The chemokine CXCL6 restricts human trophoblast cell migration and invasion by suppressing MMP-2 activity in the first trimester

H. Zhang, L. Hou, C.M. Li and W.Y. Zhang*
Department of Obstetrics and Gynecology, Beijing Obstetrics and Gynecology Hospital, Capital Medical University, No. 251 Yao Jiayuan Road, Chaoyang District, Beijing 100026, People’s Republic of China
*Correspondence address. Tel: +86-10-85968396; Fax: +86-10-85968397; E-mail: zhangwy9921@hotmail.com
Submitted on December 12, 2012; resubmitted on April 19, 2013; accepted on May 23, 2013

STUDY QUESTION: Can the chemokine CXCL6 affect trophoblast cell migration and invasion in human first-trimester placenta?
SUMMARY ANSWER: Chemokine CXCL6 inhibits trophoblast cell migration and invasion by suppressing matrix metalloproteinase (MMP)-2 activity in human first-trimester placenta.
WHAT IS KNOWN ALREADY: Several chemokines including CXCL8, CXCL12, CXCL14, CXCL16, CX3CL1, CCL14 and CCL4 can promote or inhibit trophoblast cell migration and invasion in human first-trimester placenta.
STUDY DESIGN, SIZE, DURATION: We used the trophoblast cell line HTR8/SVneo cells, primary trophoblast cells and villi explants to investigate the effect of rhCXCL6 on trophoblast cell migration and invasion.
PARTICIPANTS/MATERIALS, SETTING, METHODS: First, the CXCL6 RNA transcript level was detected in HTR8/SVneo cells derived from human first-trimester, second-trimester and third-trimester placenta by RT–PCR. Protein expression of CXCL6 and its receptors was tested in first-trimester placenta by immunohistochemistry. Secreted CXCL6 protein was detected in HTR8/SVneo cell supernatants by enzyme-linked immunosorbent assay. Secondly, the effect of rhCXCL6 on HTR8/SVneo cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Thirdly, the effect of rhCXCL6 on cell migration and invasion of HTR8/SVneo cells, primary trophoblast cells and villi explants was tested by transwell migration and invasion assays, respectively. Last, MMP-2 and MMP-9 activity in the supernatants of HTR8/SVneo and primary trophoblast cells treated by rhCXCL6 in the invasion assay was assessed by gelatin zymography.
MAIN RESULTS AND THE ROLE OF CHANCE: Abundance of the CXCL6 RNA transcript increased with pregnancy development. CXCL6 and its receptor were expressed in several cells at the human maternal—fetal interface. RhCXCL6 inhibited trophoblast cell migration and invasion by suppressing MMP-2 activity.
LIMITATIONS, REASONS FOR CAUTION: These experiments are only in vitro.
WIDER IMPLICATIONS OF THE FINDINGS: According to the literature, CXCL6 could promote tumour cell migration and invasion by accelerating MMP-9 activity. However, CXCL6 inhibited trophoblast cell migration and invasion by suppressing MMP-2 activity in human first-trimester interface. These data suggest that strict regulation of CXCL6 is required for normal migration and invasion of cells, such as those involved at the maternal—fetal interface.
STUDY FUNDING/COMPETING INTEREST(S): This study was supported by grants from the National Natural Science Foundation of China (No. 81070497), The Ministry of Education Doctoral Program Foundation of China (No. 20070025003) and the Beijing Municipal Science & Technology New Star Project of China (No. 2008B866). None of the authors has any conflict of interest to declare.
TRIAL REGISTRATION NUMBER: None.
Key words: chemokine / CXCL6 / trophoblast cells / migration / invasion
Introduction

Trophoblast migration and invasion play vital roles in the process of embryo implantation and placentation. Disturbance of embryo implantation and placentation can lead to many pregnancy complications, such as placenta increta, recurrent spontaneous abortion, pre-eclampsia and premature delivery (Huang et al., 2009). Embryo implantation and placentation is a complex process involving blastocyst adhesion to the decidua and the remodelling of maternal uterine vasculature via trophoblast migration and invasion. During the process embryos are anchored to the uterine wall, and extravillous trophoblasts substitute for vascular endothelial cells while the uterine vessels are remodelled to form the low-resistance vessels necessary to supply abundant blood for favourable fetal growth (Lunghi et al., 2007). Invasion of trophoblasts is regulated by various cytokines/chemokines, hormones and growth factors in a paracrine or autocrine pattern at the maternal–fetal interface. These factors control trophoblast adhesion, migration and invasion by regulating proteases or their inhibitory activity (Hannan and Salamonsen, 2007; Salamonsen et al., 2007). Chemokines, a large family of chemotactic small molecular weight peptides which contain conserved cysteine motifs in their NH2-terminus are known for their actions in specific leukocyte recruitment and activation of inflammation. Recent reports have revealed that chemokines are also involved in trophoblast migration and invasion or maternal–fetal immune tolerance at the maternal–fetal interface. Abnormal regulation of trophoblast invasion by chemokines has been associated with disease conditions such as pre-eclampsia and cancer metastasis (Bieche et al., 2007; Xu et al., 2009; Yoshida et al., 2010; Li et al., 2011a,b).

