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Transferrin receptor gene and protein expression and localization in human IUGR and normal term placentas

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ABSTRACT

Iron (Fe) deficiency in pregnancy is associated to low birth weight and premature delivery while in adults it can result in increased blood pressure and cardiovascular disease. Cellular Fe uptake is mediated by the Transferrin Receptor 1 (TFRC), located in the trophoblast membranes. Here, we measured TFRC mRNA expression (Real Time PCR) and TFRC protein expression and localization (Western Blotting and immunohistochemistry) in IUGR compared to control placentas. A total of 50 IUGR and 56 control placentas were studied at the time of elective cesarean section. IUGR was defined by ultrasound *in utero*, and confirmed by birth weight <10th percentile. Three different severity groups were identified depending on the umbilical artery pulsatility index and fetal heart rate. TFRC mRNA expression was significantly lower in IUGR placentas compared to controls ($p < 0.05$), and this was confirmed for TFRC protein levels. In both experiments the most severe IUGR group presented lower expression compared to the other groups, and this was also related to umbilical venous oxygen levels. TFRC protein localization in the villous trophoblast did not differ in the groups, and was predominantly present in the syncytiotrophoblast. In conclusion, these are the first observations about TFRC expression in human IUGR placentas, demonstrating its significant decrease in IUGR vs controls. Thus, Fe transport could be limited in IUGR placentas. Further studies are needed to study components of the placental Fe transport system and to clarify the regulation mechanisms involved in TFRC expression, possibly altered in IUGR placentas.

1. Introduction

The placenta is the interface between the mother and her fetus and is critical for fetal growth and nutrition. Placental transport and metabolism are major players involved in fetal nutrition and metabolism since they determine the availability of oxygen and nutrients to the fetus. Intrauterine growth restriction (IUGR) occurs when the fetus fails to achieve its full growth potential, due to reduced nutrient supply. IUGR affects 7–15% of births [1]. It can occur in isolation or in association with maternal hypertensive disorders, such as preeclampsia (PE + IUGR), and it represents a leading cause of perinatal morbidity and mortality [2]. Many studies suggest that low birth weight is associated with the development of the metabolic syndrome in adult life and this has been called "fetal programming" [3,4]. A reduction in placental nutrient transport is clearly involved in IUGR, and a specific placental phenotype has been described [5].

Iron (Fe) deficiency during pregnancy alters embryonic growth and development, and strongly increases the risk of low birth weight and preterm delivery [6,7], as well as the risk of altered newborn development and cardiovascular disease in the adult [8–12].

At a molecular level, possible effects of Fe imbalance are changes in enzyme functions and signaling pathways and alterations in oxidative stress, since iron is essential for the normal functioning of many catalytic pathways, in particular those involved in redox processes.

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Table 1
Characteristics of the population: maternal age, Body Mass Index (BMI), gestational age at birth, fetal and placental weight and maternal Hb in 56 normal and 50 IUGR pregnancies. Data are presented as mean ± standard deviation (SD) and compared by unpaired Student's *t* test.; $^{*}p < 0.05$.

	Controls	IUGR
N	56	50
Maternal age (years)	33 ± 5	34 ± 5
Body Mass Index (BMI)	22 ± 4	22 ± 5
Gestational age (weeks)	38 ± 3	33 ± 4 *
Fetal weight (g)	3230 ± 400	1390 ± 550 *
Placental weight (g)	505 ± 125	230 ± 100 *
Maternal Hb (g/dl)	10.1 ± 1.1	10.7 ± 1.9

Studies in rats have shown that Fe placental transport progressively increases during pregnancy, and is regulated by mechanisms that have only partially been clarified [13–15]. Iron is accumulated in the fetus against a concentration gradient, so that in case of maternal iron deficiency the placenta plays a protective role, establishing a hierarchy: in animal experiments, when Fe levels in the mother are reduced to 30% of control, those in the fetus remain up to about 70% of controls. Thus, in normal conditions the placenta minimizes the effect of potential deficiencies by up-regulating the proteins involved in Fe transfer [16].

