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Arterial endothelial cytokines guide extravillous trophoblast invasion towards spiral arteries; an in-vitro study with the trophoblast cell line ACH-3P and female non-uterine endothelial cells

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Abstract

Introduction—Invasion of extravillous trophoblasts (EVT) is tightly linked to appropriate cell to cell contact as well as paracrine guidance of EVT by maternal uterine cells, conducted by a variety of locally expressed cytokines. Here we investigated the interaction of the first trimester trophoblast cell line ACH-3P with adult iliac arterial (AEC) and venous endothelial cells (VEC).

Methods—The impact of ACH-3P conditioned medium (Cdm), obtained at 2.5% and 21% oxygen, on endothelial cell viability (LDH-Assay) and network formation (Matrigel-Assay) was tested. We investigated cytokine expression of AEC- and VEC-Cdm and confirmed results with ELISA analysis, and investigated the influence of Cdm on ACH-3P proliferation and invasion. Additionally, direct co-culture experiments with ACH-3P and AEC on Matrigel were performed. A subset of experiments was verified with primary trophoblasts as well as with first trimester placenta in situ specimens.

Results—ACH-3P-Cdm significantly enhanced cell viability of AEC and VEC after 72 h. ACH-3P-Cdm at 2.5% oxygen stabilized endothelial network structures in Matrigel up to 24 h, similar to the effect of a direct co-culture of AEC and ACH-3P. AEC and VEC showed a similar pattern of secreted cytokines. However, elevated levels of cytokines secreted by AEC were found for GRO, IL-6, MMP-1 and uPAR. ELISA confirmed elevated concentrations of IL-6 and uPAR in AEC compared to VEC. ACH-3P and primary trophoblasts more likely invaded towards AEC-Cdm than towards VEC-Cdm. Addition of IL-6 to Cdm increased the invasion potential of both cell types. AEC- and VEC-Cdm reduced ACH-3P cell proliferation after 24 h of culture. IL-6 was highly expressed in uterine AEC compared to VEC as visualized by immunohistochemistry.

Discussion—The presented results clearly demonstrate that cytokines of both cell types, AEC and trophoblasts, differentially contribute to successful guidance and interaction in the process of trophoblast invasion.

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Conflict of interest

No competing financial interests exist.

Keywords

Placenta; Extravillous trophoblast; Invasion; Cytokines; Spiral arteries

1 Introduction

Trophoblast invasion is a critical step during the first trimester of human pregnancy. In normal, uncomplicated pregnancies extravillous trophoblasts (EVT) invade through the uterine interstitium (interstitial trophoblasts) into spiral arteries, subsequently line and remodel them (endovascular trophoblasts), and are thus responsible for successful establishment of maternal blood flow towards the placenta. Failure of this process leads to miscarriage or pregnancy disorders like intrauterine growth restriction (IUGR) [1–3].

The interaction between spiral arteries and EVT has been studied extensively [4,5]; however, the details of the molecular interactions between spiral arteries and trophoblasts still remain unclear. Assuredly, invasion is conducted by numerous cytokines, expressed at the fetal–maternal interface. These cytokines promote trophoblast motility in an autocrine and paracrine manner [6–8].

Compared to spiral arteries, uterine veins show low degrees of EVT infiltration with no modification of the walls of these vessels [9]. Until now, it has not been clarified, why there is a targeted EVT invasion into spiral arteries rather than into veins.

Approaches to study invasion processes of primary trophoblast in vitro are restricted due to the availability of first trimester placental tissue and to the lifespan of primary first trimester trophoblasts in culture [10]. The choriocarcinoma cell line ACH-3P, generated by fusion of first trimester trophoblasts and the choriocarcinoma cell line AC1-1, comprises both trophoblast subpopulations (villous and extravillous, i.e. HLA-G negative and positive) [11], and combines their characteristics with the proliferation potential of the choriocarcinoma cell line [10]. Several groups have already used ACH-3P and confirmed their potential as a cell line that can be used as surrogate for first trimester trophoblast [11–13].

