Vitamin D improves the angiogenic properties of endothelial progenitor cells

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Grundmann M, Haidar M, Placzko S, Niendorf R, Darashchonak N, Hubel CA, von Versen-Höynck F. Vitamin D improves the angiogenic properties of endothelial progenitor cells. Am J Physiol Cell Physiol 303: C954–C962, 2012. First published August 29, 2012; doi:10.1152/ajpcell.00030.2012.—The main pathogenic feature of preeclampsia is maternal endothelial dysfunction that results from impaired angiogenesis and reduced endothelial repair capacity. In addition, preeclampsia risk is associated with vitamin D deficiency. We hypothesized that vitamin D3 stimulates proangiogenic properties of endothelial colony-forming cells (ECFCs). ECFCs were obtained and cultured from cord blood and characterized by immunocytochemistry and flow cytometry. Proliferation, total length of tubule formation on Matrigel, expression of VEGF mRNA, and pro-matrix metalloproteinases (MMP)-2 activity were assessed after treatment of ECFCs with vitamin D3. Specificity of the observed effects was tested by blocking the vitamin D receptor (VDR) or the VEGF signaling pathway. ECFCs treated with 10 nM vitamin D3 showed a 1.27 times higher tubule formation compared with vehicle-treated controls (1.27 ± 0.19) as well as a 1.36 times higher proliferation rate (1.36 ± 0.06). Vitamin D3 induced pro-MMP-2 activity (1.29 ± 0.17) and VEGF mRNA levels (1.74 ± 0.73) in ECFCs. VDR blocking by pyridoxal-5-phosphate (0.73 ± 0.19) or small interfering RNA (0.75 ± 0.17) and VEGF inhibition by Su5416 (0.56 ± 0.16) or soluble fms-like tyrosine kinase-1 (0.7 ± 0.14) reduced tubule formation and pro-MMP-2 activity (pyridoxal-5-phosphate: 0.84 ± 0.09; Su5416: 0.79 ± 0.11; or siF: 0.88 ± 0.13). This effect was neutralized by vitamin D3. Consequently, vitamin D3 significantly promoted angiogenesis in ECFCs in vitro possibly due to an increase in VEGF expression and pro-MMP-2 activity. Since angiogenesis is a crucial feature in the pathophysiology of preeclampsia these findings could explain the positive influence of vitamin D3 in reducing preeclampsia risk.

VITAMIN D, PRIMARILY KNOWN for its important role in calcium homeostasis and bone metabolism, influences the cardiovascular system through unclear mechanisms (33). Vitamin D deficiency is associated with increased all-cause and cardiovascular disease mortality, coronary heart disease, and various cardiovascular risk factors (33).

Preeclampsia, a pregnancy-specific disorder that affects 3–7% of all pregnancies, is a major cause of maternal and fetal morbidity and mortality (43) and is associated with an increased risk for cardiovascular events later in life (6). Endothelial dysfunction underlies the hypertension, proteinuria, and multiorgan damage that occur during preeclampsia. The mechanisms that contribute to the disturbed endothelial homeostasis in the pathophysiology of preeclampsia remain unclear (41, 46). Compared with normal pregnancies, preeclampsia is characterized by marked changes in vitamin D and calcium metabolism (3). Epidemiological studies have demonstrated an association between low maternal vitamin D levels and the incidence of preeclampsia (23, 27) and suggest vitamin D deficiency to be an independent risk factor for the development of preeclampsia (7). Moreover, vitamin D supplementation studies also showed protective effects on preeclampsia incidence (27, 37, 42).

Circulating endothelial progenitor cell (EPC) abundance has been proposed as a surrogate marker of vascular function and is reduced in patients with various cardiovascular risk factors (1). Decreased numbers of EPCs may be a sign of impaired endothelial repair capacity in preeclampsia (52). Late outgrowth EPCs, also referred to as “endothelial colony-forming cells” (ECFCs), have endothelial-like characteristics (50). They are highly proliferative and migrate to sites of vessel formation, possessing the ability to differentiate into mature endothelial cells (53). They are critical to, and play a complementary role, in blood vessel formation and repair (51). Vascular endothelial growth factor (VEGF) stimulates endothelial cell proliferation and migration and mediates vascular growth and angiogenesis. VEGF and its soluble receptor soluble fms-like tyrosine kinase-1 (sFLT-1) are implicated in vascular damage and repair in preeclampsia (47). Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent enzymes that play an important role in physiological as well as pathological mechanisms, being involved in angiogenesis and vascular remodeling by degrading extracellular matrix proteins (14). MMPs are implicated in the pathogenesis of vascular dysfunction associated with preeclampsia (38, 47).

