Human trophectoderm apposition is regulated by interferon γ-induced protein 10 (IP-10) during early implantation

H.Y. Sela a, D.S. Goldman-Wohl a, R. Haimov-Kochman a, C. Greenfield a, S. Natanson-Yaron a, Y. Hamani a, A. Revel a, Y. Lavy a, O. Singer b, N. Yachimovich-Cohen b, T. Turetsky b, O. Mandelboim c, B. Reubinoff b, S. Yagel a,∗

a Division of Obstetrics and Gynecology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel
b Human Embryonic Stem Cell Research Center, Goldyne Savad Institute of Gene Therapy, Hadassah-Hebrew University Medical Center, Jerusalem, Israel
c Lautenberg Center for General and Tumor Immunology, Hebrew University Hadassah Medical School, IMRIC, Jerusalem, Israel

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Abstract
Introduction: The first step in human implantation is the attraction of the blastocyst to the endometrium. We aimed to study attraction of the human blastocyst to the endometrium, and how this process is accomplished by chemokines secreted by the endometrium.

Materials and methods: Blastocyst trophectoderm cells and other trophoblast lineage cells were subjected to attraction assays by IP-10 and other chemokines using transwell migration and chemotaxis assays. Chemokine expression and secretion were investigated using immunohistochemistry, ELISA, FACS analysis, and RT-PCR on material from flushing of the uterine cavity in endometrial biopsies. Chemokine receptor expression by blastocyst trophectoderm following PGD biopsy, trophectoderm derived from hES, placental villi, and other trophoblast lineage cells were characterized by the same methods.

Results: IP-10 dramatically attracted trophectoderm derived from hES cells and other lineages by interaction with CXCR3 chemokine receptors, as shown by both chemotaxis and transwell migration. High levels of IP-10 were detected throughout the menstrual cycle at flushing of the uterine cavity. Immunohistochemistry, FACS analysis, and RT-PCR of endometrial biopsy detected IP-10 in glandular and stromal cells of the endometrium. High levels of IP-10 were detected in condition medium of the endometrial stromal and glandular cells. Of all of the chemokine/chemokine receptor combinations examined, the IP-10/CXCR3 interaction was the only cytokine that was significantly elevated.

Discussion: While they await the wandering blastocyst, IP-10 is produced by many cells of the endometrium, but not by endometrial natural killer cells.

Conclusion: Endometrial IP-10 may specifically attract human blastocyst trophectoderm cells early in implantation.

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1. Introduction

Successful embryo implantation can be divided into three processes: apposition, in which the free-floating blastocyst moves into the immediate proximity of the endometrium; adhesion, in which the trophectoderm, the outer layer of the blastocyst, attaches to the endometrial epithelium; and invasion, in which trophoblasts invade the decidua.

The non-pregnant human endometrium expresses chemokines. Those chemokines most abundant in the midsecretory phase are those most likely to play a role in implantation, and so have been most intensively investigated [1].

Endometrial chemokines are primarily detected in the glandular and luminal epithelium, leukocytes and endometrial stroma [2–11]. These data have explained the ability of endometrial chemokines to attract leukocytes to the endometrium during the implantation period.

We reported that invasive trophoblasts are attracted to the decidua by chemokine-mediated reactions [12] in human and mouse models. In addition, our group and others have demonstrated that trophoblasts express a specific set of chemokine receptors [4,5,7,10,13–15] and that chemokine receptor interactions play a role...
in controlling the migration and invasion of the trophoblast to the decidua.

Preliminary studies indicated that trophectoderm cells also express chemokine receptors [1]. Chemokines secreted by the endometrium are among the first molecules to appear during apposition [1,3].

However, the endometrial chemokines and trophectoderm chemokine receptors that participate in the mutual attraction process that draws the blastocyst into close proximity of the endometrium remain to be demonstrated.

Here, we demonstrate that the interaction of human endometrial IP-10 with trophectoderm CXCR3 regulates blastocyst trophectoderm attraction during early implantation.

