Early Human Development 91 (2015) 291–297

Contents lists available at ScienceDirect

Early Human Development

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

Immunohistochemical protein expression profiling of growth- and apoptotic-related factors in relation to umbilical cord length

Mercedes Olaya-C [a,*], Michael Fritsch [b], Jaime E. Bernal [c]

[a] Department of Pathology, The Medical School, Pontificia Universidad Javeriana–San Ignacio University Hospital, Bogota, Colombia
[b] Department of Pathology and Laboratory Medicine, Ann and Robert H. Lurie Children's Hospital of Chicago, Chicago, IL, USA
[c] Institute of Human Genetics, The Medical School, Pontificia Universidad Javeriana–San Ignacio University Hospital, Bogota, Colombia

ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 2 March 2015
Accepted 3 March 2015

Keywords:
Stillbirth
Umbilical cord
Cord accident
Immunohistochemistry

ABSTRACT

Introduction: Umbilical cord (UC) alterations are related to fetal and neonatal deaths and late neurological complications. Abnormal UC length has been recognized as the most significant abnormality linked to unfavorable outcomes. Despite its importance, causal factors resulting in abnormally long or short UCs have yet to be established. The factors that govern UC length are largely unknown. Furthermore, there is a paucity of studies that assess molecular processes involved in the establishment of UC length. We hypothesize that UC length abnormalities in UC length are associated with altered protein expression patterns of known cell growth and/or apoptosis regulators. In this study we analyze diverse protein expression patterns in different UC cell types found in UCs of normal and abnormal length.
Methods: An analytical observational study was carried out on fetal autopsies; diagnosed abnormal length UCs were compared to normal controls by gestational age. Immunohistochemical analysis of expression levels of growth and pro- and anti-apoptotic factors was performed.
Results: We performed immunohistochemistry antibody tests against FAS, BAX, Ki67, cMyc, FGF2, TGFBR3, VEGF, Bcl2, p57 and IGF2 and analyzed UC cell expression patterns. We found significant differences in specific long and/or short cord cell types in comparison to those in normal cords.
Discussion: Factors that determine UC length are still largely unknown; however, this study demonstrates significant specific cell type differences in protein expression patterns of several genes related to cell proliferation. This preliminary study provides strong supporting data to continue the search for molecular factors that determine UC length.

1. Introduction

The UC is a vital developmental transport structure between mother and fetus. Spontaneous abortion, stillbirth, unexpected perinatal death and neurological abnormalities have been linked to UC pathology [1–4]. Abnormal length (short or long) is the most frequent UC characteristic associated with such complications [5].

Several hypotheses have been proposed to explain abnormally short cords, including fetal mobility disorders (mechanical or drug-induced) [6,7], neurological disorders [8,9], chromosomal disorders [10] and twin pregnancy, all suggesting a relationship between lack of fetal movement resulting in diminished UC tension and thereby a short UC. Theories related to long cords include: uterus size [11,12], maternal parity of more than three children [5,11], male gender [10,11] and genetic [13,14] and epigenetic components [15–17]. While widely accepted that UC length increases relatively linearly throughout pregnancy, abnormally long or short cords can be documented very early in the second trimester suggesting that factors determining cord length can be disrupted relatively early in pregnancy.

Despite its importance, the molecular factors involved in determining umbilical cord length remain largely unknown. In this study, we analyzed ten protein expression patterns including pro- and anti-apoptotic factors, growth factors and indicators of cellular proliferation in seven different UC cell types. We sought to establish differences in their expression patterns in specific cell types, using collected fetal and neonatal autopsies with diagnoses of short, long and normal UC length. The goal was to establish which cell proliferation or apoptotic pathways may influence abnormal UC length.

2. Methods

In this study, we investigated umbilical cord samples obtained during postmortem examinations of stillborn or perinatal fetuses, performed at the *PUJ-HUSI* Department of Pathology from 2007 to 2013.

* Corresponding author at: Facultad de Medicina, Departamento de Patología, Pontificia Universidad Javeriana, Carrera 7ª, Bogota 40-62, Colombia. Tel.: +57 1 5946161x2440/2, +57 3106285500 (mobile).
E-mail addresses: olaya.m@javeriana.edu.co, molayac@hotmail.com (M. Olaya-C), MFritsch@luriechildrens.org (M. Fritsch), jebernal@gmail.com (J.E. Bernal).

http://dx.doi.org/10.1016/j.earlhumdev.2015.03.001
0378-3782/

In the *HUSI* perinatal pathology database, we identified diagnosis of long, short and normal UC length, paired by age. Complete case history was available for all selected cases. Autopsies had parental consent and all corresponded to natural deaths. Routinely, UC was measured and all of its features were consigned, including whether it was complete or segmented. The normal range for umbilical cord length was based upon existing tables in the literature for different gestational ages [18]. Using paraffin embedded tissue, immunohistochemistry was carried out with the antibodies listed in Table 1: All antibodies were from Santa Cruz Biotechnology Inc (USA).

The paraffin embedded sections were rehydrated and incubated for 55 min, at 20 ° C, in methanol containing 10% H_2O_2 to block endogenous peroxidase (EnVision™ FLEX + kit (Dako)). Sections were pretreated to facilitate antigen retrieval and increase membrane permeability to antibodies with the same kit and then incubated with the primary antibody. Positive reaction was visualized by 3.3-diaminobenzidine (DAB) peroxidation according to standard methods. The sections were counterstained with Harris's hematoxylin, dehydrated, coverslipped, and observed in an optical microscope. Positive and negative controls were performed and validated for each antibody.

