Trophoblast-derived chemokine CXCL12 promotes CXCR4 expression and invasion of human first-trimester decidual stromal cells

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BACKGROUND: The aim of this study was to investigate the role of the chemokine (C-X-C motif) ligand 12/chemokine (C-X-C motif) receptor 4 (CXCL12/CXCR4) axis on the crosstalk between human first-trimester trophoblast cells (TCs) and decidual stromal cells (DSCs), to contribute to a better understanding of the molecular mechanisms on the interaction between the mother and embryo during pregnancy.

METHODS: CXCR4 on human first-trimester DSC membranes was detected by flow cytometry. The effects of exogenous CXCL12 or TC-conditioned medium (TCM) on proliferation and invasion of DSCs were examined by measuring proliferating cell nuclear antigen (PCNA) and an invasion assay, respectively. Finally, a co-culture model was established to investigate the effect of CXCL12 secreted from TCs on motility of DSCs.

RESULTS: The mean (± SEM) percentage of DSCs positive for CXCR4 was 32.32 ± 7.18%. Human recombinant CXCL12 induced an increase in CXCR4 levels on DSCs via binding to CXCR4 (P < 0.01) but had no effect on the PCNA expression of DSCs. Moreover, both exogenous CXCL12 and TCM reinforced the invasive ability of DSCs via CXCR4 ligation. A co-culture model further confirmed that the enhanced invasiveness of DSCs in co-culture with TCs was inhibited by anti-CXCR4 or anti-CXCL12 neutralizing antibody (both P < 0.01).

CONCLUSIONS: Human first-trimester DSCs express membrane CXCR4 and TC-derived CXCL12 promotes CXCR4 expression and invasion of DSCs via ligation with CXCR4. Our data highlight the role of CXCL12/CXCR4 axis on the co-operation between TCs and DSCs during human first-trimester pregnancy.

Key words: CXCL12 / CXCR4 / first-trimester pregnancy / trophoblast cells / decidual stromal cells

Introduction

The successful implantation, growth and survival of a semiallograft embryo in maternal uterus depend on the integrity of a subtle materno–fetal interface established by the fetus-derived trophoblast cells (TCs) and maternal deciduas. An abnormal interaction between the mother and embryo disturbs the biological functions of multiple cells at the materno–fetal interface, which is positively related to the problems of implantation failure, miscarriage, preterm birth, pre-eclampsia, intrauterine growth restriction and so on (Salker et al., 2010; Teklenburg et al., 2010; Cecati et al., 2011; Fukui et al., 2011; Heazell et al., 2011; Norris et al., 2011). However, because of the complexity of the materno–fetal interface and limitations of current experimental approaches, there are still many unknowns regarding the particular mechanisms underlying the synchronization between the deciduas and embryo during normal pregnancy.

Decidual stromal cells (DSCs), comprising 75% of decidual cells, are the major cellular component at the materno–fetal interface. After fetal-derived extravillous cytotrophoblast cells (EVTs) penetrate the inner decidua, they locate closely to large numbers of DSCs. DSCs play multiple roles at the materno–fetal interface, including production of cytokines, the main source of extracellular matrix (ECM), antigen presentation, immune regulation, mediators of EVT invasion and hemostatic protection during TC invasion (Aplin et al., 1988; Loke et al., 1989; Zhu et al., 1992; Olivares et al., 1997; Dimitriadis et al.,...
Human decidual and villous tissue collection

Decidual and villous tissues were obtained from artificial abortions of the normal first-trimester pregnancies (gestational age 6–10 weeks) for non-medical reasons. The tissues were collected into ice-cold Dulbecco’s modified Eagle’s medium (DMEM, Gibco Products Invitrogen Corporation, Grand Island, NY, USA), then immediately transported to the laboratory after surgery, and washed in cold RPMI-1640 (Gibco Products Invitrogen Corporation) for isolation of DSCs or TCs. The study was approved by the Human Research Ethics Committee of Zhongnan Hospital, Wuhan University, and all participants completed an informed consent for the collection of tissue samples.