2. Materials and methods

2.1. Ex vivo explant culture

Villous explant cultures from first-trimester human pooled placentas (7–9 weeks gestation) were established as previously described [16] with a number of modifications. Briefly, placental tissue was placed in ice-cold saline and processed within 2 h of collection. The tissue was aseptically dissected to remove decidual tissue and fetal membranes. Small fragments of placental villi were placed on four well glass slides (LAB-TEK®, Nalge Nunc, Naperville, IL, USA) that were pre-coated with growth factor reduced-Matrigel (BD Biosciences, NJ, USA). The explants were cultured in serum-free DMEM:F12 (Biological Industries) supplemented with 160 µg/ml gentamicin and incubated at 37 °C in an atmosphere of 4–8% O2 and 5% CO2. For further investigation, the explants were fixed for 2–4 h at 4 °C in 4% (vol/vol) paraformaldehyde, embedded in paraffin and cut into 10-µm sections.

2.2. Flow cytometry analysis for JEG-3, hES and invasive trophoblast cells

The following mouse anti-human mAbs conjugated with PE were used: HLA-G (A AbD Serotec, UK), CXCR1, CXCR3 and CXCR4 (R&D Systems, Minneapolis, MN, USA). For staining and cell sorting, the cells were washed in PBS supplemented with 2% FCS and incubated with mAb on ice for 30 min, followed by washing twice. Cell sorting and fluorescence measurements were performed on a MoFlo high performance cell sorter (DakoCytometry). Data from single cell events were collected using a standard FACScanibur™ flow cytometer (Immunocytometry Systems; Beckton Dickinson).

2.3. Semi-quantitative PCR analysis

Total RNA was isolated from decidual cells and CTBs using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. cDNA was prepared according to a standard protocol. The expression of human chemokines was verified by RT-PCR. The following GAPDH primers were used: sense, 5'-TGAT-GACATCAAGAAGGTGGTGAAG-3'; and antisense, 5'-TCTTGTGAGCCATGTGGCCCAT-3'. The primers used for the detection of chemokine receptors and chemokines are indicated in Table 1.

2.4. Migration-invasion assay

Transwell polycarbonate membrane filters measuring 6.5 mm in diameter with an 8-µm diameter pore (Costar, Corning Incorporated, NY, USA) were plated with 50 µl of 75% GFR-Matrigel (Growth Factor Reduced-Matrigel BD Biosciences, NJ, USA) diluted in ice-cold 1× PBS and placed in a 37 °C incubator. Approximately 50 µl of serum- and antibiotic-free DMEM:F12 medium was added after 20 min.

Fifty microliters (2 × 10⁶/well) of single cell trophoblasts suspended in serum- and antibiotic-free DMEM:F12 medium was placed in the upper chamber. The lower chamber was filled with 600 µl of serum- and antibiotic-free DMEM:F12 medium as a control or with 100, 150, or 250 ng/ml IL-8 or 100, 250, or 500 ng/ml IP-10 (human recombinant IP-10, R&D Systems). The concentration of the chemokines was chosen in accordance with the manufacturer’s ED₅₀. Heat denatured IP-10 (boiled for 10 min) was used to clarify the effect of IP-10. The chamber was placed in a 37 °C incubator in an atmosphere of 5–8% O₂.

The cells were allowed to migrate/invade for 4 days. The media with uninvaded cells and Matrigel were then removed carefully from the upper chamber. The filter with the migrated cells was stained and fixed with crystal-violet solution for 1 h. After several washes with 1× PBS, the filter was cut off the Transwell apparatus and placed on a glass slide, with the underside of the filter facing upward. For quantification, the cells on the lower surface of the filter were counted under a microscope at 400× magnification in three different fields. The assay was performed in triplicate and each experiment was repeated three times. Medium alone was used for control.