Two investigators, with no previous knowledge of the case, independently examined all immunohistochemistry slides. For each antibody, the entire available sections were evaluated (at least two UC pieces sampled). Stain intensity (semi-quantitative) and extension [% of involved cells] (quantitative) were scored as shown in Table 2.

Obtained data were transferred to Excel® for further study. Raw odds ratio analysis was performed using the Toronto Evidence Based Medicine Tool. Statistical significance: p value <0.05, confidence intervals (95%), odds radio and chi-square were used for analyzing association variables.

3. Results

We analyzed 11 long UC, 11 short UC and 9 normal UC lengths. Clinical features are shown in Table 3.

Individual variability between UC lengths is shown in Table 4. The table displays the categorization in each group (long, short or normal), expected length (interval), measured length (real) and gestational age.

Protein expression was analyzed within seven UC cell types: artery endothelial cells, vein endothelial cells, stromal cells, perivascular cells, arterial smooth muscle cells, vein smooth muscle cells and amniotic epithelium (Fig. 1). Perivascular cells are defined as the more tightly packed stromal cells immediately surrounding the smooth muscle cells of the vessel wall. Table 5 is a summary of the results. For each growth factor or apoptotic factor, the most representative cell types with their different expression patterns (intensity and/or extension relative to controls) are shown ("Criteria" column). The p values were calculated and are shown comparing intensity or extension between abnormal length (both long and short) to normal length UCs. Cell types not shown had no significant stain differences. Negative controls were made (Fig. 2).

Table 2
Immunohistochemistry evaluation: Intensity and spreading.

Extension (% involved)		Intensity	
•0	Grade 0	•Not expressed	0
• < 30%	Grade 1	•Weak intensity	1
•30–60%	Grade 2	•Moderate intensity	2
• > 60%	Grade 3	•Strong intensity	3

Major differences in cell-type-specific protein expression intensity among long and/or short cords compared to normal cords are shown in Table 6. Weak intensity staining of p57 in the amnion was more common in long cords compared to normal length cords. Weak intensity staining for TGFBR3 in stromal cells was more common in both short and long cords combined compared to normal length cords. The absence of Ki67 staining in the amnion was associated with both short and long cords combined or with just long cords compared to normal length cords.

4. Discussion

This study utilized a cohort of autopsy fetuses with representative features for their ages. Most were neither malformed nor macerated; only one mother in each group was over 35 and five women were younger than 18. Acute chorioamnionitis was the most frequent diagnosis; which was expected because most women were in their second trimester, which is when this disease is common [19]. Only one case of multiple gestation was collected with a short cord. Interestingly, the hypothesis exists that if the uterus has scant space, such as in the case of twin pregnancy, the fetus hardly moves, and consequently, the umbilical cord is short [20]. A genetic component regulating umbilical cord length has also been proposed as women giving birth to a fetus with an abnormally long UC are at increased risk in future pregnancies to have another fetus with a long cord [13,14]. One case with a short cord corresponded to Limb–body wall malformation whose UC measured 6 cm. An association between malformed fetuses and abnormal short UCs is also well established [21]. Two other malformed fetuses in our study had long UCs, one showed atlanto-axial malformation, hypoplastic hemisphere and omphalocele, and the second one showed macrocephaly, brachydactyly and contractures.

Several umbilical cords in our study showed other related UC alterations such as hypercoiling (one short, two long and one normal), abnormal insertion (marginal—two short cords) and velamentous insertion (one short and one long) and cord entanglement (two long cords). One of them, a long combined UC, in addition to its abnormal length, exhibited velamentous insertion and nuchal cord entanglement. An association among UC abnormalities has been described [22–24]. UC accident as a cause of death in this series was seen in one short and one long cord, brought about by cord entanglement and velamentous cord insertion, as has previously been reported [25,26].

We compared antibody expression levels (intensity and extension) between short and/or long UCs and normal UCs, with significant

Table 1
Panel of antibodies utilized in the study.

Antibody against	Code	Action	[]	C	Control	Pre-treatment
BAX	(Δ 21:) sc-6236	Proapoptotic	1:50	P	Breast	Boiling in high pH
FAS	(C-20): sc- 715	Proapoptotic	1:25	P	Liver	Boiling in high pH
bcl2	(7): sc-130308	Anti-apoptotic	1:50	M	Lymph node	Boiling in high pH
p57	SPM308:sc-56456	Arrest of cell proliferation	1:50	M	Placenta	Boiling in high pH
cMyc	(9E11): sc-47694	Cell proliferation	1:1,000	M	Colon	Boiling in high pH
IGF2	(H-103) sc 5622	Cell proliferation	1:50	P	Pancreas	Boiling in high pH
FGF2	(147): sc-79	Cell proliferation	1:50	P	Kidney	Boiling in high pH
VEGF	(J-14I): sc-80442	Cell proliferation	1:50	M	Placenta Heart	Boiling in high pH
TGF*BR 3*	(A-4): sc-74511	Cell growth	1:50	M	Kidney	Boiling in high pH
Ki67	(Ki-67): sc-23900	Cell growth	1:50	M	Lymph node	Boiling in high pH

[]: Concentration of primary antibody; C: clonality, M: monoclonal, P: polyclonal.