In this study we investigated the interaction process of first trimester trophoblasts with maternal endothelial cells using the combination of ACH-3P cells and adult endothelial cells. We focused on the cytokine expression pattern of arterial and venous endothelial cells and investigated the differences between the two subpopulations. We hypothesized that cytokines, secreted by arterial endothelial cells, are more likely to attract trophoblasts compared to those secreted by venous endothelial cells.

2 Material and methods

2.1 Trophoblast cell line, primary trophoblasts and endothelial cells

ACH-3P is a fusion product of primary human first trimester trophoblasts (week 12 of gestation) with a human choriocarcinoma cell line (AC1-1). Cells were kindly provided by Gernot Desoye (Department of Obstetrics and Gynecology, Medical University of Graz, Austria). For detailed characterization see Ref. [10]. Cells were cultured in Dulbecco's

Modified Eagle Medium/Nutrient Mixture F- 12 (DMEM/F12, Life Technologies, Carlsbad, USA) supplemented with 10% fetal calf serum (FCS; GE Healthcare Life Sciences, Vienna, Austria) and antibiotics, and used in different passages (p57 – p66).

Placental tissue (gestational age 10 weeks, n = 4) was obtained after pregnancy termination for psychosocial reasons, informed consent was obtained from each woman with approval of the ethical committee of the Medical University of Graz.

Primary trophoblasts were isolated as previously described [10] and kindly provided by the Department of Obstetrics and Gynecology, Medical University of Graz (Austria). For immunohistochemistry invaded decidua basalis was selected, fixed in 4% v/v paraformaldehyde, embedded in paraffin and serial sectioned.

Human iliac arterial endothelial cells (AEC) and human iliac venous endothelial cells (VEC) were isolated from iliac artery and vein derived from the same healthy female adult organ donors by a previously described method [14] and cultivated in EGM-MV in 1% gelatin (PAN-Biotech, Aidenbach, Germany) coated flasks. The endothelial identity was confirmed by positive staining for the endothelial marker vWF and lack of markers against fibroblasts (CD90) and smooth muscle cells (smA and desmin) (data not shown). The purity of the isolated endothelial cells was 99% (CD90 and smA staining 1%).

Ethical approval was granted by the ethics committee of the Medical University of Graz.

Medium was changed every 2–3 days. All cells were cultivated under standard culture conditions (37 °C, 5% CO₂, 21% O₂). For specific experiments ACH-3P cells were also cultivated at reduced oxygen conditions (2.5% O₂).

2.2 Preparation of conditioned medium (Cdm)

Confluent ACH-3P cells were washed with HBSS (Life Technologies) and incubated with 12 ml DMEM/F12 (2.5% O₂ and 21% O₂) supplemented with 10% FCS and antibiotics. Control medium DMEM/F12 was prepared in parallel in culture flasks without cells. AEC (passage 2) and VEC (passage 2) were incubated with 12 ml EGM-MV medium (21% O₂), also supplemented with 10% FCS and antibiotics. Control medium EGM-MV was prepared in parallel without cells. After incubation for 48 h, Cdm and control medium were collected and aliquots were stored at –80 °C. Prior to application, Cdm and control medium were thawed and centrifuged at 300 × g for 10 min.

2.3 LDH-assay

AEC and VEC were seeded in a 96-well plate (6000 cells per well) and incubated for 72 h under standard culture conditions with ACH-3P-Cdm 2.5% and ACH-3P-Cdm 21% and unconditioned control medium (n = 12 of three independent experiments). Subsequently, medium were collected and centrifuged for 10 min at 300 × g. Supernatants were transferred to a 96-well plate in duplicates and incubated with the reaction mixture provided in the LDH Cytotoxicity Detection Kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions.

