The Cytokine Gene CXCL14 Restricts Human Trophoblast Cell Invasion by Suppressing Gelatinase Activity

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Well-controlled trophoblast invasion into uterine decidua is a critical process for the normal development of placenta, which is tightly regulated by various factors produced within the trophoblast-endometrial microenvironment. CXCL14 is involved in tumor growth and metastasis, and its expression in placenta is temporally regulated during pregnancy. However, the role of CXCL14 in trophoblast function during human pregnancy is not clear. In this study, by using RT-PCR through human pregnancy, we found that CXCL14 was selectively expressed at early but not late pregnancy. Immunostaining revealed that CXCL14 proteins were strongly expressed in villous cytotrophoblasts and moderately in decidualized stromal cells but very weakly in syncytiotrophoblasts and extravillous trophoblasts. The effect of CXCL14 on trophoblast invasion were examined by using human villous explants cultured on Matrigel and further proved by invasion and migration assay of primary trophoblast cells and trophoblast cell line HTR-8/SVneo. Our data showed that CXCL14 significantly inhibited outgrowth of villous explant *in vitro*; this effect is due to suppression of trophoblast invasion and migration through regulating matrix metalloproteinases activities, whereas the trophoblast proliferation was not affected. Moreover, because a receptor for CXCL14 has not been identified, we performed further cell-specific CXCL14 binding activities with regard to different cell types within the maternal-fetal interface. Our data revealed that CXCL14 could specifically bind to trophoblast cells but not decidual cells from the maternal-fetal interface. These results suggest that CXCL14 plays an important role in regulating trophoblast invasion through an autocrine/paracrine manner during early pregnancy. **(*Endocrinology* 150: 5596–5605, 2009)**

The invasion of uterine decidua and maternal vasculature by trophoblast cells is a key process in the establishment of successful pregnancy. After trophoblast invasion, the embryos are anchored in the uterine wall, and extravillous trophoblasts further remodel the uterine spiral arteries to form low-resistance vessels necessary to ensure an adequate blood supply for optimal fetal growth (1, 2). Invasion of uterine tissues and spiral arteries by trophoblast cells is regulated in a paracrine or autocrine pattern by different hormones, growth factors, and cytokines at the maternal-fetal interface. These factors regulate cell adhesion and migration, modulate protease activity, and/or induce cell proliferation and apoptosis (3–5).

Chemokines, a large family of chemotactic cytokines, are known for their roles in leukocyte recruitment and activation (6). Recent reports have revealed that chemokines were also involved in various reproductive processes. Abnormal regulation of chemokines has been associated with disease conditions such as recurrent miscarriage and cancer (5, 7, 8). *CXCL14*, also known as

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; EVT, extravillous trophoblast; FCS, fetal calf serum; h, human; MMP, matrix metalloproteinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; r, recombinant.

BRAK, is a member of the CXC chemokine family preferentially expressed in liver, small intestine, and uterus without the necessity of inflammatory stimulation (9–11). The expression of CXCL14 in tumors was heterogeneous, the majority of malignant tissues showing loss of CXCL14 expression (9, 12–15). It was also demonstrated that CXCL14 was a potent mediators of neoangiogenesis and tumor growth, invasion, metastasis (13, 15–20). *In situ* hybridization analyses revealed that *CXCL14* mRNA was expressed in human placenta and that its expression is regulated during gestation (21, 22). Intense signals were detected in second-trimester placenta tissues, but little or no signal was found at term. By using oligonucleotide microarrays, CXCL14 was found to be the most highly upregulated gene (61-fold) when comparing the midsecretory phase of endometrium with the early secretory phase of endometrium (23).

Based on the evidence that *CXCL14* mRNA was temporally and spatially expressed in the human maternal-fetal interface and the striking similarities between trophoblast cells and tumor cells with regard to proliferative and invasive properties (24), we hypothesized that the decidual and/or trophoblast CXCL14 might regulate trophoblast invasion during pregnancy. Here we investigated spatiotemporal expression of CXCL14 at the human maternal-fetal interface and its effects on trophoblast outgrowth of villous explants. We also investigated the potential cell-specific CXCL14 binding activity within maternal-fetal interface.

Materials and Methods

Tissue collection

Samples of placental tissues and deciduas from the first trimester were obtained from healthy women undergoing suction termination of pregnancy (5–9 wk) for nonmedical reasons; term placentas (n = 4) were collected after uncomplicated pregnancy and vaginal delivery. Ethical approval was granted by Ethical Committee of Chinese Academy of Sciences and HaiDian hospital in Beijing. All patients completed an informed consent to collect tissue samples. The tissues were collected and stored in ice-cold DMEM (Invitrogen, San Diego, CA), transported to the laboratory within 30 min after surgery, and washed with ice-cold PBS for cultures or fixation.

Isolation and culture of trophoblast cells

Primary trophoblast cells were isolated as described previously (25). In short, placental tissues (n = 4–5) from the first trimester were pooled, dissected into small pieces and digested with 0.25% trypsin (Amresco Inc., Solon, OH) and 15 IU/ml DNase I (Sigma, St. Louis, MO) for 5 min at 37 C with gentle agitation. Then the digested suspension was discarded, and the residual tissue was subjected to four cycles of 10-min digestion. The four resultant cell suspensions were pooled and centrifuged over a discontinuous Percoll gradient. Isolated trophoblast cells were cultured in DMEM supplemented with 2 mM glutamine, 0.2% FCS, 25 mM HEPES, 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37 C in 95% air-5% CO_2.

Because of the restricted availability of primary human trophoblast cells, we first used a human first-trimester cytotrophoblast line (HTR-8/SVneo cell) to examine cellular responses to CXCL14. HTR-8/SVneo cells with cytotrophoblast properties (26) were cultured in RPMI 1640 (Invitrogen) media containing 10% FCS. Before passage for experiments conducted in serum-free media, cells were transferred to RPMI 1640 media containing 0.2% FCS (experimental media).