2.5. Immunohistochemistry

Immunohistochemistry was performed using the Histostain-Plus kit (Zymed Laboratories Inc., South San Francisco, CA, USA). Briefly, frozen placental tissue/cell sections (5–6 µm) were fixed on ice-cold acetone for 10 min and quenched with 3% hydrogen peroxide to eliminate endogenous peroxidase activity. The slides were washed, blocked and incubated at room temperature with primary antibodies using the dilutions stated below. The primary antibodies used were as follows: mouse polyclonal anti-human HLA-G (1:25) [4H84, kind gift of M. McMaster], mouse monoclonal anti CD56 (1:50) (Zymed Laboratories), mouse monoclonal anti CXCR3 (1:50) (R&D Systems), mouse monoclonal anti IL-8 (CXCR1) (1:50) (Zymed Laboratories), and mouse monoclonal anti IP-10 (1:25) (Zymed Laboratories). EnVision peroxidase mouse/rabbit (DAKO Corporation, Glostrup, Denmark) was used as the secondary antibody. The slides were then developed with a substrate-chromogen solution of aminoethylcarbazole (Sigma, St Louis, MO, USA). The immunohistochemical analysis was repeated four times. For negative controls, we used each of the secondary antibodies as well as pre-immune serum.

For paraffin embedded samples, the antigen was retrieved prior to the immunohistochemistry procedure. Antigen retrieval was performed by microwaving the section in 10 mmol sodium citrate for 5 min.

2.6. Fluorescent immunohistochemistry

Following incubation with primary antibodies, the samples were incubated with the anti mouse Rhodamine Red-X conjugated secondary antibody (1:100) (Jackson Immunoresearch Laboratories, USA) and mounted with medium containing DAPI (Santa Cruz Biotechnology, USA). The samples were analyzed by confocal microscopy.

For the initial characterization of the antibodies, isotype matched controls or pre-immune serum was used to assure the absence of nonspecific binding.

2.7. Uterine flushing

For sampling the endometrial secretion of chemokines, 1 ml of NaCl was introduced into the uterine cavity. After 10 s, 400 µl of the uterine fluid was aspirated for ELISA analysis.

2.8. Human endometrium sample collection

The use of human tissue was approved by the Hadassah Medical Center Institutional Review Board. Women with regular menstrual cycles whose partners exhibited severe male infertility were recruited to study from the IVF unit. Endometrial samples from 10 normally cycling women (mean age, 31.4 years; range, 26–34 years) were obtained using a Pipelle curette (Laboratories Prodimed, Neuilly-en-Thelle, France) from the uterine cavity at days 10–11 and 21–22 of the menstrual cycle.

Table 1

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Sense primer</th>
<th>Anti-sense primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-8</td>
<td>TACCTTCCAACCTGCGGCT</td>
<td>GGAATTTGTGACCAAACACT</td>
</tr>
<tr>
<td>GCP</td>
<td>GGCTCCCTTCTGCGGGCT</td>
<td>ACTTCCACCTGGACACCT</td>
</tr>
<tr>
<td>IP-10</td>
<td>CATGATGATCAGCTGATT</td>
<td>TCAGATGATCAGCTGATT</td>
</tr>
<tr>
<td>MIG</td>
<td>AAGGGTGCTTTTTCCTTCT</td>
<td>TTCTCTCTCTTCTGACCTT</td>
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<tr>
<td>I-TAC</td>
<td>ATAGTGGCTGAGGCTATG</td>
<td>GGATTTGGCATGCTGCTC</td>
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<td>ATAGAACCAAGGACCTGTCG</td>
<td>TCTCTCTCTTCTGACCTG</td>
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<tr>
<td>SDF-2</td>
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</tr>
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<tr>
<td>ELC</td>
<td>TACTGACATATGACCTGCTTCT</td>
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2.9. Trophoderm cells from human blastocysts

PCD embryos were donated by couples undergoing IVF treatment at the IVF unit of the Hadassah Medical Center of The Hebrew University. Embryos that were diagnosed as genetically abnormal were recruited for the study subject to informed consent by the couples. The study was approved by the ethics committee at the Hadassah Medical Center and the Israel Ministry of Health National Helsinki Committee for Genetic Research in Humans.