So far, the relationship between vitamin D and function of ECFCs has not been investigated. We tested the hypothesis that vitamin D stimulates the proliferation and development of capillary-like tubules of ECFCs in cell culture models and that the effect of vitamin D is mediated by VEGF. We also investigated the effect of vitamin D on pro-MMP-2 activity of ECFCs.

PATIENTS, MATERIALS, AND METHODS

Patients. Ten healthy women with uncomplicated pregnancies were included in the study. These women were normotensive, without proteinuria or hyperuricemia throughout gestation, and delivered healthy babies at term. Patients with multiple gestations, chronic hypertension, diabetes, chronic renal disease, other significant metabolic disorder, evidence of infection, or a history of illicit drug use were excluded. Diagnosis

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of uncomplicated pregnancy was confirmed by chart review by a jury of research and clinical investigators. Cord blood samples were collected immediately after delivery. The local Ethic Committee of Hannover Medical School approved this research, and all pregnant women gave written informed consent.

Isolation, culture, and vitamin D receptor silencing of ECFCs from cord blood. Blood was taken from the umbilical cord vein immediately after delivery and transferred into a sterile tube containing EDTA. The samples were processed within 1 h. After centrifugation at 400 g for 10 min and removal of plasma, the blood cells were diluted with plasma replacement buffer containing EDTA, penicillin, streptomycin, and PBS. The samples were further diluted with equal volumes of isolation buffer containing PBS, penicillin, streptomycin, and 2% FBS. The samples were layered on Ficoll Plus (GE Healthcare, Buckinghamshire, England) and centrifuged at 400 g for 40 min. Cells from the mononuclear cell fraction were collected and washed two times with isolation buffer. Cells were maintained in endothelial cell growth medium 1 (EGM-1; Lonza, Basel, Switzerland; supplemented with supplier-recommended concentrations of human recombinant epidermal growth factor, fibroblast growth factor, VEGF, ascorbic acid (vitamin C), hydrocortisone, and recombinant insulin-like growth factor) with 10% FBS at 5 × 10^7 cells/well on collagen-coated sixwell plates (BD Bioscience, Heidelberg, Germany) and incubated at 37°C in an atmosphere of 5% CO₂. Medium was changed daily for 10 days and then every other day. Colonies of ECFCs appeared between 5 and 20 days of culture and were identified as well-circumscribed monolayers of cobblestone-appearing cells (29). Endothelial cell colonies were enumerated by visual inspection using an inverted microscope (Olympus, Tokyo, Japan) under ×4 magnification (Fig. 1). ECFCs derived from the colonies were plated in 75-cm² tissue culture flasks and used at 80–90% confluence. Passages 2–4 were used in experiments.

For vitamin D receptor (VDR) silencing, the ECFCs were transiently transfected with predesigned, site specific VDR small interfering (si)RNA (ON-TARGETplus, Dharmacon D-003448–02-0005) diluted in EGM-10% FBS medium (without antibiotics) containing Dharmafect 1 transfection reagent (Dharmacon). Transfection reagent-siRNA complexes (final concentration of siRNA 20 μM) were added to each well of a sixwell plate with ECFCs grown to 90% confluence. After 24 h of incubation, the media were replaced with regular growth medium (EGM-1 supplemented with 10% FBS and antibiotic) and cells were used for further experiments. Western blot was used to confirm that VDR silencing was effective.

Immunophenotyping of endothelial cells. To assess the endothelial phenotype, immunocytochemistry was performed using fluorescein isothiocyanate-labeled Ulex europaeus agglutinin I (lectin; Sigma-Aldrich, Steinheim, Germany) as cell surface staining and acetylated low-density lipoprotein (Dil-Ac-LDL; Biomedical Technologies, Stroughton, MA) to examine the cells for uptake of Dil-Ac-LDL. Cells were treated with 5 μg/ml Dil-Ac-LDL and incubated for 4 h at 37°C. Then, cells were permeabilized with Tergitol-type NP-40 for 1 min. After fixation in 4% paraformaldehyde for 10 min, cells were counterstained with 10 μg/ml lectin for 1 h. DAPI (Thermo Scientific, Rockford, IL) was used for staining nuclei. Fluorescence images were taken by a Leica EL600 fluorescence camera (Leica Microsystems, Wetzlar, Germany). Lectin was excited at 488 nm and Dil-Ac-LDL at 456 nm.

