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► To cite this version:

Isabelle Ligi, Stéphanie Simoncini, Edwige Tellier, Isabelle Grandvullemin, Maxime Marcelli, et al.. Altered angiogenesis in low birth weight individuals: a role for anti-angiogenic circulating factors. *Journal of Maternal-Fetal & Neonatal Medicine*, 2013, 27, pp.233 - 238. 10.3109/14767058.2013.807237 . hal-04018028

HAL Id: hal-04018028

<https://hal.science/hal-04018028v1>

Submitted on 7 Mar 2023

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ORIGINAL ARTICLE

Altered angiogenesis in low birth weight individuals: a role for anti-angiogenic circulating factors

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Abstract

Objective: Low birth weight (LBW) is a risk factor for hypertension at adulthood. Endothelial progenitor cells (EPCs) dysfunction has been characterized in LBW neonates. We hypothesized that changes in soluble, plasma pro- or anti-angiogenic factors are associated with EPCs dysfunction and impaired angiogenesis in LBW neonates.

Method: Venous umbilical cord blood was collected from 42 normal, term neonates and 75 LBW neonates. Cord blood endothelial colony forming cells (ECFC) from control patients were cultured in the presence of 10% of serum obtained from both groups.

Results: The proliferation and the migration of ECFC were significantly reduced when cultured with 10% of serum of LBW neonates compared to serum of control neonates. Matrigel invasion assay was not significantly altered. Umbilical vein plasma VEGF concentration was significantly reduced in LBW neonates while that of sVEGFR and PF4 were significantly higher. Addition of VEGF corrected the inhibitory effect of LBW serum on normal ECFC proliferation.

Conclusions: Serum obtained from LBW babies contains factors that exhibit an antiangiogenic effect on ECFC proliferation and migration. VEGF/sVEGF/PF4 pathway seems to be involved in the EPCs dysfunction in LBW neonates.

Keywords

Endothelial progenitor cells, hypertension programming, low birth weight, VEGF

History

Received 26 March 2013

Accepted 17 May 2013

Published online 20 June 2013

Introduction

Coronary heart disease and atherosclerosis are the leading causes of death all around the world. Low birth weight (LBW) either due to preterm birth or intra-uterine growth restriction is now considered as a major independent risk factor for cardiovascular-related diseases [1]. But the mechanisms explaining the developmental programming of cardiovascular disease in adulthood are incompletely understood. Aside the demonstrated long term effects of nephron number reduction and hypothalamic-pituitary-adrenal axis hyperactivity, factors which directly affect the vessel structure and function may have an important role [2]. Indeed, LBW presented an alteration of both structure and function of large and small vessels with an increased arterial stiffness, a microvascular rarefaction due to an incomplete angiogenesis and an endothelial dysfunction with an impairment of endothelium-dependent vasodilatation [3–6]. The endothelium controls vascular permeability and regulates vascular growth. A critical component of the circulating endothelium is endothelial progenitor cell (EPCs). EPCs play a pivotal role during

organogenesis, growth, tissue repair and postnatal angiogenesis under the control of angiogenic factors.

EPCs were first described in blood by Asahara et al. [7]. These bone-marrow derived immature cells circulate in adult peripheral blood and are enriched in umbilical cord blood of healthy term neonates. The EPCs have a great clonogenic and proliferative potential and the capacity to differentiate into mature cells in situ. These capacities confer to the EPCs a critical role in neovascularisation processes via vasculogenesis. In vitro, Ingram et al. characterized circulating EPCs as endothelial colony forming cells (ECFC) [8]. Indeed, ECFC present a cobblestone-like morphology in culture, express endothelial cell surface markers and display functional properties like clonogenic cell clusters, self-renewal and de novo vessel formation in vivo capacities of progenitor cells.