CXCL6, also known as granulocyte chemotactic protein 2 (GCP-2), was originally isolated from the osteosarcoma cell line MG-63 as a 5–6 kDa protein (Proost et al., 1993) and is a member of the CXC ELR+ chemokine family. It is located in the chromosomal region 4q21 and contains 77 amino acids (Bieche et al., 2007). The NH2-terminal glutamic acid-leucine-arginine (ELR) motif is characteristic of CXC chemokines that interact with the G-protein-coupled receptors CXCR1 and CXCR2 (Kebschull et al., 2009). CXCL6 is expressed by epithelial cells of the airways, eyes, gastrointestinal tract, mammary glands, tonsils, macrophages and mesenchymal cells, in particular during inflammation (Linge et al., 2008). In the absence of inflammatory stimulation, it is highly expressed in breast cancer (Bieche et al., 2007), colorectal cancer (Xu et al., 2009; Yoshida et al., 2010), osteosarcoma (Yoshida et al., 2010), non-small cell lung cancer (Arenberg et al., 1997; Lunghi et al., 2007) and endometrial cancer. It has also been demonstrated that CXCL6 is a potent mediator of neoangiogenesis (Keeley et al., 2011) as well as of tumour growth, invasion and metastasis. RT–PCR analyses have revealed that the chemokine receptors CXCR1 and -2 mRNAs are expressed in human placenta (Wu et al., 2004). As one of the ligands of chemokine receptors CXCR1 and -2, it has also been found that IL-8 is expressed in human placenta and can promote trophoblast migration and invasion in an autocrine/paracrine manner during early pregnancy (Jovanovic et al., 2010). Furthermore, some other chemokines may promote or suppress first-trimester trophoblast proliferation, migration and invasion, such as CXCL12, CXCL14, CXCL16, CX3CL1, CCL14 and CCL4 (Wu et al., 2004; Hannan et al., 2006; Huang and Li, 2006; Huang et al., 2006, Zhou et al., 2008; Kuang et al., 2009a,b; Jovanovic et al., 2010; Li et al., 2011a,b). Reports have demonstrated that the trophoblast cell line HTR8/SVneo and late proliferative phase endometrial stromal cells can secrete CXCL6. Moreover, the concentration of secreted CXCL6 varied under the control of other cytokines or reproductive hormones, which suggests that CXCL6 may be involved in the normal and pathological processes of human reproduction (Mine et al., 2003; Cavanagh et al., 2009). Thus, we wanted to investigate the expression of CXCL6 at the maternal–fetal interface by using RT–PCR and immunohistochemistry. Results revealed a weak signal in placenta tissues at early stage of pregnancy that increased with pregnancy development.

Based on the evidence that CXCL6 mRNA is differentially expressed at the human maternal–fetal interface at different gestational ages and because of the similarities between trophoblasts and tumour cells with respect to proliferative and invasive properties (Huang et al., 2009), we hypothesized that CXCL6 secreted by decidual or trophoblast cells might regulate trophoblast invasion into the decidua during pregnancy. Here, we investigate the expression of CXCL6 and its receptors at the human maternal–fetal interface. We also investigate its effects on trophoblast outgrowth in villous explants, as well as its effects on migration and invasion of both the extravillous trophoblast cell line HTR8/SVneo (Graham et al., 1993) and primary trophoblast cells.

Materials and Methods

Sample collection and ethical approval

Samples of placental tissues and decidua from the first trimester were obtained from healthy women undergoing suction termination of pregnancy (5–8 weeks) for non-medical reasons; term placentas (n = 20) were collected following pregnancy without complications. Ethical approval was granted by the Ethical Committee of the Chinese Academy of Sciences and Beijing Gynecology and Obstetrics hospital. All patients completed an informed consent form to collect tissue samples. Collected tissues were stored in ice-cold Dulbecco’s modified eagle medium (DMEM) (Invitrogen), transported to the laboratory within 30 min after surgery or delivery, and washed with ice-cold DMEM for cultures, RNA extraction or fixation.

