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Premature birth is associated with not fully differentiated contractile smooth muscle cells in human umbilical artery

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ABSTRACT

Smooth muscle cells (SMCs) participate to the regulation of peripheral arterial resistance and blood pressure. To assume their function, SMCs differentiate throughout the normal vascular development from a synthetic phenotype towards a fully differentiated contractile phenotype by acquiring a repertoire of proteins involved in contraction. In human fetal muscular arteries and umbilical arteries (UAs), no data are available regarding the differentiation of SMCs during the last trimester of gestation. The objective of this study was to characterize the phenotype of SMCs during this gestation period in human UAs. We investigated the phenotype of SMCs in human UAs from very preterm (28–31 weeks of gestation), late preterm (32–35 weeks) and term (37–41 weeks) newborns using biochemical and immunohistochemical detection of α-actin, smooth muscle myosin heavy chain, smoothelin, and non-muscle myosin heavy chain. We found that the number of SMCs positive for smoothelin in UAs increased with gestational age. Western blot analysis revealed a higher content of smoothelin in term compared to very preterm UAs. These results show that SMCs in human UAs gradually acquire a fully differentiated contractile phenotype during the last trimester of gestation and thus that premature birth is associated with not fully differentiated contractile SMCs in human UAs.

1. Introduction

Vascular smooth muscle cells (SMCs) alter the lumen diameter of mature muscular arteries thanks to their contraction and relaxation activities, and participate to the regulation of peripheral arterial resistance and blood pressure. To assume their function, SMCs differentiate throughout the normal vascular development from a synthetic phenotype towards a fully differentiated contractile phenotype [1]. While SMC phenotype appears to be genetically programmed, SMC differentiation during development is also regulated by biochemical factors, extracellular matrix components and hemodynamic strains [2].

The identification of SMC phenotype is commonly based on the expression of a repertoire of proteins involved in contraction. During the vascular development, the acquisition by SMCs of smooth muscle α-actin (SM α-actin), smooth muscle myosin heavy chain (SM MHC) and smoothelin, concomitantly with the loss of proteins associated with synthetic phenotype such as non-muscle myosin heavy chain (NM MHC), is recognized as a hallmark of their differentiation towards a fully contractile phenotype [1]. In human, only few studies have characterized the phenotype of SMCs in muscular arteries during the first and second trimesters of gestation and soon after birth [3,4]. Therefore, to date, no data is available regarding the differentiation of SMCs in human muscular arteries during the last trimester of gestation when premature birth can occur. In umbilical artery (UAs), which is considered as a unique, useful and accessible source of human fetal muscular arterial tissue, only sparse results in the literature reported the expression pattern for SM α-actin, SM MHC and smoothelin from 10 to 16 weeks gestation fetuses [3–5] and at birth [4,6,7]. The objective of this study is to characterize the phenotype of SMCs in human UAs during the last trimester of gestation. To this end, we have investigated the expression of SM α-actin, SM MHC, smoothelin and NM MHC in human UAs from very preterm, late preterm and term newborns.

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2. Materials and methods

2.1. Samples origin

Human umbilical cords were obtained from 31 alive newborns, whose gestational age ranged between 28 and 41 weeks, delivered at the Clinic of Obstetrics, University Hospital of Marseille, France. Gestational age was classified as very preterm (28–31 weeks, 7 newborns), late preterm (32–35 weeks, 9 newborns) and term (37–41 weeks, 15 newborns). The exclusion criteria were umbilical cord anomalies (e.g. one single UA) or factors that could have altered vascular development including maternal diabetes, maternal cardiovascular history and infants born with congenital cardiopathy, polyformative syndrome or with an APGAR score less than six at 5 min after birth. Newborns that suffered of restricted fetal growth were also excluded of the study using the AUDIPOG database birth weight modeling [8]. The maternal and newborns clinical information are presented in Table 1. All tissues were collected in agreement with French law and the study was approved by the local ethics committee. Written informed consent was obtained from all parents.

