Molecular changes in signal transduction pathways in preeclampsia and HELLP syndrome

PhD thesis

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1. INTRODUCTION

Preeclampsia and HELLP syndrome are major causes of maternal and perinatal mortality and morbidity worldwide. Unfortunately, no specific therapies are available for their treatment, and thus, their only effective therapy is the delivery of the placenta. Therefore, the main aims of current international studies are to promote the development of new screening tools for women at high risk of preeclampsia and new therapies that would enable the prolongation of pregnancy without harming the health of the mother and baby in preeclamptic pregnancies.

Emerging evidence suggests that abnormal placentation in early pregnancy leads to the hypoxic and ischemic injury of the placenta and villous trophoblast, causing angiogenic/anti-angiogenic imbalance and the development of these severe obstetrical syndromes. Therefore, our studies on preeclampsia and HELLP syndrome focused on the investigations of the placenta. As a continuation of our earlier studies, here we studied signaling molecules that are important in placental functions, cell proliferation, migration and apoptosis. We also investigated the expression patterns of genes which may play a role in placental malfunctioning in preeclampsia. Moreover, we examined the activation of various villous trophoblastic signaling pathways, the stress conditions which could alter these signaling pathways in the trophoblast, and the impact of these stresses on placental gene expresion.

Among the molecules which can affect placental signaling pathways, we chose syndecan-1 for our investigations, since it is highly expressed in the syncytiotrophoblastic layer of the human placenta, and it is involved in the regulation of signaling pathways by binding to various angiogenic and growth factors. The exact placental functions of syndecan-1 and its possible involvement in the development of placental pathologies are still obscure, however, because of syndecan-1 is highly expressed in the placenta, and it localizes to the syncytiotrophoblast, it may has a significant role in the maintenance of healthy pregnancies.

Our studies also focused on the investigations on how various stress conditions and kinase signaling pathways may affect *FLT1*, *GCM1*, *LEP*, and *PGF* expression in the villous trophoblast. An earlier investigation of our research group conducted a microarray study on placentas from women with preeclampsia, HELLP syndrome and controls. In this study we first revisited our placental microarray dataset for four selected genes (*FLT1*, *GCM1*, *LEP*, and *PGF*), which were found to be predominantly expressed by the villous trophoblast, differentially expressed in the placenta in preterm preeclampsia, and causally implicated in its pathogenesis.

Based on published evidence, we supposed that there is a complex interplay between various stress conditions, the alterations in various signaling pathways in the villous trophoblast, as well as gene expression and functional changes in the placenta in preeclampsia. One of the main objectives of our study was to investigate the placental expression changes of syndecan-1 in various subforms of preeclampsia and HELLP syndrome. In addition, we were also interested in investigating the possible changes in the expression and activities of members of the Akt-1 and MAPK signaling pathways in preeclampsia and HELLP syndrome, and in studying how certain stress conditions may affect the function of these kinases and gene expression in the villous trophoblast.

2. OBJECTIVES

The objectives of my study were:

1. The comparative study of syndecan-1 expression in placentas from patients with preeclampsia and HELLP syndome as well as matched controls;
2. The comparative study of maternal serum syndecan-1 concentrations in women wih preeclampsia and normal pregnant women;
3. The investigations of the effect of ischemia and actin citoskeleton damage on syndecan-1 expression and release in BeWo cell models;
4. The investigations of the changes in the expression and activitiy of pAkt-1, pERK1/2, pJNK and pp38 kinases in placentas from women with preeclampsia and HELLP syndrome;
5. The study on the regulatory role of the cAMP signaling pathway in BeWo cells on the expression of *FLT1*, *GCM1*, *LEP*, and *PGF*, genes associated with the development of preeclampsia;
6. The investigations of the expression of *FLT1*, *GCM1*, *LEP*, and *PGF* genes and the activitation of Akt-1, ERK1/2, JNK és p38 kinases in BeWo cells in hypoxic, ischaemic and pro-inflammatory stress conditions;
7. The examinations of the effect of Akt-1, ERK1/2, JNK and p38 kinase inhibitors on the gene expression signature in BeWo cells in hypoxic, ischamic and pro-inflammatory stress conditions.