Flow cytometry. To further characterize the isolated ECFCs and to confirm their phenotype, we conducted flow cytometric analyses using surface markers CD31, CD34, CD133, VEGFR-2, and CD45 as well as appropriate isotype controls as described by Duda et al. (19) with minor modifications. In short, cultivated ECFCs were detached from culture plasticware using 5 mM EDTA (in PBS). Then, 0.5 × 10⁶ cells were used for each FACS tube and solubilized in FACS buffer (0.1% BSA and 0.02% sodium azide in DPBS) before Fc-receptor blocking reagent (Miltenyi, Auburn, CA) was added to each tube. Then specific amounts of isotypes or antibodies (as in Ref. 19) were added to the preparations. After being washed, the cells were analyzed using the BD flow cytometer LSR-II and Software BD FACSDiva.

Western blot. Western blot was performed to demonstrate that ECFCs express VDR. 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 aprotinin). The membranes were incubated with a 1:500 dilution of the rabbit anti-VDR N-20 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). After overnight incubation at 4°C, membranes were washed three times with PBS and incubated for 2 h at 4°C with a 1:5,000 dilution of anti-rabbit IgG (GE Healthcare, Buckinghamshire, UK). The blots were washed 5 min in Pierce solution, and bands were visualized by ECL Western blotting analysis system (GE Healthcare).

In vitro angiogenesis assay. To test whether in vitro angiogenesis in ECFCs is stimulated by 1,25(OH)₂ vitamin D₃, cells were pretreated with 0.1 and 10 nM of 1,25(OH)₂ vitamin D₃ (Sigma-Aldrich, St. Louis, MO) for 24 h in endothelial basal medium (without supplements) containing 0.1% FBS or 2.5% FBS. The employed concentrations of 1,25(OH)₂ vitamin D₃ approximate physiological levels in pregnancy (18, 24). In vitro vascular network formation was tested by seeding 8,000

Fig. 1. Photomicrograph of endothelial colony-forming cells (ECFCs). Representative bright field image of ECFCs derived from umbilical cord blood (4× magnification) after 16 days of culture. Scale bar = 1 mm.
p pretreated cells/well in 96-well plates coated with 30 µl growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) and observed after 6 h by visual microscopy at ×2.5 magnification. ImageJ software (NIH Image) was used for quantification of capillary-like structures, and tubule length was analyzed by two independent observers. Each experiment was done in quartet.

Proliferation assay. The effect of 1,25(OH)2 vitamin D3 on ECFC cell growth was determined using a proliferation assay. Then, 1 × 10^4 cells per well were seeded onto 24-well culture plates with EGM-10% FBS and stimulated with 0.1 or 10 nM 1,25(OH)2 vitamin D3. After 24, 48, and 72 h of treatment, the cell number was counted by Neubauer chamber with 1:2 trypan blue dilution. To estimate population-doubling time the following equation was used: population doubling time = t \log 2/ \log (Nt/No), where t is time period (h), Nt is number of cells at time t, and No is initial number of cells.

Gelatin zymography. Gelatin zymography was performed as previously described with minor modifications (55). To examine the activity of pro-MMP-2 in ECFCs that were treated with 0.1 or 10 nM 1,25(OH)2 vitamin D3 for 24 h, conditioned media were collected and the proteins were concentrated using a centrifugal filter unit (Millipore, Bedford, MA). Equal amounts of protein (7.5 µg) for each sample were mixed with sample buffer containing 10% glycerol, 10 mM Tris·HCl, and 1% sodium SDS at a volume of 10 µl. The samples were loaded onto 10% SDS gel incorporated with 2.3 mg/ml gelatin for electrophoresis. The stacking gel was washed with 2.5% Triton-X-100 for 30 min and incubated in a collagenase buffer (50 mM Tris·HCl, pH 7.0, and 5 mM CaCl2) at 37°C overnight. Gels were stained for 1 h with 0.4% Coomassie brilliant blue and destained with a methanol-glacial acetic acid solution. The activity of pro-MMP-2 was visualized as a white band against a blue background, and densitometric quantification of gelatinolytic activity was analyzed using Image J software.

