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A novel three-dimensional in vitro system to study trophoblast-endothelium cell interactions

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Abstract

Introduction: Pregnancy complications have been linked to improper trophoblast migration and failure of spiral artery transformation. Endothelial cells play an essential role in directing trophoblast migration and transformation, although by an unknown mechanism. We describe a novel in vitro model to evaluate endothelial–trophoblast interaction and signaling in a three-dimensional system.

Method of study: Immortalized human endometrial endothelial cell line and first trimester trophoblast cells were co-cultured. Endothelial transformation into vessel-like structures occurred in MatrigelTM OpenLab Image Analysis software was used to monitor labeled trophoblast migration and endothelium transformation. Cytokine/chemokine production was determined using Multiplex.

Results: Trophoblast migrates toward endothelial cells in Matrigel, aligns on top of the endothelium within 4–8 hours and achieves complete replacement of the endothelium by 72–96 hours. Lipopolysaccharide treatment damages the endothelium and disrupts endothelium–trophoblast interaction.

Conclusion: We report a novel three-dimensional in vitro and in vivo system of trophoblast-endothelium cell interaction. Significant changes in endothelial cells' phenotype are observed upon differentiation in Matrigel. These changes may be necessary for endothelium to direct trophoblast migration and transformation.

Keywords

endothelial cells; placental bed; spiral arteries; transformation of the spiral arteries; Trophoblast

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Introduction

Normal human placental development is characterized by trophoblast invasion into the decidua and subsequent transformation of spiral arteries to help support increased blood flow to the intervillous space.[1,2] During early stages of pregnancy, extravillous trophoblast migrate into the decidua (interstitial) surround the spiral arteries, migrate into the lumen (endovascular) and replace the endothelial cells lining the vessels, a process known as physiologic remodeling of the spiral arteries.[2,3]

This endovascular invasion mainly occurs during the first 20 weeks of gestation and is mediated by a subset of trophoblast cells known as interstitial trophoblast.[4] This major event involves the migration of the trophoblast along the spiral arteries as far as the first third of the myometrium, with partial loss of endothelium, disappearance of medial smooth muscle and replacement of the muscle-elastic media of the vessels with amorphous fibrinoid material, converting muscular vessels into flaccid sinusoidal sacs.[5,6]

The appropriate interaction between the trophoblast and the endothelium is critical for the proper blood support of the fetus.[7] However, the fundamental events in this process are not clearly defined.[8] Studies based on immunohistochemistry have shown that the trophoblast and endothelial cells transiently coexist in the walls of partially modified spiral arteries.[9] Other studies proposed that the trophoblast migrate along the vessel lumen and adopt an endothelial-like cell phenotype as they replace the endothelium.[10] The authors proposed that failure of the trophoblast to invade the blood vessels and acquire an “endothelium-like phenotype” contribute to pregnancy complications such as preeclampsia, intrauterine growth restriction, and spontaneous abortion.[11–15] These conflicting reports highlight the need for further studies of the mechanism regulating trophoblast migration toward the vessels; including the role of the endothelium in this process and the fate of the endothelium upon arrival of trophoblast. These questions are relevant for understanding the complexity of trophoblast-endothelial cells interaction and its role in normal and abnormal pregnancy.

The existing in vitro models of trophoblast and spiral artery interactions use perfusion systems in which isolated myometrial arteries are co-cultured with either primary trophoblast or trophoblast cells lines.[16,17] A major limitation of this model is that it uses third trimester placentas when the first interaction between the trophoblast and the endothelium occurs during the first trimester, and the variability between preparations.

In order to address outstanding questions in a more systematic manner, we have developed an in vitro model that allows the monitoring of trophoblast migration, study of endothelial-trophoblast interaction and the analysis of the factors involved in these processes. We demonstrate that endothelial cells induce trophoblast migration and that damage of the endothelium perturbs this process.

Materials and methods

Reagents

Lipopolysaccharide (LPS) isolated from *Escherichia coli* (0111.B4) was purchased from Sigma-Aldrich (St Louis, MO, USA). Interleukin-8 (IL-8), migration inhibitory factor (MIF), growth-related oncogene-α (GRO-α), monocyte chemoattractant protein-1 (MCP-1), Regulated on Activation, Normal T-cell Expressed and Secreted (RANTES), and interleukin-6 (IL-6) multiplex beads were purchased from Chemicon/Upstate (Temecula, CA, USA). Green fluorescent linker dye PKH67 and red fluorescent linker dye PKH23 were purchased from Sigma-Aldrich. Matrigel™ derived from EHS mice tumor was purchased from BD Biosciences (San Diego, CA, USA).

Cell culture

Human endometrial endothelial cells (HEECs) were isolated from endometrial specimens treated with 1 mg/mL of biotinylated UEA-1 (*Ulex europaeus*) lectin and immortalized by telomerase-mediated transformation.[18] HEECs were maintained in EMB-2 growth media purchased from Cambrex (East Rutherford, NJ, USA) supplemented with an additional 8% fetal bovine serum (FBS) purchased from Gemini Bio-Products (Woodland, CA, USA). The first trimester human extravillous trophoblast cell line HTR8, which from now on will be referred to as H8, was a gift from Dr Charles Graham (Queens University, Kingston, ON, Canada).[19] The monocytic cell line THP-1 was a gift from Dr P. Guyre (Dartmouth Medical School, Lebanon, NH, USA). Both H8 and THP-1 cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 media purchased from Gibco (Gaithersburg, MD, USA) supplemented with 10% fetal FBS. RPMI media was additionally supplemented with 10 mmol/L HEPES, 0.1 mmol/L minimum essential medium non-essential amino acids, 1 mmol/L sodium pyruvate, and 100 IU/mL penicillin/streptomycin, all purchased from Gibco.[20] All cell lines were maintained in 5% CO_2 at 37°C.