3. METHODS

3.1. Studies on syndecan-1

3.1.1. Patient groups, clinical samples, and definitions

The Health Science Board (Budapest, Hungary) and the Institutional Review Boards of Wayne State University (Detroit, Michigan, USA) and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD), National Institutes of Health (NIH), U.S. Department of Health and Human Services (DHHS) (Bethesda, Maryland, and Detroit, Michigan, USA) approved the collection and use of materials for research purposes. Informed consent was obtained from all women prior to sample collection. Specimens and data were collected and stored anonymously.

Placental specimens were collected at the First Department of Obstetrics and Gynecology, Semmelweis University (Budapest, Hungary). Women were enrolled in the following groups: (1) late-onset preeclampsia (*n*=8); (2) early-onset preeclampsia (*n*=7); (3) early-onset preeclampsia with HELLP syndrome (*n*=8); (4) preterm controls (*n*=5); and (5) term controls (*n*=9). Maternal blood specimens (n=81) were selected from the Bank of Biological Materials of the Perinatology Research Branch, NICHD/NIH/DHHS (Detroit, Michigan). Patients were included in the following groups: (1) preterm and term preeclampsia (*n*=49) and (2) gestational age-matched controls (*n*=32). Patients with fetal congenital anomalies and multiple gestations at each site were excluded from the study. Blood specimens were collected within a week before delivery, and sera were collected by centrifuging blood at 1,300xg for 10min at 4°C, and then stored at -70°C.

Preeclampsia was defined as new-onset hypertension (systolic or diastolic blood pressure ≥140 or ≥90mmHg, respectively, measured at two time points, 4h to 1 week apart) developing after 20 weeks of gestation along with proteinuria (≥300mg during a 24h urine collection, or two random urine specimens obtained 4h to 1 week apart containing ≥1+ by dipstick, or one dipstick of ≥2+ protein). HELLP syndrome was defined by the presence of hemolysis (serum LDH >600IU/l; bilirubin >1.2mg/dl; presence of schistocytes in peripheral blood), elevated liver enzymes (serum ALT and/or AST >70IU/l), and thrombocytopenia (platelet count <100,000/mm^3), developing after 20 weeks of gestation. The term "small-for-gestational–age" (SGA) was defined as neonatal birth weight below the 10^{th} percentile for gestational age according to the national birth weight distribution curves. No medical or obstetrical complications were detected in term controls who delivered a neonate with a birth weight appropriate for gestational age. Preterm controls had preterm deliveries with no

clinical or histological signs of chorioamnionitis, and delivered a neonate with a birth weight appropriate for gestational age.

3.1.2. Placental specimens and histopathological evaluations

Placentas were obtained following Caesarean deliveries. For microarray studies, villous tissue samples were excised from central cotyledons, and microarray analysis was performed as previously described. Placentas were examined according to a standard histopathological protocol that describes the topography and size of macroscopic lesions, and then were fixed in formalin. For tissue microarray, five representative histological blocks from each placenta were paraffin-embedded to include central and peripheral cotyledons and the maternal side of the placenta. For microscopic examinations, 4μm sections were cut from these blocks and mounted on SuperFrost/Plus slides (Gerhard Menzel GmbH, Braunschweig, Germany). After deparaffinization, slides were rehydrated, stained with hematoxylin-eosin, and evaluated in 10 randomly selected microscopic fields. Macroscopic and microscopic lesions were defined according to published criteria.

3.1.3. Placental tissue microarray construction and syndecan-1 immunostainings

Representative cores of 2mm in diameter were collected from all tissue blocks of each placenta and placed into recipient blocks. Five-micrometer-thick tissue sections were cut and mounted on SuperFrost/Plus slides and stored at $+4^{o}$C until the staining. Immunostaining was carried out using a Leica BOND-MAX autostainer (Leica GmbH, Nussloch, Germany). Slides were dewaxed in Bond Dewax Solution (Leica Microsystems) and rehydrated in Bond Wash Solution (Leica Microsystems). Antigen retrieval was performed at pH6 using Bond Epitope Retrieval 1 Solution (Leica Microsystems) for 30min at 100°C. Slides were incubated for 20min at room temperature with a mouse monoclonal anti-syndecan-1 antibody (clone MI15; DakoCytomation, Glostrup, Denmark; 1:50). Primary antibody binding to tissue sections were visualized using biotin-free Bond Polymer Refine Detection (Leica Microsystems). After post-primary amplification (30min) and detection with the Novolink Polymer Detection System using 3,3'-Diaminobenzidine (DAB, Novocastra Laboratories; 1:50), slides were counterstained with hematoxylin.