VDR blocking and VEGF pathway inhibition. To study the specificity of functional effects of 1,25(OH)2 vitamin D3, we blocked the VDR with the VDR antagonist pyridoxal-5-phosphate (0.5 mM) or downregulated the VDR with siRNA (20 µM). In addition the VEGF pathway was inhibited by Su5416 (0.5 µM, VEGF pathway inhibitor) or sFlt (5 nM, VEGF pathway inhibitor) or sFlt (5 nM, VEGF pathway inhibitor). Western blot analysis was performed (as described above) in the presence or absence of 1,25(OH)2 vitamin D3 (10 nM).

Real-time RT-PCR. For mRNA expression analyses, treatment groups were used as described in previous experiments [0.1%/2.5% FBS and 0.1 nM/10 nM of 1,25(OH)2 vitamin D3]. Total RNA isolation was performed using the GTC-method of Chomczynski and Sacchi (13) with minor modifications. After removal of medium and being washed with PBS, cells were centrifuged at 2,000 g for 5 min at 4°C. Cell pellets were homogenized in 1 ml guanidinium isothiocyanate with phenol (ABGene, Cambridge, England) and cooled on ice for 5 min. Then, 200 µl chloroform were added and the organic phase was mixed with the acidic aqueous phase by shaking. The samples were centrifuged at 12,000 g, and the supernatant was transferred into a new tube and mixed with 100% isopropanol. After centrifugation for 15 min, RNA was washed with 75% UVAZOL (Merck, Darmstadt, Germany). RNA products were run on 1% agarose gel to confirm that RNA was present. High-capacity cDNA reverse transcription kit (Invitrogen, Carlsbad, CA) was used for cDNA synthesis, and 2 µg RNA were reverse transcribed in a 20-µl assay. Real-time RT-PCR was performed on the Rotor Gene 6000 PCR instrument (Corbett Research, Hilden, Germany) using three-stage program parameters as follows: 30 s at 95°C, 45 s at 64°C, and 45 s at 72°C for 40 cycles. Each sample was tested in triplicate. The following VEGF-A primers were used according to already published sequences (36): forward VEGF-A 5'-CTGGAGTGTGTGCCCCACTGA; reverse VEGF-A 5'-TCCTATGTGCTGGCCTTTGGT. For normalization, β-actin served as housekeeping gene. Primer sequences were as follows: forward β-actin 5'-CCC TAA GGC CAA CCG TGA AAA GATG, reverse β-actin 5'-GAA CCG CTC ATT GCC GAT GTG ATG (Eurogentec, Seraing, Belgium).

Lactate dehydrogenase assay. Viability of cells was assessed by the release of lactate dehydrogenase into the medium. Lactate dehydrogenase measurement was carried out according to the manufacturer’s instructions using the in vitro toxicity kit (Sigma-Aldrich). There was no difference in cell viability between the treatment and control groups (data not shown).

Statistical analysis. Effects of individual experimental treatments were compared with untreated controls using Wilcoxon signed-rank test due to nonparametric distribution of the results. Data are presented as medians of fold changes and SE compared with untreated controls. GraphPad InStat 3 software was used to perform the statistical analyses. P values at <0.05 were considered statistically significant.

RESULTS

Characterization of ECFCs. The endothelial phenotype was confirmed by immunocytochemistry using fluorescein isothiocyanate-labeled Ulex europaeus agglutinin I to examine the cells for uptake of Dil-Ac-LDL. Flow cytometric results also show that the cultured population of ECFCs through passage 10 was homogenous and had a confirmed endothelial phenotype, being CD31+, CD45−, and CD133−. CD34 is reported to be positive on freshly isolated cells, but the expression of CD34 decreases with increasing culture time (21), CD34 being negative at passage 10–15. We confirmed this finding in our flow cytometric experiments. Western blot analysis found the VDR to be expressed by ECFCs, and siRNA knockdown to be effective in silencing VDR (see Fig. 6).