Isolation of first trimester primary trophoblast

Primary trophoblast cells were isolated from a first trimester placentas as previously described[21,22] with a few modifications. In brief, placentas were washed twice with cold Hanks' balanced salt solution (HBSS; Gibco) to remove excess blood and a final wash in Ham's-F12 (Sigma-Aldrich). Cells were removed from the membranes by scraping and incubated with a final concentration of 0.25% trypsin-EDTA (Gibco) digestion buffer and incubated at 37°C for 10 minutes with shaking. FBS (Gemini) was added at a concentration of 20% to inactivate the trypsin. This mixture was vortexed for 20 seconds, allowed to sediment and the supernatant was collected. The two previous steps were repeated twice and the collected supernatant was centrifuged at 800 g for 10 minutes. Contaminating red blood cells were removed by re-suspending the cellular pellet in 10 mL Ham's-F12, and layering this suspension over 5 mL of lymphocyte separation media (ICN Biomedicals, Inc., USA) and centrifuging the gradient at 1000 g for 30 minutes. The interface, containing the trophoblast cells, was removed by transfer pipette, washed in 10 mL Ham's-F12, and centrifuged at 800 g for 10 minutes. The pellet was then re-suspended and cultured in MEM D-valine supplemented with 10% human serum (Gemini Bio-Products, USA) at 37°C in 5% CO_2.

Endothelial cell tube formation

Undiluted Matrigel™ was plated into 24-well tissue culture plates at 300 μL/well and polymerized for 30 minutes at 37°C. HEECs were then stained with green fluorescent linker dye PKH67 and plated at 1.0×10^5 in 250 μL of EBM-2 + 10% FBS media. Formation of tube-like structures was monitored by fluorescent microscopy.

Trophoblast migration toward differentiated endothelial cells

Human endometrial endothelial cells (1.0×0^5) stained with green fluorescent linker dye PKH67 were seeded into a 24-well tissue culture plate and cultured 4–8 hours in 250 μL EBM-2 + 10% FBS until vessel formation occurred. Upon vessel formation, EBM media was removed and first trimester trophoblast cells, stained with red fluorescent linker dye PKH26, were seeded at 8.0×10^4 cells/well in 500 μL of RPMI 1640 + 10% FBS. The co-culture was incubated over a period of 24–96 hours. During this incubation, cell migration was tracked by fluorescent microscopy.

LPS treatment and collection of conditioned media

Human endometrial endothelial cells in Matrigel were plated alone or in co-culture with first trimester trophoblast and treated with either medium or LPS (5–50 μg/mL) for 24 hours. Supernatant was then collected and spun down at 800 g. Cell-free supernatant was then transferred to a fresh 1.5 mL Eppendorf tube and stored at −80°C until further use.

Extraction of lysate from Matrigel

To examine protein expression from cells plated in Matrigel, cells were extracted from the Matrigel with Cell Recovery Solution (CRS) (BD Biosciences, San Diego, CA, USA), according to the manufacturer instructions. Briefly, after medium was removed from the wells, cells were washed three times with 500 μL cold 1X PBS. CRS (500μL) was then added to each well, and transferred into cold 1.5 mL Eppendorf tubes. An additional 250 μL of CRS was added to each well to ensure all cells/Matrigel were collected. Tubes were then inverted three to four times, placed on a rocker at 4° for 30 minutes, and then kept on ice for an additional 30 minutes. The tubes were then spun down at 1000 g at 4°C for 5 minutes and the supernatant discarded. The cell pellet was then incubated with lysis buffer supplemented with 0.2 mg/mL phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA), for 25 minutes on a rocker at 4° and then centrifuged for 15 minutes at 12,000 g at 4°C. The supernatant was transferred to a fresh 1.5 mL Eppendorf tube with 2 μL PIC and 2 μL PMSF/tube and stored at −40°C until further use. Protein concentration was measured using the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA), according to the manufacturer's protocol. Matrigel background was subtracted.

Determination of cytokine/chemokine expression

Chemokine and cytokine production and release was assessed using a multiplex bead immunoassay (Chemicon/Upstate). Briefly, wells of a Multiscreen HTS 96-well filter plate (Millipore, Billerica, MA, USA) were loaded with either 50 μL of prepared standard solution, cell-free supernatant, or whole-cell lysate, followed by 25 μL of multiplex beads

and incubated on an orbital shaker at ±500 rpm for 2 hours in the dark at room temperature. After three washes, 25 μL of the biotin reporter antibody cocktail was added to each well, followed by a 1.5-hour incubation in the dark with constant shaking. Finally, 25 μL of Streptavidin-Phycoerythrin was added to each well, and after a final 30-minute incubation, the plate was read using the Bio-Rad 100 IS instrument (Hercules, CA, USA).

Statistical analysis

Data are expressed as mean ± S.D. Statistical significance ($P < 0.05$) was determined using one-way ANOVA with Bonferroni correction.

Results

Differentiation of endometrial endothelial cells promotes chemokine secretion

Previously, we described the ability of the telomerase-immortalized HEECs to differentiate in Matrigel and form tubes, resembling blood vessels.[23] As seen in Figure 1, cells remained as a monolayer in wells without Matrigel (**A**) while confirmation of in vitro tube formation of HEEC was observed in cells plated on Matrigel (**B**). As cell-to-cell communication is mediated mainly by cytokines and chemokines, our next objective was to characterize the cytokine profile of HEECs grown as a monolayer or after differentiation in Matrigel; we determined cytokine release by evaluating the cell-free culture supernatants using a multiplex bead assay. The cytokine profile of HEECs grown as a monolayer or differentiated in Matrigel is similar and is characterized by the production of chemokines such as IL-8, MCP-1, and GRO-α. However, upon differentiation and tube formation in Matrigel, HEECs released higher levels of IL-8, MCP-1, and GRO-α (Table I). Additionally, HEECs cultured in Matrigel released IL-6 and MIF, which were seen at very low levels in HEECs plated in a monolayer (Table I). Matrigel alone did not produce measurable levels of any of these cytokines.