Isolation and culture of the first-trimester primary trophoblast cells

Primary trophoblast cells from first-trimester chorionic villi were isolated as described previously (Tarrade et al., 2001) with minor modifications. In short, placental tissues (n = 15–20) from early pregnancy (5–8 weeks) were pooled, fetal membranes were removed and villus tissues were dissected into small pieces and digested with 0.25% trypsin (Invitrogen) and 200 IU/L DNase I (Sigma, USA) for 10 min at 37°C with 138 rpm agitation. The digested suspension was collected and suspended in 5% fetal bovine serum (FBS). Residual tissue was subjected to a further two cycles of 10 min digestion. The three resultant cell suspensions were pooled, layered over a discontinuous Percoll (GE Healthcare, USA) gradient (15, 25, 30, 40, 50, 60 and 70%) and centrifuged at 1100g for 10 min. The 25–40% layer was collected for trophoblast isolation. Isolated trophoblast cells were cultured in DMEM supplemented with 2 mM glutamine, 0.2% FBS, 25 mM Hepes, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C in 95% air, 5% CO2.

Indirect immunofluorescence for characterization of primary trophoblast cells

Primary trophoblast cells (Fig. 1a and b), cultured on coverslips for 24 h, were fixed in a mixture of methanol and acetone (1:1) for 30 min at room temperature (RT) (Fig. 1b). After fixation, cells were permeabilized in phosphate-
buffered saline (PBS) containing 0.1% Triton X-100 (15 min). Cells were incubated in 3% bovine serum albumin for 1 h at RT. Then, cells were incubated with mouse anti-human cytokeratin 7 (1:200; Santa Cruz Biotechnology, USA; Fig. 1d), mouse anti-human β-hCG (1:100; Santa Cruz Biotechnology; Fig. 1f) or mouse anti-human vimentin (1:100; Santa Cruz Biotechnology; Fig. 1h) at 4°C overnight followed by fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate labelled secondary antibody (1:200) for 30 min at 37°C. Nuclei were stained with 1 μg/ml 4',6-diamidino-2-phenylindole (Fig. 1c, e and g) for 10 min. Finally, cells were viewed under a fluorescence microscope (Leica, Heidelberg, Germany).

Figure 1 Characterization of primary trophoblasts. Isolated primary trophoblasts (a and b) were immunohistochemically stained with CK-7 for cytotrophoblast cells (c and d), β-hCG for syncytiotrophoblast cells (e and f) and vimentin for mesenchymal cells (g and h). Original magnification, ×200.

Culture of the trophoblast cell line HTR8/SVneo

Because of the distinctive similarities between the trophoblast cell line HTR8/SVneo and primary human trophoblast cells from first-trimester chorionic villi, we first examined the level of the CXCL6 RNA transcript as well as secreted CXCL6 protein in the supernatants of cultured HTR8/SVneo cells or primary human trophoblast cells collected 48 h after serum starvation. HTR8/SVneo cells with cytotrophoblast properties were cultured in RPMI 1640 (Invitrogen) media containing 10% FBS. Before passage for experiments conducted in the serum-free media, cells were transferred to RPMI 1640 media containing 0.5% FBS (experimental media).

Enzyme-linked immunosorbent assay

Quantitative concentration determination of CXCL6 in the supernatant of HTR8/SVneo cells and primary trophoblast cells cultured for 48 h in serum-free media was performed according to the instructions of the Human CXCL6/GCP-2 Immunoassay kit (R&D Systems, Inc., USA). Briefly, preparation of all reagents, working standards and samples was done as instructed. One hundred microlitres of Assay Diluent RD1W was added to each well. Then, 50 μl of Standard, control or sample (supernatant) was added per appropriate well, and allowed to incubate for 2 h at RT. The contents of each well were then aspirated and washed, and the process was repeated three times for a total of four washes. After the
last wash, 200 μl of GCP-2 Conjugate was added to each well, and the plate was incubated for 2 h at RT. Washes were repeated as before; then, 200 μl of Substrate Solution was added to each well and allowed to incubate for 30 min at RT, protected from light. Next, 50 μl of Stop Solution was added to each well and the optical density of each well was determined within 30 min, using a microplate reader set to 450 nm, with wavelength correction set to 540 or 570 nm. This subtraction will correct for optical imperfections in the plate. The sensitivity of the assay is 1.6 pg/ml, and the inter-assay and intra-assay coefficients of variation were 6.8–7.9 and 5.0–5.9%, respectively, for each condition. The enzyme-linked immunosorbent assay was carried out in duplicate, in three separate experiments.

