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Increased placental angiogenesis in late and early onset pre-eclampsia is associated with differential activation of vascular endothelial growth factor receptor 2

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ABSTRACT

Introduction: Placentas from both early-onset (EOPE) and late-onset pre-eclampsia (LOPE) exhibit signs of underperfusion, which in turn, may be associated with altered angiogenesis. Tyrosine 951 (Y951) and Y1175 phosphorylation of the vascular endothelial growth factor receptor 2 (VEGFR2) induced by VEGF triggers the angiogenesis process. Endothelial markers such as CD31 and CD34 have been used for estimating angiogenic processes in several tissues, including placenta. We asked whether vascular density in placental villi was related to Y951/Y1175 phosphorylation of VEGFR2 in LOPE or EOPE.
Methods: We obtained placental samples from women with normal pregnancies ($n = 22$), LOPE ($n = 13$), EOPE ($n = 15$) and preterm deliveries ($n = 10$). Slices from placental tissue were used for CD31 immunostaining. We estimated the expression of CD31, CD34, VEGF, and VEGFR2 by western blot and quantitative PCR. Y951 phosphorylation of VEGFR2 was estimated by western blot, whereas Y1175 phosphorylation was analyzed by ELISA.
Results: Vessel density in terminal villi and CD31 and CD34 protein abundance were increased in LOPE and EOPE compared to normal pregnancy. However, mRNA levels for CD31 and CD34 were lower in LOPE than in normal pregnancy and VEGF mRNA was higher in EOPE. VEGFR2 protein concentration was not different among the studied groups. Y951 and Y1175 phosphorylation of VEGFR2 was higher in LOPE than in the normotensive group, but only Y951 exhibited greater phosphorylation in EOPE compared to normal pregnancy.
Discussion: Changes in vessel formation in the pre-eclamptic placenta are controversial. Our study suggests a pro-angiogenic state in both LOPE and EOPE. These changes are however, associated with differential expression of endothelial markers and VEGFR2 activation.
Conclusion: There is evidence of increased placental angiogenesis in LOPE and EOPE that is associated with differential activation of VEGFR2.

1. Introduction

Pre-eclampsia affects nearly 7% of all pregnancies, worldwide [1,2]. It is a leading cause of maternal-neonatal morbidity and mortality [2,3], particularly when it occurs <34 weeks of gestation [4]. Early-onset pre-eclampsia (EOPE) and late-onset pre-eclampsia (LOPE) [5] are characterized by failed remodeling of maternal vessels perfusing the placenta [6–11], which in turn, is associated with high velocity inflow of maternal blood to the placental villi [6].

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor type 2; Y951, tyrosine 951; Y1175, tyrosine 1175; LOPE, late-onset pre-eclampsia; EOPE, early-onset pre-eclampsia; IL-8, interleukin 8.

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Changes in vessel formation within the pre-eclamptic placenta are nevertheless controversial. Qualitative analysis [12,13] and estimation of the expression of the progenitor and mature endothelial cell marker CD34 [14] suggest elevated angiogenesis in the pre-eclamptic placenta [12,13]. However, other semi-quantitative measurements, using stereology [15–17] or immunohistochemistry for endothelial markers such as CD31 or von Willebrand factor suggest reduced angiogenesis in pre-eclamptic placentas [18–23].

These conflicting findings may be related to several issues in the selection of the pre-eclamptic placenta for analysis [11], including gestational age (early vs. late onset), the presence of intrauterine growth restriction, differences in newborn sex, and inclusion of samples before or during labor. The importance of controlling for gestational age is indicated by the observation that EOPE exhibits reduced terminal villi volume and surface area, while with LOPE, there is no evident difference in peripheral villi or the vasculature when compared to findings in normotensive controls [24].

Five vascular endothelial growth factor (VEGF) ligands, which occur in different splice variants and processed forms, have been identified in mammals [25,26]. Among these isoforms, VEGF type A (denoted as VEGF or VEGFA or $VEGF_{165}$) is the prototype for VEGF ligands involved in the angiogenic process [25,26]. VEGF binds and activates two tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1) [27,28].

VEGFR2 is expressed most prominently in vascular endothelial cells and their embryonic precursors [see Ref. details in [25]]. This receptor has potent tyrosine kinase activity and transduces the major signals for angiogenesis [25,26]. In particular, tyrosine 951 (Y951) and tyrosine 1175 (Y1175) are rapidly autophosphorylated after exposure to VEGF, leading to activation of VEGFR2, with consequent cell migration, proliferation, and angiogenesis [25–29]. Therefore, changes in the phosphorylation of VEGFR2 could be more directly associated with variations in angiogenesis than the total protein expression of this receptor or its principal ligand, VEGF.

This is particularly relevant in pre-eclampsia, since the expression of VEGF and its receptors in feto-placental tissues is controversial. Elevated, reduced or unchanged expression has been described [30–40]. However, reduced Y951 phosphorylation in placentas from pre-eclampsia suggests a reduced pro-angiogenic state compared to normal pregnancy [41]. By contrast, mRNA for VEGFR2 in placenta is high in the hemolysis, elevated liver enzymes, and low platelet count (HELLP) syndrome compared to normal pregnancy [38]. We propose that placental angiogenesis is increased in pre-eclampsia and could be associated with VEGFR2 activation.

