RESEARCH ARTICLE

Role of vitamin D in cell-cell interaction of fetal endothelial progenitor cells and umbilical cord endothelial cells in a preeclampsia-like model


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Submitted 1 April 2019; accepted in final form 22 May 2019

Brodowski L, Schröder-Heurich B, Hubel CA, Vu TH, von Kaisenberg CS, von Versen-Höynck F. Role of vitamin D in cell-cell interaction of fetal endothelial progenitor cells and umbilical cord endothelial cells in a preeclampsia-like model. Am J Physiol Cell Physiol 317: C348–C357, 2019. First published June 5, 2019; doi: 10.1152/ajpcell.00109.2019.—Maternal endothelial dysfunction is a central feature of preeclampsia (PE), a hypertensive disorder of pregnancy. Factors in the maternal circulation are thought to contribute to this endothelial dysfunction. Although understudied, factors in the fetal circulation may influence fetal endothelial cell interactions with endothelial progenitor cells as critical steps in placental angiogenesis. We hypothesize that cell-cell interactions that are important for pregnancy health are impaired by fetal serum from PE pregnancies and that 1,25(OH)2-vitamin D3 attenuates the negative effects of this serum on cell function. We tested the ability of fetal cord blood-derived endothelial progenitor cells [endothelial colony-forming cells (ECFCs)] to invade into established monolayers and capillary tube-like structures of human fetal umbilical venous endothelial cells (HUVECs), while in the presence/absence of fetal cord serum from uncomplicated or PE pregnancies, and tested the ability of 1,25(OH)2-vitamin D3 to modulate the serum-mediated effects. PE cord serum reduced the invasion of fetal ECFCs into HUVEC monolayers or tube networks. Vitamin D attenuated these effects of PE serum on endothelial functional properties. Immunocytochemical studies revealed involvement of VE-cadherin contacts in interactions between ECFCs and mature fetal endothelial cells. PE cord serum reduces the ability of fetal endothelial progenitor cells to transmigrate to fetal endothelial cell networks. Physiologic concentrations of vitamin D reverse these PE serum-mediated effects. These data appear consistent with lines of evidence that vitamin D has antipreeclampsia effects.

fetal endothelial progenitor cells; offspring health; preeclampsia; vitamin D

INTRODUCTION

Preeclampsia (PE), a pregnancy-specific hypertensive disease, is a significant cause of maternal and fetal morbidity and mortality (14). Impaired placentation is involved in the pathophysiology of the disease (43, 45) and manifests as suboptimal placental vascularization with deficits in the interaction of endothelial and trophoblast cells (43). Maternal endothelial dysfunction (45), the characteristic clinical feature of PE, is reflected by increased numbers of circulating endothelial cells (22) and widespread endothelial damage (44). Exposure of endothelial cells in culture to plasma or serum from women with PE alters endothelial function in ways that mimic the pathophysiology of PE, suggesting a role for circulating factors in development or progression of the disease (9).

Focusing on the offspring these changes seem to trigger a fetal response with the development of endothelial dysfunction, similar to what occurs in the mother. Prenatal exposure to PE has been reported as an independent risk factor for long-term cardiovascular morbidity of the offspring, e.g., stroke (14, 27, 28, 37, 39). School age children and adolescents who were exposed to a PE intrauterine environment show higher systolic and diastolic blood pressure levels (14, 17, 39, 50) and increased stiffness in the pulmonary and peripheral vascular system (10, 18).

Endothelial progenitor cells (EPCs) contribute to angiogenesis and vasculogenesis and are markers of endothelial regeneration capacity (2). Both reduced circulating numbers and impaired function of EPCs have been reported in patients with cardiovascular diseases (29) including PE (11). Recent data point to the ability of human fetal EPCs, but not fetal differentiated endothelial cells, to transmigrate to the maternal bloodstream and home to locations of maternal uterine vasculogenesis, suggesting a role of fetal EPCs in maternal endothelial function during pregnancy (48). We previously reported impaired fetal EPC and human umbilical vein endothelial cell (HUVEC) functional capacity in preeclamptic pregnancies (9).

Besides its well-known role in calcium homeostasis and bone metabolism, vitamin D is involved in the regulation of the cardiovascular and the immune system (6, 13, 19, 52). Furthermore, vitamin D deficiency is linked to increased all-cause and cardiovascular disease mortality, cardiovascular risk factors (30), as well as PE risk (7).