The Transferrin Receptor (TFRC) is an essential protein for Fe transfer across the placenta; it is an N- and O-glycosylated transmembrane protein mediating the cellular iron uptake by binding and internalization of diferric transferrin [6], and it is located in the trophoblast plasma membranes [17,18]. TFRC gene and protein levels are regulated by several factors, giving rise to the great importance of Fe for fetal growth, but many of the transcriptional and post-transcriptional regulation mechanisms still remain to be elucidated.

TFRC expression has been shown to be reduced in placentas from preeclamptic pregnancies compared to those from normal pregnancies and in 1st trimester miscarriage, but no data are available in IUGR pregnancies [19].

The aim of this study was to measure TFRC mRNA and protein levels in normal and IUGR placentas and to evaluate if they are related to the severity of intrauterine growth restriction.

2. Material and methods

2.1. Patients

Studies were performed in three University Hospitals located in Milano: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico; Ospedale Luigi Sacco, Department of Clinical Sciences; Ospedale S. Paolo, Department of Medicine, Surgery and Dentistry. The study was approved by the local Institutional Review Board (IRB), and all patients gave informed consent.

A total of 106 unrelated pregnant women were enrolled at the time of elective cesarean section: 50 IUGR and 56 controls.

IUGR was diagnosed *in utero* by means of serial ultrasound examinations, when abdominal circumference measurements were less than the 10th percentile of the age-related reference values, and/or also showed a decrease of more than 40 percentiles from the reference ultrasound growth curve [20]. Growth restriction was confirmed at birth if neonatal weight was less than the 10th percentile according to italian birth weight for gestational age standards [21].

Three severity groups were identified depending on the umbilical artery pulsatility index (PI) and fetal heart rate (FHR), as previously described [22]. IUGR1 ($n = 18$) presented both normal PI and FHR; IUGR2 ($n = 14$) had an abnormal PI and a normal FHR; IUGR3 ($n = 18$) showed both abnormal parameters.

Normal pregnancies were defined as healthy mothers with normal Body Mass Index (BMI), normal pregnancy without obstetrics complications and normal fetal growth confirmed by birth weight between the 10th and the 90th percentile for Italian references [21].

Exclusion criteria for both groups were fetal or maternal infections, maternal drugs or alcohol abuse, multiple pregnancies, fetal malformations, chromosomal abnormalities, maternal chronic hypertension, maternal cardiovascular or autoimmune diseases and diabetes.

In all pregnancies gestational age was calculated from the last menstrual period and confirmed by an ultrasound examination performed at 11–13 weeks gestation [23].

Maternal and fetal characteristics for controls and the IUGR group are presented in Table 1. Maternal age and BMI were not significantly different among the two groups. As expected, gestational age and fetal and placental weights were significantly lower in the IUGR group. Maternal Hemoglobin (Hb) was not significantly different in the two groups.

2.2. Procedures

2.2.1. Samples collection

Placental samples were collected at the time of caesarean section for RNA and protein extraction; fragments were washed using PBS (*Dulbecco's Phosphate Buffered Solution, Euroclone*), cut into small pieces which were then checked by optical microscope in order to eliminate any residual maternal decidua fragment. Selected chorionic villi were then frozen in liquid nitrogen for protein extraction, or put in RNA stabilizing solution (*RNA later, Ambion, Austin TX, USA*) for RNA extraction; in this latter case samples were stored at 4 °C for 24 h, and then stored at −20 °C.

For immunohistochemistry experiments, placental fresh full thickness samples were fixed in 10% neutral buffered formalin for 12–24 h before paraffin embedding.

2.2.2. Biochemical analyses

Umbilical venous and arterial blood was sampled in 22 IUGR and 24 controls for pH, oxygen saturation and content, and pO_2 measurements, and in 24 IUGR and 26 controls for Hb, lactate and glucose measurements, from a doubly clamped segment of the cord immediately after fetal extraction. All samples were collected in heparinized syringes and kept on ice until the end of analysis. Blood gases (pO_2 and pCO_2), pH, Hb concentration and oxygen saturation were measured on a GEM Premier 3000 (Instrumentation Laboratory). Oxygen content was calculated according to the following formula [44]: Oxygen Content (mmol/l) = Hemoglobin (g/l) × Oxygen Saturation × 0.005982.