Isolation of decidual cells

Decidual tissues from three to four separate patients undergoing suction termination of pregnancy (5–9 wk) for nonmedical reasons were pooled and minced into small pieces, then digested with 0.1% collagenase type IV (Invitrogen) and 15 IU/ml DNase I (Sigma) in DMEM containing 100 IU/ml penicillin and 100 μg/ml streptomycin in a 37 C shaking water bath for 40 min. Collagenase digestion was stopped with 10% FCS. Cell suspensions were filtered with 100-μm filters and then washed three times with sterile PBS. Cells were resuspended in DMEM, grown to confluence on polystyrene tissue culture dishes. The purity of isolated trophoblast and decidual cells was assessed by staining for cytokeratin 7 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) or vimentin (1:100; Santa Cruz Biotechnology).

RNA extraction and RT-PCR

Total RNA was extracted using TRIzol (Invitrogen) and reverse-transcribed in 25 μl of reaction mixture containing 30 U of avian myeloblastosis virus reverse transcriptase (Promega). The PCR was conducted in a total volume of 50 μl for 25 cycles of denaturation at 94 C of 30 sec, annealing at 56 C for 30 sec, and extension at 72 C for 45 sec, with a final extension step of 10 min at 72 C. The primers used in this study include CXCL14 (5′-AATGAAGCCAAAGTACCCGC-3′; 5-AGTCCTTTGCACAAGTCT) and GAPDH (5′-GCCAAGGTCATCCATGACAAC-3′; 5′-GTCCACCACCCTGTTGCTGTA-3′). The amplified products were analyzed by electrophoresis on 1% agarose gels stained with ethidium bromide.

Immunohistochemistry

Tissues were fixed in Bouin's solution. The sections were first boiled in citrate buffer (0.01 M, pH 6.0) for 15 min. Nonspecific binding was blocked in 5% bovine serum albumin for 60 min. Then, the sections were incubated in the goat antihuman CXCL14 (1:150; Santa Cruz Biotechnology) or mouse antihuman cytokeratin (1:100; Santa Cruz) overnight at 4 C. After washing in PBS, the sections were incubated with secondary antibody for 1 h at room temperature. The primary antibody was detected with diaminobenzidine solution. For some sections, primary antibodies were replaced with goat or mouse preimmune IgG as a negative control.

Indirect immunofluorescence

Trophoblast cells were fixed in 4% paraformaldehyde for 30 min at room temperature. After fixation, cells were permeabilized in PBS containing 0.1% Triton X-100 (15 min). Cells were incubated in 5% BSA for 30 min at room temperature. Then, cells were incubated with goat antihuman CXCL14 antibody

(Santa Cruz Biotechnology) or mouse antihuman cytokeratin 7 (Santa Cruz Biotechnology) or mouse antihuman vimentin (Santa Cruz Biotechnology) at 4 C overnight followed by fluorescein isothiocyanate-labeled secondary antibody for 1 h at room temperature. Nuclei were stained with 5 μg/ml propidium iodide for 10 min. Finally, cells were viewed under a laser-scanning confocal microscope (Leica, Heidelberg, Germany).

Explant cultures

The explant culture was performed as described previously (27). In brief, small pieces of tissue (2–3 mm) from tips of placental terminal villi were dissected. For preparation of Matrigel-coated plates, Matrigel was diluted in explant medium to a final concentration of 8 mg/ml and then placed in the center of a 24-well culture dish. Recombinant human CXCL14 (rhCXCL14; R&D Systems, Minneapolis, MN) (25, 50, or 100 ng/ml) was added to the extracellular matrix solution. After formation of gels (30 min at 37 C), the dissected tissue pieces were carefully put on the top of each gel drop, and incubated for 3 h to allow anchorage. Subsequently, explants were supplemented with 0.5-ml medium in the absence or presence rhCXCL14 (0–100 ng/ml). For each condition, 20 explants were analyzed, and experiments were repeated three times with different placentas. The extent of migration (*i.e.* the distance from the cell column base to the tip of the outgrowth) was measured at defined positions with ImageJ software (http://rsbweb.nih.gov/ij/).

CXCL14 binding assay

CXCL14 binding assay to detect potential CXCL14 receptor of cells surface were performed as described previously (28). In brief, recombinant human CXCL14 (rhCXCL14) protein was biotinylated by EZ-link biotinylation reagent (Pierce) before experiments. Cells prepared for CXCL14 binding assay were first washed twice with PBS and harvested by trypsinization. One million cells per milliliter were resuspended in 0.4 ml of cold culture medium, supplemented with 300 μg/ml chondroitin sulfate C. Then, cells were split into two fractions, half was incubated with 3 μM unlabeled-rhCXCL14 in binding buffer for 60 min on ice, and the other half remained untreated within the binding buffer. After 60 min, 50 nM of biotinylated-rhCXCL14 was added to each fraction of cells for another 60 min. Finally, each fraction of cells was washed twice and CXCL14 binding activity was detected by flow cytometry analysis (FACS; BD) after incubation with streptavidin-phycoerythrin (Bio-Legend, San Diego, CA) for 45 min on ice. The fraction of cells incubated with an excess of unlabeled-rhCXCL14 served as negative control as a result of the competitive binding activity by biotinylated-rhCXCL14 with unlabeled-rhCXCL14 (cold inhibition).

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) assay

MTS assay (Promega) was performed according to manufacturer's instructions. Cells were seeded at 10^3 cells/well in 96-well plates and grown to preconfluence. Cells were rinsed with PBS, and culture medium was replaced with experimental media for 24 h. Thereafter, rhCXCL14 (0–100 ng/ml) or 10% FCS (positive control) was added to culture system for 48 h at 37 C. Then 20 μl of CellTiter 96* AQueous One Solution (Promega, Madison, WI) reagent was added into each well of the 96-well assay plate containing the samples in 100 μl of culture medium, and the plate was incubated for 2–4 h at 37 C in a humidified, 5% CO_2 atmosphere. The absorbance was measured at 490 nm.