Isolation and culture of human first-trimester DSCs

After the removal of blood clots, DSCs were isolated according to our previous method with minor modifications (Zhou et al., 2008). Briefly, the specimens were cut into small pieces (~1 mm³) and digested for two to three cycles of 20 min using 0.25% trypsin (Ameresco, Solon, OH, USA) at 37°C. The cell digest was successively passed through the 200 and 38 μm sieves, then purified by centrifugation at 1000g for 20 min using a discontinuous Percoll Gradient (20, 40 and 60%) (Pharmacia, Stockholm, Sweden). Then the cells were plated into 24-well or 6-well plates and maintained in DMEM containing 10% fetal bovine serum (FBS, Gibco, USA), 100 UI/ml penicillin and 100 μg/ml streptomycin. Samples of decidua from different patients were not mixed, to avoid alterations in the DSCs phenotype resulting from allogeneic reaction and secretion of cytokines by lymphocytes that initially contaminate DSCs cultures. The primary DSCs used in the present study contained 1% contaminating cells and the detailed information on the purity and characteristics of DSCs is displayed in our previous publication (Zhou et al., 2008).

Isolation and culture of human first-trimester TCs

The villous tissue was treated by repeated trypsin digestions according to our previous method (Zhou et al., 2007, 2008). Briefly, the placental tissues were collected from five or six separate individuals and digested using 0.25% trypsin (Ameresco) and 2.5 K unit/ml DNase type I (Sigma, St. Louis, MO, USA) at 37°C with gentle agitation for 10 min. Then the digested suspension was collected and the residual tissue was subjected to two to three further cycles of 10 min digestion. The cell suspensions obtained in each digestion were mixed, and carefully layered over a discontinuous Percoll Gradient (65–20%, in 5% step), and centrifuged at 1000g for 20 min. The cells sedimenting at densities between 1.048 and 1.062 g/ml were collected and washed with DMEM. These cells were then diluted to 5 × 10⁵ cells/well, and maintained in DMEM complete medium (2 mM HEPES, 100 UI/ml penicillin and 100 mg/ml streptomycin), supplemented with 15% FBS and incubated in 5% CO₂ at 37°C. This method supplies a 95% purity of TCs and the characterization of TCs could be obtained in our previous publication (Zhou et al., 2008).

Preparation of TC-conditioned medium

The freshly isolated TCs were seeded at a density of 2 × 10⁶ cell/ml × 2 ml per well in 6-well plates pre-coated with ECM (Sigma) and cultured for 48 h. The supernatants, namely TC-conditioned medium (TCM), were collected and centrifuged at 2000g, then stored at −70°C. The supernatants from culture medium without TCs were collected as a control.

CXCR4 on first-trimester human DSCs by flow cytometry

The freshly isolated DSCs were seeded in culture flasks and cultivated for 2–3 days. To protect the membrane localization of CXCR4 to the greatest extent possible, the cells, at 70–80% cell confluence, were digested with 0.25% trypsin only for 30–50 s, then blown off gently and washed with phosphate-buffered saline (PBS). After blocking with 10% FBS, the recovered cells were mixed with mouse anti-human CXCR4-PE-CY5 monoclonal antibody or mouse immunoglobulin (Ig)G2α-PE-CY5 isotype (ebioscience, San Diego, CA, USA). After incubation in darkness for 1 h at room temperature, the cells were analyzed immediately by flow cytometry (FCS00, Beckman Coulter, USA). The experiments were repeated ten times.

After detecting the membrane localization of CXCR4 protein on DSCs, we also analyzed the effects of CXCL12 on CXCR4 in DSCs using flow cytometry. Briefly, the cultured DSCs were starved using DMEM free of FBS for 12 h, then recombinant human (rh) CXCL12 (100 ng/ml) (Peprotech, Rocky Hill, NJ, USA) was added. Some of the cells were also treated...
with neutralizing antibody for CXCL12 (25 μg/ml) or CXCR4 (20 μg/ml) (R&D Systems, Inc.) at the same time as rhCXCL12. After incubation for 48 h, the cells were digested with 0.25% trypsin and then treated with 70% ethanol and 0.1% Triton X-100, each for 20 min. The method for CXCR4 measurement by flow cytometry was the same as described above. The same sample was analyzed by flow cytometry twice and the experiments were repeated three times.

