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Oxygen regulates human cytotrophoblast migration by controlling chemokine and receptor expression

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ABSTRACT

Introduction: Placental development involves the variation of oxygen supply due to vascular changes and cytotrophoblast invasion. Chemokines and their receptors play an important role during placental formation. Herein, the analysis of the chemokine/receptor pair CXCL12/CXCR4 and further chemokine receptors, such as CCR1, CCR7 and CXCR6 expression in human cytotrophoblasts was conducted.
Methods: Human cytotrophoblasts were examined directly after isolation or after incubation with different oxygen tensions and a chemical HIF-stimulator for 12 h with realtime PCR, immunoblot, immunohistochemistry. Conditioned media of placental villi, decidua, and endothelial cells was used for ELISA analysis of CXL12. Cytotrophoblast migration assays were conducted applying conditioned media of endothelial cells, a CXCL12 gradient, and different oxygen level. Endometrial and decidual tissue was stained for CXCL12 expression.
Results: An upregulation of CXCL12, CXCR4, CCR1, CCR7 and CXCR6 was observed after cytotrophoblast differentiation. Low oxygen supply upregulated CXCR4, CCR7 and CXCR6, but downregulated CXCL12 and CCR1. In contrast to the HIF associated upregulation of the aforementioned proteins, downregulation of CXCL12 and CCR1 seemed to be HIF independent. Cytotrophoblast migration was stimulated by low oxygen, the application of a CXCL12 gradient and endothelial cell conditioned media. CXCL12 was detected in endometrial vessels, glands and conditioned media of placental and decidual tissue, but not decidual vessels.
Discussion/conclusion: Taken together, oxygen supply and cytotrophoblast differentiation seem to be regulators of chemokine and receptor expression and function in human cytotrophoblasts. Therefore, this system seems to be involved in placental development, directed cytotrophoblast migration in the decidual compartment and a subsequent sufficient supply of the growing fetus.

1. Introduction

Human placentation requires trophoblast proliferation, migration, differentiation, and invasion. In the beginning of pregnancy, trophoblast cells 'plug' uterine arteries and minimize the maternal blood flow to the placenta, which leads to a physiologically low oxygen environment [1].The partial pressure of oxygen in the intervillous space and within the endometrium at this time is estimated to be as low as 18 and 40 mmHg [2,3], compared to the exposure of the placenta to the maternal blood which exhibits 90–100 mmHg at 10–12 weeks of gestation [4]. The differentiation process involves fusion to the syncytiotrophoblast, but also development into invasive, extravillous cytotrophoblasts which conquer the maternal vessels and lead the maternal blood into the intervillous space. Insufficient trophoblast invasion and remodeling of the uterine arteries in the first trimester can lead to increased blood pressure with high velocity of the blood flow, which can damage the placental architecture and subsequently be followed by ischemia due to impaired placental perfusion and disturbed pregnancy development [5,6].

Abbreviations: CM, conditioned media; CM EC, conditioned media of endothelial cells; DFX, desferrioxamine; GFM, growth factor media; HIF, hypoxia inducible factor; O_2, oxygen; SFM, serum free media.

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In addition, to the regulation of placental growth according to oxygen levels [7], chemokines and chemokine receptors can regulate human placentation. Human cytotrophoblasts express the chemokine receptors CXCR4, CXCR6, CCR1, CCR5, CCR7 and the ligands CXCL12, HCC-1, and CXCL16 [8–11]. CXCL12, CXCL16, and CCL21 stimulate cytotrophoblast migration *in vitro* [12].

When considering a link between chemokine and chemokine receptor expression with oxygen dependent signaling, it is important that chemokines/receptors, such as CXCL12 and CXCR4 are target genes of the hypoxia inducible factors (HIFs) [13]. It is known that HIFs mediate response to hypoxia [14] and are expressed in the placenta with expression peaking at 7–10 weeks of gestation [15,16], also showing dynamic modulation between the 9–12 week of gestation when the maternal blood flow is slowly establishing a circulatory network [17].

CXCR4 transcription is increased by low oxygen in several different cell types (e.g. monocytes, endothelial, or cancer cells) [18,19], which is paralleled by elevated chemotactic responsiveness to its ligand, CXCL12 [13]. Further, CXCR6 and CCR7 are also known to be involved in breast- and lung cancer cell migration and seem to experience an oxygen and HIF-dependent upregulation [20,21]. In contrast, CCR1 is scarcely expressed by cytotrophoblasts cultured in low oxygen [8].