5-Bromo-2′-deoxyuridine (BrdU) incorporation assay

HTR-8/SVneo cells or primary trophoblast cells at a density of 5×10^5 cells were seeded in 24-well culture plates, grown to preconfluence (60%), washed with PBS, and then transferred into experimental media for 24 h. Thereafter, rhCXCL14 (0–100 ng/ml) or 10% FCS (positive control) was added to culture system for 24 h at 37 C. BrdU (10 mM) (Roche Applied Science, Indianapolis, IN) was added to each well and incubated for 4 or 24 h. Anti-BrdU antibody (Zhongshan Biotechnology, Zhongshan, China), and appropriate fluorescein isothiocyanate-labeled secondary antibody were used. Staining was visualized using confocal microscope (Leica, Heidelberg, Germany). Cells were quantified by counting the number of BrdU-positive cells in five to eight alternative areas.

Invasion assay

Invasion assays were performed in this study with modification as described previously (29). We used Transwell plates (6.5-mm diameter; Corning Life Sciences, Lowell, MA) containing polycarbonate filters with 8.0-μm pore size. The transwell inserts were first coated with 40 μl of 1 mg/ml Matrigel matrix at 37 C for 4 h for gelling according to the manufacturer's recommendations. HTR-8/SVneo cells or purified trophoblast cells were serum-starved overnight, trypsinized, and seeded at a density of 2×10^5 cells in 200 μl experimental medium without FCS on the upper chamber. rhCXCL14 was added at various final concentrations of 0, 25, 50, and 100 ng/ml. The lower chamber was filled with 600 μl experimental medium with 10% FCS. Then HTR-8/SVneo cells and primary trophoblast cells were incubated in 95% air-5% CO_2 at 37 C for 24 or 48 h, respectively. The inserts were removed and washed in PBS, and the nonmigrating cells in the upper chamber were removed with a cotton bud. The inserts were then fixed in methanol and acetone (1:1) for 10 min at room temperature and stained with hematoxylin. Filters were excised and mounted in Aquatex (Merck, Darmstadt, Germany). Cells invaded to the lower surface were counted in eight fields at a magnification of ×200. For the blocking experiments, before addition to the upper chamber, rhCXCL14 (50 ng/ml) was preincubated at 37 C for 1 h with 10 μg/ml anti-BRAK/CXCL14 antibody (R&D Systems) or control rat IgG. The assay was repeated three times, and the results are represented as fold change in cell invasion compared with control.

Gelatin zymography

Matrix metalloproteinase-2 (MMP-2) and MMP-9 secretion was evaluated by substrate gel zymography. The conditioned medium of cells treated with rhCXCL14 was collected at 24 and 48 h. Protein content of conditioned media was measured according to the Bradford method, and equal amounts of protein were subjected to electrophoresis in a 10% polyacrylamide gel containing 0.5 mg/ml gelatin (Sigma). Prestained molecular mass standards were used to determine the molecular mass of proteolytic activity.