RNA extraction and RT–PCR
Total RNA was extracted using TRIzol (Invitrogen) and reverse-transcribed in 13.5 μl of reaction mixture containing 2 μg total RNA and 25 U of supercript reverse transcriptase (Invitrogen). The PCR was conducted in a total volume of 25 μl for 23 (glyceraldehyde 3-phosphate dehydrogenase, GAPDH) or 35 (CXCL6) cycles of denaturation at 94°C for 30 s, annealing at 59°C for 45 s and extension at 72°C for 30 s, with a final extension step of 6 min at 72°C. The primers used in this study included CXCL6 (sense: 5′-CTGCTGGTCCTGTCTCTGCT-3′; antisense: 5′-GTTTCTTCTGGTCTTCCAC TGTC-3′) and GAPDH (sense: 5′-TGGAATCCCATCACCATCT-3′; antisense: 5′-GTTTCTTGGTGGCAGTGAT-3′). The amplified products were analysed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Data shown are representative of ten independent experiments for different tissue samples.

Immunohistochemistry
Tissues (villi or decidua, n = 10) were fixed in formalin solution. The sections were first boiled (92–98°C) in Tris–EDTA buffer (10 mM Tris, 1 mM EDTA-Na2) for 15 min. Non-specific binding was blocked by incubation with 5% goat serum albumin for 30 min, except goat primary antibody. Then, sections were incubated with the following antibodies: goat anti-human CXCL6 (1:30; Santa Cruz Biotechnology), mouse anti-human CXCR1 (1:200; LifeSpan Biosciences, USA), rabbit anti-human CXCR2 (1:200; LifeSpan Biosciences), overnight at 4°C. After washing in PBS containing 0.05% Tween, the sections were incubated with secondary antibody for 30 min at 37°C. The immunoreactions were detected with diaminobenzidine solution. For some sections, primary antibodies were replaced with goat, mouse or rabbit preimmune IgG as negative controls.

Villus explant cultures
The explant culture (n = 3) was performed as described previously (Bauer et al., 2004). In brief, small pieces of tissue (2–3 mm) from the tips of placental terminal villi were dissected. For preparation of Matrigel coated plates, undiluted Matrigel was placed on the internal surface of an insert (BD Biosciences, USA) and placed within a 24-well culture dish. After formation of gels (30 min at 37°C), the dissected tissue pieces were carefully put on top of each gel drop and incubated for 3 h to allow anchorage. Explants were next supplemented with 0.2 ml medium in the absence or presence of different doses (10, 50, 100 or 500 ng/ml) of recombinant human CXCL6 (rhCXCL6; R&D Systems). For each condition, 20 explants were analysed, and experiments were repeated three times with different placenta. 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/).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed according to manufacturer’s instructions. Briefly, cells were seeded at 2 × 10⁵ cells/well in 96-well plates. Cells were rinsed with PBS and the culture medium was replaced with experimental media for 24 h. Thereafter, rhCXCL6 (0, 10, 50, 100, 200 ng/ml) or 10% FBS (positive control) was added to the culture system for another 24, 48 or 72 h at 37°C. Next, 100 μl of MTT reagent (Applygen Technologies Inc., China) 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 4 h at 37°C in a humidified, 5% CO₂ atmosphere. One hundred microlitres of dimethyl sulfoxide (Sigma) was then added to induce the colorimetric reaction and the absorbance was measured at 570 nm. The experiment is representative of three independent experiments.

Migration and invasion assay
Invasion assays were performed in this study as described previously (Prast et al., 2008) with some modifications. We used transwell plates (6.5-mm diameter; Corning Life Sciences, USA) containing polycarbonate filters with a pore size of 8.0 μm. The transwell inserts were first coated with 50 μl of 1 mg/ml Matrigel matrix at 37°C for 4 h to allow gelling according to the manufacturer’s recommendations. HTR8/SVneo cells or purified primary trophoblast cells were serum-starved overnight, trypsinized and seeded at a density of 1.5 × 10⁵ cells in 200 μl of medium without FBS on the upper chamber. RhCXCL6 was added at 0, 1, 5, 10, 25, 50 and 100 ng/ml. The lower chamber was filled with 600 μl of experimental medium with 10% FBS. Then HTR8/SVneo cells and primary trophoblast cells were incubated in 95% air–5% CO₂ at 37°C for 24 h. The inserts were removed and washed in PBS, and the non-migrating cells in the upper chamber were removed with a cotton bud. The inserts were then fixed in cold methanol for 10 min at RT and stained with haematoxylin. Cells that invaded the lower surface were counted in ten fields at a magnification of ×200. For block experiments, before addition to the upper chamber, rhCXCL6 (50 ng/ml) was preincubated with 10 μg/ml anti-CXCL6 antibody (R&D Systems) or control mouse IgG at 37°C for 1 h. The assay was repeated three times, and the results are represented as a fold change in cell invasion compared with non-stimulated control.