2. Patients and methods

2.1. Patients

The Ethical Committee of the Universidad del Bío-Bío approved this cohort study. Informed consent was obtained from each participant. Pregnant women who were attended by members of the Obstetric and Gynecology Department of the Herminda Martin Clinical Hospital, Chillán, Chile, for their delivery were included in this study. Inclusion and exclusion criteria were previously published [42]. The women were classified as having (1) normal pregnancy (maternal blood pressure $<$ 140/90 mm Hg, absence of proteinuria, and no medical complications), (2) pre-eclampsia (new-onset hypertension, defined as blood pressure $\geq$ 140/90 mm Hg, with at least two measurements, 6 h apart, and proteinuria $>$ 300 mg/24 h after 20 weeks of gestation), or (3) preterm delivery ($<$37 weeks of gestation, without any clinical history of premature rupture of membranes or pathological chorioamnionitis). The pre-eclamptic group was divided into EOPE ($\leqq$35 weeks) or LOPE ($>$35 weeks). Blood pressure was measured at 27–35 weeks.

2.2. Sampling of placentas

Within 20 min after delivery, placentas were weighed and examined macroscopically to exclude clots, fibrosis, or areas of infarction. Four random full-depth samples of the placentas were then taken from each tissue. Some samples were fixed in paraformaldehyde (4%) in phosphate buffer solution (NaCl 13.7 mM, KCl 2.7 mM, Na_2HPO_4 0.9 mM, KH_2PO_4 1.8 mM, pH 7.4, 4 °C) and embedded in paraffin as previously described [43]. Other samples were immersed in liquid nitrogen and maintained at −80 °C until used for mRNA and protein extraction.

Table 1
Primers used for Q-PCR.

Protein	Sequence	Tm (melting point)	Product size	Annealing
CD31	F: 5-CAACAGACATGGCAACAAGG-3 R:5-TTCTGGATGGTGAAGTTGGC-3	85	240	55
CD34	F: 5-AATGAGGCCACAACAAACA TCACA-3 R:5-CTGTCCTTCTTAAACTCCGC ACAGC-3	82	380	59
VEGFR2	F: 5- AGACCAAAGGGGCACG ATTC -3 R:5-GTCTGGTCTTTTGGTGTTTT GCTGT-3	84	490	57
$VEGF_{165}$	F: 5-GGGCAGAATCATCACG AAGTG-3 R:5-ATTGGATGGCAGTAGCTGCG-3	78	65	60
IL-8	F: 5-TGCCAAGGAGTGCTAAAG-3 R:5-TCCACAACCCTCTGCAC-3	72	197	60
Cyclophilin	F: 5-CTCCTTTGAGCTGTTTGCAG -3 R:5-CACCACATGCTTGCCATCC-3	79	350	60

2.3. Microscopic analysis and immunohistochemistry

Paraffin-embedded tissue sections were cut into 5-μm slices to use for immunodetection of CD31 (rabbit polyclonal antibody; Thermo Scientific, Waltham, MA) using a commercial kit (Vector Laboratories, Burlingame, CA). Antigen-antibody reaction was visualized by diaminobenzidine reaction, and densitometry was performed using Image-Pro Plus software (Media Cybernetics, Rockville, MD) in ten random pictures from each preparation. This approach, allowed us to measure the perimeter of every vessel and its corresponding villi. These were expressed as mean vessel perimeter, in micrometers, divided by the respective villous perimeter.

Stem villi were defined by the presence of large vessels (more than 20% of the entire villi), with evident smooth muscle layers. Smooth muscle was identified morphologically and by location (encircling blood vessels). Diffusion villi (i.e., microcirculation) were defined, as previously described [24], as the villi containing vessels without evident smooth muscle layers.

The intensity of staining in the pictures was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) [44]. The luminance of the incident light was calibrated for each section to assign pixel values from 0 (no light transmission) to 255 (full light transmission). The immunostained signal was then extracted from the images with a color deconvolution algorithm integrated into the ImageJ software. Values are expressed as the ratio between the area of positive brown stain and the total area of the reference field. Analysis of the section was blinded.

2.4. Western blot

Samples were prepared in an Ultra-Turrax homogenizer (Daihan Scientific, Seoul, South Korea) with a lysis buffer (Tris HCL, pH 8, 20 mM; NaCl 137 mM; EDTA 2 mM; glycerol 10%, Nonidet P-40 1%) and protease inhibitor cocktail (Thermo Scientific). Homogenates were centrifuged at 14,000× g at 4 °C for 10 min. The supernatant was used for western blot analysis, where protein extracts (70 μg) were separated by SDS-PAGE (10%), transferred to nitrocellulose membranes, and probed with primary anti-CD31, anti-CD34, and anti-VEGF (Abcam, Cambridge, MA); anti-VEGFR2 and phospho-VEGFR2 in Y951 (Cell Signaling Technology, Danvers, MA); and anti-β-actin (Sigma–Aldrich, St. Louis, MO) antibodies. A horseradish peroxidase-conjugated secondary antibody was used for visualization.

2.5. ELISA for VEGFR2 tyrosine phosphorylation

The PathScan Phospho-VEGFR-2 (Tyr 1175) sandwich ELISA kit (Cell signaling Technology) was used to measure Tyr 1175 phosphorylation of VEGFR2 in total placental homogenate (300 μg) following the manufacturer's instructions.

2.6. Quantitative PCR

Reverse transcription and quantitative PCR were performed as previously described [45], using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions.