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

Yasushi Hirota, Yutaka Osuga,[1] Kaori Koga, Osamu Yoshino, Tetsuya Hirata, Chieko Morimoto, Miyuki Harada, Yuri Takemura, Emi Nose, Tetsu Yano, Osamu Tsutsumi, and Yuji Taketani

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.

I mplantation 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 (MgSO₄), 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

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% CO₂/95% 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 ~2×10^5 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 ~1×10^4 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 MgSO₄, 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% CO₂/95% 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,

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.
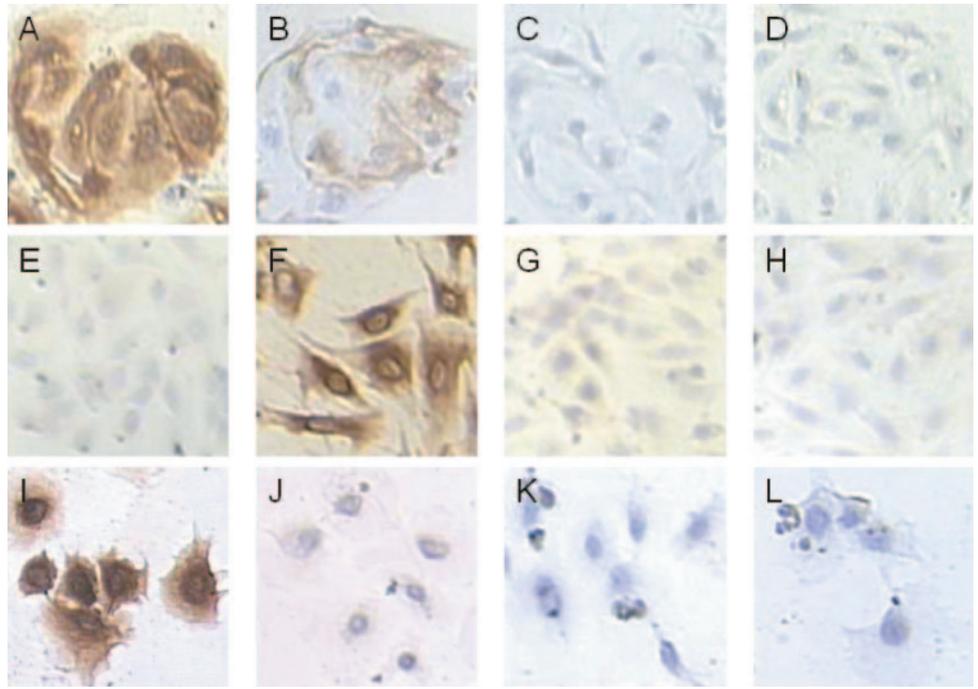
5′-TCACAAAAGAGTTCCTGCCA-3′; antisense, 5′-AAGAGGAGGCT GTAGAGGGC-3′) were chosen to amplify a 241-bp fragment. Expression of CXCL11, CXCR3, and CXCR3-B mRNA was normalized to RNA loading for each sample using GAPDH mRNA as an internal standard. Human GAPDH primers (Toyobo) were chosen to amplify a 452-bp 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 $\pm$ SEM of three separate experiments using different EEC and ESC preparations. Logarithm of the values was used in statistical analysis.
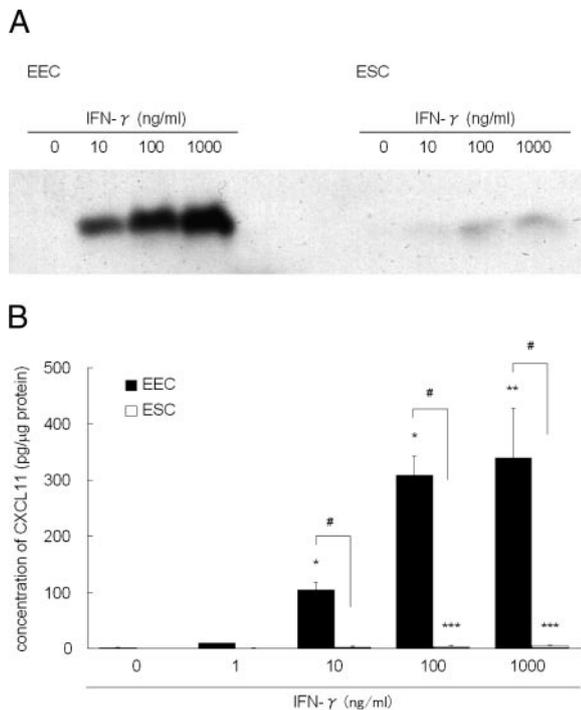[b] $p < 0.0001$, each vs. control of EEC.
[c] $p < 0.0001$, each vs. control of ESC.

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 phosphatidyl-serine-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.

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*).

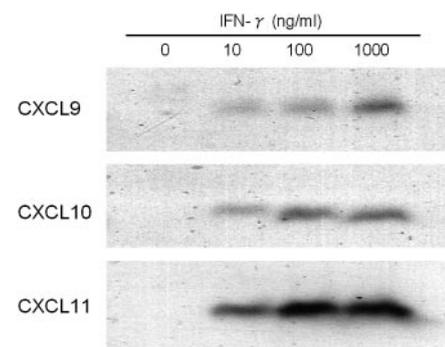### IFN-γ-induced protein and mRNA expression of CXCL11 in EEC and ESC

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 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 $\pm$ 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.

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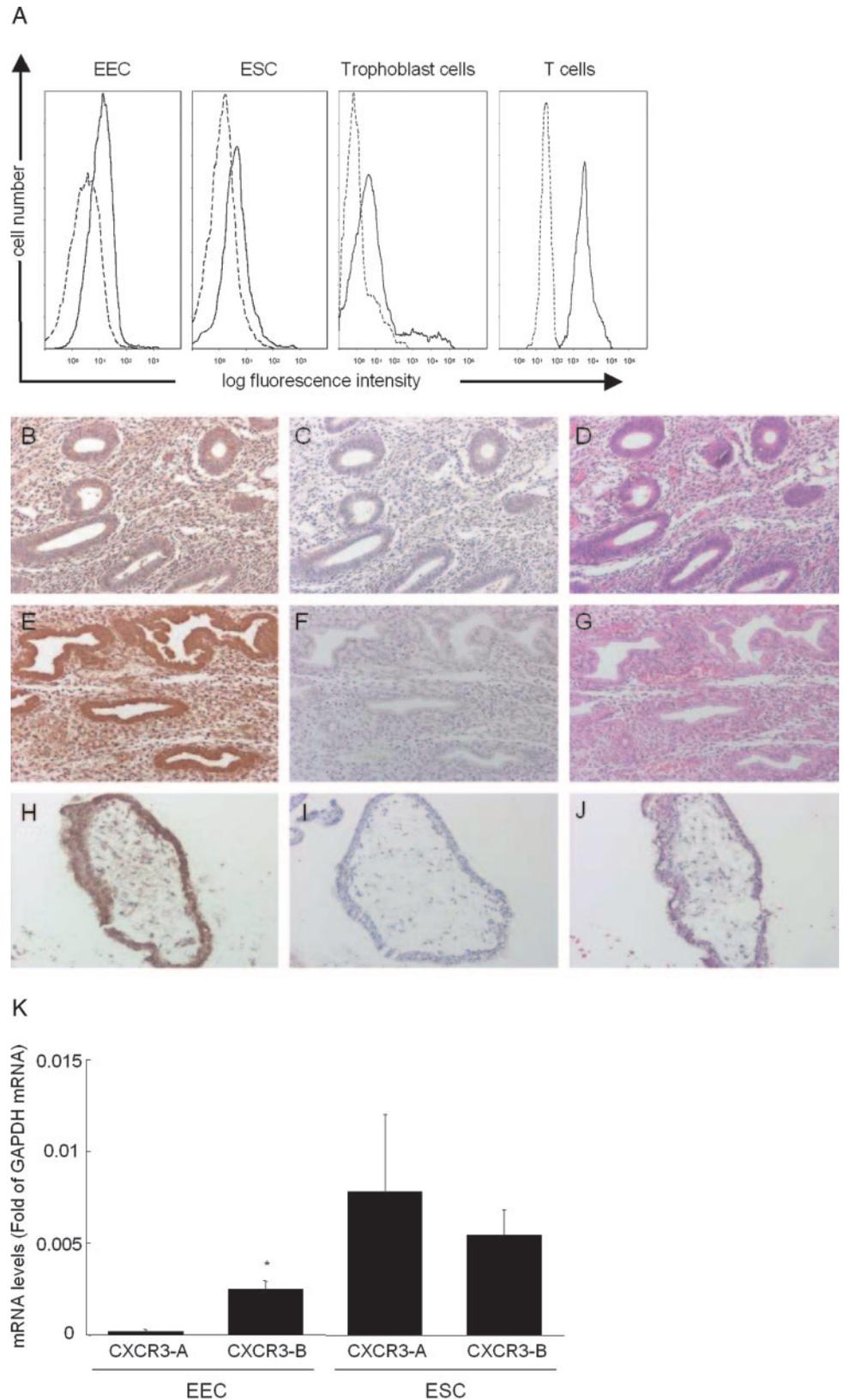
(36, 37). As shown in Fig. 4*A*, flow cytometry using an anti-CXCR3 Ab demonstrated that all the four types of cells, i.e., EEC, ESC, trophoblast cells, and Th1 cells, expressed CXCR3 on the cell surface.