Increased angiogenic activity of ECFCs treated with 1,25(OH)2 vitamin D3. Under low serum concentration (0.1% FBS), 1,25(OH)2 vitamin D3 significantly stimulated formation of capillary-like structures by ECFCs in Matrigel (Fig. 2A). After treatment with 10 nM 1,25(OH)2 vitamin D3, ECFCs showed 1.27 times higher tube length than vehicle-treated controls (1.27 ± 0.19; P < 0.05; n = 38). ECFCs treated with 0.1 nM 1,25(OH)2 vitamin D3 also showed increased tube formation; however, this did not reach significance levels (Fig. 2B). These differences could clearly and significantly be seen at a low concentration of FBS (0.1%) in treatment media, a condition in which baseline tube formation was submaximal. In contrast, no significant difference was observed between control and both 0.1 and 10 nM 1,25(OH)2 vitamin D3-treated groups in tube formation when using 2.5% FBS in culture medium (data not shown).
Enhanced proliferation of ECFC treated with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3}. 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} increased proliferation of ECFCs compared with vehicle-treated controls (Fig. 3). Cells treated with 10 nM 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} showed a 1.31 and 1.36 times higher proliferation rate at day 2 and 3 compared with vehicle-treated controls (1.31/\pm 0.04 and 1.36/\pm 0.06; \(P < 0.05; n = 6\)), both media containing 10% FBS. The population doubling time of ECFCs treated with 10 nM 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} was shorter compared with vehicle-treated controls [control: 37.35 h; 10 nM 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3}: 35.07 h].

Increased pro-MMP-2 activity in ECFCs treated with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3}. Treatment with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} significantly promoted the activity of pro-MMP-2 in ECFCs (Fig. 4A). Cells treated with a concentration of 10 nM 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} showed 1.29 times higher pro-MMP-2 activity compared to control (Fig. 4A).

Fig. 2. Effect of 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} on tube formation. ECFCs were cultured in endothelial basal medium (EBM)-0.1% FBS and treated with 0.1 or 10 nM 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} or without vitamin D\textsubscript{3} for 24 h. Capillary-tube formation was determined by seeding 8,000 ECFCs on 30 \(\mu\)l Matrigel and tube length was analyzed after 6 h by visual microscopy at \(\times 2.5\) magnification. A: Relative quantification of tube length. Results represent median fold change \(\pm\) SE of 38 independent experiments; *\(P < 0.05\). B: formation of vascular networks in vitro with ECFCs treated with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3}. a: control; b: 0.1 nM vitamin D\textsubscript{3}; c: 10 nM vitamin D\textsubscript{3}.

Fig. 3. Effect of 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} on ECFC proliferation. ECFCs were treated with 1,25(OH)\textsubscript{2} vitamin D\textsubscript{3} (0.1 or 10 nM) in EBM-10% FBS. Cell numbers were counted at 24, 48, and 72 h after treatment by Neubauer chamber. Bars represent median fold change \(\pm\) SE of 6 independent experiments; *\(P < 0.05\) vs. control.
activity than vehicle-treated controls \((1.29 \pm 0.17; P < 0.05; n = 14)\), both media containing 0.1% FBS. Figure 4B shows that the thickness of the lytic band in zymography gel was increased markedly in the vitamin \(D_3\) group. These data indicate that vitamin \(D_3\) stimulates ECFCs to secrete pro-MMP-2.

Effect of 1,25(OH)\(_2\) vitamin \(D_3\) on VEGF mRNA expression in ECFCs. To analyze the mechanisms underlying the proangiogenic effects of 1,25(OH)\(_2\) vitamin \(D_3\) on ECFCs, VEGF mRNA levels were measured after 24 h of vitamin \(D_3\) treatment by use of real-time RT-PCR. ECFCs that were treated with 1,25(OH)\(_2\) vitamin \(D_3\) showed higher expressions of VEGF than the vehicle-treated controls. At a concentration of 10 nM, 1,25(OH)\(_2\) vitamin \(D_3\) induced VEGF mRNA levels in ECFCs markedly \((1.74 \pm 0.73; P < 0.05; n = 6;\) Fig. 5). As with capillary tube formation in Matrigel assay, no significant effects of vitamin \(D\) were seen in the presence of higher concentrations of FBS (2.5%) in cell culture media (data not shown).