The donated embryos were incubated to the blastocyst stage. The blastocysts were removed from the zona pellucida and identifiable ICMs were separated from the trophoderm cells as previously described [19]. Isolated trophoderm cells or whole blastocysts, in cases where an ICM was not observed, were plated on mitomycin-C-inactivated (10 μg/ml for 2.5 h; Sigma) human foreskin fibroblasts (a gift from M. Revel, Rehovot, Israel). For the preparation of feeder layers, 105 mitotically inactivated feeders were plated in center-well tissue culture dishes (Falcon, cat. no. 353037, Becton Dickinson) pre-coated with 0.1% gelatin (Sigma) in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS; Biological Industries, Beit-Haemek, Israel); 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM l-glutamine (Invitrogen) at 37 °C in 5% CO2. The trophoderm cells were plated on the feeders in the same medium supplemented with 20% FCS or hESC medium comprised of 20% Knockout DMEM supplemented with 20% Knockout Serum Replacement (KSR) and 1% nonessential amino acids, 50 U/ml penicillin, 50 mg/ml streptomycin, 0.1 mM l-mercaptoethanol (all from Invitrogen Corporation, Grand Island, NY, USA) and 4 ng/ml basic fibroblast growth factor (bFGF) (PeproTech Inc., Rocky Hill, NJ, USA). After 48–72 h, the TE cells had adhered to the plate. The medium was removed, and the TE cells were rinsed with PBS three times for 5 min each time and subsequently fixed with 4% paraformaldehyde for 20 min at room temperature. The plates were then rinsed twice with PBS for 5 min and the fixed TE cells on these culture dishes were stored with PBS at 4 °C.

2.10. hESCs differentiation into trophoderm like cells

hESCs were cultured as previously described [20], and directed to TE fate according to the protocol of Xu et al. 2002 [21] with some modifications [20].

2.11. Enzyme-linked immunosorbent assay (ELISA)

A commercial ELISA kit was used for the detection of IP-10 (R&D Systems). Briefly, a 96-well microplate was coated with the anti-IP-10 capture antibody. After being washed, the plate was blocked using blocking reagent to avoid nonspecific adherence. Then, 100 μl of sample or standards was added to the plate for 2 h, followed by incubation with an anti-IP-10 antibody. After incubation with substrate solution, the optical density of the reaction at 450 nm was measured using a microplate reader (Biotek, USA). The results were calculated using a standard curve from known concentrations of IP-10.

2.12. Cell culture and stable transfection

ECC1 cells were derived from a well-differentiated human endometrial adenocarcinoma [22] and provided as a generous gift from Dr B van den Burgh (Hubrecht Laboratory, Utrecht, The Netherlands). The cells were transfected with pLP-10 or pIRE2-EGFP (as a negative control) using Lipofectamine reagent according to the manufacturer’s instructions. IP-10 expression in the transfected cells was measured by ELISA.

2.13. Recombinant plasmid pLP-10

The IP-10-cDNA was cloned into pIRE2-EGFP in the EcoRI-BamHI sites (Geneart, Germany).

2.14. Cell lines

JEG cells represent a human placental choriocarcinoma cell line.

2.15. Attraction/chemotaxis assay

Attraction assays were performed using μ-slides chemotaxis (ibidi, Martinsried, Germany) according to the manufacturer’s instructions. Briefly, the slides were precoated with 10% Matrigel in PBS. Six microliters of a cell suspension (3 × 104 cells) was seeded onto a pipette adapter. After the air was aspirated from the opposite adapter, the cells were flushed inside to fill the entire observation channel. The slide was incubated in a humid atmosphere for attachment. Each reservoir was gently filled with the adapter with 40 μl of medium. To fill the second chamber, another 40 μl of medium was then injected into the filled adapter. At this point, the chamber was completely filled, and the cells grew in the observation area. We then applied 18 μl of chemoattractant onto one reservoir and aspirated the same amount of liquid from the opposite side. This removal of liquid allowed flushing of the chemoattractant into the reservoir. After a short time, the chemoattractant diffused to establish a linear concentration profile. Finally, using an inverted microscope, cell movement and direction were observed, measured, videotaped, and analyzed.

