycles, and none had received hormonal treatment for at least 6 months before surgery. The tissues collected under sterile conditions were processed for primary cell cultures. The phases of the menstrual cycles were determined and classified as early, mid, and late proliferative and secretory phases according to the last and next menstrual period, basal body temperature, ultrasound findings on the endometrium and ovarian follicles, and standard histological criteria by Noyes *et al.* (23). Subcutaneous fat tissues of the abdomen were obtained from three women during hysterectomy. The tissues for mRNA extraction and Western blot analysis were snap frozen in liquid nitrogen and stored at −80 C. The tissues for *in situ* hybridization were fixed overnight in 10% formalin neutral buffer solution, subsequently dehydrated with a series of ethanol washes, and embedded in paraffin. The tissues for cell culture experiment were provided for additional preparation.

The experimental procedures were approved by the institutional review board of the University of Tokyo, and signed informed consent for use of the sample was obtained from each woman.

Isolation and culture of human endometrial stromal and epithelial cells

The isolation and culture of human endometrial stromal cells (ESCs) and epithelial cells (EECs) were processed as described previously (20, 21, 24). Fresh endometrial biopsy specimens collected in a sterile medium were rinsed to remove blood cells. The tissues were minced into small pieces and incubated in DMEM/F-12, containing 0.25% type I collagenase and 15 U/ml deoxyribonuclease I, for 60 min at 37 C. The resultant dispersed endometrial cells were separated by filtration through a 40-μm nylon cell strainer (Becton Dickinson and Co., Franklin Lakes, NJ). The endometrial epithelial glands that remained intact were retained by the strainer, whereas the dispersed ESCs passed through the strainer into the filtrate.

ESCs in the filtrate were collected by centrifugation and resuspended in phenol-red-free DMEM/F-12 containing 10% charcoal-stripped FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 mg/ml amphotericin B. The 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 six- or 48-well culture plates (Becton Dickinson) at a density of 2×10^5 cells/ml. The cells reached confluence in 2 or 3 d and then were used for the experiments.

EECs were collected by backwashing the strainer with DMEM/F-12 containing 10% charcoal-stripped FBS, seeded in a 100-mm plate, and incubated at 37 C for 60 min to allow contaminated ESCs to attach to the plate wall. The nonattached EECs were recovered and cultured in the culture medium at a density of 2×10^5 cells per well into a 12-well culture plate. The cells that reached confluence in 3 or 4 d were used for the experiments.

The purity of both the stromal and epithelial cell preparations was more than 95%, as judged by positive cellular staining for vimentin and cytokeratin, respectively.

Treatment of the cells

When the ESCs and EECs were approaching confluence, the complete media were removed and replaced with fresh media and antibiotics, and the cells were cultured in serum-free media for an additional 12 h. To examine whether AdipoR1 and AdipoR2 functioned in ESCs and EECs, the cells were incubated with adiponectin for 0–60 min or 1 mM AICAR, an experimental tool to activate AMPK, for 1 h. To evaluate the effects of adiponectin on the IL-1β-induced production of IL-6, IL-8, and MCP-1 in ESCs, the cells were incubated with or without 50 μg/ml adiponectin in serum-free media for 24 h and then stimulated with 5 ng/ml IL-1β in serum-free media for 24 h, according to our previous study (22).

RNA extraction, RT, standard PCR, and real-time quantitative PCR of adiponectin, AdipoR1, and AdipoR2

Total RNA was extracted individually from the endometrial tissue, ESCs, and EECs using an RNeasy minikit (QIAGEN, Hilden, Germany). One microgram of total RNA was reverse transcribed in a 20-μl volume using ReverTra Ace-α (TOYOBO, Osaka, Japan). Standard PCR was performed using ReverTra Dash (TOYOBO) according to the manufacturer's instructions. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (TOYOBO) were used to ensure the quality and amounts of RNA. For negative controls, RNA without RT was used. Adiponectin primers (sense, 5′-AACATGCCCATTCGCTTTAC-3′; antisense, 5′-ATTACGCTCTCCTTCCCCAT-3′) were chosen to amplify a 288-bp fragment. AdipoR1 primers (sense, 5′-AAACTGGCAACATCTGGACC-3′; antisense, 5′-GCTGTGGGGAGCAGTAGAAG-3′) were chosen to amplify a 300-bp fragment. AdipoR2 primers (sense, 5′-ACAGGCAACATTTGGACACA-3′; antisense, 5′-CCAAGGAACAAAACTTCCCA-3′) were chosen to amplify a 267-bp fragment. Both AdipoR1 and AdipoR2 primers span introns. PCR conditions for amplifications of adiponectin, AdipoR1, and AdipoR2 were 30 cycles at 98 C for 10 sec, 60 C for 2 sec, and 74 C for 15 sec. PCR products were analyzed by agarose gel electrophoresis with ethidium bromide.

