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Placental antiangiogenic prolactin fragments are increased in human and rat maternal diabetes

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ABSTRACT

Introduction/objectives: The role of the placenta in diabetic mothers on fetal development and programming is unknown. Prolactin (PRL) produced by decidual endometrial cells may have an impact. Although full-length PRL is angiogenic, the processed form by bone morphogenetic protein-1 (BMP-1) and/or cathepsin D (CTSD) is antiangiogenic.
The objectives were to investigate the involvement of decidual PRL and its antiangiogenic fragments in placentas from type-1 diabetic women (T1D) and from pregnant diabetic rats with lower offspring weights than controls.
Methods: PRL, *BMP-1*, and *CTSD* gene expressions and PRL protein level were assessed in T1D placentas ($n = 8$) at delivery and compared to controls ($n = 5$). Wistar rats received, at day 7 of pregnancy, streptozotocin (STZ) ($n = 5$) or nicotinamide (NCT) plus STZ ($n = 9$) or vehicle ($n = 9$). Placental whole-genome gene expression and PRL western blots were performed at birth.
Results: In human placentas, *PRL* ($p < 0.05$) and *BMP-1* ($p < 0.01$) gene expressions were increased with a higher amount of cleaved PRL ($p < 0.05$) in T1D than controls. In rats, diabetes was more pronounced in STZ than in NCT–STZ group with intra-uterine growth restriction. Decidual prolactin-related protein (*Dprp*) ($p < 0.01$) and *Bmp-1* ($p < 0.001$) genes were up-regulated in both diabetic groups, with an increased cleaved PRL amount in the STZ ($p < 0.05$) and NCT–STZ ($p < 0.05$) groups compared to controls. No difference in CTSD gene expression was observed in rats or women.
Conclusions: Alterations in the levels of the PRL family are associated with maternal diabetes in both rats and T1D women suggesting that placental changes in these hormones impact on fetal development.

1. Introduction

Maternal diabetes (MD) accounts for a variety of fetal adverse effects, including spontaneous abortion, intrauterine fetal death, and maternal and perinatal complications (preeclampsia, prematurity, neonatal respiratory distress syndrome) associated with abnormal birth weight [1], and depends on the severity of diabetes [2], especially when vascular complications are present [3]. The perinatal period represents a critical window of vulnerability to the environment [4] and can modulate health conditions later in life [5,6]. MD induces damage to placenta [7,8] but the molecular mechanisms involved are still elusive.

The placenta produces numerous hormones including prolactin (PRL). PRL is released from decidualized endometrial cells, and is thought to regulate the immune system [9,10], amniotic-fluid homeostasis [10,

Abbreviations: BMP-1, bone morphogenetic protein-1; CTSD, cathepsin D; D, day of gestation; dPRL, decidual prolactin; DPRP, decidual prolactin-related protein; DT1, type 1 diabetes; IUGR, intra uterine growth restriction; MD, maternal diabetes; MMP, metalloproteinase; NCT, nicotinamide; PAS, Periodic Acid Schiff; PRL, prolactin; RIN, RNA integrity number; SGA, small-for-gestational-age; STZ, streptozotocin

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11], and placental angiogenesis [12,13] at the feto-maternal interface. PRL stimulates cell migration and invasion in human trophoblast in vitro [14]. Angiogenesis is crucial for trophoblast invasion [15] and feto-placental growth [16]. Findings report that endothelial cells produce and release PRL [17].

In this regard, full-length proangiogenic PRL (23-kDa) proteolysis generates N-terminal fragments, products known to be antiangiogenic and proapoptotic, and are also known as vasoinhibins [13,17–20]. Several candidate proteases have been reported in rodents and/or in humans: i.e., certain metalloproteinases (MMPs) [21], bone morphogenetic-protein-1 (BMP-1) in neutral conditions [22], or cathepsin D (CTSD) in acid environment [23,24].

PRL and/or its vasoinhibins are thought to be involved in preeclampsia [12,13,25,26]. More recently, in a cohort of 501 preeclamptic women, the risk of small-for-gestational-age (SGA) infants was increased when vasoinhibins were present in the amniotic fluid [13]. Immune maladaptation to pregnancy may contribute to predominant 16-kDa PRL decidual production [25].

The decidual production coupled to the involvement in placental angiogenesis and preeclampsia, with an increased risk for SGA infants, suggests that decidual PRL (dPRL) and/or its fragments could play a role in MD at the feto-maternal interface.

The aim of our study was to investigate the involvement of dPRL and its antiangiogenic fragments in placentas from type-1 diabetic women (T1D) and pregnant diabetic rats with lower offspring weights than controls.

2. Methods

2.1. Collection of human placentas

Placentas were obtained from controls ($n = 5$) and women with T1D ($n = 8$) at birth, who were recruited from the Hospital Regional Center (CHRU) of Lille (France). For each placenta, samples were obtained from 4 various locations between the decidual and chorionic plates in order to limit the tissue heterogeneity, near the umbilical cord on the fetal side and pooled for gene expression or protein analyses or used individually for immunohistochemical analyses. The sampling location was uniform, performed by the same technical assistant.