2.16. Cell maintenance and preparation of conditioned medium

The HES-1 and HES-2 cell lines were derived and characterized as previously described [20]. The H7 cell line was obtained from The National Stem Cell Bank (Madison, Wisconsin). hESCs were maintained on human foreskin fibroblasts (a gift from M. Revel, Rehovot, Israel), treated for 2.5 h with 10 μg/ml mitomycin-C (Sigma–Aldrich, St. Louis, MO), and plated in gelatin-coated 9.5-cm2 well plates (Nunc, Glostrup, Denmark; 3 × 105 feeders/well). hESCs were routinely cultured in 85% Knockout DMEM supplemented with 14% knockout serum replacement, 1 mgl/l glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 1% nonessential amino acids (10 mM of each amino acid), 100 μM β-mercaptoethanol (Life Technologies; Carlsbad, CA) and 4 ng/ml basic fibroblast growth factor (Cytolab, Rehovot, Israel). The medium was changed every day. The cells were passaged weekly as small clusters following digestion with collagenase type IV (1 mg/ml; 200 U/ml) for 1 h. For FACS analysis, the urea quantification assay, and immunofluorescence staining, single cells were obtained by washing hESC colonies twice with PBS without calcium and magnesium and incubating them in 0.05% EDTA in PBS (Biological Industries, Beit-Haemek, Israel) for 10 min. The cells were then triturated into single cells and separated from the foreskin feeder cells. The medium used to propagate the foreskin feeder cells and adult fibroblasts from skin biopsies of healthy donors (a gift from H. Ben-Bassat, Jerusalem, Israel) was composed of high-glucose DMEM (Life Technologies) supplemented with 20% charcoal-treated fetal bovine serum and 2 mM l-glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin (Life Technologies).

E14 mESCs (a gift from H. Cedar, Jerusalem, Israel) and C57BL/6 mESCs (a gift from D. Elad, Jerusalem, Israel) were routinely cultured in 85% Knockout DMEM (Life Technologies) supplemented with 15% ES approved FCS (Biological Industries), 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, amino acids (10 mM of each amino acid), 1 mM sodium pyruvate, 100 μM β-mercaptoethanol (all supplements were purchased from Life Technologies), and 105 U/ml LIF (Chemicon, Huisken, Netherlands). The cells were grown on gelatin-coated 95-cm2 well plates, and the medium was changed every day. The cells were passaged every 3–4 days as single cells using 0.04% trypsin (Life Technologies) digestion for 2 min. All cells were grown at 37 °C in a 5% CO2 and 4% O2 atmosphere. Three milliliters of hESC- and mESC-conditioned medium (CM) was collected on days 7 and 3 after ESC plating, respectively. At this stage, the 95-cm2 wells contained ca. 5 × 106 hESCs and mESCs well per. In some experiments, several conditions of Nα-(ω-hydroxy-nor-L-arginine (Noha, Calbiochem, San Diego, CA) were added to the hESC or mESC culture 24 h prior to collection of the CM. To prepare the CM, cells were depleted by centrifugation, and media were filtered through a 0.22-μm filter to remove any remaining cell debris.

For the analysis of hESC-CM, media were collected as described above and placed into 1350, 3500, and 12,000–14,000 kDa pore size dialysis bags (Medicell International, London, UK). CM was then dialyzed for 24 h at 4 °C against fresh hESC medium at a 1:10 ratio of fresh medium to CM volume. Two rounds of 24-h dialysis were performed.