To assess adiponectin, AdipoR1, and AdipoR2 mRNA expression, real-time quantitative PCR and data analysis were performed using LightCycler (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions. Expression of adiponectin, AdipoR1, and AdipoR2 mRNA was normalized to RNA loading for each sample using GAPDH mRNA, for which expression was substantially constant during the menstrual cycle, as an internal standard. The primers for adiponectin, AdipoR1, AdipoR2, and GAPDH were the same as those used for standard PCR. PCR conditions were as follows: for adiponectin, 40 cycles at 95 C for 15 sec, 65 C for 8 sec, and 72 C for 12 sec; for AdipoR1, 35 cycles at 95 C for 15 sec, 65 C for 8 sec, and 72 C for 12 sec; for AdipoR2, 35 cycles at 95 C for 15 sec, 65 C for 8 sec, and 72 C for 11 sec. All these PCR conditions were followed by melting curve analysis.

Each PCR product was purified with a QIAEX II gel extraction kit (QIAGEN), and their identities were confirmed using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA).

In situ hybridization

To prepare the digoxigenin (DIG)-labeled RNA probes for adiponectin, AdipoR1, and AdipoR2, the 288-, 300-, and 267-bp fragments of the

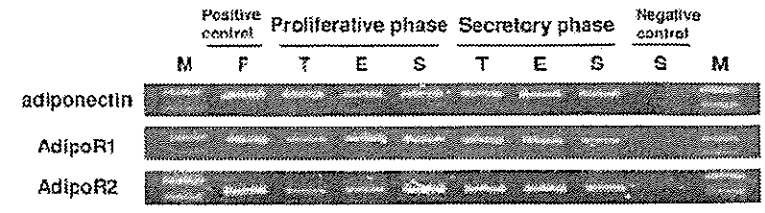

FIG. 1. Expression of adiponectin, AdipoR1, and AdipoR2 mRNA in the endometrium, as detected by standard RT-PCR. Total RNA was extracted from endometrial tissues, cultured EECs, and ESCs in the proliferative phase and the secretory phase. Subcutaneous fat tissues were used for positive controls. For negative controls, RNA without RT was used. Lane T, endometrial tissues; lane E, EECs; lane S, ESCs; lane F, sc fat tissues; lane M, DNA molecular weight standards. The data shown are representative of three different samples in each phase.

human adiponectin, AdipoR1, and AdipoR2 cDNA, obtained by RT-PCR with the primers described above, were subcloned into the appropriate restriction sites of the PCR II-TOPO vector (Invitrogen). After linearization of plasmid with an appropriate enzyme, the linearized vectors were used as templates for the synthesis of DIG-labeled RNA probes using SP6 or T7 RNA polymerase.

In situ hybridization was performed using an ISHR Starting kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. The paraffin-embedded specimens were sliced at a 6-μm thickness. These sections were mounted on poly-L-lysine-treated slides, deparaffinized, and rehydrated. They were further digested with 5 mg/ml proteinase K for 10 min at room temperature, treated with 0.17% acetic anhydrate, and then subjected to treatment with prehybridization solution containing 50% formamide and 2× standard saline citrate (SSC) (1× SSC consists of 0.15 M NaCl and 0.015 M sodium citrate) for 30 min at 42 C. The probe was diluted to a concentration of 0.5 μg/ml in hybridization buffer. Hybridization was carried out by applying the diluted probe (150 μl) to each slide section. Each section was incubated in a humidified chamber overnight at 42 C.