In adults, numerous studies have demonstrated a negative correlation between circulating ECFC numbers and vascular disease risk and progression. ECFC are now considered as the strongest biomarker to assess endothelial dysfunction [9]. Dysfunction of ECFC underlies many pathological conditions such as diabetes mellitus or preeclampsia. In neonates, there is growing evidence of ECFC dysfunction in vascular pathologies such as bronchopulmonary dysplasia, intrauterine growth restriction and in offspring diabetic or pre-eclamptic mothers [10–12]. Recently, Ingram et al. demonstrated that

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Table 1. Clinical characteristics of mothers and neonates.

Characteristics	Normal birth weight term neonates	Low birth weight neonates	p Value
Maternal data			
Number	34	44	0.69
Age* (y)	30.4 (± 6.2)	30.9 (± 6.2)	0.96
Primigravida, n (%)	9 (26 %)	15 (34 %)	0.01
Primiparity, n (%)	10 (29 %)	19 (43 %)	0.13
Multiple pregnancies, n (%)	4 (12 %)	11 (25 %)	<0.0001
Antenatal steroid therapy, n (%)	0 (0 %)	27 (61 %)	
HTA, Preeclampsia, n (%)	3 (9 %)	12 (25 %)	0.09
Premature rupture of membranes, n (%)	2 (6 %)	10 (20 %)	0.09
Elective cesarean section, n (%)	23 (68 %)	35 (80 %)	0.35
Infants data			
Number	34	49	
Gestational age, weeks	40 (±1.2)	33 (±4)	<0.0001
Birth weight, g	3510 (±471)	1690 (±564)	<0.0001
Male	22 (65 %)	28 (57 %)	0.505
Small for gestational age, n (%)	0 (0 %)	14 (29 %)	<0.0001
Median Apgar score at 5 min	10	9	<0.0001

*Data are presented as means ± SD.

the diabetic intrauterine environment affects the angiogenic capacity of cord blood ECFC from offspring of gestational diabetic mothers [12]. This endothelial dysfunction included a decreased number of circulating ECFC, a reduced colony formation and self-renewal capacity and an impairment of morphogenesis. In LBW, data are scarce. Javed et al. observed a lower number of ECFC colonies in very preterm infants (24–31 weeks) [13]. We recently demonstrated that LBW ECFC presented a reduced angiogenic potential. This was associated with a switch towards angiostatic factors in gene expression in LBW ECFC [14]. However, the origin of LBW ECFC angiogenic alteration is not known. As LBW is associated with an impaired angiogenesis and a reduced number of ECFC, we hypothesized that changes in soluble, plasma pro- or anti-angiogenic factors are associated with EPCs dysfunction and impaired angiogenesis in LBW neonates.

Materials and methods

Study population and sample collection

Sex-matched control (C, healthy newborns, gestational age >37 weeks) and LBW (birthweight <2500g either due to prematurity or intrauterine growth restriction) newborns were recruited from the department of Obstetrics and Gynecology, La Conception Hospital, Marseille, France. Small for gestational age (SGA) was defined as the birth weight of <10th percentile for corresponding gestational age using individualized AUDIPOG curves. Pregnancies associated with fetal malformation or chromosome anomaly were excluded from the study. Sera and citrate-plasma samples were collected from the umbilical vein immediately after delivery of the placenta. All blood samples were rapidly centrifuged and aliquoted and stored at –80 °C until in vitro functional assay (sera) or enzyme immunoassay (sera and plasma).

The study was approved by the local ethic Committee of our teaching hospital and informed consent was acquired from the two parents.