2.2. Removal of the umbilical cord and dissection of the UAs

Immediately after delivery of the placenta, segments of the umbilical cords, 5 cm in length, were cut at the middle part between the newborn and the placenta. Samples were placed in a physiological saline solution and were transported to the laboratory in ice. All steps of the dissection were made at low temperature on an ice bath. Given the tortuosity of UAs inside the umbilical cord, isolation of UAs was performed to permit an optimized orientation that is essential to cut workable transverse sections of UAs. The Wharton's jelly was carefully separated from the umbilical cord vessels and the UAs were isolated and washed with physiological saline solution to remove the blood of the vessels. Arteries were cut into 1 cm long pieces, that were rapidly frozen in isopentane precooled in liquid nitrogen and stored at −80 °C for histology and immunohistochemistry analysis, or that were frozen in liquid nitrogen and stored at −80 °C for biochemical analysis.

2.3. Histology and immunohistochemistry

Serial transverse sections (8 μm) of the frozen UAs were cut at −20 °C in a cryostat (Leica, CM 1900), air-dried for 10 min and stored at −80 °C. For histology, sections were subjected to (+) catechin staining to specifically characterize elastic structures [9] and to Masson's trichrome staining.

For immunohistochemistry, the tissue sections were air dried for 30–40 min and were preincubated for 1 h in PBS containing 1% bovine serum albumin (BSA, Sigma) and 10% normal goat serum in order to reduce non-specific background staining. After washings in PBS, the sections were incubated with the appropriate primary antibody for 90 min, with the exception of smoothelin C6 G clone antibody with which the sections were incubated overnight at 4 °C. Primary antibodies used in this study were mouse monoclonal antibodies directed against smooth muscle myosin heavy chain (Dakocytomation, SMMS-1 clone, 2.3 μg/mL), smooth muscle α-actin (Dakocytomation, 1A4 clone, 19,6 μg/mL), smoothelin (Gene Tex, Inc., R4A clone, 10 μg/mL; Clinisciences, C6 G clone, 10 μg/mL) and non-muscle myosin heavy chain (NM MHC, Abcam, 3H2 clone, 1:3000), a marker of synthetic phenotype of SMCs [10]. In control staining procedures, the primary antibody was omitted and isotype-matched control antibodies were used at the same final concentrations (Abcam, MOPC-21, NCM1, MG2a-53 and MPC-11 clones respectively for SM MHC, smoothelin, SM α-actin and NM MHC). The sections were washed with PBS and incubated with a peroxidase-conjugated $F(ab')^2$ fragment goat anti-mouse IgG for 1 h (Beckman Coulter, PN IM0817, 1:100), with the exception of sections incubated with smoothelin C6 G clone antibody which were incubated with biotinylated goat anti-mouse IgG for 2 h (Vector Laboratories, 1:100) and developed with the ABC reagents provided in the kit. All washes and incubations were carried out in a humidified chamber at room temperature. Peroxidase activity was detected with 3,3′-diaminobenzidine (Sigma). No endogenous peroxidase activity was detected on the control tissue sections. Sections were washed first in PBS and then in distilled water. For each tested antibody, half of sections were counterstained with Harris hematoxylin. Finally, the sections were dehydrated in graded concentrations of alcohol followed by Savesolv (LABOnord) and mounted with Savemount (LABOnord). Sections were examined by 3 independent investigators blind to the experimental conditions using a Nikon TE 2000-U inverted microscope and photographed with a Nikon DXM 1200F digital camera. NIS Elements 3.0 browser software was used for image acquisition. Images were processed using the Adobe Photoshop® image processing software.