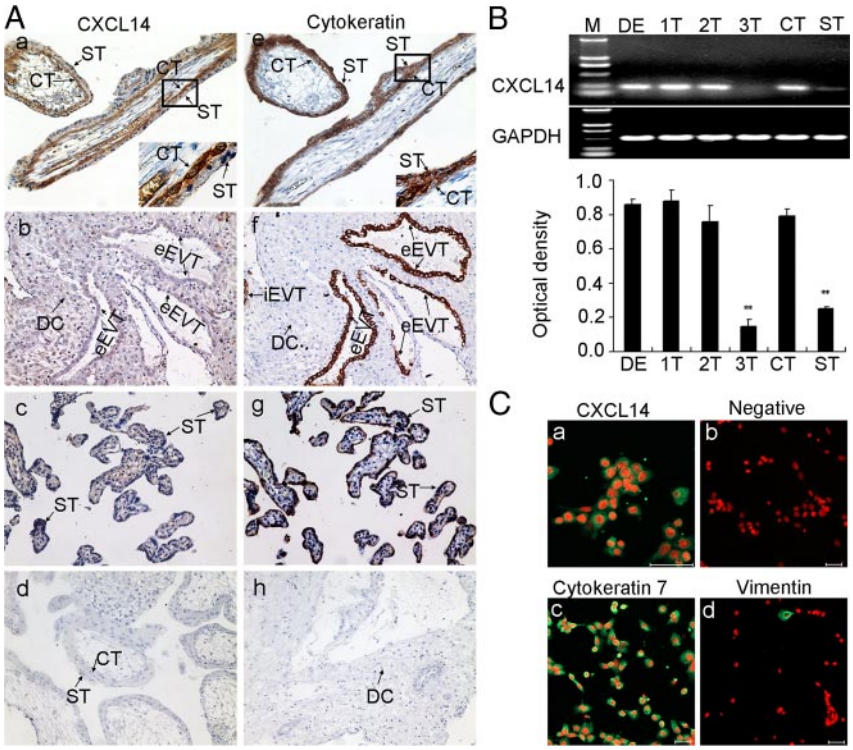

FIG. 1. CXCL14 expression at human maternal-fetal interface. A, Immunohistochemical localization of CXCL14 protein at maternal-fetal interface. Human placentas from the first trimester (a, d, e; n = 6) and term (c, g; n = 4) and human uterine deciduas from the first trimester (b, f, h; n = 6) were stained with CXCL14 (a–c), cytokeratin (e–g) antibodies or without primary antibodies (d, h). Cytokeratin stainings were confirmed trophoblast cells identity (e–g). Original magnification, ×200. B, Analysis of CXCL14 mRNA expression level. *Top*, Representative analysis of CXCL14 mRNA expression level at maternal-fetal interface by RT-PCR. *Bottom*, Relative expression intensity of CXCL14 mRNA. The results (mean ± SEM, n = 4) were calculated from values of CXCL14 mRNA relative to that of GAPDH. **, $P < 0.01$ *vs.* 1T. C, Immunofluorescence of CXCL14 in human primary trophoblast cells. Human primary trophoblast cells isolated from first-trimester placental tissues were stained with CXCL14 (a), cytokeratin 7 (c), and vimentin (d) antibodies. The purity of isolated trophoblasts was assessed by staining for cytokeratin 7 (positive) and vimentin (negative). Negative control (b), without primary antibodies. *Scale bar*, 50 μm. CT, cytotrophoblast; ST, syncytiotrophoblast; DC, decidual cell; eEVT, endovascular extravillous trophoblasts; iEVT, interstitial extravillous trophoblasts. DE, decidual tissue of first trimester; 1T, placental villi of first trimester; 2T, placental villi of second trimester; 3T, placental villi of third trimester; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M, marker.

Statistical analysis

Values were presented as means ± SEM. Statistical analysis was performed using one- or two-way ANOVA followed by *post hoc* least significant difference test, and a value of $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS 13.0 (SPSS Inc., Chicago, IL).

Results

CXCL14 expression at human maternal-fetal interface

To investigate immunolocalization of CXCL14 protein at the maternal-fetal interface, human placental tissue sections from first trimester and full term were stained. In first trimester, the CXCL14 protein was intensely stained at villous cytotrophoblasts and blood vessel of villous stroma, whereas very weak staining was observed in syncytiotrophoblasts of villi (Fig. 1A, a). In the maternal compartment of placenta, moderate staining was seen in decidualized stromal cells but was very weak in endovascular extravillous trophoblast cells (EVTs) and invading interstitial EVTs (Fig. 1A, b). Trophoblast identity was confirmed by staining for cytokeratin on a separate serial section (Fig. 1A, e–g). At term, signal of CXCL14 protein was weak or undetectable in cytotrophoblasts and syncytiotrophoblasts of villi (Fig. 1A, c), in accordance with RT-PCR results (Fig. 1B). In addition, the isolated first-trimester trophoblast cells also showed a strong staining of CXCL14 in the cytoplasm of primary trophoblast cells by indirect immunofluorescence (Fig. 1C).

CXCL14 inhibits cytotrophoblasts outgrowth from villous explants

Given the evident expression of CXCL14 on trophoblast cells and established function in tumor growth and metastasis (13, 17), we next investigated whether CXCL14 can regulate trophoblast outgrowth of villous explants on Matrigel. Culture medium and Matrigel were supplemented with increasing doses (0–100 ng/ml) of recombinant human CXCL14, and the outgrowth distance of villous explant trophoblast at the Matrigel surface was monitored at 24, 48, and 72 h. At 24 h of culture, anchorage and outgrowth of villous explants took place, but no significant difference was observed between control and CXCL14-treated groups ($P > 0.05$) (Fig. 2, A–D and M). At 48 and 72 h of *in vitro* culture, CXCL14 had significantly inhibited trophoblast outgrowth in a dose-dependent manner compared with the control group ($P < 0.01$) (Fig. 2, E–M).

CXCL14 shows no obvious effects on trophoblast cell proliferation

The effects of rhCXCL14 on trophoblast cell viability/proliferation were investigated by MTS assay and BrdU incorporation assay. HTR-8/SVneo cells and primary trophoblast cells were serum-starved for 24 h and then treated with recombinant human CXCL14 (0–100 ng/ml) for 48 h. MTS assay demonstrated that addition of CXCL14 had no obvious effect on the number of viable