Isolation, cultivation and immuno-phenotyping of controls ECFC

ECFC were obtained as previously described by Ingram et al. [8]. Briefly, UCB samples were collected from the umbilical vein of controls newborns (C) by gravity flow in heparin-coated tubes before expulsion of placenta. Blood was diluted 1:1 in PBS (Life technologies) and mononuclear cells (MNCs) were isolated using a density gradient centrifugation over lymphocyte separation medium (Eurobio, Courtaboeuf, France) for 30 min at 2000rpm, washed 3 times in RPMI medium (Life technologies, Carlsbad, CA) 10% FCS and then resuspended in complete endothelial basal cell culture medium-2 (complete EBM2 supplemented with MV SingleQuots (EGM2-MV), Clonetics, Walkersville, MD). Six-well tissue culture plates pre-coated with 0.2% gelatin were seeded with 5×10^6 cells/well and cultured in EGM2-MV in a 5% CO₂ incubator at 37 °C. Media were changed every 2 days. Colonies of endothelial cells appeared between 5 and 14 days of culture and were identified as well-circumscribed monolayers of cobblestone-appearing cells. Single colonies were replated on a six-well plate after trypsinization and grown to 80% confluency. ECFCs were used at passage 1–3.

Endothelial lineage was assessed by flow cytometry using antibodies against human CD31, CD146, CD144 and KDR

In vitro functional studies on normal birth weight term neonates ECFC

BrdU proliferation assay

ECFC were plated at 7000 cells/well in 96 well plates coated with gelatin in EGM2-MV medium during 8 h. After removing EGM2-MV, cells were washed twice with PBS. Cells were deprived overnight in non supplemented EBM2. After removing the medium, ECFC were cultured in EBM2 medium with different conditions (10% control serum ± 25 ng/mL VEGF or with 10% LBW serum ± 25 ng/mL VEGF) depending of experiments. After 12 h, BrdU was added to the

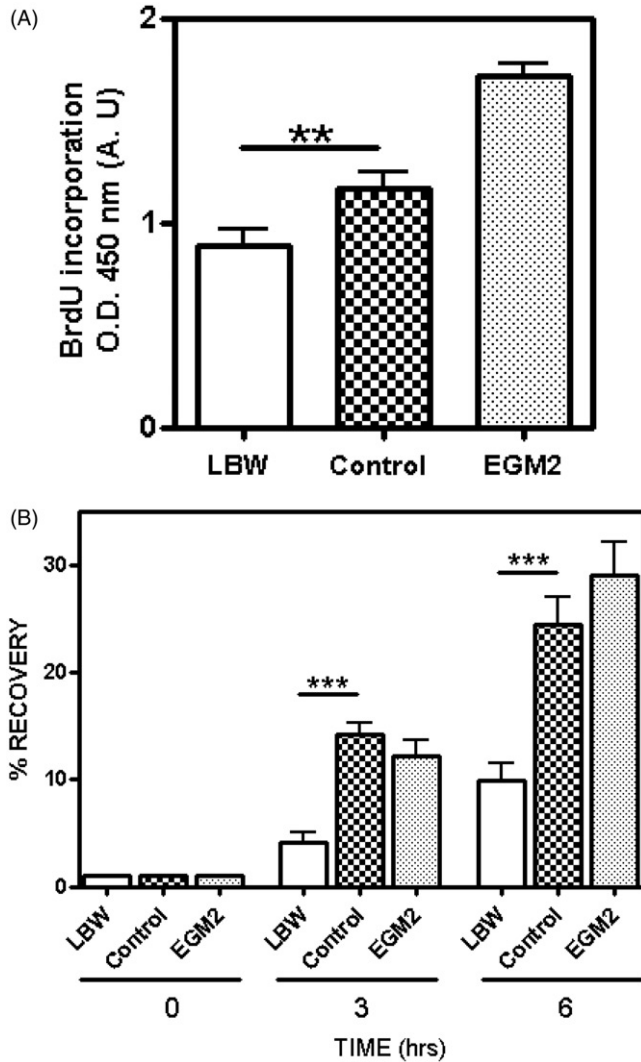


Figure 1. (A–B). Alteration of normal term ECFC proliferation and migration by LBW serum. (A) Proliferation was assayed by BrdU incorporation in 7000 ECFC. Results were expressed in arbitrary units of spectrophotometric measurements. Data represent mean \pm SEM of 79 independent experiments (using 10 control sera and 17 LBW sera on 5 different normal term ECFC). (B) quantification migration in wound healing assays of normal term ECFC cultured with 10% LBW or control serum. Results represent mean \pm SEM of 22 (13 with LBW serum, 9 with control serum) independent experiments performed in triplicate. ** $p < 0.01$, *** $p < 0.001$.

different culture media and proliferation was assessed after overnight incubation, using the Cell proliferation assay kit according to the manufacturer's instructions (Roche Diagnostic, Mannheim, Germany). Incorporation was measured by spectrophotometry using an optical density of 450 nm.