3.1.4. Immunostaining evaluation

Syndecan-1 immunostaining in the syncytiotrophoblast was quantified by the Pannoramic Viewer v1.15 of MembraneQuant software (3DHISTECH Ltd., Budapest, Hungary), which is suitable for unbiased semi-automated analysis of digital image objects based on color,

intensity, and size. After the detection algorithm was calibrated, MembraneQuant found approximately 90% of the villi with a diameter of 20μm–150μm in each core. Staining intensities (+1, +2, or +3) of all scored villi in a given core were averaged, and this score was used as the representative data for that core.

In addition, visual evaluation of immunostaining was also performed by three examiners who were blinded to the clinical information and used an Axioskop 2 plus light microscope (Carl Zeiss MicroImaging GmbH, Gottingen, Germany). Subsequently, TMA slides were digitally scanned with a high-resolution scanner (Pannoramic Scan, 3DHISTECH Ltd.), and image data were used for virtual microscopic evaluation with the Pannoramic Viewer 1.15. Virtual slides were independently evaluated by the same examiners. In both cases, immunostaining of the syncytiotrophoblast brush border membrane was semi-quantitatively scored using an immunoreactive score modified from that previously described. Briefly, at least 25 terminal or intermediate microvilli with a diameter of 20μm-150μm were evaluated in each core. Staining intensities (0, +1, +2, or +3) of villi in a given core were averaged, and this score was used as the representative data for that core.

3.1.5. BeWo cell cultures

BeWo cells (American Type Culture Collection, Manassas, VA, USA) were cultured with F12 medium (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin (P/S). Cells were either plated on 6-well plates ($0.5x10^6$/plate) or on Nunc Lab-Tek II chambered coverglass slides ($0.3x10^6$/chamber) (Thermo Scientific, Wilmington, DE, USA), and were used for various experiments: (1) to test the effect of trophoblast differentiation on syndecan-1 expression, cells were treated either with Forskolin (25μM; Sigma-Aldrich Co. LLC, St. Louis, MO, USA) or with a vehicle (dimethyl sulfoxide, DMSO, Sigma-Aldrich) for three days; (2) to test the effect of actin cytoskeleton disruption on syndecan-1 expression and release from the trophoblast, cells were treated with 25μM Forskolin for three days, and one-half of the samples were treated with 25μM Latrunculin between hours 60-72; and (3) to test the effect of ischemic stress on syndecan-1 expression and release, cells were treated with 25μM Forskolin for three days, and one-half of the samples was kept in 20% O_2, while the other one-half was kept in alternating O_2 concentrations (20% for 6h, 1% for 6h; 4 cycles) in an OxyCycler C42 (Biospherix Ltd., Lacona, NY, USA) between hours 48-72.

3.1.6. RNA isolation, cDNA synthesis, quantitative real-time reverse transcription PCR (qRT-PCR)

Total RNA was isolated from cells in 6-well plates with the RNeasy Mini Kit and RNase-Free DNase Set (Qiagen, Valencia, CA, USA). The RNA concentrations were measured with the NanoDrop1000 Spectrophotometer (Thermo Scientific). Total RNA (500ng) was reverse-transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen). TaqMan Assays (Applied Biosystems, Life Technologies Corporation) for *SDC1* (Hs00896423_m1) and *RPLP0* (large ribosomal protein; Endogenous Control; 4326314E) were used for gene expression profiling on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems).

3.1.7. Immuncytochemistry and confocal microscopy

Cells in chambered coverglass slides were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), blocked with Protein Block (Dako North America, Inc., Carpinteria, CA, USA), and stained with a mouse monoclonal anti-human syndecan-1 antibody (clone MI15; 5ug/ml; Novus Biologicals, LLC, Littleton, CO, USA) overnight at 4^{o}C. After repeated washes, Alexa Fluor 488 goat anti-mouse IgG (Invitrogen) was added at 1:1000 dilution in PBS with 10% normal Goat Serum (MP Biomedicals, LLC, Santa Ana, CA, USA). Cells were mounted with ProLong Gold antifade reagents and 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and followed by confocal microscopy using a Zeiss LSM 780 spectral confocal system (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA).

3.1.8. Syndecan-1 immunoassays

Cell culture supernatants were collected from 6-well plates and used for immunoassays. Similar to maternal serum specimens, cell culture supernatant were also measured for their syndecan-1 concentrations with a human syndecan-1 ELISA Kit (Cell Sciences, Canton, MA, USA), according to the manufacturer's instructions. The sensitivity of the assay was <2.56 ng/ml, and the co-efficients of intra-assay variation and inter-assay variation were 7.6% and 6.8%, respectively, according to the manufacturer.