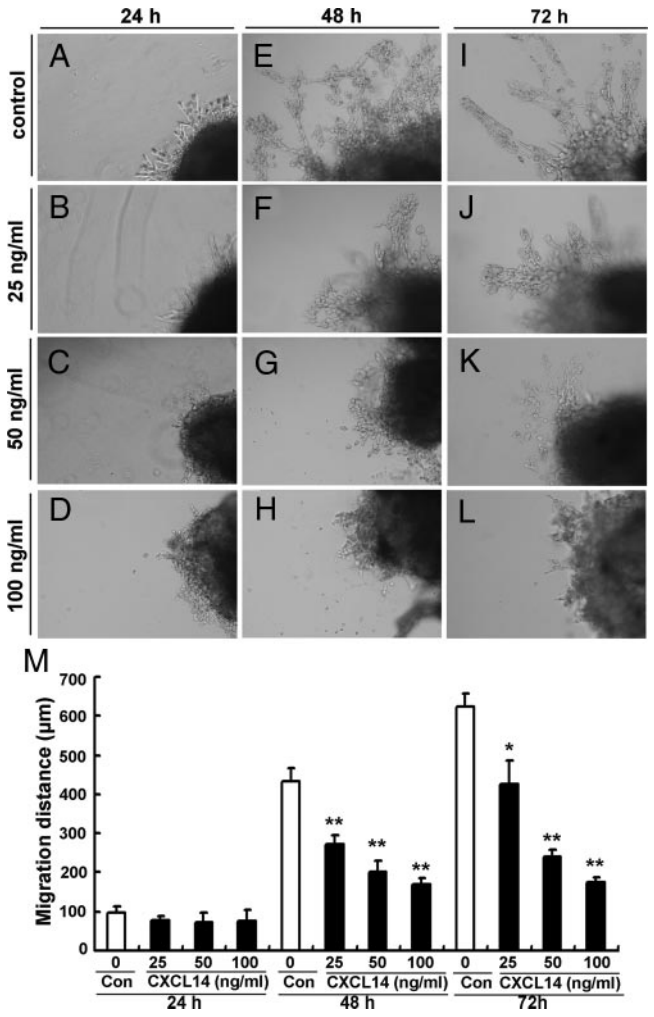

FIG. 2. CXCL14 inhibited trophoblast outgrowth and migration in first-trimester human villous explants. Villous explants (n = 20 explants per group) from 5–9 wk gestation were maintained in culture for 24, 48, and 72 h in the absence (A, E, I) or presence of 25 ng/ml (B, F, J), 50 ng/ml (C, G, K), and 100 ng/ml (D, H, L) recombinant human CXCL14 (rhCXCL14). Serial pictures of villous explants were taken under the light microscope after 24, 48, and 72 h of culture *in vitro*. Recombinant human CXCL14 treatment significantly suppressed trophoblast outgrowth from the distal end of the villous tips from 48 h of culture *in vitro* compared with control villous explants. Original magnification, ×100. The migration distance of villous tip was shown in M at 24, 48, and 72 h of culture. Migration distance of villous explant was significantly reduced already from the dosage of 25 ng/ml rhCXCL14 compared with control (con) at both 48 and 72 h (M). Data are presented as mean ± SEM of one experiment representative of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ *vs.* control.

HTR-8/SVneo cells (Fig. 3A) and primary trophoblast cells (Fig. 3B) compared with controls, whereas 10% FCS stimulated a 95.6 ± 4.6 and 101.2 ± 5.2% increase, respectively, in the numbers of HTR-8/SVneo cells and primary trophoblast cells compared with controls ($P < 0.01$) (Fig. 3, A and B). CXCL14 also had no effect on DNA synthetic rate of HTR-8/SVneo cells over 48 h compared with controls, but 10% FCS (positive control) stimulated a 41.6 ± 3.1% increase in the rate of BrdU incorporation compared with controls ($P < 0.01$) (Fig. 3C). Likewise, CXCL14 treatment also did not affect primary trophoblast cells proliferation (Fig. 3D), suggesting that CXCL14 did not influence cell cycle progression.

CXCL14 significantly inhibits trophoblast cell invasion

Because trophoblast viability/proliferation was unaffected by CXCL14 treatment, these data suggest that inhibition of trophoblast outgrowth is most possibly due to a blockade of trophoblast invasion and migration. The effects of rhCXCL14 on trophoblast cell invasion were observed in Matrigel-coated transwells. The results indicated that rhCXCL14 could significantly reduce the invasion ability of both primary trophoblast cells and HTR-8/SVneo cells (Fig. 4, A–D). Moreover, immunoneutralization experiments using CXCL14 antibody reduced the inhibitory activity of rhCXCL14 as shown in Fig. 4. We have also done parallel transwell assays without Matrigel coating to examine the effect of CXCL14 on trophoblast cell migration. The results are similar to that of invasion assay as shown in supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

CXCL14 specifically binds to trophoblast cells but not decidual cells from maternal-fetal interface

Because the receptor(s) of CXCL14 have not yet been identified, here we investigate the CXCL14 binding activities within maternal-fetal interface, to predict the localization of its potential receptor(s). By flow cytometry analysis using biotinylated recombinant human CXCL14 as probe, we found that the biotinylated CXCL14 uniformly stained HTR-8/SVneo cells and primary trophoblast cells, but not primary decidual cells isolated from maternal-fetal interface (Fig. 5, A–C), suggesting that the trophoblast cells are the bioactive target of CXCL14 and would express the unidentified CXCL14 receptor(s).

Effect of CXCL14 on secreted MMP-2 and -9 and TIMP-1 and -2 levels and activities

Among numerous factors regulation of cell motility, proteolysis play a crucial role. To investigate the role of proteolysis in CXCL14-mediated inhibition of trophoblast invasion, MMP-2 and MMP-9, and their inhibitors TIMP-1 and TIMP-2, were examined. Gelatin zymography was performed to detect the gelatinase MMP-2 and MMP-9 activity, TIMP-1 and -2 levels were detected by ELISA. The results revealed that Pro-MMP-9 (92 kDa) levels were significantly suppressed in supernatants of rhCXCL14-treated HTR-8/SVneo compared with controls at 48 h ($P < 0.05$), whereas active MMP-9 and MMP-2 enzymes were not detected (Fig. 6, A and B). Production of