Effect of VDR blocking or VEGF pathway inhibition on angiogenic and pro-MMP-2 activity of ECFCs. We found a promoting effect of 1,25(OH)\(_2\) vitamin \(D_3\) on tube formation compared with vehicle control \((1.35 \pm 0.09)\). A significant inhibiting effect on tube formation was observed when the VDR was knocked down by siRNA \((0.75 \pm 0.17)\) or the VEGF pathway was blocked by Su5416 \((0.56 \pm 0.16)\). Similar, albeit nonsignificant, reductions in tube formation were also seen after treatment with pyridoxal-5-phosphate \((0.73 \pm 0.19)\) or sFlt \((0.7 \pm 0.14)\). However, addition of 1,25(OH)\(_2\) vitamin \(D_3\) neutralized the inhibiting effects on ECFC tube formation \([\text{pyridoxal-5-phosphate and } 1,25(OH)_2 \text{ vitamin } D_3: 1.09 \pm 0.15; \text{Su5416 and } 1,25(OH)_2 \text{ vitamin } D_3: 1.03 \pm 0.47; \text{sFlt and vitamin } D_3: 1.36 \pm 0.14;\) Fig. 6].

Blocking the VEGF signal by Su5416 \((0.88 \pm 0.43)\) decreased pro-MMP-2 activity. The incubation in combination with 1,25(OH)\(_2\) vitamin \(D_3\) rescued the negative effect of VEGF inhibition (Fig. 7).

DISCUSSION

Current theoretical approaches describe preeclampsia as a two-stage disorder with reduced placental perfusion followed by maternal endothelial dysfunction \((43, 45, 56)\). Several epidemiological studies suggest that preeclampsia is associated with vitamin \(D_3\) deficiency \((7, 23)\) and that oral vitamin \(D_3\) supplementation markedly reduces the risk for preeclampsia \((27, 28, 42)\). However, the mechanisms by which vitamin \(D_3\) might exert its preventive effect have not been investigated. We demonstrate a significant positive impact of vitamin \(D_3\) on angiogenic potential of ECFCs in vitro. ECFCs treated with vitamin \(D_3\) show a significantly higher formation of the whole
expression was increased by human glioma cells transfected with MT1-MMP while MMP-inhibition downregulated their VEGF expression (17). Thus the stimulatory effect of vitamin D₃ treatment on pro-MMP-2 activity might also be mediated by VEGF expression. Our experiments using VEGF pathway inhibitors support this hypothesis. However, the relation between VEGF and especially pro-MMP-2 has not been studied so far and further research and experiments focusing on this field are needed.

To test the specificity of the observed functional effects, we blocked the VDR chemically or by VDR silencing with siRNA. In addition, we inhibited the VEGF pathway at two different levels. Blocking of VDR or of the VEGF signaling cascade reduced the formation of capillary-like structures and pro-MMP-2 activity of ECFCs after vitamin D₃ treatment. There is evidence for a reciprocal relation between VEGF and MMPs in different cell types (9). MMPs have been implicated in endothelial cell migration and differentiation in vitro. Various studies (38–40) also indicate an alteration in MMP levels in preeclamptic women. Pro-MMP-2 affects endothelial properties and plays a crucial stimulatory role in angiogenesis (49). We demonstrate a significant enhancement of pro-MMP-2 activity in ECFCs after vitamin D₃ treatment.

length of capillary-like structures on Matrigel compared with vehicle-treated controls. In addition, we show that vitamin D₃ increases cell proliferation of ECFCs. VEGF and its receptor have been shown to be implicated in vascular damage in preeclampsia. We found a 1.74-fold increase of VEGF mRNA expression after treating ECFCs with vitamin D₃. This finding suggests that the demonstrated increase of capillary-formation after vitamin D₃ treatment in ECFCs could be mediated by the increased expression of VEGF that is known to stimulate endothelial cell migration and differentiation in vitro. Various studies (38–40) also indicate an alteration in MMP levels in preeclamptic women. Pro-MMP-2 affects endothelial properties and plays a crucial stimulatory role in angiogenesis (49). We demonstrate a significant enhancement of pro-MMP-2 activity in ECFCs after vitamin D₃ treatment.

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Endothelial cells vitamin D3 induces inhibition of tube formation and, to some extent, proliferative activity of endothelial cells and, to some extent, proliferative mediates the rapid actions remains controversial (20).

The role of the nongenomic actions in most changes in gene expression and appear to be mediated by cell surface receptors. However, these effects are not seen in endothelial cells isolated from healthy tissue (4). Consequently, the effect of vitamin D3 on angiogenesis may depend on the cell type.

Cell proliferation is also an important step in angiogenesis. There is evidence that vitamin D3 influences cell cycling and proliferation through a VEGF-mediated pathway. This effect was detected before in vascular smooth muscle cells (12). The fact that vitamin D3 increases cell proliferation of ECFCs in our study is consistent with the hypothesis that vitamin D3 has a stimulating function in the process of angiogenesis.