3.1.9. Statistical analyses

Demographic data were analyzed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Comparisons among the groups were performed using Chi-square and Fisher's exact tests for proportions, Kruskal-Wallis and Mann-Whitney tests for non-normally distributed continuous variables, and the t-test for normally distributed continuous variables. Pearson coefficients and associated p-values were calculated for the correlation between maternal serum syndecan-

1 concentrations and clinical/demographic parameters using R (www.r-project.org). A linear model was used to reveal group differences in maternal serum syndecan-1 concentrations after adjusting for gestational age and birth weight. The level of significance was set to 5%.

3.2. Evaluations of kinases pathways

3.2.1. Patient groups, clinical samples and definitions

Patient groups, clinical samples and definitions were the same as described in 3.1.1.

3.2.2. Placental tissue specimens

Placental tissue specimens were the same as described in 3.1.2.

3.2.3. Placental TMA construction and immunostainings

Placental TMA construction was the same as described in 3.1.3. Then, five μm-thick tissue sections were cut from TMA blocks and mounted on SuperFrost/Plus slides, which were dewaxed and rehydrated. Endogenous peroxidase was blocked with H_2O_2 (10%) for 20min at room temperature (RT), and then slides were heated at 100°C for 45min in 10mM Tris containing 1mM EDTA (pH=9.0) for antigen retrieval. After washing, unspecific binding was blocked (10min, RT) with an appropriate buffer (NovoLink Polymer Detection System, Novocastra Laboratories, Newcastle, UK). Immunostainings were carried out using phospho Akt-1 (Cell Signaling Technology, Inc., Danvers, MA, USA), phospho ERK1/2 (Cell Signaling), phospho JNK (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), phospho p38 (Cell Signaling) antibodies 4°C overnight. After the postprimary block (30min), slides were visualized by Novolink Polymer Detection System using DAB (Novocastra in 1:20 dilution), and counterstained with hematoxylin.

3.2.4. Evaluation of immunostainings

TMAs were digitally scanned by a high-resolution Pannoramic Scan scanner, and then digital slides were used for virtual microscopic evaluation by two examiners blinded to the clinical information using the Pannoramic Viewer 1.15. At least 25 terminal or intermediate villi with a diameter of 20μm-150μm were evaluated in each core. Immunostainings were semi-quantitatively scored for villous trophoblasts with an immunoreactive score modified from that previously published. The intensity of the immunostainings (0, +1, +2, +3) was averaged

within a core, and this score was used as the representative data for that core. Immunoscores were then averaged at the placenta and the patient group levels.

3.2.5 Primary trophoblast cultures

At Wayne State University, the modified method of Kliman et al. was used to isolate villous cytotrophoblasts from normal term placentas collected from healthy pregnant women who delivered an AGA neonate at term (n=4). Approximately 100g villous tissues from each placenta were cut into pieces, rinsed in PBS, and digested at 37^{o}C for 90 minutes with a solution containing 0.25% Trypsin (Invitrogen, Carlsbad, CA, USA) and 60U/ml DNAse I (Sigma-Aldrich). Subsequently, dispersed cells were filtered through Falcon nylon mesh cell strainers (100μm, BD Biosciences, San Jose, CA). Erythrocytes were lysed with 5ml NH_4Cl (Stemcell Technologies, Vancouver, BC, Canada); then washed and resuspended cells were layered over 20-50% Percoll gradients, followed by centrifugation for 20 minutes at 1,200g. From the collected trophoblast containing bands, non-trophoblastic cells were excluded by negative selection with anti-CD14 (20ug/ml) and anti-CD9 (20ug/ml) mouse monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) and MACS anti-mouse IgG microbeads (Miltenyi Biotec, Auburn, CA, USA). The isolated primary trophoblasts were plated on collagen-coated 12-well plates (BD Biosciences; $3x10^6$ cells/well) in triplicate and kept in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 5% human serum and 1% P/S (Invitrogen) for seven days. Total RNA was isolated on each day in triplicate for gene expression profiling.