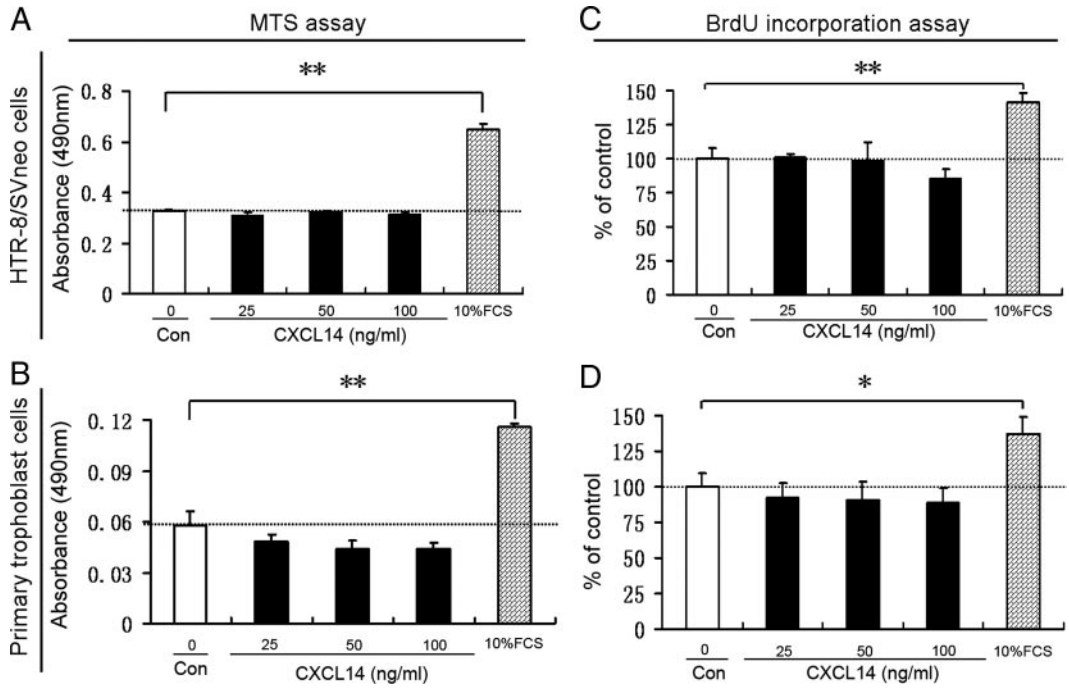

FIG. 3. Effects of recombinant human CXCL14 on trophoblast cell proliferation. A and B, MTS assays of HTR-8/SVneo cells (A) and primary trophoblast cells (B), respectively. rhCXCL14 (25–100ng/ml) added exogenously to the culture media had no effect on total viable cell numbers by either HTR-8/SVneo cells (A) or primary trophoblast cells (B) compared with controls (con). Positive controls, 10% FCS significantly increased viable cell number (**, $P < 0.01$ *vs.* control). Data represent mean ± SEM of quadruplicate (representative of three independent experiments). C and D, BrdU incorporation assays of HTR-8/SVneo cells (C) and primary trophoblast cells (D), respectively. Increasing concentration of CXCL14 had also no effect on the rate of DNA synthesis by either HTR-8/SVneo cells (C) or primary trophoblast cells (D) over 48 h compared with controls, whereas 10% FCS (positive control) stimulated BrdU incorporation compared with control (*, $P < 0.05$; **, $P < 0.01$ *vs.* control). Values are expressed as mean ± SEM fold change compared with control of quadruplicate (representative of two independent experiments).

soluble TIMP-1 in supernatants of HTR-8/SVneo cells did not change upon addition of rhCXCL14 (supplemental Fig. 2A), whereas TIMP-2 was undetectable by ELISA. Furthermore, gelatin zymography revealed that addition of rhCXCL14 (0–100 ng/ml) to primary trophoblast cells cultures considerably decreased secretion of pro (72 kDa) and active (62 kDa) forms of MMP-2 at 24 and 48 h (Fig. 6, C and D). Pro-MMP-9 levels were too low to allow accurate quantification. Soluble TIMP-1 and TIMP-2 levels did not evidently change in supernatants of CXCL14-treated primary trophoblast cells compared with control at 24 and 48 h (supplemental Fig. 2, B and C). Based on the observed changes in MMP-2 or MMP-9 activity, we have performed further ELISA detection to examine the changes in MMP-2 or -9 protein abundance in primary trophoblast cells or HTR-8/SVneo cell line. As shown in supplemental Fig. 3, the changes in protein level are consistent with the changes in gelatinase activity. Moreover, MMP-2 or -9 neutralization experiments were performed to determine whether they could mimic the effect of CXCL14 on trophoblast invasion. As shown in supplemental Fig. 4, the blockade of MMP-2 or -9 (with 10 μg/ml antibody) significantly inhibit trophoblast cell invasion similar to that of rhCXCL14 (100 ng/ml) in primary trophoblast cells or in the HTR-8/SVneo cell line, respectively.

Discussion

The present study has shown for the first time an expressional profile of CXCL14 protein at the human maternal-fetal interface. Our data demonstrated that CXCL14 was strongly expressed in the villous cytotrophoblasts, moderately in the decidualized stromal cells, and very weakly in the syncytiotrophoblasts and the extravillous trophoblast in human first trimester implantation sites. We showed further that recombinant CXCL14 could significantly inhibit cytotrophoblasts outgrowth of villous explants through Matrigel *in vitro*, which was associated with a suppression of trophoblast invasion and decreased trophoblast gelatinolytic activity without affecting trophoblast viability/proliferation. These findings were almost consistent using both HTR-8/SVneo cells and primary trophoblast cells. In addition, biotinylated CXCL14 uniformly stained HTR-8/SVneo cells and primary trophoblast cells by flow cytometry assay, but not primary decidual cells. These results suggest that CXCL14 expressed at the maternal-fetal interface may play an important role in regulating trophoblast invasion through binding to its potential receptor(s) of invading trophoblast cells during early pregnancy.

A number of chemokines and their receptors have recently been identified at the maternal-fetal interface, sug-