In the present study, we aimed at investigating the effect of cell differentiation and oxygen tension on the chemokine and receptor pair CXCL12 and CXCR4 in human cytotrophoblasts. Further, the investigation of additional chemokine receptors, such as CXCR6, CCR1, and CCR7 were included to show an overall regulation pattern, which might be of importance for placenta development.

In addition, the source of CXCL12 at the maternal fetal interface was analyzed suggesting a CXCL12 gradient, which might influence the chemotactic behavior of cytotrophoblasts, which is further modulated by chemokine receptor expression and the existing oxygen supply.

We hypothesize that the regulation of chemokine and receptor expression due to changes in oxygen supply at the maternal–fetal interface and cytotrophoblast differentiation to the invasive, extravillous phenotype modulate their chemotactic responses and that specific CXCL12 expression as chemoattractant are implemented during this process. These regulatory events might be of great importance for placental development and growth.

2. Material and methods

2.1. Human tissue collection

Tissue collection was approved by the University's ethical board and the patients gave informed consent. Placentas from elective terminations of pregnancy (5–12 weeks) were prepared for further cytotrophoblast isolation and placenta villous explant culture. Additionally, these tissues (villi and decidua) and also endometrial samples throughout the menstrual cycle were fixed and embedded in paraffin [22]. Endometrial samples were obtained from regularly cycling women undergoing hysterectomy or endometrial biopsy for benign conditions. The samples were correlated to the proliferative ($n = 3$) or secretory phase ($n = 3$) (6 different women) evaluated by the cycle day and by a pathologist.

2.2. Cytotrophoblast isolation and culture

Cytotrophoblasts were isolated from the placentas ($n = 6$) as published before [23,24]. Placental villi were subjected to a series of enzymatic digests, which detached cytotrophoblasts progenitors from the villi cores. Afterwards purification with a Percoll gradient was performed. Cytotrophoblasts were cultured in serum free medium (SFM; Dulbecco's modified Eagle's medium, 4.5 g/l glucose (Sigma, St. Louis, MO, US) with 2% Nutridoma (Roche, Indianapolis, IN, US), 100 μg/ml penicillin/streptomycin, 1% sodium pyruvate, 1% HEPES and 1% gentamicin, UCSF Cell Culture Facility). Except for the chemotaxis and migration assays, remaining leukocytes were removed by using CD45 coupled to magnetic beads (Dynal beads, Invitrogen, Grand Island, NY, US) [25].

Cytotrophoblasts were maintained under standard tissue culture conditions ($20\%O_2/5\%CO_2/95\%$air) or placed in a Bactron anaerobic incubator (Sheldon Manufacturing Inc., Cornelius, US) with a $2\%O_2/93\%N_2/5\%CO_2$ environment for 12 h. Desferrioxamine (DFX, Sigma) at a concentration of 160 μM was used as a chemical HIF stimulator for 12 h [26].

For the differentiation assay, which provides information about the expression patterns of the invasive, extravillous phenotype, cytotrophoblasts were collected for protein analysis immediately after isolation and after 12 h of culture on Matrigel® (BD biosciences, Sparks, MD, US) in standard culture conditions [27].

For the analysis of the oxygen influence cells were collected for RNA and protein analysis after 12 h of culture in standard conditions (control value) or low oxygen condition or after treatment with DFX ($n = 6$).

2.3. Immunoblotting

The cells were lysed in a modified RIPA buffer (1% DOC, 0.1% SDS, 1% NP-40, 150 mM NaCl, 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10% glycerol) containing protease inhibitors (Pierce, Rockford, IL USA). Protein concentration was measured using D_C Protein assay kit (BIO-RAD, Hercules, CA, US). Equal amounts of proteins were separated on 10% Tris–HCl gels and transferred to nitrocellulose (BIO-RAD). The membrane was blocked with 5% BSA/PBS-Tween for 15 min and probed using the following antibodies: polyclonal rabbit anti-human CXCR4 (1 μg/ml; Abcam), polyclonal goat anti-human CXCL12 (0.1 μg/ml), monoclonal mouse anti-human, CCR7 (1 μg/ml), CXCR6 (1 μg/ml) (R&D Systems), polyclonal rabbit anti-human CCR1 (1 μg/ml; Abcam), mouse monoclonal anti-human HIF-2α (1 μg/ml; Novus Biologicals, Littleton, CO, US), rabbit polyclonal anti human HIF-1α (0.1 μg/ml; Bethyl Laboratories, Montgomery, TX, US) and monoclonal mouse-anti-human actin (0.1 μg/ml; Sigma). Lysates of a Jurkat cell line (ATCC, Manassas, VA, US) and HUVECs (ATCC) were the positive control. Non-specific mouse or rabbit IgG was used as negative control.