3.2.6. BeWo cell cultures

BeWo cells were plated into 6-well plates ($5x10^5$ cells/well) and cultured in F12 medium (Invitrogen) supplemented with 10% FBS and 1% P/S. For differentiation experiments, BeWo cells were treated for 96h either with the cAMP-analogue Forskolin (25μM, Sigma-Aldrich) or with DMSO (Sigma-Aldrich) as a control, and then cells were harvested for total RNA isolation and gene expression profiling. For kinase activity experiments, BeWo cells were treated for 36h with 25μM Forskolin, and then were split into four treatment groups for 24h: 1) kept under normoxia (20%O_2); 2) kept in hypoxia (2%O_2); kept in ischemia (1% and 20%O_2 alternating for every 6h) in an Oxycycler C42 (BioSpherix, Lacona, NY, USA); or 4) treated with 10ng/ml IL-1β (R&D Systems) in normoxia. After treatments, BeWo cells were lysed and collected for total RNA and total protein. All experiments were run in triplicate.

3.2.7. RNA isolation, cDNA generation, qRT-PCR

Total RNA was isolated from BeWo cells and primary trophoblasts with the Qiagen RNeasy kit (Qiagen). RNA concentrations were measured with NanoDrop1000 (Thermo Scientific, Wilmington, DE, USA). The 28S/18S ratios and the RNA integrity numbers were assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Total RNA (500ng) was reverse-transcribed with the SuperScript III First-Strand Synthesis System (Invitrogen) and oligo(dT)20 primers (Invitrogen). TaqMan assays (Applied Biosystems) were used for gene expression profiling on the Biomark qRT-PCR system (Fluidigm, San Francisco, CA, USA) according to the manufacturer's instructions.

3.2.8 Protein isolation and phospho-kinase assays

BeWo cells were homogenized with 120µL lysis buffer (50mM Tris-HCl, pH7.5; 150mM NaCl; 1% NP40; 5mM EDTA) and incubated for 1h on ice. Homogenates were centrifuged (15min at 10,000 rpm) to pellet non-soluble debris; supernatants containing soluble or solubilized proteins were collected, and their total protein contents were measured by BCA assay (Thermo Scientific). The Proteome Profiler 96 Human Phospho-RTK Array 1 (R&D Systems, Minneapolis, MN, USA), which employs a sandwich immunoassay technique, was used to simultaneously detect phosphorylated kinases in each sample on 96-well microplates according to the manufacturer's recommendation. After 3h of incubation, raw chemiluminescence signal data were obtained with a Fujifilm LAS-4000 Cooled CCD Camera Gel Documentation System (Fujifilm North America Corp., Valhalla, NY, USA).

3.2.9. Statistical analyses

Demographic, clinical and immunoscore data were analyzed using SPSS v.12.0 (SPSS Inc., Chicago, IL, USA). Comparisons among the groups were performed using the Fisher's exact test for proportions, t-test for normally distributed and Mann-Whitney test for non-normally distributed continuous variables. A p=0.05 cutoff was applied for statistical significance. All other data were analyzed in the R statistical language and environment. To analyze qRT-PCR data, gene expressions were calculated relative to the *RPLP0* housekeeping gene. The ΔΔCt method was used to determine the magnitude of expression changes and pooled variance t-test was used to determine the significance of differences. A p=0.05 cutoff was applied to infer statistical significance. For the analysis of Proteome Profiler data, raw chemiluminescence signals were adjusted for noise by subtracting the background signal. Then, adjusted chemiluminescence signal data were log-transformed and normalized by subtracting the signal from the heat shock protein 60 (HSP60) internal control reference values. The

estimated mean +/- standard error (SE) values were summarized, and t-tests were used to determine differential expression. False discovery rate (FDR) correction was applied on the nominal p-value to obtain q-values. A q=0.1 cutoff was applied for statistical significance.

4. RESULTS

4.1. Results of the syndecan-1 studies

4.1.1. Demographic and clinical data

In the placental study, peak systolic and diastolic blood pressures were significantly higher in all patient, but not control, groups. Proteinuria was detected in all cases but not in the control groups. Although term and preterm controls were matched to cases within two weeks of gestational age, the median gestational age of term controls was slightly higher than that of cases with late-onset preeclampsia. In the maternal serum study, peak systolic and diastolic blood pressures were higher in women with preeclampsia compared to the controls. Proteinuria was present in all cases, and HELLP syndrome developed in 10 cases of preeclampsia. Primiparity was more frequent and birth weight was lower in patients with preeclampsia than in controls.

