Placenta 34 (2013) 222–230

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

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

ARTICLE INFO

Article history:
Accepted 5 December 2012

Keywords:
Blastocyst attraction
Chemokines
Endometrium
IP-10
Trophectoderm

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.

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

* Corresponding author. Center for Human Placenta Research, Department of Obstetrics and Gynecology, Hadassah-Hebrew University Medical Center, Mt. Scopus, P.O. Box 24035, Jerusalem 91240, Israel. Tel.: +972 2 5844111; fax: +972 2 5815370.
E-mail address: simcha.yagel@gmail.com (S. Yagel).

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http://dx.doi.org/10.1016/j.placenta.2012.12.008

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% O_2 and 5% CO_2. 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 (DakoCytomation). Data from single cell events were collected using a standard FACSCalibur™ 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′-TGATGACATCAAGAAGGTGGTGAAG-3′; and anti-sense, 5′-TCCTTGGAGGCCATGTGGGCCAT-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 (2×10^5/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 ED_{50}. 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% O_2.

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 peroxidase 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 mounting 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 the study from our 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
Primers used for the detection of chemokine receptors and chemokines.

Chemokine	Sense primer	Anti-sense primer	Product (bp)
IL-8	TGACTTCCAAGCTGGCCGT	GGATGTTTGTTACCAAAGCATC	159
GCP	GGTCCTGTCTCTGCTGTGCT	ACTTCCACCTTGGAGCACT	128
IP-10	CCATGAATCAAACTGCGATT	TCAGACATCTCTTCTCACCC	228
MIG	AAGTGGTGTTCTTTTCCTCT	TTTCTTTTGGCTGACCTGTT	283
I-TAC	ATGAGTGTGAAGGGCATGG	GGATTTAGGCATCGTTGTCC	230
SDF-1	ATGAACGCCAAGGTCGTGGTCG	TGTTGTTGTTCTTCAGCCG	202
SDF-2	ATGAACGCCAAGGTCGTGGTCG	CGGGTCAATGCACACTTGTC	220
Mip1a	ACTGCAGGTCTCCACTGCTG	CTGCCGGCTTCGCTTGGTTA	214
Mip1b	ATGAAGCTCTGCGTGACTGT	CTTGCTTCTTTTGGTTTGGA	213
SLC	ATGGCTCAGTCACTGGCTCT	CCTTTCTTGCCAGTCTTGGA	347
ELC	TACTGGCCCTCAGCCTGCTGGTTCT	CGGCGCTTCATCTTGGCTGA	272