Slides were washed three times in washing solution (50% formamide and 2× SSC) for 20 min each at 42 C, treated with ribonuclease for 30 min at 37 C, and washed three times in 0.1× SSC for 20 min each at 42 C. After being blocked with blocking solution, the sections were incubated with an anti-DIG, alkaline phosphate-conjugated antibody (1:500; Roche) for 60 min at room temperature, and washed three times in washing buffer. Color development was carried out by overlaying them with 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate (NBT-BCIP; Roche), and they were incubated in a humidified chamber in the dark for 12 h at room temperature. All sections were evaluated under light microscope. Sense probe hybridization was used as a control for background level.

Western blot analysis

Cultured cells and endometrial tissues were homogenized in the lysis buffer containing 50 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue. The lysates were further diluted with lysis buffer to give a final concentration of 1 mg total protein/ml. Samples of 20 μg protein per lane were resolved by 10% (for total AMPK-α and phospho-AMPK-α) and 14% (for AdipoR1 and AdipoR2) SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane and incubated with antirabbit antibodies to AdipoR1 (5 μg/ml) and AdipoR2 (5 μg/ml), total AMPK-α (1:1000), or phospho-specific AMPK-α (1:1000) as primary antibodies and antirabbit horseradish peroxidase as a secondary antibody (1:1000). Immune complexes were visualized by use of the ECL Western blotting system (Amersham Biosciences).

Measurement of IL-8, IL-6, and MCP-1

Concentrations of IL-6, IL-8, and MCP-1 were measured using a specific ELISA kit (Quantikine; R&D Systems) according to the manufacturer's protocol as described previously (22, 25). The sensitivities of the assays were 3.12, 15.6, and 31.2 pg/ml for IL-6, IL-8, and MCP-1, respectively. The intraassay and interassay coefficients of variation were less than 5% in these assays.

Statistical analysis

Data were checked for normal distribution using Bartlett test and evaluated using ANOVA with *post hoc* analysis (Fisher's protected least significance) for multiple comparisons. $P < 0.05$ was accepted as statistically significant.

Results

Expression of adiponectin, AdipoR1, and AdipoR2 mRNA in endometrial tissues, EECs, and ESCs

The expression of adiponectin, AdipoR1, and AdipoR2 mRNA in endometrial tissues, EECs, and ESCs were detected by standard RT-PCR analysis (Fig. 1). A signal of the same size was detected in control sc fat tissues.