Wound healing assay

Confluent ECFC monolayers were obtained after plating 20 000 cells on gelatin-coated 24-well culture plates. Confluent ECFC were wounded with a 100 μ l tip, washed with RPMI and completed with EGM2-MV, EBM2 10% C serum or EBM2 10% LBW serum. All experiments were performed on wounds of equivalent sizes. Wound repair was assessed using video microscopy coupled with the NIS-element software to measure lesion area. Using the data from time 0 (T0), a percentage of recovery was calculated.

Capillary tube formation

ECFC were seeded at a cell density of 20 000 cells/well in 96 well plates coated with 50 μ L of cold growth factor reduced Matrigel (6 mg/mL; BD Biosciences, San Jose, CA). Cells were cultured in EBM2 and 10% C serum or LBW serum was added. At 6 and 24 h, images were collected using Nikon Eclipse TE2000-U video imaging inverted microscope with 4X magnification objective, acquired using the NIS-Element AR 3.0 software for Nikon. The number of closed loops was measured to quantify tube formation using ImageJ software.

For each functional study, EGM2-MV medium was considered as positive control. Each experiment was done in duplicate.

Angiogenic (VEGF) and antiangiogenic (VEGFR-1, endoglin, PF4, TSP-1) factors determinations in LBW or control sera and plasma

Enzyme linked immunosorbent assays (ELISAs) for VEGF, sVEGFR-1, human soluble endoglin, platelet factor 4 (PF4) and thrombospondin-1 were conducted in duplicate according to manufacturer's instructions (R&D Systems, Minneapolis, MN). Mean minimal detectable doses were 9.0 pg/mL for VEGF, 3.5 pg/mL for sVEGFR-1, 0.007 ng/mL for endoglin and 0.355 ng/mL for TSP-1.

Statistics

Data are given as means (\pm SEM), median, or frequency (percentages) as appropriate. We analysed categorical variables with the χ^2 test (or Fischer's exact test if the expected value was less than 5). Spearman test was used to test non parametric correlations. Median's test was used to compare medians in two populations (with no normality variables). Analyses were conducted using the statistical software program SPSS version 15 (SPSS Inc, Chicago, IL). p Values < 0.05 were considered as statistically significant.

Results

Characteristics of the populations

27 (C = 10, LBW = 17) sera samples and 56 (C = 24, LBW = 32) plasma samples were respectively collected. Characteristics of the population are described in Table 1.

LBW serum impairs angiogenic properties of normal term ECFC *in vitro*

Proliferation of ECFC cultured with 10% LBW serum was significantly decreased compared to that of ECFC cultured with 10% C serum ($p = 0.0062$; Figure 1A). There was no correlation between proliferation and birth weight or gestational age or perinatal conditions. We also investigated the capacity of ECFC to migrate in a scratched wound assay. After 6 h, LBW serum significantly impaired ECFC repair capability ($p < 0.0001$; Figure 1B). At 6 h, the percentage of recovery was correlated to gestational age ($r = 0.674$, $p = 0.012$) and birth-weight ($r = 0.57$, $p = 0.044$). However, the formation of capillary-like structures on Matrigel was not significantly reduced in ECFC cultured with 10% LBW serum at 6 ($p = 0.25$) or 24 h ($p = 0.16$). Altogether these findings indicate that LBW serum contains circulating inhibitors of ECFC functions.