All the diabetic women were managed with standardized protocols regarding the treatment of diabetes, especially insulin therapy and prenatal care. Control women were defined by having normal glucose tolerance and a newborn with a normal birth weight. This study was approved by the ethics committee of the CHRU of Lille (DG7 2007-0340/CCP07/43). All women involved received verbal information about the study and gave their written informed consent before examination.

2.2. Animal study

Female Wistar rats (200–250 g, Janvier, Le Genest Saint Isle, France) were housed individually with a controlled 12/12 h light/dark cycle, at 22 ± 1 °C, and were fed ad libitum with a standard chow diet. On the 7th day (D) of gestation, three groups of animals were formed, i.p. injected sequentially after 15 min: a diabetic group with severe hyperglycemia (the STZ group: $n = 5$, received vehicle (NaCl 0.9%) and streptozotocin (STZ) [65 mg/kg, dissolved in citrate buffer 0.1 mmol/l, pH 4.5; Sigma-Aldrich, St Quentin-Fallavier, France]); a diabetic group with moderate hyperglycemia (NCT–STZ group: $n = 9$, received nicotinamide [NCT, Sigma-Aldrich, 75 mg/kg dissolved in NaCl 0.9%] and STZ [65 mg/kg]); and a control group (C group: $n = 9$, received the same volume of two vehicles). By injecting STZ and NCT at day 7 of gestation, we aimed to prevent the early formation of the fetal pancreas. In addition, at this stage, STZ induces MD without directly affecting fetal pancreas. The GLUT2 glucose transporter, through which STZ triggers beta cell destruction, is not expressed in the early pancreas development [27,28].

Daily capillary blood-glucose (ACCU-CHEK Performa Glucometer, Roche Diagnostics, Mannheim, Germany) and weight were evaluated from D7–18 in dams. At D19, for all groups, an oral glucose-tolerance test was performed after 16 h of fasting. Animals received 2 g/kg of glucose, and blood samples were collected from the tail vein at 0, 30, 60, 90, and 120 min to assess glucose (ACCU-CHEK Performa glucometer) and insulin (insulin Rat kit ELISA, Mercodia, Uppsala, Sweden) levels.

All animal experiments were conducted in accordance with the European Communication Council Directive of November 24, 1986 (86/609/EEC).

2.3. Collection of blood and tissue samples from rats

Pregnant rats were sacrificed before delivery at D21, corresponding to the end of pregnancy, and maternal blood was collected to assess glucose (ACCU-CHEK Performa glucometer), insulin (insulin rat kit, ELISA), and PRL (rat prolactin ELISA kit, CUSABIO BIOTECH, China) plasma levels. Fetuses were removed with their respective placentas and separately weighed. Placentas were washed several times in Phosphate Buffered Saline (PBS) to eliminate red blood cells and transferred in RNAlater (Qiagen, Courtaboeuf, France) for gene expression analysis, or were snap-frozen in liquid nitrogen and stored at −80 °C for protein analysis, or were fixed in 4% paraformaldehyde (without previous washing in PBS) for histological analysis. Fetuses' pancreases were removed and fixed in 4% paraformaldehyde. Placentas and pancreases, fixed in 4% paraformaldehyde, were paraffin-embedded (Paraplast Plus, McCormick LLC, St. Louis, MO, USA). Fetuses' blood samples were collected for the determination of glucose levels (ACCU-CHEK) and plasma samples in each litter were pooled to assess insulin levels (insulin rat kit, ELISA).

2.4. Gene-expression analyses of human and rat placentas

2.4.1. Extraction of RNA

Placental samples from women were transferred in RNAlater (Qiagen, France). For rats, five placentas were randomly selected from each dam and placentas from each group were pooled together for microarray analysis or used individually for qRT-PCR validation.

Total RNA was isolated using the RNeasy® kit (Qiagen). The quantification and quality of RNA were assessed by spectrophotometric analysis with NanoDrop (Thermo Fischer Scientific, Illkirch, France) and by Agilent Bioanalyzer capillary electrophoresis system (Agilent technologies France, Massy, France), respectively. Measurements were carried out with a Bioanalyzer using RNA integrity number (RIN): the correlating electropherogram and gel-like image were generated for each sample. The RIN was obtained prior to array hybridization. Only high-quality RNA (RIN > 8) was used for analysis.

2.4.2. Quantitative RT-PCR (qRT-PCR) gene-expression analysis of human placentas

For humans, total RNA from each placenta was reverse-transcribed to cDNA using a cDNA synthesis kit (ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis, Invitrogen, France). qRT-PCR was run in duplicate using a LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics, Meylan, France), primers (Table 1), and a real-time PCR system (Thermocycler LightCycler LC 2.0 Roche, France). Gene expression was normalized with the *TATA box-binding protein* gene.

2.4.3. Rat placenta microarray procedure and qRT-PCR gene-expression analysis

Whole-gene expression profiling, using the Illumina Rat Ref-12 BeadChip (22523 probes for a total of 21792 genes from NCBI RefSeq; Illumina, San Diego, CA, USA), was carried out according to standard procedures. Aliquots of 300 ng of total RNA were reverse-transcribed to cDNA, transcribed to cRNA, amplified and biotin-labeled with a