Differentiated HEECs promote first trimester trophoblast migration

As differentiation of HEECs upregulates secretion of chemokines, we hypothesized that differentiated endometrial endothelial cells may promote trophoblast migration. Therefore, the first trimester trophoblast cell line HTR-8 (H8) was co-cultured with differentiated HEECs and migration evaluated. Fluorescent imaging revealed trophoblast migration is specific toward differentiated HEECs. As early as 2 hours, trophoblast cells began migrating toward the differentiated endothelial cells (Figure 2a, 2c), by 4 hours trophoblast cells are distributed along the walls of the tubes (Figure 2d, 2e), and by 8 hours trophoblast cells are found covering the endothelial cells (Figure 2f), while no trophoblast cells were observed in the lumen of the tubes after 48 hours (Figure 2g–2h).

Up to this time point, the co-culture appeared as a double cell layer formed by endothelial cells at the bottom and trophoblast cells at the top (Figure 3a and 3b). However, using double color montage, we observed that after 18–24 hours of co-culture, both the trophoblast and the HEECs had begun to come together as indicated by the appearance of bright yellow spots (Figure 3c). Complete migration of trophoblast cells and consequent transformation of endothelial cells was seen between 48 and 96 hours (Figure 3d).

These newly formed trophoblast-endothelial structures were found to be highly stable, as the integrity of the tubes structures was maintained for several days (up to 21 days) (Figure 4a) while non-transformed HEECs begin to breakdown after 48 hours (Figure 4b).

Next we evaluated whether the migration and transformation observed with the trophoblast cell lines is also observed with primary cultures of trophoblast cells isolated from first trimester placentas. As shown in Figure 4c, after 24 hours of co-culture with differentiated HEECs, first trimester primary trophoblast cells migrate on top of differentiated HEECs and the double color was also observed after 48 hours, as seen with the trophoblast cell line (Figure 4d).

Migration of monocytes (THP-1) toward trophoblast-HEEC tubes

To evaluate the specificity of the migratory effect induced by HEECs on first trimester trophoblast cells, we incubated differentiated HEECs with the THP-1 monocytic cell line. Following 24 hours of co-culture, THP-1 cells accumulated at the top of the Matrigel forming a single clump of cells (Figure 5a). Similarly, when we co-cultured endothelial cells with epithelial ovarian cancer cells, no migration of the cancer cells was observed confirming the specificity of the endothelium-induced trophoblast migration (data not shown).

However when THP-1 cells were added to the transformed trophoblast-HEEC tubes, monocytes migrated toward the lining of the lumen and distributed parallel to the trophoblast-HEEC cells. It is important to note that contrary to what we observed with trophoblast migration, monocytes did not cover the walls of the tubes; their distribution was always at the lumen, lining the cellular structure (Figure 5b).

Transformation of endothelial cells by trophoblast shifts cytokine production

The next step was to characterize the cytokine profile of the transformed trophoblast-endothelial tubes. Supernatants from HEEC alone, trophoblast alone, or co-cultured were collected after 48 hours and cytokines levels analyzed by a multiplex bead immunoassay. The transformed co-culture showed a similar cytokine profile as those expressed by HEEC and trophoblast; in addition, the levels of secretion were significantly higher than levels observed in the supernatant of either the trophoblast or HEEC cells alone. Endothelial cells produce high levels of MCP-1 and IL-8 while the levels of GRO-α and IL-6 are approximately in the same range as compared to the trophoblast alone. Following transformation, the levels of all these cytokines were significantly higher than those produce by the single cell cultures (Table II).

Effect of LPS stimulation on HEEC cells

To determine the effect of pathological conditions, such as the presence of infection, on trophoblast-endothelium interaction, we treated endothelial cells with bacterial LPS. Differentiated (Matrigel) and undifferentiated (monolayer) HEECs were cultured in the presence or absence of LPS at 5 or 50 μg/mL for 24 hours. As shown in Fig. 6, differentiated HEECs lose their structure in a dose-dependent manner, while no morphological changes were seen in undifferentiated HEECs cells.

We then evaluated the cytokine profile on these cultures; treatment with 50 μg/mL LPS increased IL-8, MCP-1, IL-6, and GRO-α production in differentiated and undifferentiated HEECs (Figure 7a, 7b). However, a differential response in MIF levels was seen in endothelial cells cultured in Matrigel. Differentiated HEECs stimulated with 50 μg/mL LPS cells showed a 2.1F decrease in MIF production, correlating with breakdown of the tube-like structures; while MIF levels further increased in cells grown in a monolayer (Figure 7c).

Treatment of trophoblast cells with 50 μg/mL LPS for 24 hours did not affect the cytokine profile (Figure 8a). However, when the same concentration of LPS was added to the transformed trophoblast/HEECs culture, a significant cytokine response was observed, similar to endothelial cells alone treated with LPS, characterized by high levels of GRO-α, IL-8, and MCP-1 (Figure 8b).

Interestingly, transformed cultures after 5 days, a time when HEECs alone are not viable, had high levels of cytokine production; however, if we stimulated the cultures with 50 μg/mL LPS, only IL-8 showed a significant increase (Figure 8d), suggesting that the transformed cells maintain some of the original HEECs capacity to respond to LPS, a characteristic that is not observed in the long-term trophoblast cells (Figure 8c).