2.17. Derivation of trophoderm cells from hESCs

hESC-derived trophoblast cells were prepared using an established protocol [20]. hESCs were detached from foreskin plates using EDTA as described above and plated on Permanox slides (Nunc) coated with 75% Matrigel. The cells were grown for 14 days in 4 (PeproTech b) mm morphogenic protein 4 (PeproTech b) in hESC medium and then either fixed with 4% PFA/PBS for arginase 1 immunofluorescence staining or detached from the Matrigel for HLA-G staining and flow cytometric analysis using 0.04% trypsin (Life Technologies).

3. Results

Immuno histochernistry of both cytotrophoblasts and HLA-G-positive invasive trophoblasts revealed the expression of both CXCR1 and CXCR3 in first-trimester placental sections (data not shown, see Fig. 1 in Supplemental Material). Using FACS analysis, JEG choriocarcinoma cells were positive for CXCR3 but not CXCR4 (Fig. 1a). Cytotrophoblast cells (C TBs) isolated from first-trimester placenta were positive for CXCR1, CXCR3, CXCR4, CCR5, CCR7 using...
Fig. 1. Chemokine receptor expression on trophectoderm cells (a) FACS analysis of chemokine receptors expressed on JEG cell lines. Only CXCR3 is expressed. (b) RT-PCR of chemokine receptors in cytotrophoblast cells (CTBs) isolated from first-trimester placental villi and chemokines in endometrial cells. Bold text indicates chemokine/chemokine receptor pairs that were further investigated. Shaded rows indicate chemokines that were not; positive pairs (+) showed between a 2-fold and 10-fold increase above control, while negative pairs (−) showed less than a 2-fold increase. (c) Immunohistochemistry of villi, invading trophoblast stained with anti-HLA-G (magnification 400). FACS analysis of invasive trophoblasts (HLA-G positive) cultured on Matrigel and isolated by sorting for HLA-G. CXCR1 and CXCR3 were expressed (other chemokine receptors were negative, data not shown). (d) RT-PCR of CXCR1 and CXCR3 in trophectoderm cells that were differentiated from hESCs. (e) FACS analysis of CXCR1 and CXCR3 on HLA-G positive and HLA-G-negative trophectoderm cells. (f) Representative HLA-G and CXCR3 immunohistochemistry staining of trophectoderm cells derived from hESCs. (g) Representative immunofluorescence of CXCR3 and HLA-G on trophectoderm cells grown on Matrigel. Negative control using the secondary antibody. Light-field microscopy and DAPI staining are depicted. (h) Representative HLA-G, CXCR3, and CXCR1 immunohistochemistry of blastocyst-derived trophectoderm cells grown on a feeder layer after the removal of the ICM (inner cell mass).
RT-PCR (Fig. 1b). FACS analysis on HLA-G positive invasive trophoblasts grown on Matrigel for 4–6 days confirmed the expression of CXCR1 and CXCR3 on these cells (Fig. 1c). The following were negative: CXCR2, CX3CR1, CCR1, CCR2, CCR5, CCR6, CCR7. Trophoderm cells generated from hESCs were positive for CXCR1 and CXCR3 by semi-quantitative RT-PCR (Fig. 1d). However FACS analysis on purified HLA-G positive trophoderm cells grown on Matrigel primarily expressed CXCR3 and low level of CXCR1. These cells were also CXCR3 positive by immunohistochemistry (Fig. 1e,f).

3.1. Endometrial cells produce specific chemokines

For endometrial cells to attract blastocyst trophoderm cells, the endometrial cells must express and secrete chemokines that match the chemokine receptors expressed by the trophoderm cells. As demonstrated above, invasive trophoderm cells express CXCR1 and CXCR3 chemokine receptors. Endometrial samples expressed CXCR1 and CXCR3 ligands, IL-8 (CXCL8), IP-10 (CXCL10) and MiG (CXCL9) (Fig. 2a). ITAC (CXCL11), GCP2 (CXCL6) and NAP2 (CXCL7) were not detected (data not shown).