We hypothesize that inhibitory effects on cell-cell interactions between EPCs and mature endothelial cells can be demonstrated under PE-like conditions and that 1,25(OH)2-vitamin D3 can inhibit these effects.

MATERIAL AND METHODS

This study was performed at the Department of Obstetrics and Gynecology, Hannover Medical School (Germany). The Ethical Committee at Hannover Medical School approved the study, and informed written consent was obtained from each participant.

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**Study participants.** Six healthy women with uncomplicated, normotensive pregnancies (controls) and seven women with PE provided umbilical cord and cord blood samples. All had singleton pregnancies. Women with uncomplicated pregnancies were normotensive and without proteinuria throughout gestation and delivered healthy babies. Patients with PE had gestational hypertension and proteinuria beginning after 20 wk of pregnancy with resolution of clinical symptoms postpartum. Gestational hypertension was recognized as an absolute blood pressure of ≥140 mmHg systolic and/or ≥90 mmHg diastolic after 20 wk of gestation. Proteinuria was defined as ≥300 mg per 24-h urine collection, ≥2+ protein on a voided urine sample, ≥1+ protein on a catheterized urine specimen, or a protein-creatinine ratio of ≥0.3. All patients were nonsmokers by self-report and were without clinical history of preexisting renal, vascular, or metabolic disease. Patients were matched for gestational age, body mass index (BMI), and race. Clinical and demographic data describing these pregnant subjects, all of whom delivered at Hannover Medical School, are presented in Table 1.

**Blood sample collection.** Umbilical cord venous blood was drawn into sterile collection tubes for serum and EDTA plasma, immediately after delivery. Serum tubes were centrifuged between 30 min and 1 h after collection, for 5 min at 2,000 g at room temperature. The EDTA-containing tubes were centrifuged at 400 g for 10 min within 1 h of delivery. The serum and plasma were stored at −80°C for later use. The EDTA anticoagulated blood was used for isolation of blood cells. Once all samples were acquired, two separate pools of serum were created, namely an uncomplicated pregnancy and a PE pool. The PE and normal pregnancy pools were created by combining equal volumes of samples/pool group, n = 7 and n = 6, respectively. The serum pools were used for the functional in vitro assays at a dilution of 5% vol/vol in culture media.

**Endothelial colony-forming cell isolation and culture.** Endothelial colony-forming cells (ECFCs), also referred to as late-outgrowth EPCs, are a subpopulation of EPCs that closely resemble endothelial cells in terms of transcriptome and proteome (32); they are capable of proliferation, differentiation, and migration and contribute to endothelial repair and angiogenesis in vitro and in vivo and, in contrast to mature endothelial cells, play a major role in neovascularization (42). For these reasons, we isolated the ECFC subpopulation from the cord blood samples of healthy, uncomplicated pregnancies. After the plasma layer was removed, the EDTA-containing tubes were refilled with an equivalent volume of plasma replacement media containing EDTA, penicillin, streptomycin, and PBS. After further doubling the volume by adding media consisting of penicillin, streptomycin, PBS, and 2% (vol/vol) fetal bovine serum (FBS), the samples were layered on Ficoll Plus (GE Healthcare, Buckinghamshire, UK) and centrifuged at 400 g for 30 min. The mononuclear cell fraction was collected and washed twice with isolation buffer. Cells were cultured in endothelial growth medium-1 (EGM, Lonza, Basel, Switzerland; supplemented with supplier-recommended concentrations of human recombinant epidermal growth factor, VEGF, ascorbic acid, hydrocortisone, and recombinant insulin-like growth factor) with 10% vol/vol FBS and 1% penicillin/streptomycin at 5 × 10^5 cells/well on collagen coated six-well plates (BD Bioscience, Heidelberg, Germany). The cells were incubated at 37°C and 5% CO₂. The growth media were changed daily until colonies of ECFCs appeared as well-circumscribed monolayers of cobblestone-appearing cells (21). Colonies were identified by visual inspection using an inverted microscope (Olympus, Tokyo, Japan). ECFC colonies were further characterized by immunophenotyping and flow cytometry as described previously (21). Colonies were isolated as described previously (22) and seeded into 75-cm² tissue culture flasks and utilized for experiments at 80–90% confluence. All experiments were run with ECFCs at passage 3 to 5.