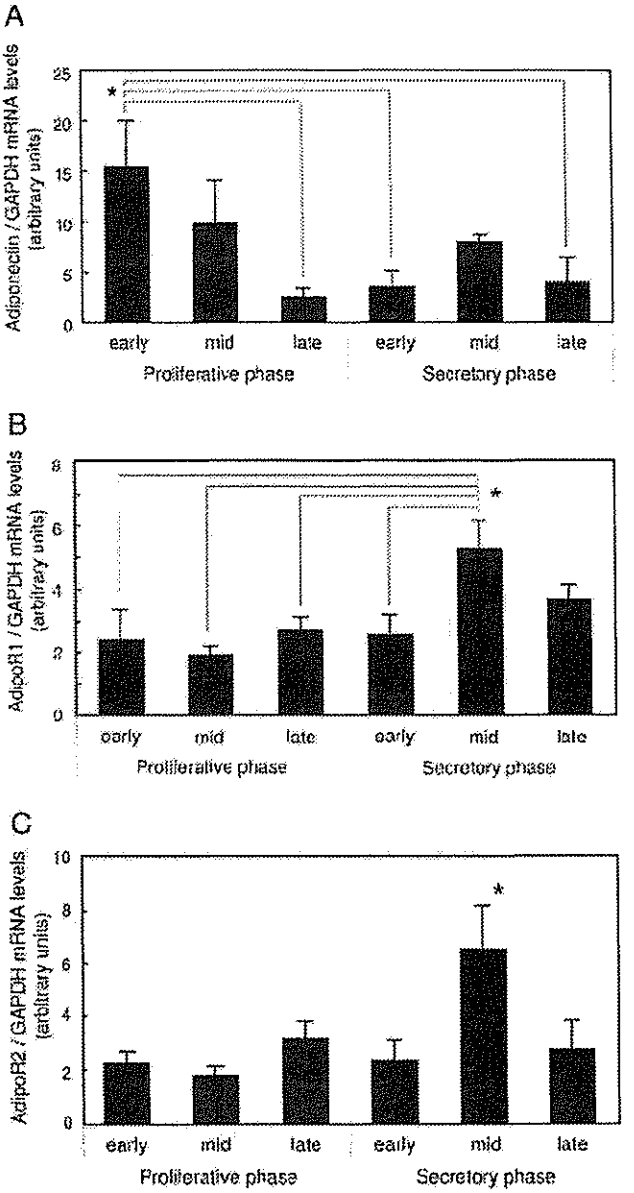

FIG. 2. Expression of adiponectin (A), AdipoR1 (B), and AdipoR2 (C) mRNA in the endometrium throughout the menstrual cycle. Endometrial tissues were obtained from 28 women (early proliferative and mid-secretory phase, n = 4; mid and late proliferative and early and late secretory phase, n = 5). Total RNA isolated from the endometrial tissues was reverse transcribed and amplified by real-time PCR using primers for adiponectin, AdipoR1, or AdipoR2. The data were calculated by subtracting the signal threshold cycles (C_T) of the internal standard (GAPDH) from the C_T of adiponectin, AdipoR1, or AdipoR2. Values are the mean ± SEM. A, *, $P < 0.01$ *vs.* late proliferative and early and late secretory phase; B, *, $P < 0.01$ *vs.* early, mid, and late proliferative, and early secretory phase; C, *, $P < 0.05$ *vs.* all other groups.

Expression of adiponectin, AdipoR1, and AdipoR2 mRNA in the endometrium throughout the menstrual cycle

Real-time quantitative PCR analysis showed adiponectin, AdipoR1, and AdipoR2 mRNA were expressed in endometrial tissues throughout the menstrual cycle (Fig. 2). The

expression levels of adiponectin mRNA were significantly higher in the early proliferative phase compared with those in the late proliferative phase and the early and late secretory phases (Fig. 2A). The expression levels of AdipoR1 mRNA were significantly higher in the mid-secretory phase compared with the early, mid, and late proliferative phases and the early secretory phase (Fig. 2B). The expression levels of AdipoR2 mRNA were significantly higher in the mid-secretory phase compared with all other phases (Fig. 2C).

In vivo expression of adiponectin, AdipoR1, and AdipoR2 mRNA in the endometrium

In situ hybridization demonstrated that AdipoR1 and AdipoR2 were all expressed in glandular and luminal epithelial cells and stromal cells in all phases of the menstrual cycle (Fig. 3). Expression levels of AdipoR1 and AdipoR2 mRNA in the mid-secretory phase seemed to be higher than those in the other phases. The expression levels of each molecule appeared slightly higher in glandular and luminal epithelial cells than stromal cells. Adiponectin mRNA was also detected in glandular and luminal epithelial cells and stromal cells (Fig. 4). No specific hybridization products were observed when using the sense riboprobes.

Expression of AdipoR1 and AdipoR2 proteins in endometrial tissues, EECs, and ESCs

Using Western blotting, the expression of AdipoR1 and AdipoR2 proteins were detected as a bond at 42.4 and 35.4

