Endocrine gland-derived vascular endothelial growth factor in etiology of pre-eclampsia – our experiences

MICHAŁ SZUBER1, WIESŁAW MARKWITZ1, HENRY N. JABBOUR2, FIONA DENISON3, GRZEGORZ H. BRĘBOROWICZ1

Abstract
Background: Pre-eclampsia (PE) is a hypertensive complication of 7-10% of all pregnancies worldwide. Endocrine gland-derived vascular endothelial growth factor (EG-VEGF, prokineticin 1 – PK-1) and its receptor (PKR1) has been identified. The main function of EG-VEGF is angiogenesis, and therefore it may play a crucial role in impaired endothelial functioning in pre-eclamptic pregnancies. Materials and methods: We designed two study groups: pregnant PE-patients and healthy controls. The samples were obtained during elective caesarean sections of the consented patients. We have collected myometrial and placental biopsies from 40 patients (19 were PE-patients). Tissue was collected and fixed or frozen. RNA was extracted and subjected to quantitative PCR and fixed tissue was used for immunohistochemistry. A sample of every tissue was cultured in vitro, following stimulation with EG-VEGF and the activation of the intracellular pathways was assessed. Results: There is a peak response in 30 minutes after adding ligand (EG-VEGF) to the pre-incubated tissue. This pattern is alike in myometrium and placenta of the controls whereas in the PE-patients the placental response is blurred. The response to the ligand is stronger in placentas of the controls than in their myometriums whereas in PE-patients it is completely opposite. In immunohistochemistry slides greater presence of PK1 and PKR1 was seen in endothelial cells of the controls. RT-PCR shows greater expression of the PK1 in placentas of the controls than those of the PE-patients. Conclusions: From this stage of our research we can conclude that signaling and expression of EG-VEGF in PE-patients are disturbed.

Key words: pre-eclampsia, prokineticin-1 (PK1), endocrine gland-derived vascular endothelial growth factor

Background
Pre-eclampsia (PE) is a hypertensive complication of 7-10% of all pregnancies worldwide, is responsible for approximately 40% of iatrogenic preterm deliveries and is associated with significant perinatal morbidity and mortality of the newborns. In Europe, hypertensive disorders of pregnancy are the second most common cause of maternal mortality whereas PE increases the future risk of developing hypertension, coronary heart disease and cerebro-vascular disease in mothers. In spite of recent findings, the pathophysiology of PE still remains unclear. The pathogenesis of PE is thought to act at three levels: defective placentation, placental ischemia and endothelial cell dysfunction of which the latest is considered to be a key factor associated with PE. Endothelial cell dysfunction is believed to be responsible for several changes, such as increased endothelial-mediated vasoconstriction, increased vascular permeability and increased endothelial-mediated platelet aggregation leading to maternal hypertension, proteinuria and thrombocytopenia.

Prokineticin-1 (PK1) [also known as endocrine gland derived vascular endothelial growth factor (EG-VEGF)] is a recently described protein with a range of physiological and pathological functions [1]. The cognate receptors for PK1 are two closely related G protein-coupled receptors, PK receptor (PKR)-1 and PKR2. These couple to either Gi or Gq [2-4], activating downstream signaling pathways that include calcium mobilization, stimulation of phosphoinositide turnover, and activation of MAPK. PK1 is expressed in steroidogenic tissues including the placenta [5, 6], ovary [7, 8], and adrenal [3]. It mediates tissue specific vascular effects, which include capillary endothelial cell survival, proliferation, differentiation, and induction of fenestrae [3]. In contrast to VEGF, PK1 has no effect on endothelial cells derived from aorta, umbilical vein, or cornea [5]. More recently, mouse studies have demonstrated that PKR1 gene activation promotes angiogenesis in cultured cardiac endothelial cells without increasing VEGF levels [9]. PK1 is also thought to have a role in immune regulation, affecting differentiation of human bone marrow cells into a distinct mono-