2.4. Western blot analysis

One cm long pieces of UAs segments were cut into small pieces and homogenized with an Ultra-turrax® in a cold Tris/Triton buffer pH 7.4, containing Tris (100 mM), NaCl (276 mM), KCl (5.4 mM), Triton X-100 (0.1%) and NaN_3 (0.02%). After a centrifugation for 5 min at 12,000 g, triton extracts were collected and the pellets were washed in PBS. After a centrifugation for 5 min at 12,000 g, the pellets were dissolved at 95 °C for 5 min in a reducing buffer containing dithiotreitol (5%) and SDS (2%). For western blot analysis, four triton and four reduced extracts corresponded to UAs from four different newborns were run for western blotting were separated on 4–12% SDS-polyacrylamide gel, blotted on cellulose C+ membranes and blocked in 3% BSA-TBS overnight at 4 °C. Membranes were probed with mouse monoclonal antibodies directed against SM MHC (Dakocytomation, SMMS-1 clone, 1 μg/mL), SM α-actin (Dakocytomation, 1A4 clone, 1 μg/mL), and smoothelin (Gene Tex, Inc., R4A clone, 2 μg/mL). Bound antibodies were visualized using peroxidase-conjugated goat anti-mouse $(Fab')^2$ antibody (Jackson laboratories) and ECL reagent. Membranes were stripped and reprobed with monoclonal antibody directed against human β-actin as a charge control using a standard procedure described earlier [11]. Prestained rainbow molecular weight markers (MultiMark® Multi-Colored Standard, Invitrogen) were also run on each gel.

3. Results

3.1. Histology of the UAs

The structural patterns described above were consistent in all umbilical arteries in each group.

In term UAs, the main structural component of the UA was the smooth musculature. The intima was limited to a monolayer of endothelial cells. In the media, two parts can be clearly distinguished. The inner part of the media consisted of small cells with little cytoplasm, poorly defined outlines, and loosely disposed in an abundant ground substance. The outer part showed bundles of tightly packed SMCs with distinct outlines [Fig. 1a]. The respective surface area of the inner and of the outer parts of the media relative to the cross sectional area of UA was not different between the three groups (data not shown). No clear adventitial layer was observed. Neither nerves or vasa vasorum were present. UAs were poor in elastic structures. Furthermore, no internal elastic lamina was observed [Fig. 1b].

3.2. Distribution of SMCs markers in UAs

The staining patterns described above were consistent in all umbilical arteries in each group.

In term UAs, SM α-actin and SM MHC showed similar pattern of staining. Except the endothelial cells, all cells of UAs stained strongly with SM α-actin [Fig. 2a] and SM MHC [Fig. 3a] antibodies. NM MHC was detected in all cells of the media, with a strong

Table 1
Maternal and newborns clinical information.

Characteristics	Term newborns	Late preterm newborns	Very preterm newborns
Maternal data			
Number	7	8	9
Age (mean ± SD), years	33.5 (+/−5)	34 (+/−4)	29 (+/−3.6)
Primigravidia, *n* (%)	0 (0%)	2 (25%)	3 (21%)
Primiparity, *n* (%)	1 (14%)	5 (63%)	10 (71%)
Multiple pregnancies, *n* (%)	0 (0%)	1 (13%)	1 (7%)
Antenatal steroid herapy, *n* (%)	0 (0%)	4 (50%)	14 (100%)
HTA, preeclampsia, *n* (%)	0 (0%)	2 (25%)	2 (14%)
Premature rupture of membranes, *n* (%)	0 (0%)	4 (50%)	11 (79%)
Elective cesarean section, *n* (%)	7 (100%)	7 (88%)	14 (100%)
Newborn data			
Number	7	9	15
Gestational age (mean ± SD), weeks	39.6 (+/−1.7)	33.5 (+/−1.5)	30 (+/−1.2)
Birth weight (mean ± SD), g	3390 (+/−550)	1644 (+/−550)	1360 (+/−340)
Male *n* (%)	5 (71%)	4 (44%)	6 (40%)
Small for gestational age, *n* (%)	0 (0%)	0 (0%)	0 (0%)
Median Apgar score at 5 min	10	9	9