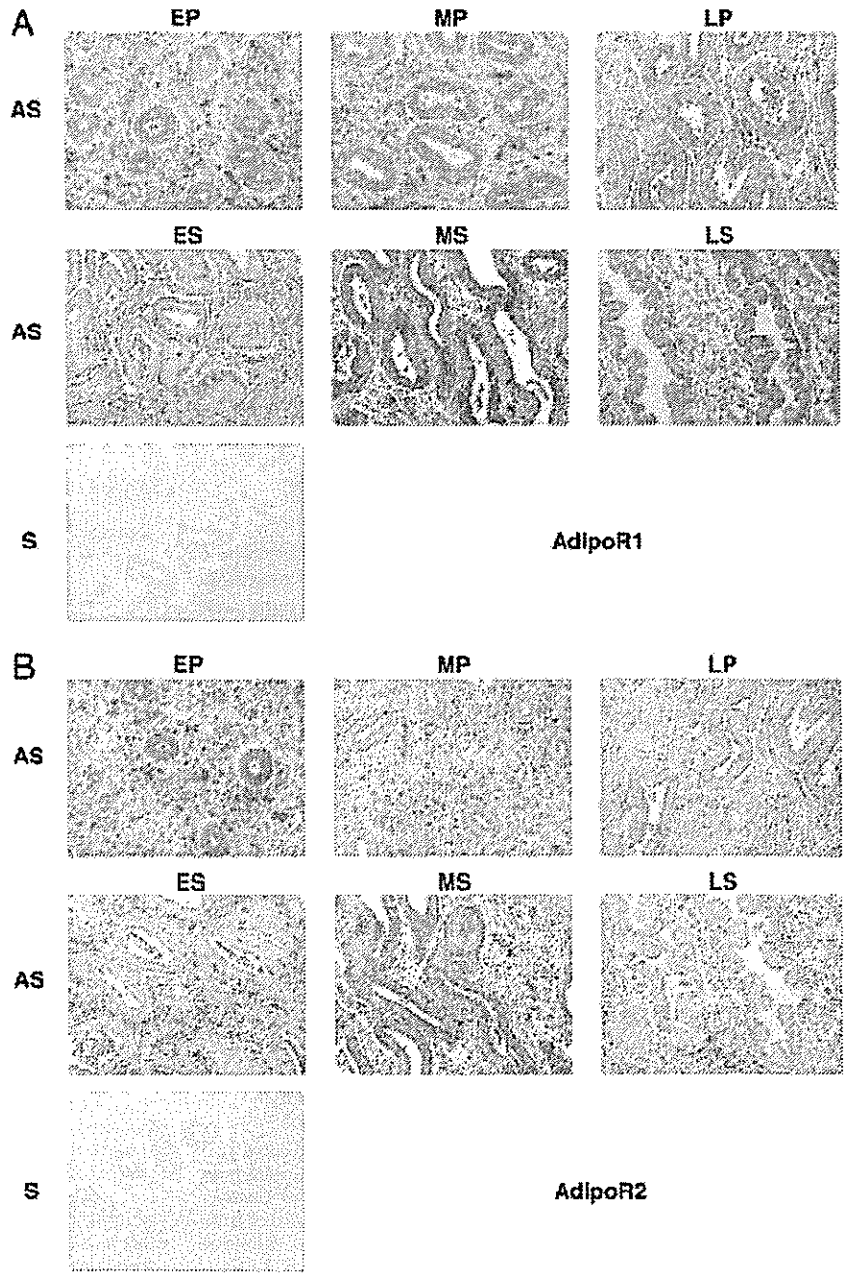

FIG. 3. *In situ* hybridization for AdipoR1 (A) and AdipoR2 (B) in the human endometrium throughout the menstrual cycle. Endometrial tissues obtained from 24 women [early (EP), mid (MP), and late proliferative (LP) phase, n = 3; early (ES) and late secretory (LS) phase, n = 4; mid-secretory (MS) phase, n = 7] were analyzed. The endometrial sections were hybridized with DIG-labeled antisense (AS) or sense (S) riboprobes. The pictures shown are representatives in each phase (AS) and MP (S). Magnification, ×100.

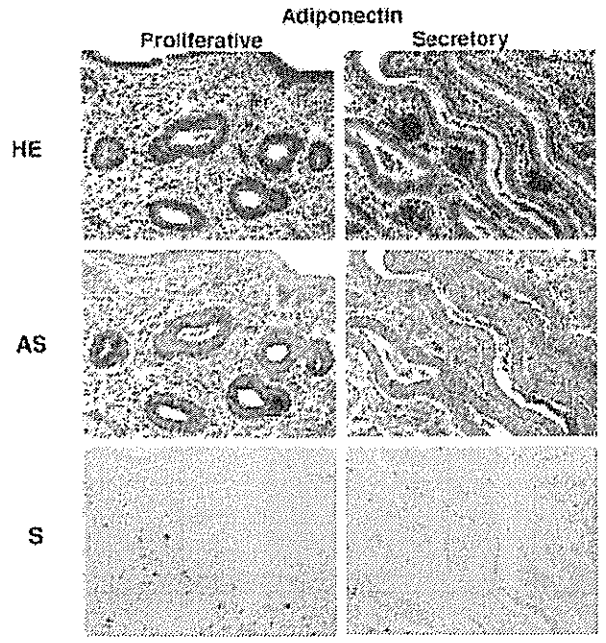

FIG. 4. *In situ* hybridization for adiponectin in the human endometrium. Endometrial sections were stained with hematoxylin and eosin (HE) or hybridized with DIG-labeled antisense (AS) or sense (S) riboprobes. The sections are of proliferative and secretory phases of the menstrual cycle. Magnification, ×100.

kDa, respectively, in endometrial tissues, EECs, and ESCs (Fig. 5).

Adiponectin-induced AMPK phosphorylation in ESCs and EECs

As shown in Fig. 6A, phosphorylation of AMPK was induced by adiponectin in ESCs in a dose-dependent manner. The phosphorylation of AMPK was apparent at 5 min and most prominent at 10 min (Fig. 6B). The phosphorylation levels were lower with 50 μg/ml adiponectin than with 1 mM AICAR, a positive control (Fig. 6C). Phosphorylation of AMPK was also induced by 50 μg/ml adiponectin in EECs (Fig. 6D).

Effects of adiponectin on IL-1β-induced IL-6, IL-8, and MCP-1 production in ESCs

Time-course experiments were conducted to determine the effect of adiponectin on the production of IL-6, IL-8, and MCP-1 in ESCs (Fig. 7). The concentrations of IL-6, IL-8, and MCP-1 in all samples were above the lower limits of the assays. Preincubation with adiponectin significantly decreased IL-1β-induced IL-6, IL-8, and MCP-1 production in ESCs with time, up to 24 h in culture, compared with the controls. Significant decreases were seen at 8 h for IL-8 and 12 h for IL-6 and MCP-1.

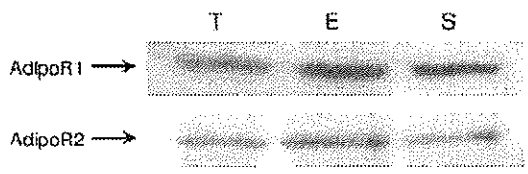

FIG. 5. Expression of AdipoR1 and AdipoR2 proteins in endometrial tissues, EECs, and ESCs. Expression of AdipoR1 and AdipoR2 proteins were examined by Western blotting in endometrial tissues, EECs, and ESCs. The result is representative of six (AdipoR1) or nine (AdipoR2) separate experiments using samples from different women. Lane T, endometrial tissues; lane E, EECs; lane S, ESCs.

Discussion

The present study demonstrated the expression of AdipoR1 and AdipoR2 in the human endometrium for the first time. RT-PCR analysis of the endometrial tissues revealed that the gene expression of both AdipoR1 and AdipoR2 was significantly increased in the mid-secretory phase of the menstrual cycle. Histologically, the expression of both genes was equally observed in ESCs and in EECs. In cultured ESCs, adiponectin stimulated AMPK phosphorylation and suppressed IL-1β-induced IL-6, IL-8, and MCP-1 secretion. Adiponectin also induced AMPK phosphorylation in EECs.

The regulation of adiponectin receptors may be complex and varies depending on the cells and tissues. To date, sev-