Effect of LPS stimulation on endothelium/trophoblast transformation

Next we determined if treatment of endothelial cells with LPS would affect trophoblast migration and or transformation of the tubes. HEECs cells differentiated in Matrigel were treated with LPS (50 μg/mL) for 24 hours. Afterwards, labeled trophoblast cells were added to the culture, and migration and transformation was monitored by fluorescence microscopy. Trophoblast migration was observed in both LPS-treated and non-treated HEECs; however the distribution of the trophoblast in the culture was different in the LPS-treated group. While in the control group, as described above, the trophoblast migrated toward the endothelium and distributed on the surface of the endothelial cells (Figure 9a), in the case of the LPS-treated group, random trophoblast migration was observed and the trophoblast clumped within the lumen (Figure 9b).

Discussion

Proper characterization of endothelial-trophoblast interaction is essential in understanding the processes involved implantation and establishment of normal placentation. In the present study, we describe a novel in vitro system that enables further characterization of the mechanisms involved in endothelium-trophoblast interactions. Using this model, we demonstrate that trophoblast cells migrate in a specific manner toward the endothelium.

Our findings suggest that the endothelium plays a central role in trophoblast migration. We have found that upon differentiation, unstimulated HEECs express and secrete high levels of chemokines IL-8 and GRO-α, suggesting these cells have the capacity to induce trophoblast migration. Indeed, recent data has shown the chemokines CX3CL1 and CCL7 are expressed by endometrial epithelial cells, and trophoblast cells, in proximity, express the receptors for these chemokines suggesting endothelial chemokines may orchestrate trophoblast migration.[24] This was observed in our in vitro model where first trimester trophoblast migrate toward

HEECs in a specific manner, and trophoblast distribution follows the same pattern of the existing endothelium.

Crocker et al.[25] have suggested that uterine endothelial cells may become more receptive for trophoblast invasion by undergoing a process of conversion, which then may lead to the transformation process. This process of 'conversion' is observed in the HEECs cultured in Matrigel as seen by a significant increase in the production and secretion of chemokines.

Interestingly, treatment of the endothelial cells with LPS although had an effect on cytokine profile, it did not inhibit trophoblast migration. However, a major effect of LPS stimulation was disruption of the structure of the endothelium and also changed the distribution and organization of the trophoblast. Based on these results we could postulate that the integrity of the endothelium may not be required for migration of the trophoblast. Other cells present at the implantation site, such as NK cells, may be more relevant,[24] but it is critical for the adequate distribution of the invading trophoblast and the final transformation. As shown in Figure 9, when LPS damages the endothelium, we find clumps of trophoblast cells at the lumen of the tubes or in areas deprived of endothelial cells, very similar to what is observed in pregnancy complications such as preeclampsia.[26,27]

The significant increase on MIF expression observed in the cultures of endothelial cells treated with LPS correlates with a recent report by Chaiworapongsa et al.[28] who found that intra-amniotic infection in women with preterm labor and preterm PROM was associated with a significant increase in median amniotic fluid MIF concentration. The authors concluded that intra-amniotic infection and preterm parturition, but not term parturition, are associated with a significant increase in amniotic fluid MIF concentrations.[28] Our in vitro studies suggest that endothelial cells stimulated by bacterial products, such as LPS, may represent an important source of MIF expression.

Ashton et al. showed that introduction of first trimester trophoblast cells into segments of spiral artery leads to endothelial cell apoptosis. The authors suggested that the transformation of the spiral arteries involves the induction of cell death of the vascular smooth muscle cells and the endothelium by the trophoblast.[29] Although we do see an increase in detached endothelial cells upon the arrival of trophoblast, using the double-labeled cell model, we clearly noted a close localization of trophoblast and endothelial cells. Therefore, we seek to further determine whether the endothelium-like phenotype described by Zhou et al.[10] is the result of fusion of the invading trophoblast and the local endothelium or the result of phagocytosis. We can observe this characteristic in the long-term culture where the response of the transformed tubes follows some of the same characteristics of the original endothelium, although endothelial cells alone only survive in the culture for a maximum of 48 hours.

Regulation of trophoblast invasion must be maintained for proper implantation and placental growth, as many pregnancy complications are associated with either shallow (i.e. pre-eclampsia) or excessive invasion (accrete).[2,30–32] Invasion of the trophoblast into maternal tissue and subsequent transformation is an intricate process that is regulated by multiple factors, and the site of implantation is populated with immune cells and various cell types

that help regulate this process.[33,34] Due to the unique characteristics of human pregnancy, there is not equivalent animal system that could be used to study the cellular mechanism underlying the process of spiral transformation. The models described in this study may allow recreating and characterizing many of these cellular interactions.

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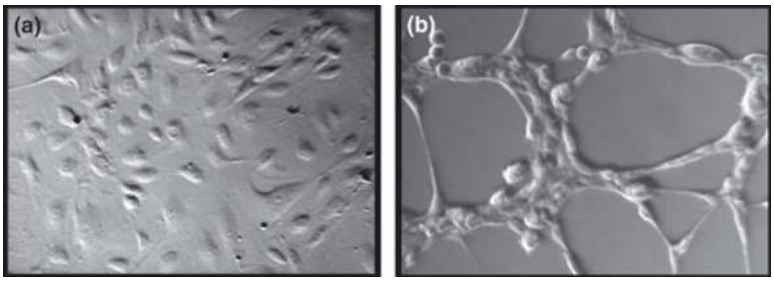

Figure 1. Endothelial tube formation.

Light microscopy images after overnight incubation of immortalized human endometrial endothelial cells (1.0×10^5) grown as a monolayer, (**a**) or in Matrigel-coated wells (**b**). 10× magnification.