2.4 Matrigel assay

96-well plates (Thermo Fisher Scientific Inc., Waltham, USA) were coated with 40 μ l Matrigel (BD Biosciences, Franklin Lakes, USA) per well. Confluent AEC were washed with HBSS, detached with accutase (both from Life Technologies Carlsbad, USA), and seeded at a density of 10^4 cells per well. Cells were resuspended with ACH-3P-Cdm 2.5% and ACH-3P-Cdm 21%, or control medium to reach a total volume of 150 μ l per well ($n = 12$ of three independent experiments). Network formation was recorded using the cell imaging platform Cell-IQ-Analyzer 2004-01 (Chipman Technologies, Tampere, Finland). Tube length of endothelial networks was quantified after 6 h, 12 h and 24 h of incubation using the Cell-IQ-Analyzer software v.AN.1.9.0. (Chipman Technologies). Since VEC only showed poor network formation on Matrigel, they were excluded from further analysis.

2.5 Co-culture of AEC and ACH-3P on Matrigel

To investigate the direct influence of ACH-3P cells on AEC, 96-well plates were coated with 40 μ l Matrigel per well. Confluent AEC were harvested and seeded at a density of 10^4 cells/well. AEC were allowed to form networks at 37 °C under standard oxygen conditions. Confluent ACH-3P cells were labeled with CellTracker™ Green CMFDA (1:1000, Invitrogen) for 30 min at 37 °C and added to AEC (ratio EC: ACH-3P – 10,000:5000 cells/chamber) at different time points ($t = 0$ h, $t = 6$ h), to reach a total volume of 200 μ l per well ($n = 9$ of three independent experiments). Cells were observed and analyzed after 6, 12, 24 and 72 h using the Cell-IQ system.

2.6 Angiogenic protein analysis

The presence of angiogenic cytokines in AEC- and VEC-Cdm ($n = 9$ of three independent experiments) was determined using the Raybio Human Angiogenesis Antibody Array C Series 1000 (RayBiotech, Norcross, USA). 1 ml of respective Cdm was applied per membrane and membranes were processed according to the manufacturer's instructions. Chemiluminescent imaging was performed using the FlourchemQ system and signal densities were analyzed with AlphaView software version 2.0.1.1. (both from AlphaInnotech, Cell Biosciences, Santa Clara, USA). Ratios of the respective protein and the internal standard densities were determined, and protein signals from the AEC-Cdm were presented as fold change of VEC-Cdm, which was set to 1.

In addition, we selected the proteins with the highest densities (IL-6 and uPAR) for validation of the angiogenesis data using ELISA quantification (Quantikine Human basic Immunoassay, R&D Systems Inc., Biomedica, Vienna, Austria). Cdm were analyzed in triplicates according to the manufacturer's instructions.

2.7 Invasion assay

The cell invasion assay was performed using cell culture inserts with 8 μ m pore-sized polycarbonate membranes coated with a thin layer of polymerized collagen I in a 24-well plate (Merck KGaA, Darmstadt, Germany). AEC-Cdm, VEC-Cdm and control medium were placed in triplicates into the wells ($n = 9$ of three independent experiments). For respective experiments 10 ng/ml recombinant IL-6 (Preprotech, Rocky Hill, USA) was added to Cdm and control medium. ACH-3P cells and primary trophoblast cells were transferred

onto the filter of the insert (500,000 cells/insert). After 24 h incubation at 37 °C, non-migratory cells were removed from the upper side of the filter. Migratory cells were stained and removed from the filter with the extraction solution provided in the kit and optical density was measured using a microplate reader (Anthos, Krefeld, Germany).

