OBSTETRICS

Preeclampsia: novel insights from global RNA profiling of trophoblast subpopulations

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BACKGROUND: The maternal signs of preeclampsia, which include the new onset of high blood pressure, can occur because of faulty placentation. We theorized that transcriptomic analyses of trophoblast subpopulations in situ would lend new insights into the role of these cells in preeclampsia pathogenesis.

OBJECTIVE: Our goal was to enrich syncytiotrophoblasts, invasive cytotrophoblasts, or endovascular cytotrophoblasts from the placentas of severe preeclampsia cases. Total RNA was subjected to global transcriptional profiling to identify RNAs that were misexpressed compared with controls.

STUDY DESIGN: This was a cross-sectional analysis of placentas from women who had been diagnosed with severe preeclampsia. Gestational age-matched controls were placentas from women who had a preterm birth with no signs of infection. Laser microdissection enabled enrichment of syncytiotrophoblasts, invasive cytotrophoblasts, or endovascular cytotrophoblasts. After RNA isolation, a microarray approach was used for global transcriptional profiling. Immunolocalization identified changes in messenger RNA expression that carried over to the protein level. Differential expression of non—protein-coding RNAs was confirmed by in situ hybridization. A 2-way analysis of variance of non-coding RNA expression identified particular classes that distinguished trophoblasts in cases vs controls. Cajal body foci were visualized by coilin immunolocalization.

RESULTS: Comparison of the trophoblast subtype data within each group (severe preeclampsia or noninfected preterm birth) identified many highly differentially expressed genes. They included molecules that are known to be expressed by each subpopulation, which is evidence that the method worked. Genes that were expressed differentially between the 2 groups, in a cell-type—specific manner, encoded a combination of molecules that previous studies associated with severe preeclampsia and those that were not known to be dysregulated in this pregnancy complication. Gene ontology analysis of the syncytiotrophoblast data highlighted the dysregulation of immune functions, morphogenesis, transport, and responses to vascular endothelial growth factor and progesterone. The invasive cytotrophoblast data provided evidence of alterations in cellular movement, which is consistent with the shallow invasion often associated with severe preeclampsia. Other dysregulated pathways included immune, lipid, oxygen, and transforming growth factor-beta responses. The data for endovascular cytotrophoblasts showed disordered metabolism, signaling, and vascular development. Additionally, the transcriptional data revealed the differential expression in severe preeclampsia of 2 classes of non-coding RNAs: long non-coding RNAs and small nucleolar RNAs. The long non-coding RNA, urothelial cancer associated 1, was the most highly up-regulated in this class. In situ hybridization confirmed severe preeclampsia-associated expression in syncytiotrophoblasts. The small nucleolar RNAs, which chemically modify RNA structure, also correlated with severe preeclampsia. Thus, we enumerated Cajal body foci, sites of small nucleolar RNA activity, in primary cytotrophoblasts that were isolated from control and severe preeclampsia placentas. In severe preeclampsia, cytotrophoblasts had approximately double the number of these foci as the control samples.

CONCLUSION: A laser microdissection approach enabled the identification of novel messenger RNAs and non-coding RNAs that were misexpressed by various trophoblast subpopulations in severe preeclampsia. The results suggested new avenues of investigation, in particular, the roles of PRG2, Kell blood group determinants, and *urothelial cancer associated 1* in syncytiotrophoblast diseases. Additionally, many of the newly identified dysregulated molecules might have clinical utility as biomarkers of severe preeclampsia.

Key words: biomarker, cytotrophoblast, laser microdissection, mRNA, non-protein-coding RNA, placenta, preeclampsia, syncytiotrophoblast

In many ways the placenta steers the course of pregnancy.[1] Preeclampsia is one of the great obstetrics syndromes that also include intrauterine growth restriction, preterm labor, preterm premature rupture of membranes, late spontaneous abortion, and placental abruption.[2] They share the common feature of being associated with defects in deep placentation; the depth to which cytotrophoblasts invade the uterus is relatively shallow compared with normal pregnancies. This oftentimes is accompanied by aberrations in physiologic transformation of the spiral arteries.[2,3] How this common denominator diverges into the disparate signs of each condition is not understood. Preeclampsia, which affects 3—7% of pregnancies in developed countries, is diagnosed according to the maternal signs that include the new onset of high blood pressure and, variably, proteinuria.[4] Severe (and early-onset) preeclampsia (sPE) entails exacerbation of the hypertension or new findings such as thrombocytopenia, impaired liver function.[5,6] Preeclampsia is also a major cause of premature birth and low birthweight.[7] Beyond pregnancy, the development of a pregnancy complication that is associated with faulty placentation, such as preeclampsia, positively correlates with an elevated relative risk of

Cite this article as: Gormley M, Ona K, Kapidzic M, et al. Preeclampsia: novel insights from global RNA profiling of trophoblast subpopulations. Am J Obstet Gynecol 2017;217:200.e1-17.