PCNA levels in first-trimester human DSCs by flow cytometry

PCNA (proliferating cell nuclear antigen) exists in the nucleus of normal proliferating cells or tumor cells and is closely linked to DNA synthesis in early G1 and S phases, acting as an excellent marker for the proliferative ability of cells. In the present study, to explore the role of CXCL12/CXCR4 axis on cell proliferation, the pre-starved DSCs were treated with CXCL12 (25 μg/ml) or CXCR4 (20 μg/ml) neutralizing antibody, then stimulated with rhCXCL12 (100 ng/ml) for 48 h. To permeabilize the membranes, digested cells were treated with 70% ethanol and 0.5% Triton X-100 for 20 min, respectively, then centrifugation followed by blocking with 10% FBS. The collected cells were incubated with mouse anti-human PCNA-phycocerythrin (PE) monoclonal antibody or mouse IgG2a-PE isotype (eBioscience) in the dark for 1 h, then analyzed immediately by flow cytometry. The same sample was measured by flow cytometry twice and the experiments were repeated three times.

Invasion assay

The invasive ability of DSCs across ECM was evaluated objectively with transwell plates based on our previous matrigel invasion assay performed in human first-trimester TCs (Zhou et al., 2007, 2008). The cell culture inserts (8 mm pore size, 6.5 mm diameter; Corning, NY, USA) coated with 20 μl ECM were placed in a 24-well plate and two sets of invasion assays were performed as follows.

First, the freshly isolated DSCs (2 × 10⁵ in 200 μl DMEM) were treated with rhCXCL12 (100 ng/ml) or various dilutions of TCM (1/5, 1/4, 1/3 and 1/2 TCM). Before this stimulation, some of the wells with cells were pre-incubated with CXCR4 (20 μg/ml) or CXCL12 (25 μg/ml) neutralizing antibody. The lower chamber was filled with 800 μl DMEM with 10–15% FBS. The cells were then incubated at 37°C for 48 h.

Second, the co-culture invasion model of TCs and DSCs was established to observe the invasiveness of DSCs regulated by TC-derived signals. Briefly, the isolated TCs (2 × 10⁵ in 800 μl DMEM with 15% FBS), together with CXCL12 neutralizing antibody (25 μg/ml) or vehicle control, were seeded in the lower chambers and cultured for 48 h at 37°C. Then the freshly isolated DSCs (2 × 10⁵ in 200 μl DMEM) blocked with CXCR4 neutralizing antibody (20 μg/ml) or vehicle control were plated in the upper chamber. The cells were then incubated at 37°C for another 48 h. Cultures of DSCs alone were used as control.

The inserts were removed, washed in PBS and the non-invading cells together with the ECM were removed from the upper surface of the filter by wiping with a cotton bud. The inserts were then fixed in 4% formalin, stained with hematoxylin and observed using an inverted phase contrast microscope (Olympus, Tokyo, Japan). The number of cells which had migrated to the lower surface was counted at a magnification of ×200. To eliminate the individual variability, the results were assessed by two independent researchers and the invasive index was calculated as the proportion of the migrated cells of the experiment group to that of its own control. Each experiment was performed in duplicate and experiments were repeated three times.

Statistics

The post hoc Dunnett’s t-test and Dunnett’s T3 test were employed to compare the significance levels between the control and different treatment groups, when appropriate. Unless stated otherwise, all data were analyzed using the Statistical Package for the Social Sciences 17.0 and presented as mean ± SEM. The differences were accepted as significant at P < 0.05.

Results

Human first-trimester DSCs expressed membrane CXCR4

We used flow cytometry to detect the membrane expression of CXCR4 on human first-trimester DSCs in 10 independent samples. CXCR4 protein could be detected in all of the analyzed samples with the percentage of CXCR4-positive cells varying from 9.4 to 71.1%, with an average of 32.32 ± 7.18% (Fig. 1).