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*.

A

B
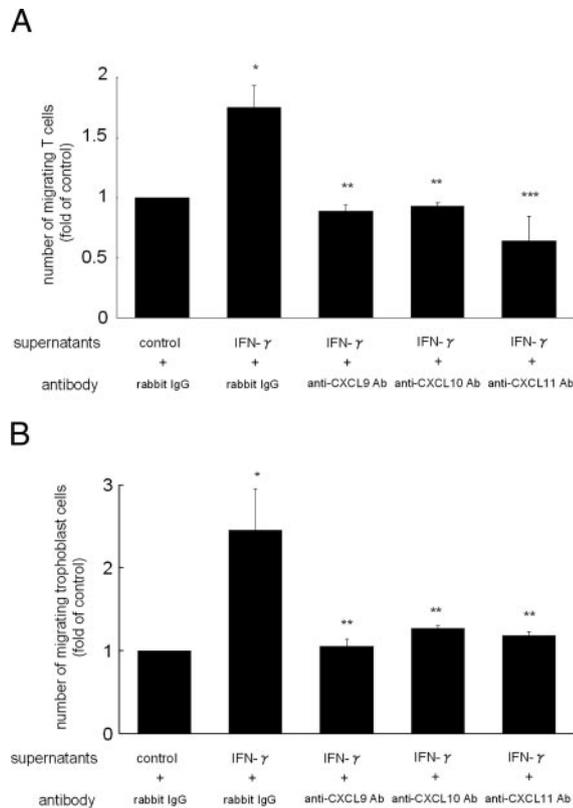
FIGURE 5. Effects of conditioned medium of IFN-γ-stimulated EEC on the migration of T cells and trophoblast cells. Migration assay was performed to study whether the migration of T cells and trophoblast cells was affected by endometrial CXCL11 expression. Supernatants of EEC either stimulated or not 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. 5×10^6 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