4.1.2. Syndecan-1 expression is dependent on villous trophoblast differentiation

In term control placentas, syndecan-1 immunostaining was confined to the syncytiotrophoblast apical membrane, displaying a linear granular structure. A moderate amount of these syndecan-1 immunopositive granules was also detected in the syncytiotrophoblast cytoplasm. No syndecan-1 immunostaining was detected in the cytotrophoblast or in other cell types of the villi.

Forskolin-treated BeWo cells expressed an increasing amount of *SDC1* during morphological and biochemical differentiation (Day 1: no change; Day 2: 3.2-fold increase, $p=8.3x10^{-4}$; Day 3: 3.4-fold increase, $p=5.3x10^{-5}$). Furthermore, differentiating BeWo cells released significantly higher amounts of syndecan-1 into the cell culture medium on Day 2 (4.3 ± 0.5ng/ml, p=0.028) and Day 3 (15.2 ± 2.0ng/ml, p=0.008) than on Day 1 (under the detection limit of 2.56ng/ml).

When compared to non-treated BeWo cells, Forskolin-treated BeWo cells, especially multinucleated fused cells, had strong cell membrane syndecan-1 immunostaining on Day 3

of the cultures. In accord with placental findings, we also detected a punctuate pattern of cell membrane and cytoplasmic syndecan-1 immunostaining in these BeWo cells.

4.1.3. Placental syndecan-1 mRNA expression is not affected by preeclampsia

We did not find any significant difference in placental *SDC1* expression for early-onset preeclampsia, either complicated with (1.43-fold down-regulation, adjusted p=0.74) or without (1.56-fold down-regulation, adjusted p=0.65) HELLP syndrome when compared to the control group.

4.1.4. Placental syndecan-1 immunoreactivity is increased in preeclampsia and HELLP syndrome

The most profound alteration in syndecan-1 immunostainings was observed in cases with early-onset preeclampsia where the cytoplasm of the syncytiotrophoblast gave a strong immunoreaction, and the syncytiotrophoblast apical membrane was immunonegative for this proteoglycan in several cases. Similar but less intensive syndecan-1 immunostaining of the syncytiotrophoblast cytoplasm was observed in late-onset preeclampsia specimens. In cases of early-onset preeclampsia with HELLP syndrome, the syncytiotrophoblast apical membrane showed strong linear positivity. The syncytiotrophoblast also showed signs of syndecan-1 release to the intervillous space in 29%-55% of cases in all study groups, and to a somewhat larger extent in cases with early-onset preeclampsia and HELLP syndrome than in controls.

To objectively quantify placental syndecan-1 immunostaining on scanned TMA slides, we used the newly developed Pannoramic Viewer v1.15 of MembraneQuant and found that: (1) syndecan-1 immunostaining did not change with gestational age as immunoscores in preterm and term control groups were similar (2.33 ± 0.33 versus 2.29 ± 0.33, p=0.66); (2) syndecan-1 immunoscores were higher in cases of late-onset preeclampsia than in the term control group (2.59 ± 0.26 versus 2.29 ± 0.33; p=0.0001); (3) syndecan-1 immunoscores were higher in cases of early-onset HELLP syndrome than in the preterm control group (2.63 ± 0.23 versus 2.33 ± 0.33; p=0.02); and (4) syndecan-1 immunoscores were higher in cases of early-onset preeclampsia than in the preterm control group (2.59 ± 0.36 versus 2.33 ± 0.33; p=0.02). These findings were supported by microscopic and virtual microscopic evaluations and semi-quantitative TMA immunoscorings, although these latter scoring systems slightly differed.

4.1.5. Maternal serum syndecan-1 concentrations are decreased in preeclampsia

Maternal serum syndecan-1 concentrations positively correlated with birth weight (R^2=0.25, p=$1.7\text{x}10^{-6}$) and gestational age (R^2=0.22, p=$1\text{x}10^{-5}$), and negatively correlated with mean

arterial pressure (R^2=0.08, p=0.012) and urine protein content (R^2=0.06, p=0.039). Maternal serum syndecan-1 concentration was lower in cases with preeclampsia (median: 673ng/ml, interquartile range (IQR): 459-1161ng/ml) than in the control group (median: 1158ng/ml, IQR: 622-1480ng/ml).