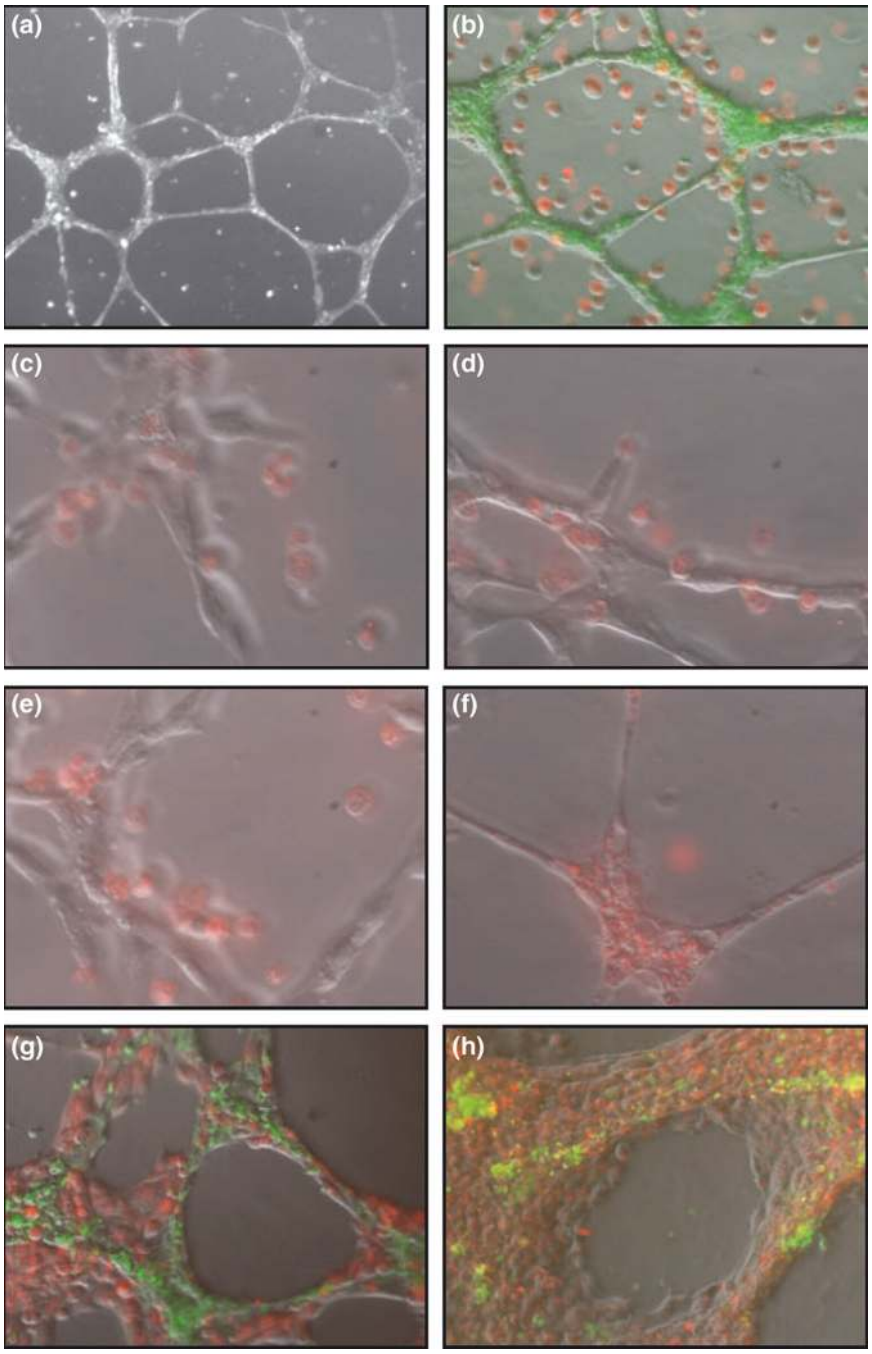

Figure 2. Trophoblast migration in the three-dimensional Matrigel system toward differentiated endothelial cells.

Human endometrial endothelial cells were plated in Matrigel overnight (1.0×10^5) and then co-cultured with PKH26 labeled first trimester trophoblast (8.0×10^4) (red). (**a**) Low magnification (25×) of the tubes; (**b**) trophoblast cells added to the culture migrate toward the endothelial cells (25×). (**c-h**) Sequential migration of trophoblast cells (100×) over a period of 2 hours (**c**), 4 hours (**d** and **e**) and 8 hours (**f**), 24 hours (**g**) and 48 hours (**h**).

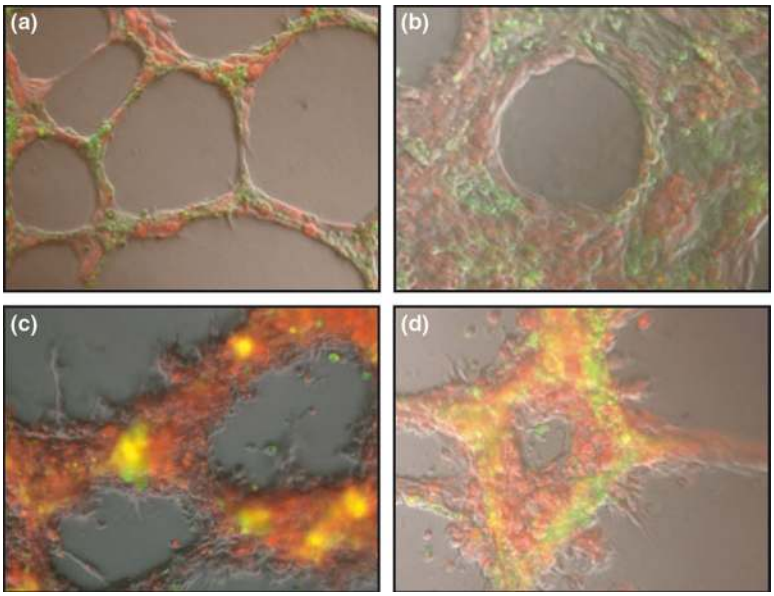

Figure 3. Trophoblast-endothelium interaction.