A

B

C
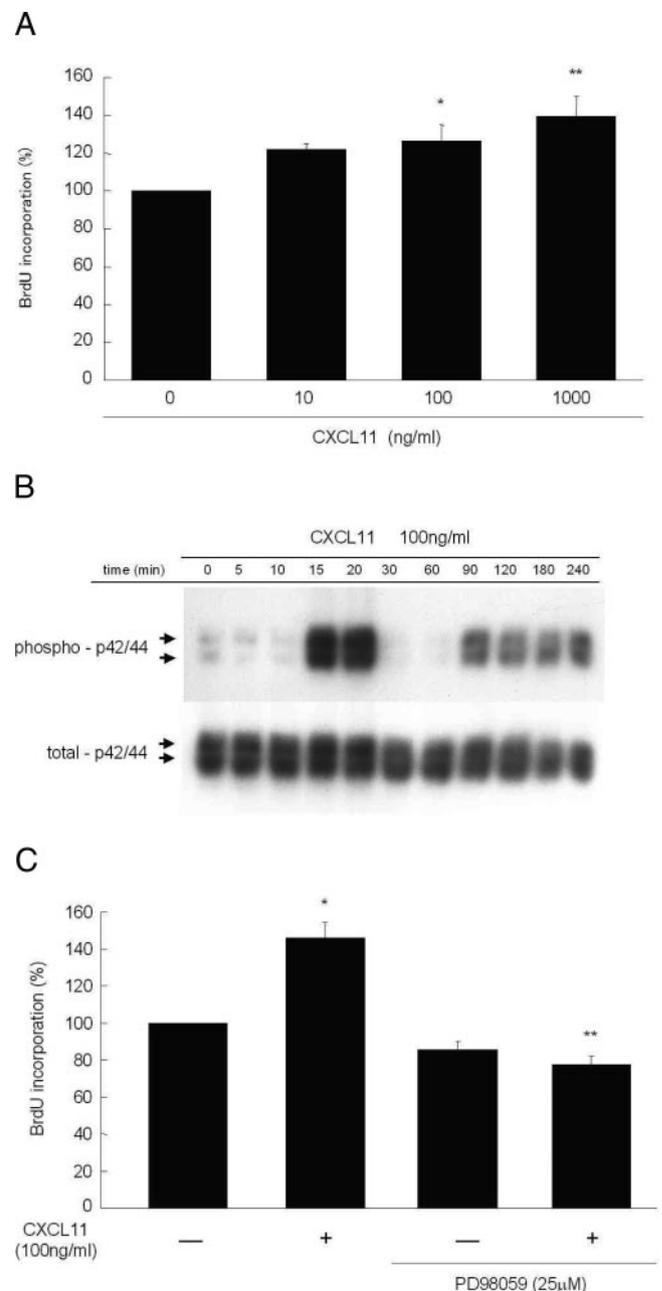
FIGURE 6. CXCL11-induced ESC proliferation via p42/44 MAPK activation. *A*, The effect of CXCL11 on the proliferation of ESC was examined by measuring BrdU incorporation into DNA by using the cell proliferation ELISA. ESC were treated with CXCL11 at different concentrations for 24 h. The values represent 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.
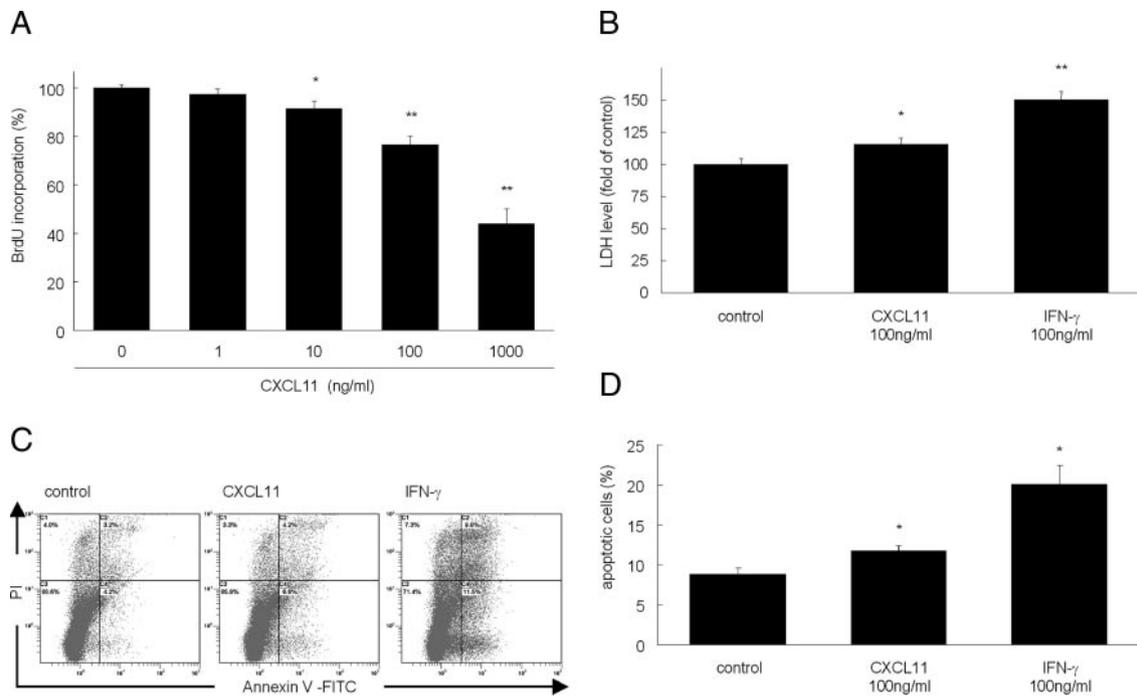
FIGURE 7. CXCL11-induced inhibition of proliferation and stimulation of apoptosis in EEC. *A*, The effect of CXCL11 on the proliferation of EEC was examined by measuring BrdU incorporation into DNA by using the cell proliferation ELISA. EEC were treated with CXCL11 at different concentrations for 24 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 dells. The percentage of apoptotic cells in CXCL11- and IFN-γ-stimulated cells was higher than that in 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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