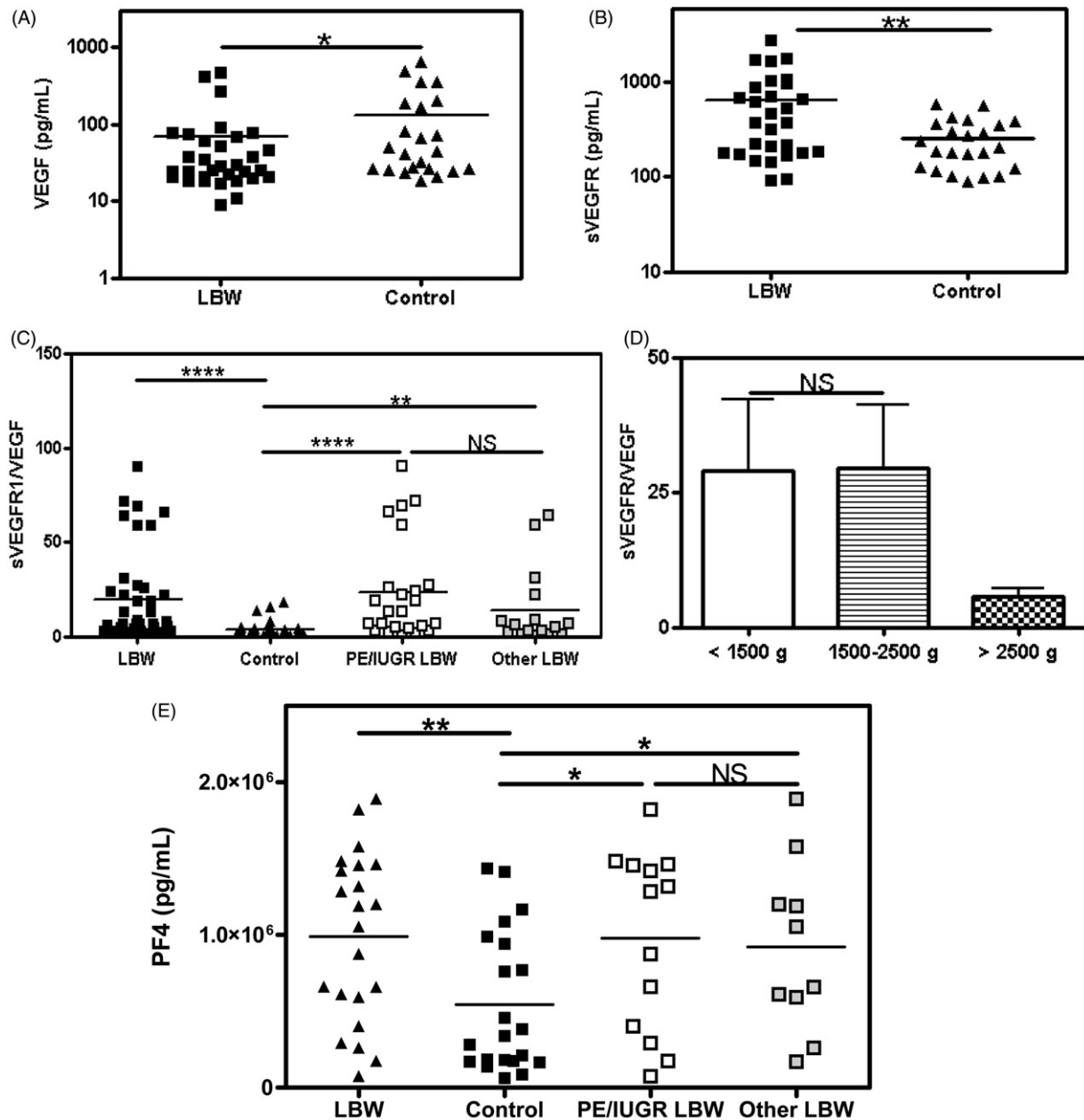


Figure 2. (A–E) Evaluation of LBW serum VEGF, sVEGFR1 and PF4 status by immunoassays. Values are given as mean \pm SEM. (A) VEGF, vascular endothelial growth factor. (B) sVEGFR, soluble vascular endothelial growth factor receptor. (C) ratio sVEGFR/VEGF according to perinatal conditions of birth. PE: pre-eclampsia, IUGR: intrauterine growth restriction, other LBW: neonates born after premature rupture of membrane or spontaneous premature labor. (D) ratio sVEGFR/VEGF according to birthweight categories. (E) PF4, platelet factor 4 according to perinatal conditions of birth. NS: not significant, * $p < 0.05$. ** $p < 0.01$. **** $p < 0.0001$.

LBW neonates display an anti-angiogenic status

Angiogenesis is a tightly regulated system that involves circulating angiogenic factors. As LBW serum inhibits ECFC function, the angiogenic status of LBW neonates was investigated. Well-known pro-angiogenic (VEGF) and anti-angiogenic (sVEGFR-1, endoglin, PF4) factors were assessed using ELISA (Figure 2). VEGF level (Figure 2A) was significantly decreased in LBW group ($p = 0.045$). The levels of sVEGFR (Figure 2B), of the ratio sVEGFR/VEGF (Figure 2C–D) and of PF4 (Figure 2E) were significantly increased in LBW group ($p = 0.014$, $p =$ and $p = 0.024$, respectively). Levels of endoglin or thrombospondin-1 did not differ between the groups ($p = 0.249$ and