Sequential distribution of trophoblast and human endometrial endothelial cells in the three-dimensional Matrigel system can be observed by labeling each cell type with a different fluorescent dye. (**a**) Trophoblast cells reached the walls of the tubes (red) and distribute between and on top of the endothelial cells (green). (**b**) Appearance of trophoblast cells (red) together with endothelial cells (green). (**c** and **d**) Composite picture of red fluorescently labeled trophoblast cells and green endothelium reveals areas of yellow color product of the combination of red and green. 10× magnification.

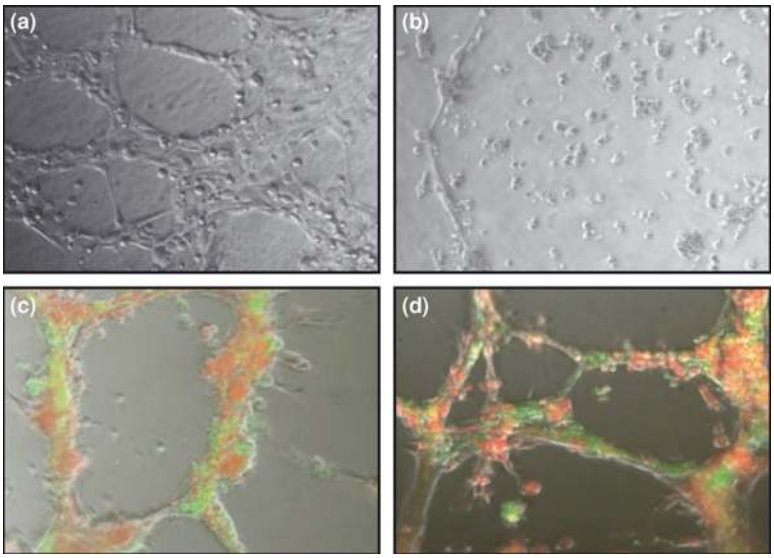

Figure 4. Co-culture of human endometrial endothelial cells (HEECs) and trophoblast maintain endothelial cell structure.

HEECs were plated in Matrigel overnight (1.0×10^5) and then co-cultured with first trimester trophoblast (8.0×10^4) for a period of 21 days. Tubes integrity remained intact in the wells containing transformed trophoblast-endothelial cells (**a**); HEECs cultured alone began to breakdown after 48 hours (**b**). Primary trophoblast migration in the three-dimensional Matrigel system. HEECs were grown in Matrigel overnight (1.0×10^5) and then co-cultured with PKH26 labeled primary culture of first trimester trophoblast (8.0×10^4) over a period of 24 hours (**c**) and 48 hours (**d**). 10× magnification.

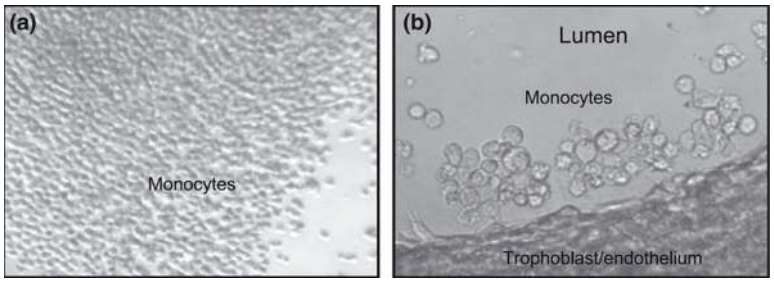

Figure 5. Specificity of the endothelium-induced migration.

Human endometrial endothelial cells were grown in Matrigel overnight (1.0×10^5) and then co-cultured with THP-1 cells and migration monitored by light microscopy. (**a**) THP-1 monocytes remained at the top of the well and did not show any migratory effect. 10× magnification (**b**) THP-1 cells incubated with transformed trophoblast/endothelium tubes migrate and distribute on the lumen of the tubes. 25× magnification.

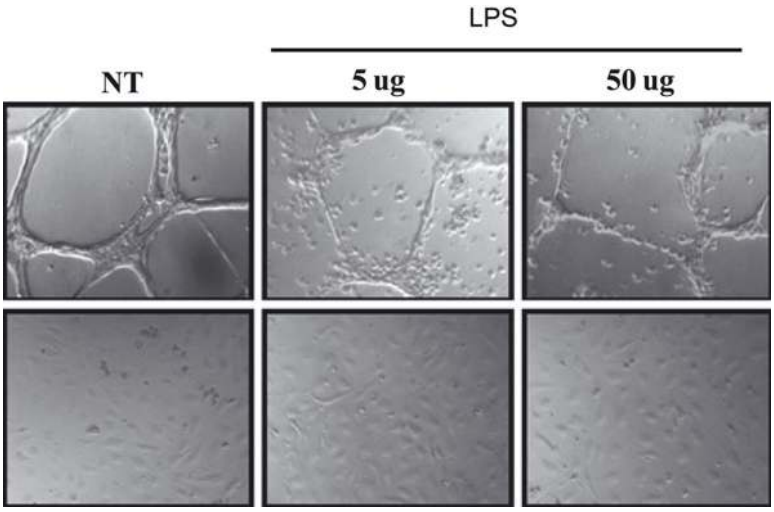

Figure 6. Effect of lipopolysaccharide (LPS) on differentiated and undifferentiated endothelial cells.

Light microscopy of human endometrial endothelial cells (HEECs) plated with Matrigel (top panel) or without Matrigel (bottom panel) and treated with LPS at 5 or 50 μg/mL for 24 hours. HEECs cultured in Matrigel treated with LPS lose their structure in a dose-dependent manner (**top panel**), while cells in monolayer do not show any morphological change on the presence of LPS (**bottom panel**).