2.8 Proliferation assay

ACH-3P cells were seeded in a 12-well plate (100,000 cells/well) and incubated with 1 ml of AEC-Cdm, VEC-Cdm or control medium for 24 h under standard culture conditions. After two washing steps with HBSS, cells were detached with accutase and counted with the Casy cell counter and analyzer (Roche Innovatis, Basel, Switzerland). With this assay the total number of viable cells as well as dead cells was measured. Since the culture conditions showed no effect on the rate of dead cells, the decrease of cell numbers at the end of the experiments could be directly linked to a reduced proliferation of cells (n = 9 of three independent experiments).

2.9 Immunohistochemistry

Immunohistochemistry was performed using the Ultravision LP detection system (Thermo Scientific, Fremont, USA) according to the manufacturer's instructions. Interleucin-6 (mouse IgG1, 5 µg/ml, Peprotech, USA) and Desmin (mouse IgG1, 111 µg/ml, Dako, Vienna, Austria) were diluted in antibody diluent (Dako). Sections were counterstained with Mayer's hemalaun and mounted with Kaiser's glycerol gelatin (Merck, Vienna Austria). Negative controls were incubated with the appropriate IgG fractions as isotype controls.

vWF (rabbit IgG, 7.1 µg/ml, Sigma–Aldrich, USA) and HLAG (mouse IgG1, 1 µg/ml, Exbio, Czech Republic) were applied for immunohistochemical double labeling (Multivision Polymer Detection system (MultiVision anti-rabbit/AP + anti-mouse/HRP polymers; Thermo scientific, Fremont, USA) according to the manufacturer's instructions.

2.10 Statistical analyses

Data are reported as means ± standard deviation (SD). Differences in conditioned medium among cell types (AEC, VEC, ACH-3P) and control were analyzed by one-way analysis of variance (ANOVA) with least significant difference post-hoc tests. Student's t-test was applied for Raybio protein expression assay, ELISA quantification and differences in ACH-3P/primary trophoblast invasion towards Cdm. In addition, non-parametric tests (Kruskal–Wallis, Mann-Whitney-U) were used to verify obtained statistical results and revealed no difference in the proposed statements. Statistical analysis was done using SPSS IBM Statistics 21. A p-value < 0.05 was considered as significant.

3 Results

3.1 Paracrine effects of ACH-3P Cdm on viability of AEC and VEC

ACH-3P-Cdm obtained at 2.5% and 21% oxygen significantly increased EC viability (measured as decreased LDH activity in the supernatant of EC cultures) in AEC (Fig. 1A, 2.5% oxygen: 1.85 fold ±0.04; 21% oxygen: 4.5 fold ±0.04) as well as in VEC cultures (Fig.

1A, 2.5% oxygen: 7.7 fold ± 0.04 ; 21% oxygen: 25 fold ± 0.03) after 72 h compared to standard control medium.

3.2 Paracrine effects of ACH-3P Cdm on endothelial network formation

ACH-3P-Cdm 2.5% and 21% significantly slowed down endothelial network formation after 6h compared with control medium as measured by endothelial tube length (Fig. 1B). However, subsequently ACH-3P-Cdm 2.5% showed a stabilizing effect on existing endothelial structures over time of culture (Fig. 1B). ACH-3P-Cdm 2.5% significantly stabilized tube length after 12 h (2.5%: $5929 \pm 123 \mu\text{m}$; control: $4405 \pm 375 \mu\text{m}$) as well as after 24 h (2.5%: $4170 \pm 156 \mu\text{m}$; control: $902 \pm 56 \mu\text{m}$). No significant differences could be detected between the two oxygen concentrations after 12 and 24 h of incubation. Iliac VEC did not develop networks on Matrigel; hence, no data can be shown for these cells.

3.3 Coculture of EC and ACH-3P cells on Matrigel

AEC were seeded on Matrigel and allowed to form network structures (Fig. 2A–D). In the coculture experiments fluorescently labeled ACH-3P cells, added at time point $t = 0$ h inhibited formation of endothelial network structures (Fig. 2E–H), whereas if added after 6 h ACH-3P cells incorporated into pre-existing endothelial networks and stabilized them up to 72 h (Fig. 2I–L). ACH-3P cells cultured without endothelial cells did not show any network formation on Matrigel (data not shown).