We tested the secretion of these factors by endometrial cells. Isolated endometrial stromal cells, glandular cells and uterine flushing samples contained significant concentration of IP-10 (Fig. 2b) and IL-8 (data not shown). However, IP-10 and IL-8 were not detected in the NK cell supernatants (data not shown, see Fig. 2 in Supplemental Materials).

Immunohistochemistry localized the expression of IL-8 and IP-10 to the glandular and stromal cells of the endometrium (Fig. 2c,d).

3.2. Endometrial chemokines direct trophoderm migration and attraction

To examine whether blastocysts are attracted by endometrial chemokines, trophoblasts and trophoderm cells were placed in the upper chamber of a Transwell apparatus and IL-8 and IP-10 were added in the lower chamber. After 3 days of incubation, we
quantified the number of trophoblasts or trophectoderm cells that had migrated through the membrane. We observed the functional ability of IP-10 and IL-8 to support human trophoblast migration in vitro (Fig. 3a, Student’s t-test, \( p < 0.01 \) (IL-8) and \(< 0.001 \) (IP-10)). IP-10 generated by ECC1 cells transfected by PLP1 also induced migration of the trophoblasts (data not shown, see Fig. 3 in Supplemental Materials). IP-10 also induced budding of invasive trophoblasts stemmed from cultured first-trimester villi (Fig. 3b). The migration of trophectoderm cells was directed by IP-10, in a dose-dependent manner, by endometrial culture medium but not by boiled IP-10 or BSA (Fig. 3c, Student’s t-test, \( p < 0.01 \) (IL-8) and \(< 0.001 \) (IP-10)). However, IL-8, the ligand of CXCR1, did not direct migration (Supplemental Fig. 4). This result may stem from the low level of CXCR1 expression in trophectoderm cells. CXCL12, the ligand of CXCR4, had no effect on the migration of either trophoblasts or trophectoderm cells (data not shown).

In addition, when endometrial culture medium was placed in the lower chamber of the Transwell apparatus it enhanced the migration of trophectoderm cells as well (Fig. 3c). More importantly, the same results were achieved with trophectoderm, which was attracted by IP-10 (Fig. 3d and Video 1 in Supplemental Materials), in the special real-time attraction chamber. In contrast, boiled IP-10 or medium alone did not have any effect on cell movement.

Supplementary data related to this article can be found at doi: 10.1016/j.placenta.2012.12.008.

4. Discussion

Implantation failure is a major cause of the low success rate of IVF treatment. All of the delicate phases of implantation must occur successfully for the floating blastocyst to anchor in the maternal endometrium, invade the decidua and develop into a viable embryo.

During the first step of implantation, the blastocyst migrates within close proximity of the endometrium. This movement enables blastocyst-maternal dialog via soluble mediators, such as growth factors and cytokines or chemokines. This dialog prepares the blastocyst and endometrium for adhesion, which is the next step of implantation. Our data provide strong evidence for the attraction of human blastocysts just before apposition by endometrial chemokines secreted into the uterine cavity.

The results of this study are in accord with scattered evidence in the literature describing the expression of chemokine receptors by blastocyst trophectoderm cells and production of chemokines by the endometrium [1,3,5–11,13]. In particular, we demonstrate, for the first time, the functional ability of endometrial chemokines to attract trophectoderm cells in a human model that is similar to the in vivo setting.

Our group and others have reported that human invasive trophoblasts express various types of chemokine receptors that are imperative for the direction of migration and invasion of the invasive trophoblasts deep into the decidua and maternal myometrium. We have also reported that the promotion of trophoblast invasion is orchestrated by a specific set of chemokines from decidual natural killer (dNK) cells [13].