0.53, respectively). When stratified by perinatal conditions (pre-eclampsia and IUGR group vs. other causes group (premature rupture of membranes and spontaneous premature labor), there was no significant differences for sVEGFR1/VEGF ratio. Level of PF4 was not correlated to perinatal conditions or to gestational age. The level of PF4 was inversely correlated to maternal age ($r = -0.478$, $p = 0.03$).

Exogenous VEGF corrects the anti-angiogenic effect of LBW serum on ECFC proliferation *in vitro*

VEGF is the most prominent angiogenic factor. The addition of exogenous VEGF to ECFC cultured with LBW serum significantly increased their proliferation capacity (Figure 3).

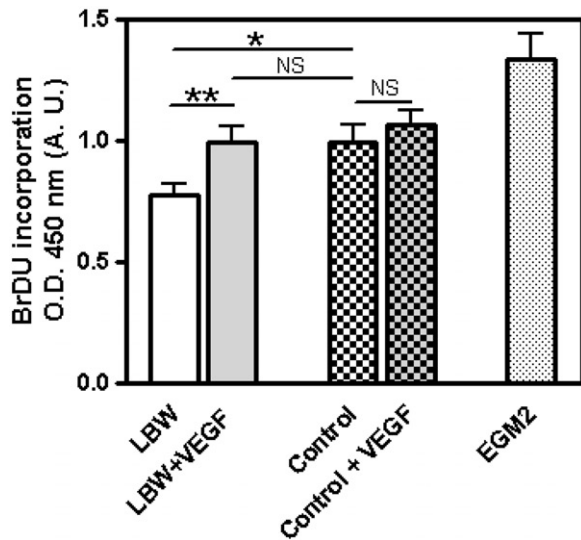


Figure 3. Exogenous VEGF corrects the anti-angiogenic effect of LBW serum on ECFC proliferation *in vitro*. Proliferation assayed by BrdU incorporation in 7000 ECFC. VEGF added at 25 ng/mL. Data represent mean \pm SEM of 30 independent experiments performed in duplicate. NS: not significant, * $p < 0.05$, ** $p < 0.01$.