**HÜVEC isolation and culture.** Human umbilical venous endothelial cells (HÜVECs) are used frequently to study the regulation of endothelial cell function, potency of angiogenesis inhibitors and stimulators, and the development of atherosclerotic plaque (40). HÜVECs were isolated according to the protocol of Jaffe et al. (26) with some modifications. Umbilical cords were collected immediately after delivery from women with uncomplicated pregnancies and stored at 4°C until the cells were isolated under sterile conditions. After incubation of the umbilical cord with collagenase-enriched cord buffer for 25 min at 37°C, the detached endothelial cells were washed in 10 ml growth medium and 1.2% penicillin/streptomycin (Biochrom, Berlin, Germany) and transferred to a T75 cell culture flask. HÜVECs were characterized by flow cytometry using the typical endothelial cell phenotypic (CD31 +, CD90 −), cultured in T75 cell culture flasks, and utilized in passage 3 to 5.

**Monolayer invasion coculture assays.** The cross talk between progenitor and mature endothelial cells is of particular importance for endothelial homeostasis and repair processes in the vascular system. To simulate this interaction a coculture model of ECFCs and HÜVECs was established. For the coculture models, HÜVECs were seeded onto gelatin-coated 24-well culture plates with growth media and 10% vol/vol FBS. At confluence, the endothelial cell monolayers were labeled with CellTracker Red CMTPX (Invitrogen). For this purpose, 50 µg Cell Tracker were dissolved in 10 µl DMSO and mixed with 5 ml growth medium. After 30 min of incubation, the cells were washed with 10 ml of fresh medium. ECFCs were concurrently labeled with CellTracker Green CMFDA (Invitrogen). After incubation for 30 min and washing with 10 ml of fresh medium, the green-labeled ECFCs were trypsinized and 1 × 10⁵ cells were added to the red-labeled endothelial cell monolayers. In separate experimental conditions, 50 Harvard University investigators performed this procedure, and the coculture models were established. The coculture models were established immediately after the ECFCs and HÜVECs were isolated and cultured in T75 cell culture flasks, and utilized in passage 3 to 5.

### Table 1. Clinical and demographic data of patients who provided blood samples

<table>
<thead>
<tr>
<th>Maternal age, yr</th>
<th>Controls (n = 6)</th>
<th>Preeclamptic Pregnancy (n = 7)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age at delivery, wk</td>
<td>36.5 ± 3.4</td>
<td>37.1 ± 5.8</td>
<td>0.17</td>
</tr>
<tr>
<td>Maternal age at delivery, wk</td>
<td>38.2 ± 0.8</td>
<td>37.3 ± 1.4</td>
<td>0.11</td>
</tr>
<tr>
<td>Maternal age at delivery, wk</td>
<td>50</td>
<td>28.6</td>
<td>0.59</td>
</tr>
<tr>
<td>Maternal age at delivery, wk</td>
<td>24.2 ± 6.1</td>
<td>27.5 ± 2.5</td>
<td>0.22</td>
</tr>
<tr>
<td>Gestational SBP, predelivery, mmHg</td>
<td>109 ± 12.4</td>
<td>165.3 ± 16</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gestational SBP before 20 wk gestation, mmHg</td>
<td>118.5 ± 7.0</td>
<td>132 ± 17.5</td>
<td>0.12</td>
</tr>
<tr>
<td>Gestational DBP, predelivery, mmHg</td>
<td>72.2 ± 13.6</td>
<td>105.4 ± 10.5</td>
<td>0.0004</td>
</tr>
<tr>
<td>Gestational DBP before 20 wk gestation, mmHg</td>
<td>72.2 ± 13.6</td>
<td>73.3 ± 5.2</td>
<td>0.35</td>
</tr>
<tr>
<td>Birth weight</td>
<td>2,998 ± 391</td>
<td>2,908 ± 560</td>
<td>0.75</td>
</tr>
<tr>
<td>Birth weight percentile</td>
<td>0.52</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Birth weight percentile &lt; 10th (n), %</td>
<td>16.7</td>
<td>14.3</td>
<td>0.07</td>
</tr>
<tr>
<td>Maternal race, white (n), %</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Infant sex, male (n), %</td>
<td>16.7</td>
<td>57.1</td>
<td>0.27</td>
</tr>
</tbody>
</table>