1 Department of Perinatology and Gynecology, University of Medical Sciences in Poznań, Poland
2 Human Reproductive Sciences Unit, Medical Research Council, Edinburgh, Scotland
3 Reproductive and Developmental Sciences, Simpson Centre for Reproductive Health, Edinburgh, Scotland
cytokine storm during late pregnancy, from 20th week of gestation until the 2nd week of puerperium) with systolic and diastolic BP more than 140/90 mm Hg on at least two occasions and urinary protein more than ++ on dipstick (or more than 0.5 g/24 H, or more than 300 mg/l, or ACR > 30 mg/mmol) and otherwise healthy, pregnant women. The samples were obtained during the elective cesarean sections of the consented patients from both groups (third trimester only). Collection of placentas and myometrium samples was approved by the Ethics Committee by the University of Medical Sciences in Poznan, Poland, and followed the recommended guidelines for using human subjects. We have collected and processed myometrial and placental biopsies from 40 patients so far. Among those 19 were PE-patients. Each sample was approximately 3 by 2 cm in size. Tissue was collected and divided into three samples. One part of every sample was fixed overnight at 4°C in 4% paraformaldehyde in 10 mM PBS and wax embedded for immunohistochemical analysis. Another sample was placed in RNA later, and RNA was extracted using an RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions and subjected to quantitative PCR. The tissue was homogenized until the samples were uniformly homogeneous. The concentration of RNA was measured using a spectrophotometer, and RNA quality was confirmed on agarose gels. Real-time PCR was performed by monitoring in real time the increase in fluorescence of the SYBR Green using 7900 HT Fast real-time PCR System by Iscience. In addition, a third sample of every tissue was placed in RPMI (Sigma, Poole, UK) (containing 2 mm l-glutamine, 100 IU penicillin, and 100 μg/ml streptomycin) and transported to the laboratory for in vitro culture. Tissue was cultured in vitro, following stimulation with EG-VEGF and the activation of the intracellular pathways was assessed. After stimulation with ligand (EG-VEGF) (time course – 0, 5, 10, 20, 30 minutes after stimulation respectively) the tissues were homogenized in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml aprotinin] by using a Tissue lyser Qiagen by Retsch, until the samples were uniformly homogeneous. After centrifugation, protein concentrations of the supernatant were determined with BSA (fraction V; Sigma, St. Louis, MO) as standards. Placental and myometrial proteins were electrophoretically separated on NuPAGE 4-12% Novex Bis-Tris Gels by Invitrogen and electrically transferred onto 0.25 μm polyvinylidene difluoride membranes. The blots were washed with PBS-Tween 20 0.1% (PBS-T) and blocked in Odyssey Blocking Buffer for 1 hour. Then the blots were incubated with a mixture of antibodies against pERK (pERK rabbit phospho p44/42 MAP Kinase by Cell Signalling®) and tERK (teRK mouse p42 MAP Kinase by Cell Signalling®) for 60 minutes. Afterwards the membranes were washed in PBS Tween and incubated in the mixture of secondary antibodies against primary antibodies (IRDye 800 Conjugated Affinity Purified Anti-Mouse IgG (H&L) (Goat) by Rockland; Alexa Fluor 680 goat anti-rabbit IgG (H+L) highly cross absorbed 2 mg/ml by Molecular Probes) for 60 minutes. After several washes in PBS Tween and PBS the membranes were scanned using LI-COR Biosciences Odyssey.
Infrared Imaging System. The intensities of immunoreactive bands were measured and the image analyzed on a desktop computer using LI-COR Biosciences Odyssey software. The mean pixel density for each band was analyzed to obtain relative OD units for phosphorylation of ERK signalling proteins (Fig. 1, Fig. 2)

Results

The tissue is still being processed, and so far the signalling processes are not completed, yet. In samples that are ready (about 50% of all samples) there is a prominent pattern with peak response (fold in pERK/tERK ratio to minute “0”) in 30 minutes after adding ligand (EG-VEGF) to the pre-incubated tissue. This pattern is very much alike in both myometrium and placenta of the control group (Fig. 3, Fig. 4) whereas in the PE-patients the placental response is blurred (Fig. 5). We also discovered that the response to ligand is stronger in placentas of the control patients than in their myometriums whereas in PE-patients it is completely opposite (Fig. 6).

Apart from signalling the immunohistochemistry examination was performed. In those slides abundant presence of PKR1 but not PK1 was seen in endothelial cells of the control (Fig. 8) while in PE-patients the amount of staining was slightly lower for the receptor (PKR1) as well as for the ligand (PK1) (Fig. 7). In myometrium samples of both groups it was difficult to visualize even the receptor, not mentioning the ligand (EG-VEGF). Negative controls confirmed the physiological structures of placentas and myometriums in both control and PE-patients.
As for the RNA assessment, table 1 shows the values of PK1 and PKR1 expression in placenta and myometrium (myo), in PE-group and in controls (CTRL). The number values of the expression correspond to the reference value which was the expression of PK1 and PKR1 in normal, eutopic, second-phase endometrium.

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<th>Table 1. Values of the PK1 and PKR1 expression in placenta and myometrium (myo) in the PE-group and in the controls (CTRL)</th>
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Table 2. Placenta PKR1 controls versus PE. No statistically significant differences were detected ($P=0.471$)

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Conclusions
From this early stage of our research we can conclude that signalling pathways in PE-patients are disturbed. The relative response to the ligand is highest in 30 minutes and this pattern of signalling is predominant in controls while in PE-patients signalling is greatly disturbed. This may be due to smaller amount of the PK1 receptors (as seen on some of the slides) or their impaired ability to transform in response to the ligand (EG-VEGF). To resolve this issue we designed an experiment with inhibitors of the signalling pathways in endothelial cells such as EGFR, c-SRC, Gq, MEK, PKA, PKC; that will be conducted in the near future. There is however statistically significant difference in PK1 expression in the placenta of women from PE-group compared to controls (0.58 versus 2.04). It shows that the expression of PK1 in placenta is statistically significantly higher in healthy women compared to women suffering from pre-eclampsia which is in contrast with other papers already published [12].

Acknowledgments
We would like to show our gratitude to the team of the Human Reproductive Sciences Unit, Medical Research Council, Edinburgh, for their technical support. Special thanks goes to Sheila Wright, dr Sharon Battersby, Jemma Evans and dr Kurt Sales, all working under the supervision of dr Henry N. Jabbour, without whose help this work wouldn’t be possible.

References


Michal Szuber
Department of Perinatology and Gynecology
University of Medical Sciences in Poznań
Polna 33, 60-535 Poznań, Poland