CXCL12 increased CXCR4 levels in human first-trimester DSCs via CXCL12/CXCR4 signal pathway

After identifying the membrane existence of CXCR4, we observed the changes in CXCR4 levels after CXCL12 stimulation. Exogenous CXCL12 (100 ng/ml) increased the number of CXCR4-positive cells (P < 0.05, compared with the control). Moreover, this increase in CXCR4 protein was effectively blocked by CXCL12 or CXCR4 neutralizing antibody (P < 0.05 or P < 0.01, compared with the...
CXCL12 treatment group), which indicated that CXCL12 increased CXCR4 protein in DSCs via CXCL12/CXCR4 signals (Fig. 2).

Effects of CXCL12/CXCR4 on proliferation of human first-trimester DSCs

PCNA is a reliable marker of the proliferative ability of cells. As shown in Fig. 3, despite a tendency for an increase in PCNA expression in the CXCL12 stimulation group, there were no significant changes in PCNA level in various experimental groups (P > 0.05, compared with the control), suggesting CXCL12/CXCR4 do not participate in the modulation of DSC proliferation.

TC-derived CXCL12-promoted migration and invasion of human first-trimester DSCs in vitro

An invasion assay was performed to evaluate the role of CXCL12/CXCR4 signaling pathway on DSCs invasive activity in vitro. Similar to the action of CXCL12 on CXCR4 levels in DSCs, exogenous CXCL12 (100 ng/ml) improved the invasive ability of these cells. Compared with the control, the number of migrated cells in the CXCL12 treatment group increased (P < 0.01). The action of CXCL12 on DSC invasiveness was further demonstrated by a complete inhibition of CXCL12-enhanced DSC invasion by anti-CXCR4 or anti-CXCL12 neutralizing antibody (compared with the CXCL12 treatment group, P < 0.01). In addition, we could observe that in the control groups there were even a number of DSCs which migrated to the lower surface of the transwell inserts, indicating DSCs themselves display chemotactic and migratory behavior towards serum components. However, an unexpected result was an obvious difference in the DSCs invasive ability between the control and CXCR4 neutralizing antibody treatment group (P < 0.01). The invasive index of CXCR4 antibody treatment group was only equivalent to 64% of the control (Fig. 4A).

Furthermore, as shown in Fig. 4B and C, TCM promoted the invasiveness of human first-trimester DSCs in a dose-dependent manner and TCM at a 1/4 dilution was already sufficient to induce a stimulatory effect (compared with the control, P < 0.05 or P < 0.01). Moreover, the addition of neutralizing antibody to CXCR4 or CXCL12 inhibited the TCM-induced DSC invasiveness (compared with the CXCL12-treatment group, P < 0.01), but the invasive index in CXCR4 antibody treatment group was even lower than that of the control (P < 0.05).

To further confirm the role of CXCL12/CXCR4 axis on the interaction of TCs and DSCs, we also assessed the DSC invasive ability in a TC-DSC co-culture model. The results clearly demonstrated that...
when co-cultured with TCs, the number of migrated DSCs was increased (compared with the control, $P < 0.01$). Moreover, compared with the co-culture group, the invasive index in TC-anti-CXCL12 or DSC-anti-CXCR4 pretreatment group decreased ($P < 0.01$) but was still higher than that of the cultured DSCs alone ($P < 0.05$ or $P < 0.01$). Interestingly, when CXCL12 and CXCR4 neutralizing antibodies were added at the same time, the capacity of DSCs to migrate was dramatically impaired (compared with the co-culture group, $P < 0.01$) and even dropped to the basal level (compared with the control, $P > 0.05$), which strongly implied that TC-derived CXCL12 might be the dominant paracrine regulator of the biological functions of CXCR4-positive DSCs (Fig. 5).