3.4 Differential secretion of angiogenic cytokines by AEC and VEC

AEC and VEC showed a similar pattern of secreted cytokines. However, significantly elevated levels of cytokines secreted by AEC were found for GRO (1.6 ± 0.2 fold), IL-6 (7.2 ± 1.5 fold), MMP-1 (1.6 ± 0.2 fold) and uPAR (1.8 ± 0.3 fold) compared to VEC (Fig. 3A–B).

3.5 Quantification of IL-6 and uPAR secretion by ELISA

The concentrations of IL-6 and uPAR in EC-Cdm were quantified using ELISA. AEC showed significantly elevated concentrations of IL-6 ($441 \pm 56 \text{ pg/ml}$; VEC: $109 \pm 114 \text{ pg/ml}$) (Fig. 3C) as well as uPAR ($2409 \pm 585 \text{ pg/ml}$, VEC: $1570 \pm 248 \text{ pg/ml}$) (Fig. 3D) compared to VEC. ELISA quantification clearly validated the results from the cytokine assay.

3.6 Paracrine effects of EC-Cdm on ACH-3P cell and primary trophoblast invasion

ACH-3P cells more likely invaded towards AEC-Cdm ($157\% \pm 12\%$) than towards VEC-Cdm ($62.9\% \pm 16\%$) or control medium. The addition of IL-6 significantly increased invasion of ACH-3P cells to $302\% \pm 12\%$ (AEC-Cdm) and $250\% \pm 18\%$ in VEC-Cdm. Migration towards standard control medium was set to 100% (Fig. 4A).

Primary trophoblasts more likely invade towards AEC-Cdm ($129\% \pm 10\%$) than towards VEC-Cdm ($105\% \pm 5\%$). The addition of IL-6 increased the invasion potential of primary trophoblasts significantly towards AEC-Cdm ($164\% \pm 19\%$), but not in VEC-Cdm ($135\% \pm 32\%$) (Fig. 4B).

3.7 Paracrine effects of EC-Cdm on ACH-3P cell proliferation

ACH-3P cells showed a reduced proliferation rate when cultivated in AEC-Cdm for 24 h ($237,660 \pm 51,983$ cells/ml) compared to cells grown in control medium ($620,000 \pm 50,000$ cells/ml) (Fig. 4C). Also VEC-Cdm reduced ACH-3P cell proliferation to $330,000 \pm 100,000$ cells/ml. There was no significant difference between the effects of AEC- and VEC-Cdm.

3.8 Expression of IL-6 in decidual arteries and veins in first trimester placental tissue

The comparison of serial sections with the respective immunodouble staining with vWF and Desmin enables a clear identification of decidual arteries and veins (Fig. 5 A,B,D,E). The presence of HLAG refers to invaded regions of decidual arteries (Fig. 5A). IL-6 was found strongly expressed in invaded arterial endothelial cells (Fig. 5C), while venous endothelial cells showed only low expression of the antibody (Fig. 5F).

4 Discussion

In this study we used an *in vitro* model system to study the interaction of adult endothelial cells from artery and vein with trophoblast, using iliac AEC and VEC and the trophoblast cell line ACH-3P. We mainly focused on paracrine factors, but also evaluated effects of direct cell-to-cell contact. *In vitro* model systems are necessary tools for investigating trophoblast invasion, despite the limitations of being an artificial system. Here we used endothelial cells from the iliac artery and vein as models for adult endothelial cells. Since trophoblasts invade into adult endothelial network structures *in vivo*, we refrained from using fetal endothelial cells like human umbilical vein endothelial cells (HUVEC). A subset of experiments was verified with primary trophoblasts as well as with first trimester placenta *in situ* specimens.