Our results indicate that the expression of chemokine receptors is an important determinant of invasive trophoblasts. JEG choriocarcinoma cells, isolated first-trimester invasive trophoblasts, villi producing trophoblasts, trophectoderm cells generated from ES cells, and most importantly blastocyst trophectoderm cells all expressed specific chemokine receptors. CXCR3 is the chemokine receptor that is most consistently expressed by these cells, particularly blastocyst trophectoderm cells. These cells are the most
Fig. 3. Migration and attraction of trophectoderm cells and trophoblasts by IP-10 and IL-8. (a) Representative Transwell migration assay of trophoblasts toward 100 ng/ml IL-8 and 500 ng/ml IP-10. (One asterisk indicates significant results, Student’s t-test, *p < 0.01; 2 asterisks, **p < 0.001). (b) The effect of IP-10 (250/500 ng/ml) on budding of invading trophoblast grown on Matrigel. Budding becomes more extensive in a dose-dependent manner. Carets indicate budding. (c) Representative Transwell migration assay of trophectoderm cells grown on Matrigel by IP-10 or boiled IP-10 (Asterisk indicates significant result, Student’s t-test, *p < 0.001). (d) Attraction of trophectoderm cells by IP-10 (250 ng/ml) in μ-slide chemotaxis slides. Note the different location of the cell 8 and 13 h after start point (See also Supplemental Video 1).
relevant trophoblasts for blastocyst attraction. Of the known ligands of CXCR3, IP-10 and Mig were detected in human endometrium during all phases of the menstrual cycle; these data are in agreement with other reports [16] and references therein. Isolation of subpopulations of the endometrium and immunostaining revealed that uterine NK cells do not produce IP-10 or Mig chemokines without activation by IL15 [9]. In contrast, endometrial organ culture, endometrial stroma, and glandular cells produce significant amounts of IP-10. Notably, IP-10 was detected in uterine cavity flushings throughout the menstrual cycle, and endometrial IP-10 had a direct functional ability to attract blastocyst trophoblast cells.

Recombinant IP-10, IP-10 generated from endometrium in vivo, and IP-10 produced by pRES2-EGFP all induced the migration of trophoblast cells through Matrigel and induced attraction in a “real time” attraction assay (as described in Materials and methods, Attraction/Chemotaxis Assay). This positive effect was specific for maternal IP-10 and embryonic CXCR3 interaction in models that are similar to the in vivo setting. The paradigm presented here is that endometrial IP-10 (and perhaps other chemokines) are produced throughout the menstrual cycle in anticipation of the attraction and adherence of the wandering blastocyst to the endometrium. It is tempting to speculate that this prolonged expression allows for an extended window of implantation in the course of the cycle.

The types of chemokines involved and their temporal expression by the non-pregnant endometrium are matters of debate in the literature. This debate may stem from different methods of detection (i.e. gene array screening, immunohistochemistry, or FACs analysis) or differences in the type of endometrium (curet- tage, isolated cultured cells, or “Pipelle” sampling as utilized in our study). However, a consensus exists that chemokines are produced by glandular epithelium, endometrial stroma, and leukocytes. The role of these chemokines has primarily been attributed to the recruitment of leukocytes, such as NK cells or macrophages, to the secretory endometrium. Interestingly, unlike decidual NK cells, which have previously been reported to secrete several chemokines, the non-pregnant, nonactivated endometrial NK cells do not produce chemokines [9].

The direct role of endometrial IP-10 to attract the human blastocyst is almost identical to the mechanism for the aposition of the goat blastocyst described by Nagaoka et al. [10]. Similar to the described human blastocyst, goat endometrium produces and secretes IP-10 to attract the blastocyst via interactions with CXCR3. Moreover, feto-maternal crosstalk between activated adhesion molecules is important for the next step of implantation.

The evidence provided here may have implications for the diagnosis and treatment of infertility in assisted reproduction programs. Uterine flushing can be considered useful for determining the levels of IP-10. Moreover, in cases of endometrial “insufficiency,” agents such as interferon gamma may be used to increase the level of IP-10 in infertile cases caused by endometrial defects.

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Conflict of interest

The authors declare they have no conflict of interest.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at doi:10.1016/j.placenta.2012.12.008.

References