4.1.6. Syndecan-1 release from BeWo cells is altered by ischemia and actin cytoskeleton disruption

To model syncytiotrophoblastic syndecan-1 release or retention in preeclampsia, we placed Forskolin-treated BeWo cells in ischemic conditions for 24h, or co-treated them for 12h with Latrunculin B, a disruptor of actin cytoskeleton. We detected no difference in *SDC1* expression for the BeWo cells kept in ischemic conditions (1.14-fold change, p=0.72) or for those treated with Latrunculin B (1.07-fold change, p=0.69) compared to the controls. However, there was an increased release of syndecan-1 from BeWo cells into the culture medium in ischemic conditions (30$\pm$1.8ng/ml, p=0.042), and a decreased release after Latrunculin B treatment (17.4$\pm$3.3ng/ml, p=0.050) compared to the controls (24.0$\pm$4.2ng/ml). Immunofluorescence stainings for syndecan-1 and confocal imaging of Forskolin-treated control BeWo cells revealed the strongest cell membrane syndecan-1 immunostaining of multinucleated, fused cells. BeWo cells kept in ischemic conditions had faint cell membrane staining of syndecan-1, suggesting an increased shedding of syndecan-1 from the trophoblast cell surface into the cell culture medium, similar to the increased shedding of syndecan-1 from cell surfaces in pro-inflammatory conditions. Latrunculin B-treated BeWo cells had no (or faint) cell membrane syndecan-1 immunoreactivity; however, there was increased granular syndecan-1 accumulation in their cytoplasm, suggesting a defect in the cytoplasmic and cell membrane transport of syndecan-1, leading to its decreased release into the cell culture medium upon the disruption of the actin cytoskeleton.

4.2. Results of the studies on kinase pathways

4.2.1. Demographic, clinical and histopathologic data

Demographic, clinical and histopathologic data were the same as described in 4.1.1.

4.2.2. Placental gene expression changes in preterm preeclampsia with or without HELLP syndrome

Placental *LEP* expression was 108.9-fold ($q=6.4x10^{-5}$) and 56.9-fold (q=0.002) up-regulated in preterm preeclampsia and preterm preeclampsia with HELLP syndrome compared to preterm controls, respectively. Similarly, placental *FLT1* expression was 3.6-fold (q=0.085) and 4.3-fold (q=0.04) up-regulated in preterm preeclampsia and preterm preeclampsia with HELLP syndrome compared to preterm controls, respectively. The differential expression of *GCM1* and *PGF* did not reach significant levels according to the criteria set by our study.

4.2.3. Changes in trophoblastic signaling pathways in preeclampsia and HELLP syndrome

Among alterations in trophoblastic signaling pathways evaluated by phosphorylated Akt-1, ERK1/2, JNK, and p38 immunopositivity of the villous trophoblast, the most impact of preeclampsia was observed on the p38 signaling pathway. Among cases of preterm preeclampsia, the mean pp38 immunoscore increased in larger extent in cases with HELLP syndrome (2.15$\pm$0.43, p=0.004) than in those who did not have HELLP syndrome (1.98$\pm$0.37, p=0.01) compared to preterm controls (1.19$\pm$0.51). Pp38 immunoscore was not changed in late-onset preeclampsia (1.60$\pm$0.39, p=0.5) compared to term controls (1.33$\pm$0.53).

Similar, but less intense changes were observed in the ERK1/2 signaling pathway. Among cases of preterm preeclampsia, the mean pERK1/2 immunoscore was significantly higher in cases associated with HELLP syndrome (1.33$\pm$0.38, p=0.03), while the increase in mean pERK1/2 immunoscore did not reach statistical significance in cases who did not have HELLP syndrome (1.11$\pm$0.73, p=0.3).

4.2.4. Trophoblast differentiation affects angiogenic/anti-angiogenic gene expression balance

We examined gene expression changes during the course of trophoblast differentiation to optimize our assays. When differentiating primary villous trophoblasts obtained from normal term placentas, we observed that days 2 and 3 of differentiation represented the peak expression for the selected genes. When differentiating BeWo cells with the cAMP-analogue Forskolin, we observed a plateau in the expression of the selected genes between days 2 and 3 of differentiation. Similar to primary trophoblasts, we detected a remarkable increase in *PGF* and *LEP* expression during differentiation, while *FLT1* expression changed only modestly. Since a relatively high and stable expression of these genes was needed for further experiments with BeWo cells, we chose to use the day 2 to 3 time-window in differentiation.

