Placenta 41 (2016) 14–26

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Markers of early endothelial dysfunction in intrauterine growth restriction-derived human umbilical vein endothelial cells revealed by 2D-DIGE and mass spectrometry analyses

Andres Caniuguir [a, b, 1], Bernardo J. Krause [b, 1], Cherie Hernandez [a, b], Ricardo Uauy [b], Paola Casanello [a, b, *]

[a] Division of Obstetrics & Gynecology, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile
[b] Division of Pediatrics, Faculty of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile

ARTICLE INFO

Article history:
Received 31 August 2015
Received in revised form
20 February 2016
Accepted 24 February 2016

Keywords:
Umbilical endothelial cells
Intrauterine growth restriction
Vascular dysfunction
Proteome

ABSTRACT

Intrauterine growth restriction (IUGR) associates with fetal and placental vascular dysfunction, and increased cardiovascular risk later on life. We hypothesize that endothelial cells derived from IUGR umbilical veins present significant changes in the proteome which could be involved in the endothelial dysfunction associated to this conditions. To address this the proteome profile of human umbilical endothelial cells (HUVEC) isolated from control and IUGR pregnancies was compared by 2D-Differential In Gel Electrophoresis (DIGE) and further protein identification by MALDI-TOF MS. Using 2D-DIGE 124 spots were identified as differentially expressed between control and IUGR HUVEC, considering a cut-off of 2 fold change, which represented ~10% of the total spots detected. Further identification by MALDI-TOF MS and *in silico* clustering of the proteins showed that those differentially expressed proteins between control and IUGR HUVEC were mainly related with cytoskeleton organization, proteasome degradation, oxidative stress response, mRNA processing, chaperones and vascular function. Finally Principal Component analysis of the identified proteins showed that differentially expressed proteins allow distinguishing between control and IUGR HUVEC based on their proteomic profile. This study demonstrates for the first time that IUGR-derived HUVEC maintained in primary culture conditions present an altered proteome profile, which could reflect an abnormal programming of endothelial function in this fetal condition.

1. Introduction

Intrauterine growth restriction (IUGR) is characterized by a reduced fetal growth along with chronic fetal hypoxia and/or increased feto-placental vascular resistance [1–3]. The altered vascular function observed in IUGR is partially reflected in umbilical and placental vessels; however the associated increased cardiovascular risk in later life is of greater relevance. Studies in endothelial cells isolated from umbilical vessels (i.e. HUVEC and HUAEC) show that IUGR-derived cells present an altered expression of proteins related to L-arginine/eNOS metabolism, suggesting that changes in the vascular reactivity observed in IUGR offspring represent an altered endothelial phenotype [4,5]. Notably these specific changes persist *in vitro* even after several passages under standard culture conditions, highlighting the possibility of an epigenetic-mediated programming of the endothelium [6] leading to vascular dysfunction.

Several studies have explored the proteome of human umbilical vein endothelial cells (HUVEC) under basal culture conditions and in response to diverse stimuli in order to integrate the different pathways implicated in endothelial function (see Richardson et al., 2010 [7]). However data showing whether HUVEC obtained from pathological pregnancies present an altered proteome expression when compared with HUVEC from normal pregnancies are lacking. In this study we characterized the proteome of cultured HUVEC

Abbreviations: HUVEC, human umbilical veins endothelial cells; IUGR, intrauterine growth restriction; 2D-DIGE, 2 dimension differential in-gel electrophoresis.

* Corresponding author. Division of Obstetrics and Gynaecology, Division of Pediatrics, School of Medicine, Pontificia Universidad Católica de Chile, Marcoleta 391, Santiago, Chile.
E-mail address: pcasane@uc.cl (P. Casanello).

[1] These two authors equally contributed to this work.

http://dx.doi.org/10.1016/j.placenta.2016.02.016
0143-4004/

derived from term IUGR, and compared it with the proteome of HUVEC from term normally grown fetuses born of healthy mothers using 2-dimension differential in gel electrophoresis (2D-DIGE) along with mass spectrometry. After identification, differentially expressed proteins were further analyzed with *in silico* tools for unveil the signaling pathways where they participate and their potential effects on endothelial function. Finally, using principal component analysis, a signature of the IUGR proteome was obtained.

2. Materials and methods

A detailed description of methods can be found as Supplementary material.

2.1. Study participants

Pregnant women attending routine antenatal care at the Maternity of the Hospital Clínico Pontificia Universidad Católica-Christus and the Hospital Sótero del Río, Santiago, Chile, were invited to participate. The women included in this study were: not on regular medication, nonsmoking, normotensive, and free from preeclampsia, pregestational or gestational diabetes. All participants signed a written consent approved by the corresponding ethics committees of the Faculty of Medicine at the Pontificia Universidad Católica de Chile and Servicio de Salud Metropolitano Sur Oriente at the Hospital Sótero del Río. Maternal and newborn characteristics of comparison groups are described in Table 1. Maternal age ($p = 0.52$), parity ($p = 0.29$), gender ($p = 0.15$) and delivery mode ($p = 1.00$) were not different between IUGR and control groups. IUGR infants had a lower gestational age, birth weight and height, compared with controls ($p < 0.05$). Adequacy of birth weight for gestational age (WGA) at birth using the National Standard Curve [8] served to define the percentile score for each newborn. IUGR neonates were ranged from 2 to 10 centile in WGA confirming prenatal ultrasound based diagnose. Newborn infants in the control group had WGA centile ranging from 25 to 90 centile.