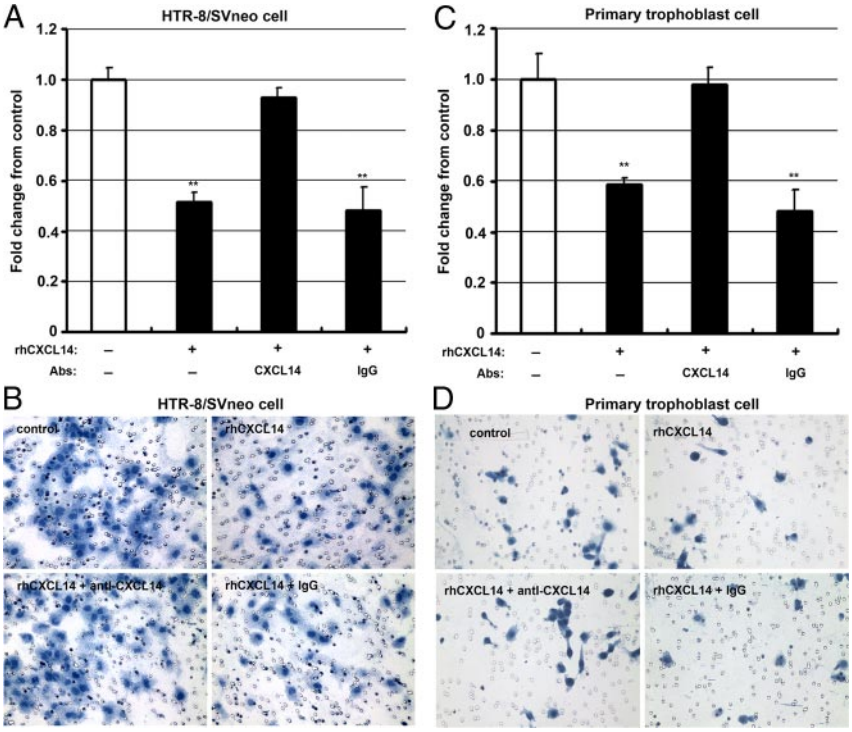

FIG. 4. CXCL14 inhibited invasion of HTR-8/SVneo cells and primary trophoblast cells. A and C, Matrigel invasion assays of HTR-8/SVneo cells (A) and primary trophoblast cells (C) in the absence or presence of CXCL14 (50 ng/ml) and anti-CXCL14 antibody (Abs). For the blocking experiments, rhCXCL14 (50 ng/ml) was preincubated with 10 μg/ml anti-CXCL14 antibody or normal rat IgG, which was then subjected to invasion assays. Results are shown as mean invasion (expressed as fold change from control) $\pm$ SEM (**, $P < 0.01$ *vs.* control). B and D, The representative microscope images of invasive HTR-8/SVneo cells (B) and primary trophoblast cells (D) in the absence or presence of CXCL14 (50 ng/ml) and anti-CXCL14 antibody and normal rat IgG. Original magnification, ×200. Data were representative of three independent experiments.

gesting a key role for chemokines in embryo implantation and placentation. Hannan *et al.* (7) indicated that CX3C and CC chemokine ligand-receptor networks were functionally presented at the human fetomaternal interface, and CX3CL1, CCL14, and CCL4 promoted human trophoblast migration. Huang *et al.* (29) demonstrated that chemokine CXCL16 could induce proliferation and invasion of first-trimester human trophoblast cells. With the use of oligonucleotide microarrays, it was revealed that *CXCL14* was most highly regulated gene in human midsecretory endometrium compared with early secretory endometrium (30). Quantitative PCR indicated that *CXCL14* mRNA expression from basal plate was gestationally regulated. Intense signals were exhibited in first- and second-trimester samples, but little or no expression was detected at term (23). Consistent with the abundant expression of *CXCL14* mRNA in human first-trimester maternal-fetal interface (23), in this study, we confirmed the mRNA expression of CXCL14 by RT-PCR and further identified its localization using immunohistochemistry. Here we clearly showed that CXCL14 was highly expressed in the villous cytotrophoblasts, moderately in the decidualized stromal cells, but not in the syncytiotrophoblasts and the extravillous trophoblast, which was in line with Northern blot results of Red-Horse *et al.* (31) and suggested its expression was regulated with trophoblast cell differentiation. Drake *et al.* (21), using *in situ* hybridization, demonstrated that *CXCL14* had a punctate expression pattern, often clustered near the basement membrane that encases the villous stroma, which is very near cytotrophoblasts of villi. The differences of CXCL14 localization in regards to mRNA and protein might implicate a posttranslational protein transport of this protein at maternal-fetal interface.

In addition to the well-known directional leukocyte chemotaxis of chemokines, some studies have showed that CXC chemokines could influence angiogenesis, growth, and metastatic potential of tumor (20, 32). *CXCL14* has

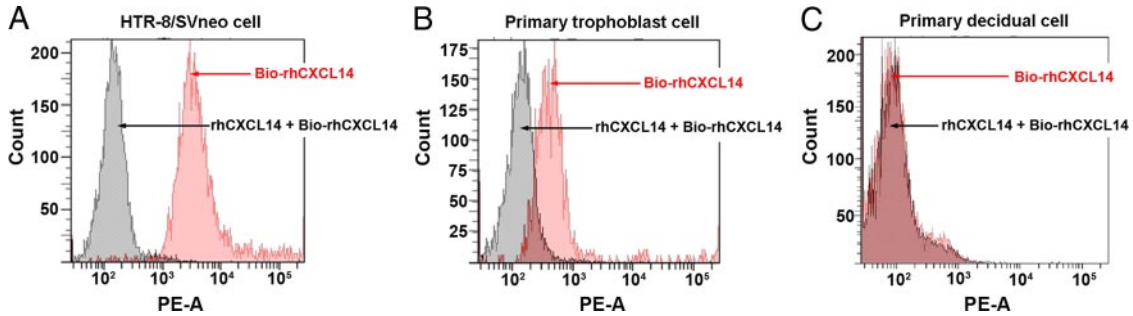

FIG. 5. Binding analysis of labeled CXCL14 to HTR-8/SVneo cells, primary trophoblast cells, and decidual cells by flow cytometry. The red histograms show biotinylated-CXCL14 (Bio-CXCL14) bound with CXCL14-putative receptor on the cells, the gray histograms depict control stainings of biotinylated-CXCL14 after competition with excess of unbiotinylated CXCL14 (rhCXCL14 + Bio-CXCL14). The panels show potential receptor expression of corresponding CXCL14. Note that HTR-8/SVneo cells (A) and primary trophoblast cells (B) stained positive for CXCL14-binding, whereas primary cultured decidual cells (C) stained negative for CXCL14. Data are representative of three independent experiments. PE-A, Phycoerythrin-Alexa.