**Discussion**

Our previous study using immunohistochemistry revealed positive staining for CXCR4 in human first-trimester DSCs (Zhou et al., 2008). As a seven transmembrane chemokine receptor, the ligand-binding sites of CXCR4 differ from other G-protein-coupled receptors and are closer to the extracellular surface (Wu et al., 2010). To further explore the role of CXCR4-mediated signal transduction at the materno-fetal interface, in the present study, we first determined the membrane expression of CXCR4 on primary cultured DSCs. Flow cytometry analysis displayed the presence of membrane CXCR4 in all samples. The data of Hess demonstrate a significant induction of CXCR4 mRNA in decidualized endometrial stromal cells (ESCs) in response to TC-secreted products but the molecules involved in this stimulatory action is unclear (Hess et al., 2007). Our previous study has confirmed the production of soluble CXCL12 by human first-trimester TCs (Zhou et al., 2008). Thus, in the present study, we also observed the effects of CXCL12 on CXCR4 levels in DSCs. Consistent with our speculations, CXCR4 protein in DSCs significantly increased after addition of CXCL12 and this increase could be effectively abolished by CXCL12 or CXCR4 neutralizing antibody. The result that the CXCR4 gene in decidualized ESCs is up-regulated after stimulation with TCM for 3 or 12 h (Hess et al., 2007), combined with our current result that CXCR4 protein increased in DSCs after 48 h incubation with rhCXCL12, indicated that trophoblast-derived CXCL12 may enhance the transcription, then translation of CXCR4 gene in DSCs. In the present study, we assessed total protein level rather than membrane expression of CXCR4 in DSCs after CXCL12 treatment. However, CXCL12, also known as stromal cell-derived factor-1, has always been thought of as the exclusive ligand for CXCR4. Only by binding with CXCL12, could CXCR4 be activated and trigger G-protein-coupled intracellular signal transduction. Therefore, it was inferred that TC-derived CXCL12 promotes the expression of CXCR4 in DSCs via CXCL12/CXCR4 axis in a paracrine manner, which might be beneficial to the CXCL12/CXCR4-mediated signal transmission as well as crosstalk and synchronization between TCs and DSCs.

In view of the stimulatory action of CXCL12/CXCR4 on the proliferation of tumor cells (Teicher and Fricker, 2010), we also analyzed the expression of PCNA in CXCL12-treated DSCs. However, the results clearly showed that there was no remarkable difference in PCNA expression in DSCs between CXCL12 treatment and control group. We have found in our previous research that CXCL12 or CXCR4 blocking antibody fails to inhibit the up-regulated viability of DSCs stimulated by TCM (Zhou et al., 2008). These results demonstrate that in spite of expression of CXCR4 in DSCs, CXCL12/CXCR4 axis did not take part in the modulation of DSC proliferation. In our previous research, it has also been shown that human first-trimester VCTs promote their own proliferative ability via CXCL12/CXCR4 signals in an autocrine manner (Zhou et al., 2008). Fetal-derived TCs include several subpopulations and different lineages display different biological functions, whereas DSCs originate from the fibroblast-like stromal cell precursors which undergo progesterone-induced decidualization after estrogen-priming. Thus, despite the expression of CXCR4 in both TCs and DSCs, CXCL12/CXCR4 might present different effects on different cells via different signaling pathways. Further study is
required to understand the biological functions of CXCL12/CXCR4 on various subpopulations of decidual cells.

The study by Gellersen et al. (2010) has demonstrated that co-culture of ESCs with AC-IM88, a hybridoma cell line characteristic of invading EVTs, greatly enhances the invasive potential as well as matrix metalloproteinase (MMP) 9 and MMP 2 production by decidualized ESCs but the detailed mechanism is still a mystery. We disclosed in the present study that both exogenous CXCL12 and TCM reinforced the migration and invasion of DSCs in vitro, and that the increased motile ability of DSCs could be reversed by blocking antibody to CXCL12 or CXCR4, suggesting that TC-secreted CXCL12 was capable of modulating the invasiveness of DSCs via CXCL12/CXCR4 axis. This conjecture was further confirmed by the co-culture experiments which unambiguously unveiled that enhanced migration of DSCs induced by TC co-culture could be effectively abolished by CXCL12 or CXCR4 neutralizing antibody. Our previous research has verified that CXCL12 not only regulates the invasive ability of VCT-derived EVTs in an autocrine manner but also induces DSC MMP 9 and MMP2 activities via CXCL12/CXCR4 signals (Zhou et al., 2008). Combining our previous study with the present results, we propose that TC-derived CXCL12 has a cascade amplification effect on the communication between TCs and DSCs: promoting VCT proliferation as well as VCT-derived EVT invasion in an autocrine manner, and modulating DSC invasion via enhanced MMP activity in an paracrine manner, which contributes to the biological functions of the various cells in a co-operative fashion and the formation of the materno–fetal immune milieu.