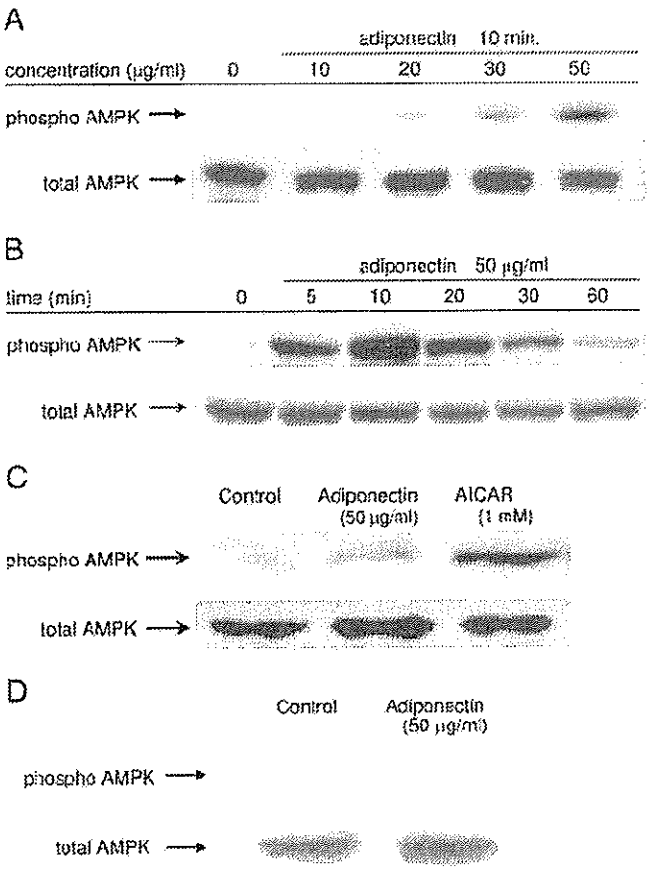

FIG. 6. Phosphorylation of AMPK by adiponectin in ESCs and EECs. Adiponectin-induced phosphorylation of AMPK was examined by Western blot analysis. Cell lysates of ESCs (A–C) and EECs (D) treated with the indicated doses of adiponectin for 10 min or for the indicated times with 50 μg/ml adiponectin underwent Western blotting using the specific antibodies for phospho-AMPK and total AMPK. AICAR was used as a positive control (C). The results are representative of at least four separate experiments using samples from different women.