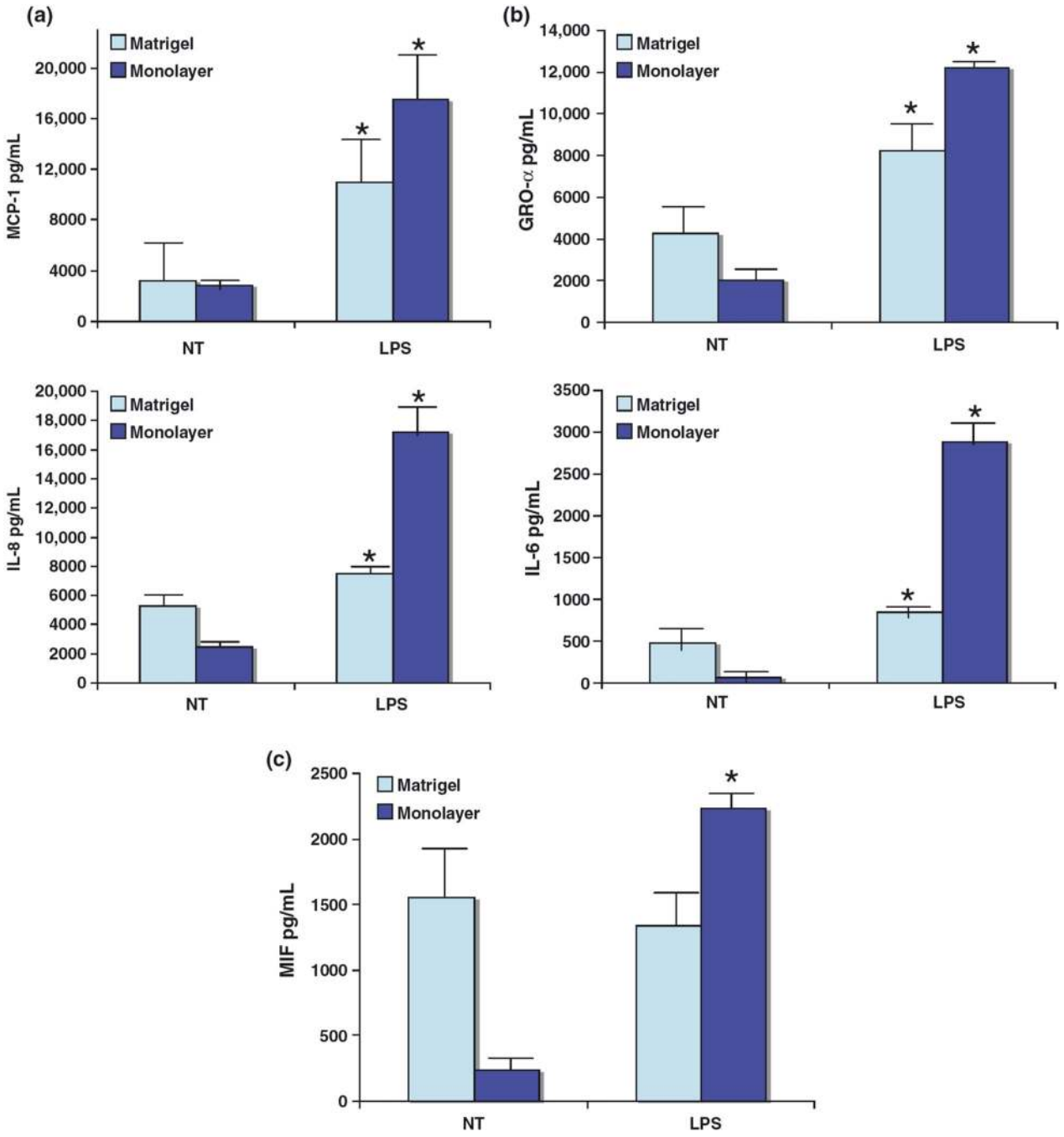

Figure 7. Human endometrial endothelial cells (HEECs)' cytokine profile in response to lipopolysaccharide (LPS) treatment.

Differentiated (Matrigel) and undifferentiated (monolayer) HEECs were treated with 50 μg/mL LPS for 24 hours after which cell-free supernatant was collected and cytokine/chemokine secretion analyzed using a multiplex bead assay. Stimulation with LPS significantly increased levels of MCP-1, IL-8 (**a**), GRO-α, and IL-6 (**b**) in both groups of cells as compared with the no treatment (*$P < 0.05$). However, a differential response was

seen in the levels of migration inhibitory factor (**c**) in cells cultured in Matrigel as compared with the no treatment (*P < 0.05).