Values are means ± SD. Distribution was examined with Shapiro–Wilk normality test. Continuous data were compared with unpaired t-test or Mann-Whitney, as appropriate. Categorical variables were compared by Fisher’s exact test. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.
ments, fetal cord serum from normal and PE pregnancies was added at 5% vol/vol concentration. The experiments were performed in the presence and absence of 10 nM 1,25(OH)2-vitamin D3. Images were taken at 0, 1, 2, 3, 4, 5, 8, 12, and 20 h at ×20 magnification. After taking phase-contrast images, the ECFCs were recorded in the FITC channel and the endothelial cells in the Alexa 456 channel. The number of cells invaded into each monolayer was measured from 0-h to 20-h time points. Invasion capacity was expressed as percentage of invaded green labeled cells in relation to total cell count using ImageJ Freeware (NIH Image). Four independent experiments were performed with each test performed in duplicate.

**Tubule integration coculture assay.** Matrigel (35 μl per well) was added into wells of a 96-well plate. HUVECs were stained with CellTracker Red CMFDA Dye. A 10-mM stock solution was prepared by dissolving the dye in DMSO. The dissolved dye was diluted 1:1,000 in serum-free medium (HUVEC growth medium as appropriate). The cells were washed with PBS and incubated with 5 ml of the diluted dye-containing media for 30 min. The cells were washed three times with PBS. After labeling, 17,000 HUVECs were added to each Matrigel-coated well. The cells were incubated for 4 h to form capillary-like structures. ECFCs were concurrently labeled with green fluorescent dye (CellTracker Green CMFDA Dye) as described above. After confirmation of HUVEC capillary-like structures at the 4-h time point, 17,000 labeled ECFCs were added to the wells containing HUVECs. In separate experiments, fetal cord serum from normal and PE pregnancies was added at 5% vol/vol concentration. The experiments were performed in the presence and absence of 10 nM 1,25(OH)2-vitamin D3 (Sigma-Aldrich, St. Louis, MO). Subsequently, the total length of the capillary-like structures per field of view was measured at ×2.5 magnification after 20 h of coincubation.

![Graph](image_url)

**Figure 1.** A: invasion of endothelial colony-forming cells (ECFCs) into the human umbilical vein endothelial cell (HUVEC) monolayer and the effect of uncomplicated pregnancy (control) fetal cord blood sera, preeclampsia (PE) fetal cord blood sera, and 1,25(OH)2-vitamin D3 on invading capacity in a coculture assay. Invasion of green labeled ECFCs into a red labeled HUVEC monolayer was analyzed after 1–20 h by visual microscopy at ×20 magnification. Data are expressed as invading ECFC fraction in relation to the basal ECFC count (at 0 h) in means ± SD. *P < 0.05 vs. control serum at the same time point; #P < 0.05 vs. PE serum at the same time point; n = 4. B: representative photomicrographs of invasion of ECFCs fluorescently labeled green into the fluorescently labeled red HUVEC monolayer after 0 to 5, 10, and 20 h.
using ImageJ Freeware (NIH Image) after fluorescence microscopy. Four independent experiments were performed with each test performed in duplicate.

**Flow cytometry.** ECFCs were pretreated with or without 10 nM 1,25(OH)2-vitamin D3 and uncomplicated pregnancy (control) sera or PE sera at 5% vol/vol for 24 h before flow analysis. Cells were trypsinized with Accutase (ThermoFisher Scientific) and resuspended in staining buffer. Cells were treated with 10 μl of intraglobin, followed by incubation with antibodies [anti-VE-cadherin (no. 348505), anti-VCAM (no. 305809), and anti-CD31 (no. 130-117-390); Biolegend, San Diego, CA] at 4°C for 30 min. Cells were then washed with wash buffer and centrifuged at 1,000 rpm. After washing and prior to measurement, propidium iodide (10 μg/ml; Sigma-Aldrich) was added. To measure endothelial cell marker expression, cells were analyzed by flow cytometry using FACS Calibur (Beckton-Dickinson, Franklin Lakes, NJ). The antibodies used in this approach were previously tested and validated (47). Six independent experiments were performed.