In fact, much attention has focused on the migratory and invasive potential of ESCs in endometrosis or endometrial carcinomas, however little is known about its significance in pregnancy. Here, we provided compelling evidence that human first-trimester DSCs display chemotactic and migratory behavior towards serum components and their invasive ability could be further enhanced by TC-derived signals. In a rat model, it is observed that after initial invasion of the uterine luminal

![Figure 4](image-url)
epithelium, the TCs come to lie adjacent to the residual basal lamina but do not penetrate it. It is the decidual cells that penetrate the basal lamina and underlie the TCs (Schlafke et al., 1985). By using an embryo co-culture model, Grewal et al. (2008) have found that decidualized ESCs become more motile and migrate away from the implantation site, allowing the embryo to spread out. Furthermore, our previous research has disclosed that elevated MMP 9 and MMP 2 activity by human first-trimester DSCs facilitates the invasion of EVTs (Zhou et al., 2008). Based on these observations, it is reasonable to propose that on the long journey of TC penetration into maternal deciduas, DSCs are not merely ‘passively’ invaded by the embryo, instead, they could ‘actively’ pave the way for TCs through ECM degradation and movement. A recent investigation by Godbole has demonstrated that conditioned medium from in vitro decidualized human ESCs even increases the invasiveness of TC cell lines and alters the expression pattern of integrins, MMPs and inhibitors of metalloproteinases (Godbole et al., 2011). In our opinion, the embryonic TCs as well as maternally derived DSCs are cells which co-operate very efficiently at the materno–fetal interface. Therefore, we still have a long way to go before understanding the subtle dialogue between the mother and embryo.

Unexpectedly, the CXCR4 neutralizing antibody completely blocked the increased invasive capacity stimulated by exogenous CXCL12 or TCM treatment. Moreover, in our co-culture model, there was no obvious difference in the invasiveness of DSCs in the presence of CXCL12 and CXCR4 neutralizing antibodies added at the same time versus the control group. Thus, CXCL12/CXCR4 might be a leading modulator in the indirect contact between TCs and DSCs. However, we could not exclude the possibility that the combination of membrane CXCR4 receptor with its neutralizing antibody disturbs the downstream common signal pathway, which results in a completely inhibitory effect. In addition, we observed CXCR4 on membranes of human first-trimester DSCs and the proportion of CXCR4-positive cells differed among samples. However, owing to the nature of the samples (collected from artificial abortions), it was impossible to track the pregnancy outcomes of these volunteers. Therefore, the relationship of inter-individual variability in CXCR4 levels to pregnancy outcome is still an open question, and awaits further studies.

In summary, we have confirmed that human first-trimester DSCs express membrane CXCR4 and that TC-derived CXCL12 is able to promote CXCR4 level and invasion of DSCs via ligation with CXCR4, which highlights the role of the CXCL12/CXCR4 axis in the co-operation between TCs and DSCs. Our research will contribute to a better understanding of the orchestration between the fetus and mother during pregnancy, which might provide intellectual insight for the clinical treatment for infertility and some pregnancy complications. A more complex picture is likely to emerge regarding the
relationship of chemokines to pregnancy, and we still have a long way to go before understanding the detailed molecular mechanisms involved in materno–fetal dialog during embryo implantation and placentation.

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Authors’ roles
L.R. was responsible for experimental execution and results analysis. Y.Q.L. participated in experimental execution. W.H.Z. was responsible for the study design, results analysis, manuscript drafting, critical discussion and communication to the editor. Y.Z.Z. participated in study design, manuscript preparation, critical discussion and support of the research.

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Conflict of interest
None declared.

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