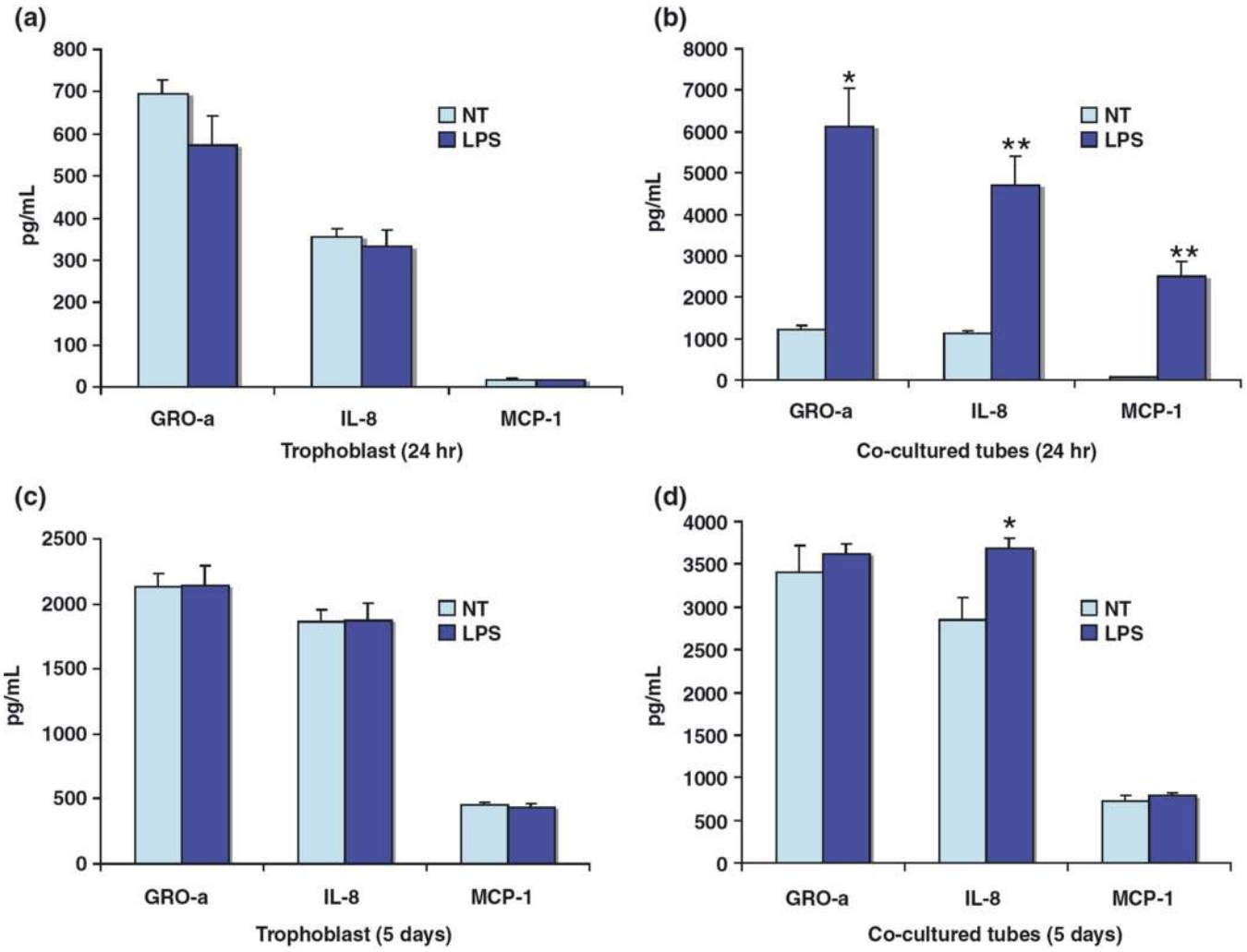

Figure 8. Long-term response of transformed trophoblast/endothelial tubes to lipopolysaccharide (LPS).

Both short-term and long-term cultured trophoblast cells stimulated with 50 μg LPS do not show any change on cytokine production (**a** and **c**); however, stimulation of short-term co-cultured tubes (24 hours) with 50 μg LPS showed a significant increase in GRO-α, MCP-1, and IL-8 secretion (**b**). The long-term co-cultured tubes (5 days) produced high levels of cytokines but only IL-8 secretion increased following LPS treatment (**d**). *P < 0.05 **P < 0.01 as compared with the no treatment control group.

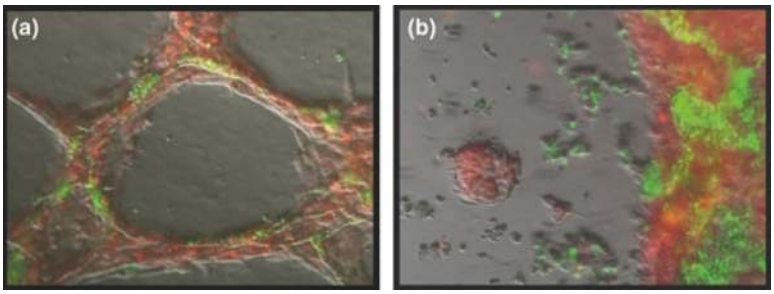

Figure 9.

Lipopolysaccharide (LPS) disrupts trophoblast migration toward differentiated human endometrial endothelial cells (HEECs). HEECs were plated (1.0×10^5) with Matrigel and treated with 50 μg/mL LPS for 24 hours and migration monitored by fluorescent microscopy. (**a**) Trophoblast co-cultured with non-treated endothelial cells migrated and aligned on top of the tubes. (**b**) Trophoblast co-cultured with LPS treated HEECs began to clump within the lumen of the tubes.

Table 1.

Secretion of chemokines by differentiated and undifferentiated endothelial cells

Chemokines	Monolayer (pg/mL)	Matrigel (pg/mL)	Fold difference
IL-6	60.27	1063	17.6
IL-8	2479	10403	4.2
MIF	238	2307	9.7
MCP-1	2868	9270	3.2
GRO-α	2003	8072	4

Table 2.

Secretion of chemokines by differentiated endothelial cells, first trimester trophoblast, and co-cultured tubes[a]

Chemokines	Endothelial (pg/mL)	Trophoblast (pg/mL)	Transformed endothelial-trophoblast (pg/mL)
IL-6	324.7 ± 13.6	211.7 ± 18.4	522.1 ± 26
IL-8	4536.5 ± 179.9	2586.9 ± 252.7	6691.6 ± 260.6
GRO-α	4595.6 ± 425.3	5476.7 ± 551.5	7921 ± 539.9
MCP-1	2711.7 ± 165.7	279.9 ± 33.7	3641.7 ± 307.7

[a]Chemokine secretion was determined using a multiplex bead immunoassay. Cell-free supernatants from endothelial cells, first trimester trophoblast, and co-cultured cells were analyzed following 48-hour incubation in the medium. Data represent three different experiments.