**Immunocytochemical staining for VE-cadherin expression.** Cells were plated on gelatin-coated coverslips and fixed with 3% paraformaldehyde and 2% sucrose after labeling with CellTracker DeepRed dye or Green CMFDA dye. Cells were then permeabilized with PBS + 0.1% Triton X-100 and incubated with primary antibody for 4 h at room temperature [rabbit- anti-human VE-cadherin (no. 2500); 1:100; New England Biolabs, Frankfurt, Germany] followed by a 2-h incubation with the secondary antibody [Alexa Fluor 488 Goat Anti-Rabbit (no. A-11034); Thermo Fisher Scientific]. Nuclei were stained using DAPI (Thermo Fisher Scientific). Probes were mounted in ProLong Gold antifade (Thermo Fisher Scientific). Images were obtained using a fluorescence microscope at ×1,000 magnification. Analysis was performed with ImageJ Freeware (NIH Image). The antibodies used in this study were previously tested and validated (47). Five independent experiments were performed with several replicates each.

**Vitamin D receptor silencing of ECFCs.** ECFCs were transfected with specific vitamin D receptor (VDR) small interfering (si) RNA (VDR silencer validated siRNA; AM51331; Ambion, Kaufungen, Germany; sense: GGAGGAAAAACAGACAAAtt; antisense: UUU-UGUCUGUUUUUCCUCCtt) rarefied in antibiotic-free EGM/10% FCS plus Dharmafect 1 transfection reagent (Dharmacon). Transfection solution was added at a final concentration of 100 μM siRNA to wells of a six-well plate with ECFCs at 70–80% confluence. Cells were incubated for 24 h, and then medium was changed to regular growth medium and cells were used for further experiments. Four independent experiments were performed with each test performed in duplicate.

**Fig. 2.** A: area of the human umbilical vein endothelial cell (HUVEC) monolayer filled by endothelial colony-forming cells (ECFCs) and the effect of uncomplicated pregnancy (control) sera, preeclampsia (PE) sera, and 1,25(OH)2-vitamin D3 on area occupied by ECFCs in a coculture assay. Area occupied by ECFCs was analyzed after 20 h by visual microscopy at ×20 magnification. Data are expressed as total area occupied by ECFCs in percentage of the microscopic field. *P < 0.05 vs. control; n = 4. The ends of the whiskers represent the maximum and minimum measured values. B: representative photomicrographs of HUVECs fluorescently labeled red, ECFCs fluorescently labeled green, and overlay channel after 20 h of ECFC invasion into HUVEC monolayer in coculture.
independent experiments were performed with each test performed in duplicate.

**Western blot analysis.** Western blot analysis of the VDR was performed to show the efficiency of VDR silencing as described previously (53). In brief, cells were lysed with Laemmli buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.2% Triton X-100, 0.1 mM PMSF, 0.1 mM Na3VO4, 25 mM Na-fluoride, 25 mM β-glycerophosphate, 2 mM EGTA, 10 mg/ml leupeptin, and 10 mg/ml aprotin).
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Fig. 4. Invasion of endothelial colony-forming cells (ECFCs) into the human umbilical vein endothelial cell (HUVEC) monolayer and the effect of non-targeting siRNA, vitamin D receptor siRNA, and 1,25(OH)2 vitamin D3 under preeclamptic condition (PE sera) on invading capacity in a coculture assay. Invasion of green-labeled ECFCs into a red-labeled HUVEC monolayer was analyzed after 1–20 h by visual microscopy at ×20.0. Data are expressed as invading ECFC fraction in relation to the basal ECFC count (at 0 h) in means ± SD. *P < 0.05 vs. control at the same time point; n = 4. Distribution was examined using Shapiro-Wilk normality test. Continuous data were compared with paired t-test or Wilcoxon test as appropriate.

After overnight incubation of the membranes at 4°C with the primary antibody detecting the VDR (1:3,000; Santa Cruz Biotechnology, Santa Cruz, CA), membranes were washed three times with PBS buffer and incubated with a 1:5,000 dilution of anti-rabbit IgG (GE Healthcare) for 2 h at room temperature. Finally, blots were washed 5 min in Pierce solution, and bands were visualized by ECL Western blotting analysis system (GE Healthcare).