Migration assays followed the same protocol as invasion assays except without Matrigel. The data are representative of three independent experiments.

Gelatin zymography
The secretion of matrix metalloproteinases (MMPs)-2/9 (MMP-2 or MMP-9) by primary isolated trophoblast cells and HTR8/SVneo cells was evaluated by substrate gel zymography. The conditioned medium of cells treated with rhCXCL6 was collected at 24 h after serum starvation. The 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.

Statistical analysis
Values are presented as means ± sem. Statistical analysis was performed using one-way analysis of variance followed by a 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 11.5 (SPSS Inc., Chicago, IL, USA).
Results

Expression of CXCL6 and its receptors, CXCR1/2, at the human maternal–fetal interface

First, using RT–PCR we found that the CXCL6 transcript significantly increased in human placenta with pregnancy development (*P < 0.05*). The extravillous trophoblast cell line HTR8/SVneo also transcribed CXCL6 RNA (Fig. 2).

Secondly, we found that the extravillous trophoblast cell line HTR8/SVneo cells and first-trimester isolated primary trophoblast cells could secrete 188 ± 5 and 118 ± 8 pg CXCL6 protein/10⁶ cells/48 h, respectively.

In addition, to identify immunolocalization of CXCL6 and its receptors, CXCR1/2, at the maternal–fetal interface, human placental and decidual tissue sections from the first trimester, was stained. In first-trimester tissues, the CXCL6 protein was intensely stained in decidual stromal cells (DSCs), glandular epithelium cells and blood vessel cells; moderately stained in invading extravillous trophoblast cells of decidual tissues and weakly stained in cytotrophoblasts and syncytiotrophoblasts of villi (Fig. 3a and b). The two receptor proteins of CXCL6, CXCR1/2, were extensively stained in villous and decidual cells (Fig. 3c–f).

Thus, CXCL6 protein can be secreted by both trophoblasts and decidual cells and the expression profile of CXCR1/2 suggests that CXCL6 might not only bind to trophoblasts but also to decidual cells at the maternal–fetal interface.

CXCL6 does not significantly affect trophoblast proliferation

The effect of rhCXCL6 on trophoblast cell proliferation was investigated by an MTT assay. HTR8/SVneo cells were serum-starved for 24 h and then treated with rhCXCL6 (0–200 ng/ml) for 0, 24, 48 and 72 h. The different concentrations rhCXCL6 had no obvious effect on the number of proliferative HTR8/SVneo cells when compared with the negative control, whereas 10% FBS (positive control) stimulated a significant increase (*P < 0.01*; Fig. 4). These data suggest that CXCL6 does not change trophoblast cell cycle progression.

CXCL6 inhibits trophoblast migration and invasion from villous explants

Villous explant growth conditions can affect trophoblast migration and invasion. Thus, we next investigated whether CXCL6 regulates ectogenic trophoblast outgrowth of villous explants cultured on Matrigel. Culture medium was supplemented with increasing doses (0–500 ng/ml) of rhCXCL6, and the outgrowth distance of villous explant trophoblasts 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 (0 ng/ml) and rhCXCL6-treated groups (*P > 0.05*). At 48 and 72 h of in vitro culture, however, CXCL6 significantly inhibited trophoblast outgrowth when compared with that of the control group (*P < 0.01*; Fig. 5).

CXCL6 significantly inhibits trophoblast migration and invasion

Because trophoblast cell proliferation was not influenced by rhCXCL6 treatment, these data suggest that inhibition of villous explant growth is possibly due to suppression of trophoblast migration or invasion. Thus, the effects of rhCXCL6 on trophoblast cell invasion or migration were tested. The results indicated that rhCXCL6 significantly decreased the migration of HTR8/SVneo cells (*P < 0.01*; Fig. 6a–i) and the invasion ability of both HTR8/SVneo cells (Fig. 7a–i) and primary trophoblast cells (Fig. 8a–d) in a dose-dependent manner, when compared with the control group (*P < 0.01*). Moreover, immunoneutralization experiments using anti-CXCL6 antibody abolished the inhibitory activity of rhCXCL6 (Figs 6 and 7).