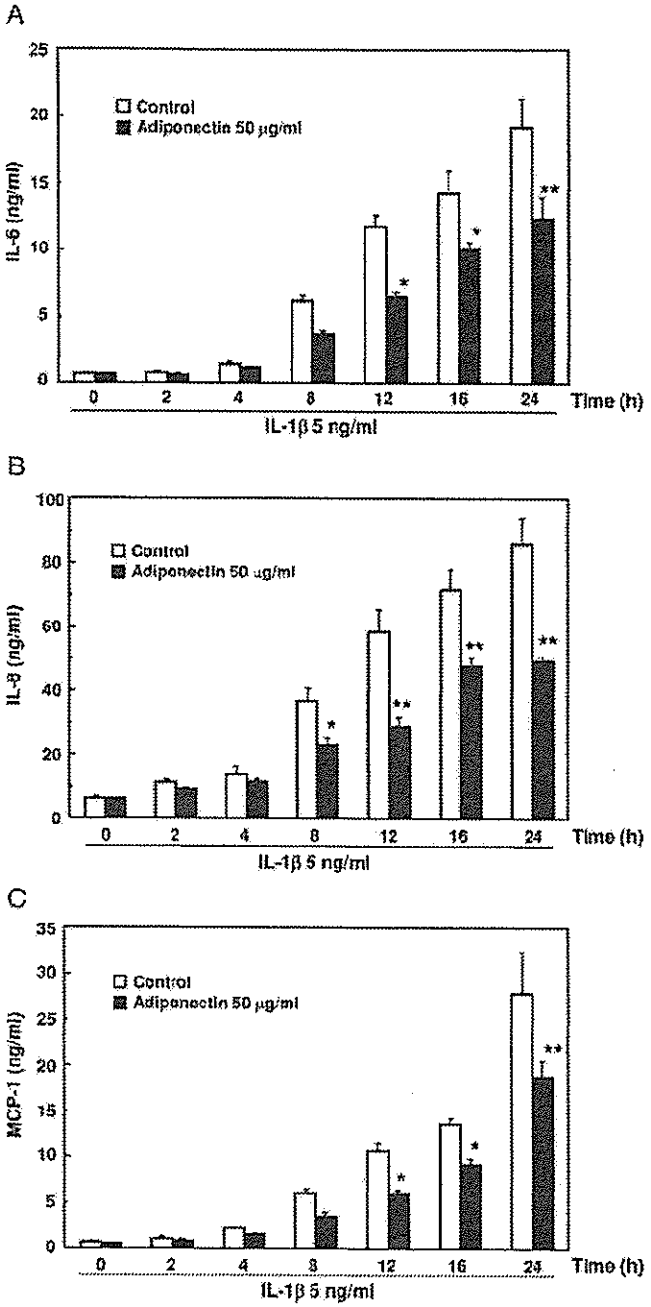

FIG. 7. Effects of adiponectin on the production of IL-6 (A), IL-8 (B), and MCP-1 (C) induced by IL-1β in ESCs. ESCs were incubated with or without adiponectin (50 μg/ml) for 24 h and then stimulated with IL-1β (5 ng/ml) for the indicated time. At the end of the incubation period, the conditioned media were collected and assayed for concentrations of IL-6, IL-8, and MCP-1 by ELISA. Values are the mean ± SEM of quadruplicate cultures. *, $P < 0.005$ *vs.* control; **, $P < 0.0001$ *vs.* control. The results are representative of at least three separate experiments using samples from different women.

eral hormones and drugs, such as insulin, GH, fenofibric acid, and troglitazone, have been shown to up- or down-regulate the expression of AdipoR1 and AdipoR2 (12, 26–29). In the present study, both AdipoR1 and AdipoR2 expression in the endometrium was increased in the midluteal phase. The midluteal phase is a period when the endometrium is receptive for the embryo and thus is called the implantation period. A myriad of genes are specifically expressed in the endometrium during the implantation period (30–32). Taken together, the increase of AdipoR1 and AdipoR2 may be a part of endometrial change for implantation, and it is intuitively speculated that the increase is affected by ovarian hormones and various cytokines that alter characteristics of the endometrium during the period.

AMPK is a fuel sensor to regulate cellular energy balance, and it also mediates effects of adipokines in modulating food intake, body weight, and glucose and lipid homeostasis (33). Adiponectin stimulates phosphorylation and activation of AMPK in the skeletal muscle, and the activated AMPK subsequently induces fatty-acid oxidation and glucose uptake. Adiponectin also stimulates AMPK in the liver and promotes fatty-acid oxidation and suppresses gluconeogenesis (10). In view of these findings, the present finding that adiponectin induced phosphorylation of AMPK in ESCs and EECs suggests that adiponectin may regulate energy supply in ESCs and EECs for an endometrial function such as reception of the embryo.

In the present study, a considerably high concentration (50 μg/ml) of adiponectin induced apparently small levels of phosphorylation of AMPK. There remains the possibility that the responses observed were cross-ligand activations.

Adiponectin has been indicated to have antiinflammatory properties. Although adiponectin inhibits phagocytic activity and LPS-induced production of TNF-α and IL-6 in macrophages (9, 34), it increases the production of the antiinflammatory mediators IL-10 and IL-1RA in monocyte, macrophage, and dendritic cells (34, 35). Antiinflammatory function of adiponectin is also suggested by its inhibiting LPS-induced IL-6 production in adipocytes (36). The present finding that adiponectin suppressed IL-1β-induced secretion of IL-6, IL-8, and MCP-1 in ESCs suggests antiinflammatory roles of adiponectin in the endometrium.

Whereas the process of implantation entails inflammation-like events (37–40), exaggerated inflammatory responses may perturb the integrity of endometrial function and lead to pathological conditions, including abortion and complicated pregnancies, such as preeclampsia and underdevelopment of the fetus. To render the endometrium favorable for implantation and ensuing fetal development, inflammatory responses of endometrial cells are suggested to be spatiotemporally fine tuned (22). In this context, the increased expression of AdipoR1 and AdipoR2 in the mid-secretory phase may subserve implantation, augmenting the adiponectin action of suppressing the production of inflammatory cytokines. However, adiponectin does not seem indispensable for pregnancy, because adiponectin-deficient mice were shown to be fertile (41).

The antiinflammatory effect of adiponectin in ESCs may be relevant to the pathogenesis of endometriosis. We have recently shown that serum and peritoneal fluid adiponectin levels are decreased in women with endometriosis (17, 42). Inflammation associated with endometriosis is suggested to promote the development of the disease. In particular, IL-1β-induced production of IL-6, IL-8, and MCP-1 in ESCs