Statistical analysis. Data are presented as means ± SD. Distribution was examined with Shapiro-Wilk normality test, and treatment groups were compared with unpaired t-test or Mann-Whitney test as appropriate. Data were analyzed with Prism 4 software package (GraphPad Software, La Jolla, CA).

RESULTS

Participant characteristics. Maternal age, maternal prepregnancy BMI, race, parity, Cesarean section rate, and infant sex were comparable between the PE and control groups. By definition, women with PE had higher systolic and diastolic blood pressures at delivery compared with controls (Table 1).

Monolayer invasion coculture assay. To test the effects of serum on the interaction of endothelial progenitor and mature endothelial cells, we measured the invasion of ECFCs into an endothelial cell monolayer of HUVECs in an in vitro coculture system exposed to fetal serum from uncomplicated (C) or PE pregnancies, in the presence or absence of 1,25(OH)2-vitamin D3. The capacity of ECFCs to invade into HUVEC monolayers was significantly impaired by PE cord serum by 10 h (C: 66 ± 3.6%; PE: 54 ± 6.4%; P = 0.03) and 20 h (C: 77 ± 0.2%; PE: 59 ± 4.7%; P = 0.03) compared with control serum. Vitamin D treatment significantly enhanced the invasion capacity of ECFCs at 10 and 20 h, respectively, and neutralized the difference between PE and control serum (Fig. 1A).

Figure 2 shows the area occupied by ECFCs of the monolayer after 20 h of incubation with exposure to PE and control cord serum. The invasion measured as area occupied by ECFCs was reduced under preeclamptic conditions in comparison to control conditions (C: 11 ± 2%; PE: 7 ± 1%, P = 0.03). After treatment of the control and PE serum-exposed groups with 1,25(OH)2-vitamin D3, vitamin D lead to an invasion boost in all groups compared with untreated control (Fig. 2A).

Integration coculture assay. The integration of ECFCs into preformed tubules of differentiated endothelial cells (HUVECs) was investigated in a coculture Matrigel assay with pooled fetal cord serum from uncomplicated or PE pregnancies and in the presence or absence of vitamin D3. Overall tubule assembly was markedly impaired in the presence of PE serum compared with control serum (P = 0.003; Fig. 3A). 1,25(OH)2-vitamin D3 (10 nM) increased tubule formation significantly by cells exposed to control or PE serum (C: P = 0.02; PE: P = 0.04). Under PE conditions, the ratio of participating HUVECs and ECFCs in building tubule-like networks shifted toward increased ECFCs compared with control conditions. This effect was significantly enhanced by treatment with vitamin D (Fig. 3, B–E).

VDR silencing. To test the specificity of the vitamin D effect, the VDR was silenced and ECFC invasion into a HUVEC monolayer was explored in the presence of pooled PE serum (Fig. 4). The increased invasion of ECFCs induced by vitamin D while in presence of PE serum was suppressed by knockdown of VDR (PE: 5 h: P = 0.38; 10 h: P = 0.97; 20 h: P = 0.94). Total ECFC invasion was not affected by the non-targeting siRNA, vitamin D receptor siRNA, and 1,25(OH)2 vitamin D3 under control conditions. This effect was significantly enhanced by treatment with vitamin D (Fig. 3, B–E).