4.2.5. Trophoblastic hypoxia and ischemia mimic gene expression changes in preterm preeclampsia

To investigate the changes in the expression of the selected genes in various stress conditions, which are relevant in inducing preeclampsia-related changes in the villous trophoblast in preeclampsia, first we differentiated BeWo cells for two days, and then applied three different stress conditions for 24 hours: 1) *In ischemia*, *LEP* expression increased by 2.1-fold (p=0.04), 2) *In hypoxia*, the expression of *FLT1* increased by 2.5-fold (p=0.056), while *PGF* was down-regulated by 1.4-fold (p=0.04). 3) *After IL-1β treatment* that mimicked pro-inflammatory changes in the placenta in preeclampsia, we observed the down-regulation of *PGF* by 1.6-fold (p=0.01); however, there was no significant change in the expression of *FLT1* or *LEP*.

4.2.6. Hypoxia and ischemia mimic kinase pathway changes in preterm preeclampsia associated with or without HELLP syndrome

We treated BeWo cells in the same way as for the qRT-PCR experiments, and kinase activity assays were run on protein lysates: 1) *In ischemia*, JNK had a 2.5-fold increased activity (q=0.03), ERK1/2 had a 2.4-fold decreased activity (q=0.02), while there was a 1.3-fold, marginally significant increase in p38 activity (q=0.17). 2) *In hypoxia*, the activity of three kinases increased significantly. There was a 1.7-fold increase in ERK1/2 activity (q=0.03), a 1.6-fold increase in JNK activity (q=0.1), and a 1.3-fold increase in p38 activity (q=0.06). 3) *After IL-1β treatment*, JNK had a 2.1-fold increased activity (q=0.03), while p38 activity was increased by 1.4-fold (q=0.04).

4.2.7. The p38 kinase signaling pathway predominantly regulate gene expression changes in hypoxic and ischemic BeWo cells

In normoxic control differentiating BeWo cells, the ERK1/2 inhibitor decreased the expression of *FLT1*, *LEP* and *PGF*, in line with the role of ERK1/2 in supporting trophoblast differentiation and the expression of trophoblastic genes. *In hypoxia*, the p38 and JNK inhibitors had the most effect on gene expression compared to normoxic control cells, in line with the role of p38 and JNK kinases in signaling environmental stress in the placenta. *In ischemia*, the p38 and Akt-1 inhibitors had the most effect on gene expression compared to normoxic control cells, while the p38 inhibitor impacted mostly gene expression after *IL-1β treatment* compared to normoxic control cells. Overall, the p38 signaling pathway had the most impact on *LEP*, *FLT1* and *PGF* expression in the three applied stress conditions.

5. CONCLUSIONS

1) Syndecan-1 was mainly localized to the syncytiotrophoblast, especially to its apical membrane in term, control placentas;
2) Syndecan-1 immunostaining of the syncytiotrophoblast and its apical membrane did not change with gestational age in the control placentas;
3) There was increased syndecan-1 immunostaining intensity of the syncytiotrophoblast in cases of late-onset preeclampsia and early-onset preeclampsia with or without HELLP syndrome;
4) *SDC1* expression did not change in preterm preeclampsia and HELLP syndrome compared to preterm controls;
5) Maternal serum syndecan-1 concentration positively correlated with gestational age and birth weight, and negatively correlated with blood pressure and proteinuria;
6) Maternal serum syndecan-1 concentration was lower in cases of preeclampsia than in gestational age-matched controls;
7) The presence of SGA neonates did not affect maternal serum syndecan-1 concentration in pregnancies with preeclampsia;
8) The disruption of the actin cytoskeleton led to the accumulation of cytoplasmic syndecan-1 in BeWo cells, while ischemic stress caused increased release of syndecan-1 from BeWo cells;
9) Trophoblastic pp38 immunoscore was higher in preterm preeclampsia, especially in cases with HELLP syndrome, than in controls;
10) Mean pERK1/2 immunoscore was higher in preterm preeclampsia with HELLP syndrome than in controls;
11) In differentiating BeWo cells, ischemia up-regulated *LEP* expression, and it increased JNK and decreased ERK1/2 activity;
12) In differentiating BeWo cells, hypoxia up-regulated *FLT1* and down-regulated *PGF* expression, and it increased ERK1/2, JNK and p38 activity;
13) In differentiating BeWo cells, IL-1β treatment down-regulated *PGF* expression, and it increased JNK and p38 activity; and
14) Among the investigated MAPK signaling pathways, the p38 signaling pathway had the most impact on *LEP*, *FLT1* and *PGF* expression in BeWo cells under stress conditions compared with control, normoxic BeWo cells.

6. PUBLICATIONS

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