Effect of CXCL6 on trophoblast-secreted MMP-2 and MMP-9

Among the numerous factors regulating cell invasiveness, proteolysis, which involves the actions of MMPs, plays a key role. To investigate the role of MMPs in CXCL6-mediated inhibition of trophoblast invasion, MMP-2 and MMP-9 were examined. Gelatin zymography was performed to detect the gelatinase MMP-2 and MMP-9 activity. The results revealed that Pro-MMP-2 (72 kDa) levels were significantly suppressed in the supernatant of rhCXCL6-treated HTR8/SVneo cells when compared with those of controls at 24 h by invasion assay (*P < 0.05*). No significant difference was found between Pro-MMP-9 (92 kDa) levels in supernatants of rhCXCL6-treated HTR-8/SVneo cells and control at 24 h by invasion assay (*P > 0.05*), while active MMP-9 and MMP-2 enzymes were not detected (Fig. 9). Likewise, gelatin zymography revealed that addition of rhCXCL6 (0, 100 ng/ml) to primary trophoblast cell cultures considerably suppressed the gelatinase activity of both HTR8/SVneo cells (Fig. 10a–i) and primary trophoblast cells (Fig. 11a–d) in a dose-dependent manner, when compared with the control group (*P < 0.01*). Moreover, immunoneutralization experiments using anti-CXCL6 antibody abolished the inhibitory activity of rhCXCL6 (Figs 10 and 11).

![Figure 2](image-url) Relative expression intensity of CXCL6 mRNA. The CXCL6 transcript increased in human placenta with pregnancy development. CXCL6 transcript levels were examined by RT–PCR in human placenta derived from the first (1, n = 10), second (2, n = 10) or third (3, n = 10) trimesters (T), as indicated, as well as in HTR8/ SVneo cells. GAPDH was used as a control for input. The pictures are representative examples from ten independent experiment for different tissue samples. The results in the bar-chart (mean ± SEM, n = 10) were calculated from values of CXCL6 mRNA relative to that of GAPDH. *P < 0.05, **P < 0.01 versus 1T.
decreased secretion of proMMP-2 at 24 h, though no significant difference in Pro-MMP-9 (92 kDa) levels in supernatants between rhCXCL6-treated primary trophoblast cells and control cells was detected at 24 h by invasion assay ($P > 0.05$); again, active MMP-9 and MMP-2 enzymes were not detected (Fig. 10).

**Discussion**

A number of chemokines and their receptors have recently been identified at the maternal–fetal interface, suggesting that they play a key role in embryo implantation and placentation. Indeed, it has been suggested that different chemokines have different expression patterns according to their function at the human maternal–fetal interface (Red-Horse et al., 2004a). Generally, the patterns fall into four categories: (i) diffuse expression emanating from DSCs, (ii) focal expression by resident leucocytes or by fibroblasts, (iii) expression by invading cytotrophoblasts or (iv) localized expression in cells lining the uterine vasculature (Red-Horse et al., 2004a). A few chemokines have more than one expression pattern (Red-Horse et al., 2004a). Chemokine CXCL14 expression, detected by RT–PCR, is gradually decreased in human placenta with the development of pregnancy (Kuang et al., 2009a). Our research indicates that chemokine CXCL6 mRNA is increasingly expressed in human placenta with the development of pregnancy. Crosstalk between trophoblast cells and uterine decidual cells has been shown to be mediated by all kinds of cytokines and chemokines in the
The microenvironment of the maternal–fetal interface (Salamonsen et al., 2007). Research has suggested that interaction between chemokine CXCL12 and its receptor, CXCR4, is involved in maternal–fetal immunological tolerance in all three trimesters of gestation and contributes to the invasion of extravillous trophoblasts during pregnancy (Yang et al., 2006a,b). We found that the chemokine CXCL6 was secreted by several uterine decidual cells and trophoblast cells. Therefore, chemokine CXCL6 possibly plays a role on trophoblast cells in the process of placentation and embryo implantation.

Several chemokines have been shown to exhibit three main functions at the maternal–fetal interface during early human pregnancy: (i) immune tolerance in the process of implantation (Perez Leiros and Ramhorst, 2013); (ii) inflammation attributed to complex pregnancy complications, such as pre-eclampsia, premature birth, dead fetus,