Fig. 3. A: assembly of tubule-like structures by endothelial colony-forming cells (ECFCs) added to preformed tubuli of human umbilical vein endothelial cells (HUVECs) and the effect of uncomplicated pregnancy (control) sera, preeclampsia (PE) sera, and 1,25(OH)2 vitamin D3 on capillary-tube formation in a cocultured Matrigel assay. Capillary-tube formation (average total tube length per microscopic field) was analyzed after 24 h by visual microscopy at ×2.5. Data are expressed as total tube length in µm. *P < 0.05 vs. control; #P < 0.05 vs. PE; n = 4. The ends of the whiskers represent the maximum and minimum measured values. B: fraction of tube-like structures by ECFCs added to preformed tubuli of HUVECs and the effect of uncomplicated pregnancy (control) sera, PE sera, and 1,25(OH)2 vitamin D3 on alteration of capillary-tube fraction in a cocultured Matrigel assay. Capillary-tube formation (average total tube length per microscopic field) of labeled ECFCs and HUVECs was analyzed after 24 h by visual microscopy at ×2.5 magnification. Data are expressed as percentages (means ± SD) of ECFCs and HUVECs, respectively, across the entire tubule network. *P < 0.05 vs. control; #P < 0.05 vs. PE; n = 4. C: representative photomicrographs of HUVECs fluorescently labeled red, ECFCs fluorescently labeled green, and overlay channel after 24 h of incubation on Matrigel (left). Representative 3-dimensional animation of the HUVECs, ECFCs and overlay channel (right). D: representative image of the interaction of HUVECs (fluorescence-labeled red) and ECFCs (fluorescence-labeled green) in the tubuli network. The thin arrows mark the gaps in the HUVEC tubule web, which are filled in by the ECFCs. The thick arrow marks the point of interaction of HUVECs and ECFCs in a branching point. E: representative photomicrographs of HUVECs fluorescently labeled red and ECFCs fluorescently labeled green and the effect of uncomplicated pregnancy (control) sera (top left) and PE sera (bottom left) and the effect of 1,25(OH)2 vitamin D3 under control (top right) and PE (bottom right) conditions after 24 h of incubation.
ing siRNA. After VDR silencing, vitamin D treatment had no effect on invasion by ECFCs.

**Flow cytometry analysis.** We assessed the distribution of endothelial cell markers and cell adhesion proteins of ECFCs by FACS analysis using flow cytometry. ECFCs expressed PCAM, VCAM, and VE-cadherin. The expression of the investigated endothelial cell markers in the control and PE group was comparable (VCAM: C: 11.6% PE: 12.5%; VE-cadherin: C: 14.1%; PE: 15.2%; PCAM: C: 9.5%; PE: 9.4%). Treatment with vitamin D increased VCAM [C + VitD: 12.9% (+1.3%); PE + VitD: 14.2% (+1.7%)] and VE-cadherin expression [C + VitD: 15.6% (+1.5%); PE + VitD: 18.1% (+2.9%)] or PCAM expression (Fig. 5).

**Immunocytochemical staining.** Cell-cell interactions between mature endothelial cells (HUECs) and endothelial progenitor cells (ECFCs) were qualitatively demonstrated by immunocytochemistry. We observed the formation of cell junctions between neighboring cells as reflected by VE-cadherin expression (Fig. 6). This demonstrates that endothelial progenitor cells in the coculture and invasion assays did not simply migrate into the monolayer but rather formed cell-cell contacts with mature endothelial cells and specifically integrate into the monolayer.

**DISCUSSION**

Endothelial cells form networks of capillary-like (“tubule”) structures when placed in a reconstituted basement membrane (Matrigel) (34). Both the tubule assay and endothelial monolayer assay have been well established as in vitro models to study the invasion/integration of endothelial progenitor cells into the endothelium. The major new finding of our study is that fetal blood-derived endothelial progenitor cells (ECFCs) exhibit markedly decreased integration and invasion into monolayers and capillary-like tubules of mature umbilical vein endothelial cells (HUVECs) in vitro when exposed to low concentrations (5% vol/vol) of fetal serum from PE pregnancies compared with fetal serum from uncomplicated pregnancies. These fetal serum-mediated inhibitory effects on ECFC behavior were substantially reversed by exogenous administration of vitamin D in the physiologic range. We also observed that endothelial progenitor cells communicate with mature endothelial cells via VE-cadherin cell-cell contact mechanisms. During integration of ECFCs into the endothelium in vivo, ECFCs differentiate into a more mature endothelial phenotype (48). Our findings implicate factors in the fetal circulation of potential relevance to mechanisms by which angiogenesis and endothelial homeostasis are disrupted in PE.

The soluble fms-like tyrosine kinase-1 (sFLT1 or sVEGFR-1), an antiangiogenic protein, is released in excess by the placenta in PE (24). SFTL1 is increased in fetal cord blood (51) and maternal blood weeks before the onset of clinical disease and correlates with the severity of the disease (31). Excess sFLT1 and other antiangiogenic or proinflammatory cytokines in the maternal and fetal circulation (1) might have contributed to the observed effects of PE serum.