2.2.3. Real Time quantitative RT-PCR analysis

2.2.3.1. TFRC mRNA levels from 49 IUGR and 41 control placentas were analysed by Real Time PCR. Total RNA was isolated from tissues using Trizol reagent (*Invitrogen, Life Technologies, Cergy, France*) following the manufacturer's instructions and treated with "DNA-free kit" (*Ambion, Austin TX, USA*) to remove potentially contaminating DNA. RNA concentration was determined by spectrophotometer NanoDrop ND1000 (*NanoDrop Technologies, Wilmington, DE*). For *TFRC* expression studies, 500 ng of total RNA were reverse transcribed by the High Capacity cDNA Reverse Transcription Kit (*Applied Biosystems, Foster City, CA*) and the obtained cDNA served as template for quantitative Real Time PCR, based on TaqMan methodology, using the 7500 Fast Real Time PCR System (*Applied Biosystems, Foster City, CA*).

The amount of *TFRC* RNA was calculated using the 2^{-DCt} method relative to hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) and succinate dehydrogenase complex-subunit A (*SDHA*) housekeeping genes, selected from a pool of tested housekeeping genes, because they showed a similar amplification efficiency in a scale of RNA concentrations [24]. All the assays were provided by Applied Biosystems (*TaqMan Gene Expression Assays*: ID#: Hs00174609_m1, Hs99999909_m1 and Hs00188166_m1 for *TFRC*, *HPRT* and *SDHA*, respectively). All samples were reverse transcribed in duplicate and cDNA was run in triplicate to allow assessment of sample homogeneity and technical variability. Log_{10}-transformed results were used to obtain normally distributed values, as previously described [25].

Real-time data were analysed using the Sequence Detector software.

2.2.4. Western blotting analysis

TFRC protein levels from 17 IUGR and 11 control placentas were analysed by Western Blotting analysis.

The placental protein lysate was obtained from the liquid Nitrogen frozen chorionic villi samples using the Tri-reagent (*Sigma Aldrich*) following the standard procedure; in order to measure TFRC protein expression in IUGR vs control placentas, 10 μg of protein lysate were diluted 2:1 in a reducing buffer, composed by 66% DTT Reducing Agent 30X (*Cell Signaling, Euroclone*) and 33% SDS blue sample buffer (*Cell Signaling*), and denaturated at 96 °C for 15 min. Proteins were then loaded in a 8% SDS-polyacrilamide gel, electrophoresed and transferred to a PVDF membrane (*Roche*) by a Trans-Blot SD Semi-Dry Electrophoresis Transfer Cell (*Biorad, USA*). The obtained membrane was blocked with 3% BSA (in TBS) for 60 min and incubated overnight at 4 °C with the primary antibody. For TFRC the monoclonal antibody (*Zymed, Palo Alto, CA*) was diluted 1:2000 in TBS containing 0.5% BSA; for *β-Actin*, used for normalization of results, the polyclonal antibody (*Genetex Inc, Irvine, CA*) was diluted 1:1000 in TBS containing 5% BSA. Afer washing with TBS containing 0.01% Tween 20 (*Sigma Aldrich*) (TBS-T), membranes were incubated with the peroxidase coniugated secondary antibody for 60 min at room temperature. For TFRC, the goat-anti mouse antibody (*Chemicon International, Inc., Temecula, CA*) was diluted 1:10,000 in TBS containing 0.5% BSA; for *β-Actin*, the rabbit antibody (*Genetex Inc, Irvine, CA*) was diluted 1:1000 in TBS containing 5% BSA. After washing again with TBS-T, visualization of the immunologically detected proteins was achieved using the Super Signal West Pico Chemiluminescent Substrate (*Pierce*). Processed blots were exposed to X-ray film (*Kodak*) for the optimum exposure time. The