Enhanced chemiluminescence (ECL) detection reagents and Hyperfilm ECL (Amersham Biosciences Pittsburgh, PA, US) were used for visualization.

2.4. Reverse transcription-PCR/real-time PCR

RNA was extracted after lyzing cytotrophoblasts with Trizol®. For Reverse Transcription-PCR, AMV reverse transcriptase (Invitrogen) was used to produce cDNA. RTmastermix contained RT buffer, dNTPs (each 100 mM), RT-random primer and DEPC-treated dH2O ad 18 μL (High capacity cDNA Archive Kit 432217, Applied Biosystems, Foster City, CA, USA). CDNA corresponding to 50 ng total RNA was used as a template in the PCR reaction. This consisted of ABI MasterMix (Applied Biosystems, Carlsbad, CA, USA) and pre-designed CXCR4, CXCL12, CXCR6, CCR1 and CCR7 TaqMan® gene expression systems which included the primers (Applied Biosystems). ABI Prism 7900HT real-time quantitative PCR instrument detected the accumulation of the PCR product. The endogenous control was eukaryotic 18S rRNA (Applied Biosystems). The condition for the PCR reaction was chosen following the instructions (http://tools.invitrogen.com/content/sfs/manuals/cms_041280.pdf, page 18).

2.5. In vitro organ culture of placental anchoring villus explants

Organ cultures of placental anchoring villi ($n = 4$) were set up as previously described [28]. First trimester anchoring villi were obtained by microdissection and cut into pieces no thicker then 5 mm to ensure even distribution of the oxygen levels in the tissue [29]. The villi were then placed on a Matrigel (BD Biosciences, San Jose, CA, US) coated 12 mm Millicell-CM culture dish inserts (Millipore, Billerica, MA, US). These were cultured for 48 h in Ham's F-12/Dulbecco's modified Eagle's medium (1:1, vol/vol) containing antibiotics/antimycotics and 10% FCS (UCSF Cell Culture Facility). For another 48 h the explants were maintained under standard or low oxygen conditions, then fixed in 4% paraformaldehyde, washed in PBS, infiltrated with increasing concentrations of sucrose (5–15%) followed by OCT compound (Miles Scientific, Princeton, MN, US) and frozen.

2.6. Immunohistochemistry

Placenta villus explants ($n = 4$) were triple stained with CXCR4 or CXCL12 (both 10 μg/ml) (R&D Systems, Minneapolis, MN, US) primary antibody, a rat anti-human cytokeratin-7 (CK7) antibody (1:100; 7D3, UCSF) and nuclear stain (Vectashield mounting medium with DAPI, Vector Laboratories, Burlingname, CA, US). Secondary antibodies were FITC- and Rhodamine-conjugated (Jackson Immuno Research, West Grove, PA, US). As a negative control, sections were incubated with non-immune serum. Staining was detected with constant exposure times with a Leica CTR5000 upright microscope and a DFC480 color camera.

Decidual ($n = 6$) and endometrial ($n = 6$) paraffin sections (both from 6 different women) were processed using the manufacturers protocol (Vector) and CXCL12 (R&D Systems), vWF and CK7 (Dako, Clostrup, Denmark) antibodies.

2.7. Conditioned media of first trimester placental villi, decidua and endothelial cells

Placental villi and decidua from the same placenta ($n = 4$) were dissected in 2–3 mm^3 big pieces, weighed (wet weight) and cultured in SFM (1 ml/g). After 24 h the conditioned media (CM) was collected and cell debris removed by centrifugation. CM of uterine microvascular endothelial cells (CM EC; $n = 3$) (Cambrex, East Rutherford, NJ, US, now the company is LONZA) was generated by incubating 300,000 cells/ml in endothelial cell growth medium (EGM-2; Cambrex; growth factor medium, GFM) containing fetal calf serum, human epidermal growth factor,