The developmental origins of disease and health paradigm suggests that adverse perinatal exposures increase the risk of cardiovascular diseases in child- and adulthood (3, 4, 20). In line with this paradigm prenatal exposure to PE has been reported as an independent risk factor for long-term cardiovascular morbidity of the offspring, e.g., stroke (14, 27, 28, 37). PE is associated with a smaller umbilical cord vein area and wall thickness. Endothelial cord cell dysfunction and advanced aging is also suggested in the preeclamptic neonate (8, 25, 36,
53). Taken together, these findings may serve as a proxy of disturbed cardiovascular development in the offspring resulting in increased cardiovascular risk.

Vitamin D deficiency has been linked to an approximate fivefold increased risk for the development of PE (7, 46, 49). In mice vitamin D deficiency during pregnancy results in maternal hypertension and adverse effects on placental and fetal development (34). Vitamin D deficiency in young rats is associated with elevated blood pressure and endothelial dysfunction (35). These data are consistent with the hypothesis that vitamin D deficiency predisposes to disturbed endothelial homeostasis. The vitamin D receptor (VDR) is expressed in human placenta, mature endothelial cells, and, as we have shown previously, in cord blood-derived ECFCs (21, 33, 41).

In this study, we found a stimulating effect of vitamin D on cell-cell interactions that may be important for endothelial homeostasis and repair.

Vitamin D is known to promote cell proliferation, migration, and invasion in different cell types (15). Potentially relevant to PE, vitamin D regulates key target genes associated with implantation, trophoblast invasion, and anti-inflammatory responses in maternal decidua and fetal trophoblast (5, 15, 16). The literature also supports a promoting effect of vitamin D₃ on trophoblast and endothelial function and homeostasis (8, 9, 21, 53). We previously showed a significant positive effect of vitamin D₃ on ECFC migration and tubule formation due to the stimulation of VEGF production (21). Vitamin D also promoted proliferation of ECFCs and neutralized the negative effects of hypoxic conditioned media on ECFC migration (9, 53). Vitamin D enhanced ECFC functionality both in a pre-eclamptic milieu and also under “normal” conditions in the present study. Here it can be assumed that the vitamin has beneficial effects on the endothelium in pregnancy independent of pathologic conditions. Therefore, a sufficient vitamin D status at conception and in early gestation, when the maternal cardiovascular system adapts to the challenges of pregnancy, might improve overall pregnancy outcome possibly via increased VEGF production (21).

The endothelium is the immediate selective barrier between blood and the surrounding tissue. Increased vascular permeability is an important event of microvascular dysfunction in pregnancies complicated by PE due to dysregulated endothelial junction integrity of the maternal vascular system (23). Factors released by the placenta into the maternal circulation, e.g., pro- and anti-inflammatory cytokines, can induce endothelial injury and dysfunction in PE. The barrier integrity is regulated by cell-cell adhesion proteins, i.e., VE-cadherin, VCAM-1, and PCAM-1. The formation of cell junctions between neighboring mature endothelial cells (HUVECs) and endothelial progenitor cells (ECFCs) was qualitatively demonstrated using immunocytochemistry as reflected by VE-cadherin expression. Although there is evidence for modulation of these adhesion molecules by vitamin D in several cell types (12), we did not show a significant effect of vitamin D on expression of these adhesion molecules by fetal endothelial cells.

In conclusion, preeclamptic cord serum impairs cell-cell interaction during invasion and integration while physiological concentrations of vitamin D overcame the inhibitory effects of PE serum. While PE is a cardiovascular risk factor for the mother and her offspring later in life (11, 14), pregnancy provides an early opportunity to recognize and potentially reduce cardiovascular risk. Even though vitamin D deficiency is only one risk factor for PE, sufficient vitamin D status at conception and throughout pregnancy might improve maternal and offspring vascular health in pregnancy and thereafter. Whether the observed cellular changes persist in the neonatal period and childhood and are a possible early marker of an increased cardiovascular risk of the progeny of PE pregnancies has to be investigated by further studies.

ACKNOWLEDGMENTS

We greatly appreciate the support of the faculty, residents, and staff of the Gynecology Research Unit and Delivery at Hannover Medical School in recruiting participants and collecting blood samples and Katja Borns for technical assistance.

GRANTS

The study was supported by German Research Foundation (DFG) Project Grant VE490/7-1 (to F. von Versen-Höynck).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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