While expression of cytokines in first trimester trophoblasts has been extensively investigated [15–17], knowledge about paracrine factors secreted by uterine arterial endothelial cells is rare. Spiral arteries are considered to attract EVT in a targeted manner. Nevertheless, the cellular details behind this mechanism still remain unclear. Beyond that, little is known about trophoblast invasion of decidual veins. Therefore, we focused on differences in cytokine expression patterns of both, arterial and venous endothelial cells to identify, why trophoblasts more likely invade into uterine arteries than veins.

At the distal end of the cell column cytotrophoblasts change their phenotype towards a non-proliferative and instead invasive phenotype [6]. This change is mandatory for successful migration and invasion of EVT towards maternal tissues. Our data are in line with this switch in phenotype: ACH-3P cells reduce proliferation in presence of endothelial cell conditioned medium (AEC-Cdm as well as VEC-Cdm), suggesting that cytokines of AEC and VEC may also contribute to this phenotypic switch.

The invasion process of trophoblasts and the concomitant spiral artery remodeling has been studied extensively within the last few years. In the present study we demonstrate that ACH-3P and primary trophoblasts invade more likely towards AEC-Cdm than towards VEC-Cdm or control medium, suggesting a contribution of AEC cytokines to stimulate

trophoblast migration/invasion. In accordance with our data, it was reported that EVT show no or just a low degree of invasion into decidual veins [18]. We speculate that a particular cytokine pattern of AEC serves as chemoattractant for trophoblasts and contributes to a directed invasion process. Further investigation of AEC- and VEC-Cdm revealed higher amounts of GRO, IL-6, MMP-1 and uPAR in AEC-Cdm compared to VEC-Cdm. These cytokines may well be involved in angiogenesis and the invasion process of EVT.

GRO in the used array detects GROa, GROb and GROg, which are identical to CXCL1, CXCL2, and CXCL3, respectively. In tumor biology it was reported that down-regulation of CXCL-1 inhibits tumor invasion [19], which might be also true for trophoblast invasion in first trimester of pregnancy.

We further found strong signals of IL-6 in AEC-Cdm. IL-6 has been suggested to be a potent pro-angiogenic protein, stimulating proliferation and growth of endothelial cells and smooth muscle cells in vitro [20]. Nevertheless, the function in trophoblast invasion is still controversially discussed. While Jovanovic and Vicovac reported stimulation of trophoblast invasion and migration on the basis of HTR-8/SVneo cells [21], Champion et al. reported the contrary [22]. Our findings support the thesis, that IL-6 stimulates trophoblast invasion, as shown by primary trophoblasts as well as ACH-3P cells. In line with these findings, immunohistochemical staining of first trimester decidual sections revealed elevated expression of IL-6 in decidual arterial endothelial cells rather than venous endothelial cells.

Another strong signal in AEC was found for MMP-1, a member of a group of matrix-metallo-proteinases. The successful invasion process is tightly linked to the ability of trophoblasts to secrete matrix-metalloproteinases [23,24]. MMP-1 is commonly expressed in cells of the maternal-fetal interface, including EVT [25], cytotrophoblasts, syncytiotrophoblast [26] and decidual cells [27]. It has been reported, that decidual endothelial cells express a low amount of MMP-1 in preeclamptic decidual tissue [28].

We could also detect and validate the presence of the soluble form of uPAR in AEC-Cdm, which is also believed to play a central role in trophoblast invasion and migration. It has been found in invasive EVT [29], perivascular smooth muscle cells and endothelial cells [30].

Development of embryo and placenta is highly dependent on physiological changes of oxygenation throughout pregnancy [31]. In the first trimester, development of the embryo occurs at physiologically low oxygen concentrations (2.5% oxygen) [32,33]. We demonstrated that ACH-3P-Cdm obtained at both (2.5% and 21%) oxygen concentrations, enhanced the viability of AEC and showed a better effect for VEC, even after 72 h of incubation. It is commonly known that endothelial cell viability is strongly influenced by cytokines. Recently, we were able to demonstrate the life-prolonging properties of cytokines from avascular and blood vessel derived mesenchymal stromal cells of the placenta on placental endothelial cells [34].