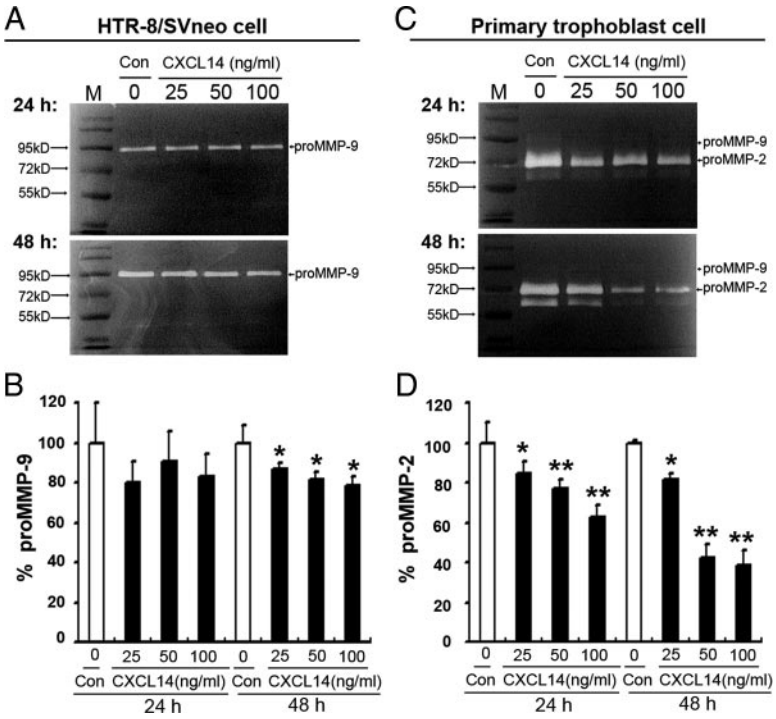

FIG. 6. Effect of CXCL14 on secreted MMP-2 and MMP-9 levels. A and C, Representative gelatin zymography of supernatants pooled from HTR-8/SVneo cells (A) or primary trophoblast cells (C) in the presence of CXCL14 (0–100 ng/ml) for 24 and 48 h, respectively. Proteolytic activity was noted for the 72-kDa gelatinase corresponding to proMMP-2, and the 92-kDa gelatinases corresponding to proMMP-9. B and D, relative densitometric analysis of proMMP-9 in the HTR-8/SVneo cells (B) and proMMP-2 in the primary trophoblast cells (D). Results are shown as the mean ± SEM fold change compared with control of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared with control (con). M, Marker.

been shown to be down-regulated in the majority of tumors, and its expression has been shown to regulate tumor growth (9, 12, 13, 17). Because there are many common features shared by trophoblast cells and tumor cells, we examined whether CXCL14 regulates trophoblast outgrowth of villous explants *in vitro*. The results clearly indicated that recombinant CXCL14 dramatically suppressed trophoblast invasion, which is similar to the suppressive function toward human oral carcinoma cells. To shed additional light on the mechanisms by which CXCL14 regulate trophoblast invasion, we next examined whether CXCL14 could regulate proliferation and migration of trophoblast. The results revealed that recombinant CXCL14 did not affect the viability and DNA synthesis of trophoblast cells, suggesting that inhibition of trophoblast outgrowth is most possible due to a blockade of trophoblast migration. This prediction has been further confirmed by our transwell experiments. On the other hand, some reports (16, 20, 33) demonstrated that the CXCL14 chemokines overexpressed in myoepithelial cells and myofibroblasts of breast cancer, and CXCL14 enhanced proliferation, migration, and invasion of breast epithelial cells, thus suggesting that CXCL14 might displayed different functions at different pathophysiological environments.

To explore whether CXCL14 directly targeted on trophoblast cells to exert its function, we further studied the cell-specific binding activity of CXCL14 at the maternal-fetal interface. The results showed that biotinylated CXCL14 specifically bound to HTR-8/SVneo cells and primary trophoblast cells but not decidual cells, suggesting that the trophoblast cell is the functional target of CXCL14 and would express the unidentified CXCL14 receptor(s). A range of chemokine receptors have recently been demonstrated to be present on human trophoblast cells, including CXCR1, CXCR4, and CXCR6; CCR1, CCR3, CCR5, CCR7, CCR8, and CCR9; XCR1; and CX3CR1 (21, 34). Nevertheless, receptor binding assays showed that CXCL14 does not bind to CXCR1 and CXCR2 (10). In this regard, our results provide a valuable model that could be further used to identify the specific receptor(s) of CXCL14.

Trophoblast invasion are closely correlative with the expression of MMPs (35, 36), which are capable of degrading extracellular matrix. Among members of MMP family, MMP-2 and MMP-9 were suggested to play the major role in the final pathway of trophoblast invasion. This led us first to examine the effect of CXCL14 on the gelatinases. Indeed, suppressed motility in primary trophoblast was associated with statistically significant decrease of MMP-2 proteolytic activity. This also supports previous findings that MMP-2 is the main gelatinase in early first trimester trophoblasts (35). Treatment of HTR-8/SVneo cells with CXCL14 significantly suppressed MMP-9 activity, suggesting that the effect of CXCL14 on the gelatinase activity was cell type dependent. In addition to MMP regulation, other mechanisms might also be operative in the process, such as increase of TIMPs or effects on the plasminogen activator/inhibitor system of serine proteases (36). However, in our defined culture system, CXCL14 was shown to have no effect on levels of TIMP-1 and TIMP-2 secretion.

In summary, the present data demonstrated that CXCL14 was temporally and spatially expressed in the human maternal-fetal interface during early pregnancy. CXCL14 restricts invasion of trophoblast cells, which is associated with decrease of gelatinases. For further investigation, it will be of great interest to study the roles of CXCL14 in the hydatidiform mole and chorionic tumors.

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