We conducted our experiments with 0.1 or 10 nM vitamin D3 as described in several publications (5, 18), approximating physiologic vitamin D3 serum concentrations in pregnant and nonpregnant women (0.1 nM; Refs. 18, 24). While the placenta produces ~50% of the circulating vitamin D3 (16), we assume higher placental vitamin D3 levels and included 10 nM vitamin D3 in our experimental setting to simulate local placental conditions.

In our experiments, FBS masks the effect of vitamin D3 on ECFCs. Similar findings have been demonstrated before in vascular smooth muscle cells (12). The likely reason is that FBS is rich in a variety of growth factors including VEGF, insulin-like growth factor, and platelet-derived growth factor. Collectively, these findings support our hypothesis that vitamin D3 stimulates angiogenic function in ECFCs through a VEGF-mediated pathway.

MMP-2 and its active proenzyme pro-MMP-2 have important intracellular functions, particularly in angiogenesis (10). Proteolytic degradation of the basement membrane of the parent vessel and ECFC immigration requires proteolytic enzymes including MMPs. MMPs are produced by a variety of cell types including ECFCs (57). Inhibition of pro-MMP-2 leads to decreased formation of capillary-like structures whereas addition of recombinant pro-MMP-2 improves the in vitro angiogenesis in human umbilical vein endothelial cells (49). The fact that vitamin D3 increases the activity of pro-MMP-2 in our study suggests that vitamin D3 thereby might stimulate the degradation of extracellular matrix proteins and thus promotes angiogenesis. One can speculate that a possible mechanism to explain this relation could again be an increase of VEGF expression that might enhance pro-MMP-2-activity.

Several hypotheses have been set forward to explain the molecular mechanisms of vascular dysfunction in preeclampsia. Impaired placentation and concomitant placental hypoxia induce the release of various factors in the maternal circulation that lead to enhanced endothelial permeability (25), altered expression of adhesion molecules, and increased intracellular free calcium content in the endothelium (26). VEGF is one of the most important growth and survival factors of endothelial cells and, free VEGF is reduced in the plasma of preeclamptic women (15), whereas its soluble receptor sFlt-1 is elevated in the serum of preeclamptic women (8). An increase of free VEGF locally or in the maternal circulation could thereby improve endothelial function and consequently might even delay or improve symptoms of preeclampsia. Hence, increased expression of VEGF by endothelial cells in the presence of vitamin D3 could plausibly explain a preventive effect of vitamin D3 intake on the development of preeclampsia in vivo.

Endothelial cells are the primary constituents of new vessels, and a variety of endothelial functions are required for angiogenesis. Cultured endothelial cells are shown to be similar to angiogenic endothelial cells in vivo (44). However, cell culture can lead to changes in growth characteristics and cell surface markers of cells (reviewed in Ref. 2). Organ culture or in vivo angiogenesis models should verify our findings in the future.

For all experiments, we used cultured ECFCs because they have been shown to form blood vessels with circulation in the placental bed during placental development (51). Although ECFCs can be isolated from well-established culture techniques and by flow cytometry, no standards and accepted means of phenotyping have yet been defined, and there are several different, partly contradictory, approaches found in the literature (extensively reviewed in Ref. 51). For cell culture experiments, mostly ECFCs derived from the umbilical cord blood are used because the concentration of ECFCs in cord blood is 15 times higher than in the adult peripheral circulation. In culture, cord blood ECFC populations can double at least 100 times without any signs of senescence, while adult ECFCs typically do not exceed >20–30 population doublings (29). We therefore used fetal ECFCs for these experiments, although it has to be noted that conclusions from our experiments have to be understood within these limits and knowledge can only partially be transferred to explain the maternal disease of preeclampsia.

In conclusion, we show that vitamin D3 stimulates angiogenesis in vitro. By increasing VEGF expression and pro-MMP-2 activity, we also demonstrate a possible mechanism by which vitamin D3 exerts its effect on ECFC function. These findings could explain the known connection between vitamin D3 deficiency and preeclampsia risk and may elucidate why vitamin D3 substitution in early pregnancy (i.e., in time of placental development) appears to reduce the risk of developing preeclampsia. Future investigations will focus on the confirmation of the observed effects of vitamin D3 on endothelial function in the in vivo situation.

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