2.2. Cell culture and protein extraction

Umbilical cords were obtained immediately after delivery and transported from the maternity ward to the laboratory. Human umbilical vein endothelial cells (HUVEC) were isolated and cultured (37 °C, 5% CO_2) to confluence up to third passage (9–12 days after isolation). Cells were serum-starved (2% sera) for 24 h and exposed (24 h, 37 °C) to a gas mixture (5% CO_2-balanced N_2) to obtain 5% O_2, [4]. HUVEC were lysed with ~100 μl of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM TCEP, and protease-phosphatase inhibitors mix) and stored a −80 °C for further analysis.

Table 1
Maternal and newborn characteristics by group.

Variable	Normal	IUGR	*p*-value
Maternal age (yo)	30.6 ± 1.77	28.7 ± 2.50	0.52
Parity	0.9 ± 0.40	0.3 ± 0.30	0.29
Gestational age (weeks)	39 ± 0.34	37.2 ± 0.64	0.02
Birth weight (kg)	3.4 ± 0.12	2.5 ± 0.11	<0.01
Height (cm)	50.4 ± 0.59	47.4 ± 0.48	<0.01
Ponderal index	2.7 ± 0.09	2.3 ± 0.08	0.01
Percentile range	25–90	2–10	
Gender (F/M)	3/7	5/2	0.15
Delivery (C/V)	6/4	5/2	1.00

Ponderal index expressed as birth weight x 100 x height-3 (g/cm3). F and M indicate total number of the female or male neonates. C and V indicates the number of cesarean (C) or vaginal (V) deliveries in each group. Values are mean ± S.E.M or frequency, *p*-value was determined by *t*-test and Fisher's exact test.

2.3. 2-D DIGE

Whole cell protein extracts were quantified and equal amounts of proteins from every sample pooled for further labelling with Cy2 fluorophore to be used as internal reference in the gels. Control and IUGR HUVEC were labelled with Cy3 and Cy5 dye, respectively. Protein labelling were carried out using Refraction-2D™ labelling kit, NH DyeAGNOSTICS GmbH, Germany). Isoelectric focusing (IEF) was performed using IPG ImmobilineTM DryStrip (24 cm, pH 3–7NL; 18 cm, pH 6–11) and subjected to isoelectrofocusing in an Ettan IPGphor 3 System (GE), applying on average 60,000 V h (Vh) per strip. Two-dimensional electrophoresis was run at 30 °C continuously overnight, power supply to 1 W/gel (continuous run) in an Ettan DALT 12 System (GE healthcare). Both IEF and SDS-PAGE were run under darkness.

2.4. Gel imaging and spot detection

Gels were scanned for Cy2, Cy3 and Cy5 with the Ettan DIGE Imager (GE healthcare) at 100 μm (pixel size) resolution. Setup, warp, detection y quantitation in 2D gel images were performed with Delta2D software (V3.3 Decodon GmbH, Germany). The software delivered the calculated spot volumes and normalized to the corresponding image and against the pooled Cy2 standard of each individual spot, in each gel.

2.5. MALDI-TOF MS analysis

Differentially expressed spots from 2D-gels were cut using a sterile pipet tip (~1000 μl) and transferred to microcentrifuge tubes for trypsin digestion (0.3 μg/μL) (Promega Corp., WI-USA. Concentrated samples were resuspended in 0.1% v/v formic acid/ 3% v/v methanol prior to analysis by mass spectrometry (MS). The mass spectra acquisitions were performed using a MALDI-TOF Microflex (Bruker Daltonics Inc., MA-USA) operated in positive ion reflectron mode detection and controlled by FlexControl 3.0 software (Bruker Daltonik GmbH, Germany). Mass range 600–4000 *m/z* was used for the identification of the most prominent peaks in each spectrum. Spectra were processed and calibrated in the Mmass software (V5.4.0) using MALDI-TOF peptide mode. Peptide mass fingerprints (PMFs) were searched using the Mascot database (MatrixScience Inc., USA), ProteinProspector MS-Fit database V5.10.9 (UCSF-USA) and ProFound (http://prowl.rockefeller.edu/prowl-cgi/profound.exe). Search parameters were: Trypsin digestión, 1 missed cleavage; fixed modification, carbamidomethyl (C); variable modification, methionine oxidation; monoisotopic mass; mass tolerance ±0.1–0.3 Da and peptide charge, +1. $p < 0.05$ was used for local PMF search considering Homo Sapiens taxonomy restrictions.

2.6. Statistics

Delta-2D Decodon-derived data were expressed as mean ± RSD; statistical analysis using independent *t*-test with $p < 0.05$ was considered significant to establish differentially expressed proteins. Univariate analysis using non-parametric Mann–Whitney *U* test was carried out for spots with at least 2 fold change (positive or negative) in their accumulation pattern. Further analysis of proteome changes were accomplished by Principal Component Analysis (PCA). False discovery rates were controlled as suggested by Delta-2D Decodon software and previous reports [9–11]. Principal Component Analysis (PCA) and Hierarchical Clustering Analysis were carried out in parallel using STATISTICA Software V7 (StatSoft.