0002-9378

http://dx.doi.org/10.1016/j.ajog.2017.03.017

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maternal cardiovascular disease years later.[8]

The association between preeclampsia and abnormal placentation has been the impetus for studies aimed at understanding the origins and dimensions of the latter defects. At a morphologic level, abnormalities in formation of the placental bed are thought to be particularly important. Cytotrophoblast invasion of the uterine wall is variably shallow rather than deep. More consistently, cytotrophoblast remodeling of uterine spiral arteries is incomplete, which has the net effect of reducing maternal blood flow to the placenta.[9] At a molecular level, cytotrophoblast differentiation along the pathway that leads to uterine invasion is abnormal, as demonstrated by deficits in the elaborate switching of stage-specific antigens that normally enable invasion, vascular mimicry, and their cohabitation of the decidua with the unusual maternal immune cells that reside in this location.[10] In addition to morphologic and targeted molecular analyses, investigators are also applying global transcriptional profiling approaches to better understand the placental defects that are associated with preeclampsia. To date, these studies have focused on chorionic villi,[11,12] the maternal-fetal interface,[13] or purified cytotrophoblasts as they differentiate along the invasive pathway in vitro.[14] These studies have the advantage of being unbiased therefore enabling the de novo discovery of misregulated genes.

However, the global transcriptional profiling approaches that have been applied to date all have certain disadvantages. In tissue-based analyses, signals from the cell types of interest are diluted by the many other cell types that contribute to the gene expression signature of the sample. Cultured cytotrophoblasts, although a more homogenous cell type, may not wholly recapitulate differentiation along the invasive pathway in the absence of maternal cells and the signals that they provide. To circumvent these problems, we applied a laser microdissection approach to placentas from sPE pregnancies. This enabled the enrichment and transcriptional profiling of specific trophoblast subpopulations: syncytiotrophoblasts (STBs), invasive cytotrophoblasts (iCTBs) and endovascular cytotrophoblasts (eCTBs). The results revealed novel aspects of gene expression in each of the cell types that we examined, which could be useful sPE biomarkers if they appear in maternal blood. The data also suggested new theories about the pathogenesis of sPE, particularly in terms of the placental manifestations.

Materials and Methods

Study design

This was a case-control study with the goal of the identification of changes in trophoblast gene expression in sPE compared with noninfected preterm birth (nPTB). The American College of Obstetrics and Gynecology criteria were used to identify patients with preeclampsia[5,15]: (1) the new onset of hypertension measured on 2 occasions of least 4 hours apart: blood pressure $\geq$140 mm Hg (systolic) or 90 mm Hg (diastolic) in a woman who was normotensive at $<$20 weeks of gestation; and (2) the appearance of proteinuria: excretion of $\geq$300 mg in a 24-hour period or protein/creatinine ratio $\geq$0.3 mg/dL or dipstick reading of 1+, which is used only if other quantitative methods are not available. SPE was also diagnosed with American College of Obstetrics and Gynecology criteria[5,15] based on additional signs and symptoms: systolic blood pressure $\geq$160 mm Hg and/or diastolic pressure $\geq$110 mm Hg on 2 occasions at least 4 hours apart while the patient is on bed rest, proteinuria of $\geq$5 g in a 24-hour period or 3+ on urine dipstick, hemolysis (peripheral blood smear and/or lactate dehydrogenase $\geq$480 U/L), elevated liver function (serum glutamic oxaloacetic transaminase $\geq$64 U/L or serum glutamic-pyruvic transaminase $\geq$80 U/L), thrombocytopenia (platelets $\leq$100,000), oliguria ($\leq$500 mL in 24 hour), creatinine $\geq$1.1 mg/dL or a doubling of the serum creatinine concentration with no known renal dysfunction/disease, cerebral or visual disturbances, and/or convulsions with no history of seizure disorders.

As controls, samples were obtained from women who delivered because of nPTB, diagnosed according to the criteria recommended by Herron et al,[16] including regular uterine contractions at $>$20 or $<$37 weeks of gestation, which are $\leq$5−8 min apart and accompanied by $\geq$1 of the following events: (1) progressive changes in the cervix, (2) cervical dilation $\geq$2 cm, and/or (3) cervical effacement $\geq$80%. Patients with evidence of inflammation were excluded on the basis of the following criteria: maternal fever $>$100.4°F, uterine tenderness, fetal tachycardia (fetal heart rate $>$160 beats per min), and/or (placental) histologic criteria compatible with inflammation.[17-19] Women with preexisting medical conditions such as thyroid insufficiency, chronic hypertension, or diabetes mellitus were excluded from the case and the control groups. Other exclusion criteria included premature rupture of membranes and/or fetal anomaly. The University of California San Francisco Institutional Review Board (Committee on Human Research) approved this study. Written informed consent was obtained from all donors.

A laser microdissection approach was used to isolate STBs, iCTBs, and eCTBs. Total RNA that was purified from each sample type was subjected to microarray analyses, which enabled the identification of misexpressed transcripts. An immunolocalization approach was used to identify sPE-associated alterations in messenger RNA (mRNA) abundance that carried over to the protein level. A 2-way analysis of variance (ANOVA) dentified changes in the expression of non-protein-coding RNAs that tracked with sPE. An in situ hybridization approach was used to verify the latter data. Immunolocalization of coilin enabled enumeration of Cajal body foci, sites of small nucleolar RNA (snoRNA) activity.

Human tissue collection

Samples were collected within 1 hour of birth, washed in phosphate-buffered saline solution (PBS), transferred to ice-cold cytowash medium (DME/H-21 Medium, 1% glutamine plus, 1% penicillin/streptomycin, 0.1% gentamycin) supplemented with 2.5% fetal bovine