We examined the ability of AEC to build network structures under the influence of ACH-3P-Cdm. ACH-3P-Cdm obtained at 2.5% oxygen stabilized existing endothelial network structures, supporting the hypothesis that cytokine expression under physiologically low

oxygen tensions positively influences the interaction with AEC. Additionally, we examined the influence of a direct cell-to-cell setup by performing co-cultures of AEC and ACH-3P cells. AEC were not able to build up network structures when co-cultured together with ACH-3P cells right from the very beginning. Referring back to the *in vivo* situation, this may explain why the endothelium of spiral arteries disappears after invasion of EVT. Interestingly, when ACH-3P cells were added 6 h after tube formation by AEC, they attached to existing network structures and stabilized them, resembling the strong interaction between the two cell types *in vivo*.

In conclusion, we were able to show that cytokines of both cell types, AEC and ACH-3P, contribute to successful contact and interaction in the process of trophoblast invasion (Fig. 6) and that IL-6 plays a crucial role in trophoblast invasion behavior as shown with primary trophoblasts and ACH-3P cells. The combinatory effect of AEC-Cdm in reducing proliferation and enhancing invasion towards AEC makes it tempting to speculate that the cytokines released from endothelial cells of spiral arteries attract EVT to only invade arteries rather than veins.

In the future it will be interesting to investigate, if spiral arteries in IUGR patients show less cytokine expression of the stated cytokines GRO, IL-6, MMP-1 and uPAR. It is tempting to speculate that insufficient cytokine attraction by spiral arteries could be an additional reason for impaired trophoblast invasion. However, it will be challenging to investigate the spiral artery cytokine expression profile *in vivo*.

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Abbreviations

AEC	arterial endothelial cells
Cdm	conditioned medium
EVT	extravillous trophoblast
IL	interleucin
uPAR	urokinase plasminogen activator receptor
VEC	venous endothelial cells

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A **B**

Fig. 1. Effect of ACH-3P-Cdm obtained at 2.5% and 21% oxygen on endothelial cell viability and network formation.

(A) Cdm obtained at 2.5% and 21% oxygen significantly enhanced cell viability of AEC, as well as of VEC after 72 h incubation compared with control medium. Results are shown as fold change of control, which was set to 1. *** $P < 0.001$ (ANOVA). (B) On Matrigel AEC were incubated with ACH-3P-Cdm 2.5%, 21% and control medium for 24 h. Compared to control medium, ACH-3P-Cdm 21% did not enhance endothelial network formation. AEC incubated with control medium showed the highest tube length after 6 h, however strongly

decreasing after 12 and 24 h. ACH-3P-Cdm 2.5% initially did not enhance network formation, however exhibited a stabilizing effect on existing network structures after 12 and 24 h of incubation. Data are means $\pm$ SD of three different cell isolations per cell type, each measured in triplicates. Median is represented as rhombus. * $P < 0.05$, ** $P < 0.01$ (ANOVA). AEC, arterial endothelial cells; Cdm, conditioned medium; SD, standard deviation; VEC, venous endothelial cells.

Fig. 2. Coculture of AEC and ACH-3P cells on Matrigel.

AEC were seeded on Matrigel and allowed to form networks (**A–D**). Fluorescent labeled ACH-3P cells were added (**E–L**) in a ratio of 2:1 (AEC:ACH-3P). When co-cultured from $t = 0$ h (**E–H**), ACH-3P cells interact with AEC and prevent them from building network structures. However, when added after 6 h (**I–L**), ACH-3P cells adhered to existing network structures and stabilized them up to 72 h. Pictures were taken after 6, 12, 24 and 72 h. Scale bar = 100 μm . AEC, arterial endothelial cells.