**Figure 5** rhCXCL6 inhibited trophoblast outgrowth and migration in first-trimester human villous explants. Villous explants (n = 20 explants per group) from 5 to 6 week gestation were maintained in culture for 24, 48 and 72 h in the absence or presence of rhCXCL6 (10, 50, 100 and 500 ng/ml). Serial pictures of villous explants were taken under the light microscope after 24, 48 and 72 h of culture in vitro. RhCXCL6 treatment significantly suppressed trophoblast outgrowth from the distal end of the villous tips from 48 h of culture in vitro compared with control (0 ng/ml) villous explants. Original magnification, ×40. The migration distance of the villous tip was shown at 24, 48 and 72 h of culture. Data are presented as mean ± SEM, n = 3 with 20 replicates per experiment. ✫<0.01 versus control (0 ng/ml).
fetal growth restriction (Cemgil Arikan et al., 2012; Gurdol et al., 2012; Hwang et al., 2012; Schmedt et al., 2012); (iii) trophoblast cell migration and invasion into the uterus giving rise to the proper remodelling of the maternal vessels of the maternal–fetal interface in order to supply abundant blood (Hannan and Salamonsen, 2007). Chemokines are also key components in the process of leucocyte recruitment from peripheral blood into the maternal–fetal interface. The interaction of different chemokines with their receptors on leucocytes allows for the selective activation and chemotaxis of neutrophils, eosinophils, lymphocytes or monocytes, which is necessary for migration to the sites of evolving innate immunity, selective immunity and immune tolerance at the maternal–fetal interface in human pregnancy (Salamonsen et al., 2007). For example, the chemokine CXCL12 was shown to be involved in immune tolerance (Yang et al., 2006a,b). Indeed, human first-trimester trophoblasts have been shown to recruit CD56brightCD16-NK cells into decidua by expressing and secreting CXCL12/stromal cell-derived factor-1 (Wu et al., 2005).

We know that trophoblast invasion into the uterus is associated with remodelling of the uterine vasculature in early pregnancy (Burrows et al., 1996; Red-Horse et al., 2004b; Lunghi et al., 2007), whereas, in late pregnancy...

**Figure 6** RhCXCL6 significantly reduced HTR8/SVneo cell migration in a dose-dependent manner. HTR8/SVneo cells were treated with rhCXCL6 at the indicated doses (1, 5, 10, 25, 50, 100 ng/ml) (d–i), with (b) or without CXCL6 blocking antibody (e) and subjected to the migration assay. Controls were not treated with rhCXCL6 (0 ng/ml) (a). Results are shown as the mean migration (expressed as fold change from control) ± SEM, n = 3. **P < 0.01 versus control (0 ng/ml). Original magnification, ×200.
pregnancy, trophoblasts mainly secrete hormones by syncytiotrophoblasts fused with cytotrophoblasts (Gauster et al., 2009). It has been indicated that a defect of trophoblast invasion is directly involved in serious and specific complications of human pregnancy, such as pre-eclampsia and placenta increta (Tantbirojn et al., 2008). Importantly, so far, many chemokines have been identified that could promote trophoblast cell proliferation, migration and invasion, including CXCL8, CXCL12, CXCL16, CX3CL1 and CCL14, while only CXCL14 has been demonstrated to restrict trophoblast cell migration and invasion (Kuang et al., 2009a,b).

Chemokines have also been associated with angiogenesis, growth and metastatic potential of tumours. In fact, many chemokines were first purified from tumour cells, for instance, CXCL6 was purified from the osteosarcoma cell line MG-63 (Proost et al., 1993). Because many common features are shared by trophoblasts and tumour cells, we examined whether CXCL6 regulates villous explant growth in vitro. In contrast to tumour cells, extravillous cytotrophoblast (EVCT) invasion is precisely regulated and controlled. EVCTs never fuse, but migrate and invade the Matrigel, and so we examined the invasive capacity of the isolated first-trimester trophoblasts and HTR8/SVneo cells. These findings were

Figure 7 RhCXCL6 significantly reduced HTR8/SVneo cell invasion in a dose-dependent manner. HTR8/SVneo cells were treated with rhCXCL6 at the indicated doses (1, 5, 10, 25, 50, 100 ng/ml) (d–i), with (b) or without CXCL6 blocking antibody (c) and subjected to the invasion assay. Controls were not treated with rhCXCL6 (0 ng/ml) (a). Results are shown as the mean invasion (expressed as fold change from control) ± SEM, n = 3. **P < 0.01 versus control (0 ng/ml). Original magnification, × 200.
highly comparable using either HTR8/SVneo cells or primary trophoblast cells. The results clearly indicate that rhCXCL6 remarkably suppresses trophoblast cell invasion. Notably, our results reveal that rhCXCL6 does not influence the proliferation of trophoblasts, suggesting that inhibition of villous explant growth is most likely due to suppression of trophoblast migration and invasion. In addition, it has been demonstrated that the production of CXCL6 leads to intra-tumoural expression of gelatinase B, an advantage for tumour growth by increased angiogenesis (Van Coillie et al., 2001). Thus, together these data suggest that CXCL6 might display different functions in different pathophysiological environments with different cell types. Furthermore, CXCL6 expression at the maternal–fetal interface possibly plays an important role in regulating trophoblast invasion through binding to its receptors on invading trophoblast cells during early pregnancy.

MMPs degrade basement membranes and extracellular matrix components and are therefore important effector molecules for cell migration or invasion (Van Den Steen et al., 2003). Trophoblast invasion is closely correlated with the expression of MMPs, which are capable of degrading extracellular matrix. Among the members of the MMP family, MMP-2 and MMP-9 have been suggested to play a major role in the final pathway of trophoblast invasion. Real-time RT–PCR quantification of trophoblast cells (AC1M-88) revealed significant changes in the mRNA transcripts of MMP12 following treatment with the chemokines CX3CL1 and CCL14 (Hannan and Salamonsen, 2008). Interaction of CXCL12, secreted by human trophoblasts, with its receptor CXCR4, enhanced the coordination between trophoblasts and DSCs, via the regulation of MMP-9 and MMP-2, which may improve the functional maternal–fetal interface (Zhou et al., 2008). CXCL14 ligand and its

---

**Figure 8** RhCXCL6 significantly reduced the invasive ability of primary trophoblasts. Primary trophoblasts were treated without (control) (a) or with rhCXCL6 (100 ng/ml) (b), with (c) or without (d) CXCL6 blocking antibody, and subjected to the invasion assay. Results are shown as the mean invasion (expressed as fold change from control) ± SEM. *P < 0.05 versus control (0 ng/ml). Original magnification, ×200.
receptor, on the other hand, have been shown to restrict human trophoblast invasion by suppressing gelatinase activity (Kuang et al., 2009a). CXCL8 contact with its receptors (CXCR1/2) has been demonstrated to stimulate trophoblast cell migration and invasion by increasing levels of matrix metalloproteinase MMP-2 and MMP-9 (Jovanovic et al., 2010). Thus, there has been considerable evidence that MMPs play roles in trophoblast invasion at the fetal–maternal interface (Kuang et al., 2009a; Zhou et al., 2009; Nissi et al., 2013). Meanwhile cultured human endometrial stromal cells are reported to secrete CXCL6 (Mine et al., 2003), which has been shown to significantly increase in amniotic fluid of infected amniotic cavities (Mittal et al., 2008). We speculate that the chemokine CXCL6 combined with receptors CXCR1/2 probably plays an important biological role on trophoblasts in an autocrine or paracrine manner. This led us first to examine the effect of CXCL6 on the gelatinases. Indeed, CXCL6-induced suppression of motility in primary trophoblasts was associated with a statistically significant decrease of proMMP-2, and similar results were observed with HTR8/SVneo cells. This also supports previous findings that MMP-2 is the main gelatinase involved in early first-trimester trophoblasts, especially in JAR and early trophoblasts (6–8 weeks). Thereafter, in late first-trimester trophoblasts (9–12 weeks), both MMP-2 and -9 participate in trophoblast invasion (Staun-Ram et al., 2004). In addition to regulation of MMPs, other mechanisms might also be operative in the process, such as increase of tissue inhibitors of metalloproteinases or effects on the plasminogen activator/inhibitor system of serine proteases.

In summary, the present data demonstrate that CXCL6 is expressed at the human maternal–fetal interface during early pregnancy. CXCL6 restricts migration and invasion of trophoblast cells, which is associated with a decrease of gelatinases in an autocrine or paracrine manner. Therefore, we propose that deregulation of CXCL6 is probably associated with pregnancy complications. For further investigation, it will be of great interest to study the roles of CXCL6 in pregnancy complications.
Acknowledgements

We thank Prof. Wang H.M. for providing the HTR8/SVneo cells and the laboratory at the institute of zoology, Chinese Academy of Sciences.

Authors’ roles

W.Y.Z. designed the project, analysed data, and wrote and edited the manuscript. H.Z. designed the project, collected tissues, performed all the experiments, analysed data, and wrote and edited the manuscript. L.H. designed the project, assisted in data analysis and edited the manuscript. C.M.L. collected tissues and assisted in some experiments. All authors gave their final approval of the submitted version.

Funding

This study was supported by grants from the National Natural Science Foundation of China (No. 81070497), The Ministry of Education Doctoral Program Foundation of China (No. 20070025003) and the Beijing Municipal Science & Technology New Star Project of China (No. 2008B66). The funding bore the cost of all kinds of laboratory reagents and antibodies.

Conflict of interest

None of the authors has any conflict of interest to declare.

References


Hannan NJ, Jones RL, White CA, Salamonsen LA. The chemokines, CX3CL1, CCL14, and CCL4, promote human trophoblast migration at the feto-maternal interface. Biol Reprod 2006;5:896–904.