Fig. 3. Differences in AEC and VEC cytokine expression.

(A) Human angiogenesis antibody array. AEC- and VEC-Cdm from three different isolations and control medium were incubated with two membranes onto which 43 proteins are blotted in duplicates. The rectangles highlight proteins, which are significantly higher expressed in AEC-Cdm than in VEC-Cdm (GRO, IL-6, MMP-1, uPAR). Blots are representatives of three independent experiments. (B) Arrays were analyzed using densitometry and normalized to the respective internal control. Protein expression is shown as fold change of VEC-Cdm, which was set to 1. (C, D) Proteins IL-6 and uPAR were further quantified using ELISA. AEC secreted significantly elevated concentrations of IL-6 (C) and uPAR (D) compared to VEC. All results are shown with control values subtracted. Median is represented as rhombus. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Student's t-test). AEC, arterial endothelial cells; Cdm, conditioned medium; GRO, growth related oncogene; IL, interleukin; MMP, matrix metalloproteinase; uPAR, urokinase plasminogen activator receptor; SD, standard deviation; VEC, venous endothelial cells.

Fig. 4. Influence of AEC- and VEC-Cdm on trophoblast invasion and proliferation.

(A) ACH-3P cells were seeded into 8 μ m pore sized membranes and allowed to invade towards AEC- and VEC-Cdm. Optical density evaluation revealed a significant better invasion of ACH-3P cells towards AEC-Cdm compared to VEC-Cdm and control medium (set to 100%). Cdm supplemented with IL-6 increased ACH-3P cell invasion towards AEC- and VEC-Cdm. * $P < 0.05$; ** $P < 0.01$ (Student's t-test) (B) Primary trophoblasts invaded more likely towards AEC-Cdm than VEC-Cdm. Addition of IL-6 significantly elevated invasion abilities of primary trophoblasts in AEC-Cdm but not in VEC-Cdm. Control

medium was set to 100%. In Proliferation Assay $*P < 0.05$ (Student's t-test) (C) AEC-Cdm as well as VEC-Cdm reduced ACH-3P proliferation compared to control medium after 24 h incubation. Data are means $\pm$ SDs of three different cell isolations. Median is represented as rhombus. $***P < 0.001$; $**P < 0.01$ (Student's t-test). AEC, arterial endothelial cells; Cdm, conditioned medium; SD, standard deviation; VEC, venous endothelial cells.

Fig. 5. Interleukin-6 expression in decidual arteries and veins.

First trimester decidua with immunohistochemical staining/double staining (gestational age 10 weeks). Rows are composed of serial sections, first column is immunodoublestained for vWF (blue, serves here as a marker for vascular endothelial cells) and HLA-G (brown, serves here as marker for extravillous trophoblasts), second row is immunostained with Desmin (brown, serves here as marker for muscle cells), third row is immunostained for IL-6. Invaded uterine artery (**A**) versus non invaded uterine vein (**D**); Desmin staining enables the distinction between artery (**B**) and vein (**E**). IL-6 staining revealed strong expression in arterial endothelial cells (**C**) and less staining in venous endothelial cells (**F**). Scale bar: 50 μ m. No nuclear counterstain in (A; B) or nuclei were counterstained with Hemalum (B,C,E,F).

Fig. 6. Proposed expression of cytokines by EC and ACH-3P cells and their influence on the opposite cell type.

(A) Pathway of endothelial cell cytokine expression. Arterial endothelial as well as venous endothelial cytokines reduce ACH-3P cell proliferation. Trophoblast invasion is triggered by arterial cytokines while venous cytokines do not stimulate this process. **(B)** Pathway of ACH-3P cell cytokine expression. ACH-3P cells secrete cytokines that stabilize arterial endothelial network formation. Additionally, the viability of both endothelial cell types is enhanced by trophoblast cytokines. AEC, arterial endothelial cells; VEC, venous endothelial cells.