Discussion

In the present study, we demonstrate that LBW serum impairs normal term ECFCs function decreasing their proliferation and migration capacities. We also show that LBW infants display an anti-angiogenic status and that addition of VEGF can reverse the pathological effects of LBW serum on normal term ECFCs function. In a previous study, we investigated functions of ECFCs extracted from umbilical cord blood of LBW infants [14]. We recently found that LBW ECFCs displayed reduced colonies formation and self-renewal capacities and reduced both *in vitro* and *in vivo* angiogenic capacities like migration and morphogenesis. We also observed a modulation in angiogenic gene expression and an anti-angiogenic profile in LBW ECFCs. Our current study suggests that LBW-ECFCs impairment is the consequence of an inadequate fetal environment during pathological pregnancies.

Angiogenesis is a strictly regulated process regulated by a balance of positive and negative factors [15]. This balance is essential for fetal vascular development. VEGF is a critical and more potent proangiogenic factor and its activity can be counterbalanced by the soluble form of VEGF receptor 1. For the first time, we showed that VEGF was significantly decreased in LBW plasma independently of maternal and perinatal conditions or infants' trophicity. Its antagonist, sVEGFR1, was significantly increased in LBW plasma. This increase was more pronounced in babies born from pre-eclamptic mothers as currently described in literature, but remained significant even in babies born from unexplained preterm labor or from PROM. Indeed, IUGR as well as pre-eclampsia are associated with an increase of circulating both maternal and fetal SVEGFR1 and a decrease of circulating maternal and fetal VEGF [16,17]. However, such studies compare level of fetal circulating angiogenic and anti-angiogenic factors between near-term or term control babies

and preterm or term babies born in preeclamptic or IUGR conditions [16–19]. So, little is known about angiogenic status of preterm neonates whatever their perinatal conditions may be. Recently, we demonstrated a decreased expression of angiogenic genes (VEGF) and an increased expression of anti-angiogenic genes of LBW-ECFC (thrombospondin1, PF4) [14]. PF4 is a negative modulator of endothelial cell proliferation and migration. This regulation occurs directly on cells or via inhibition of the interaction of angiogenic growth factors (FGF and VEGF) with cell surface receptors [20]. As PF4 is significantly increased in LBW serum, we could hypothesize that, as sVEGFR1, PF4 antagonizes the angiogenic effect of VEGF in LBW neonates. On the other hand, circulating thrombospondin-1 was not increased in LBW. This is not surprising as TSP-1 is a matricellular protein that usually acts locally.

In LBW, both ECFCs and the environment (serum and plasma) display an antiangiogenic status. We could hypothesize that perinatal conditions, stress or steroids could influence angiogenic status of the fetus. However, the mechanisms underlying anti-angiogenic imbalance in LBW neonates are still unknown. During fetal programming of cardiovascular disease, antenatal glucocorticoids (GC) exposure is one mechanistic pathway that has been extensively studied [21]. Prenatal exposure to GC modifies sometimes permanently the development of many organs through an inhibition of angiogenesis [22]. The consequences of antenatal exposure to GC are not known on EPCs function. On one hand, it has been demonstrated that GC suppressed the expression and the secretion of VEGF, an essential proangiogenic factor that regulate EPCs activity. On the other hand, Greenberger et al. described that VEGF mRNA expression in dexamethasone-treated-hemangioma EPCs was not decreased [23]. Another mechanistic hypothesis would be oxidative stress. Oxidative stress is a pathogenic element during atherosclerosis and plays a pivotal role in vascular dysfunction and remodeling [24]. Many clinical situations during pregnancy like gestational hypertension/pre-eclampsia, gestational diabetes, infection or inflammatory conditions are associated with oxidative stress [25]. Furthermore, LBW neonates are physiologically more sensitive to oxidative stress due to defect in anti-oxidative enzymes [26]. Reactive oxygen species (ROS) contribute to endothelial dysfunction in many complications of prematurity such as in utero hypertension programming, chronic lung disease [24,26,27]. It has been demonstrated that oxidative stress promotes endothelial cell necrosis, apoptosis and disturb angiogenic factors balance leading to vascular degenerative damage and microvessel rarefaction. In adult, ROS have been shown to disturb the function of EPCs [28].

A limitation of this study was that capillary tube formation studied by matrigel assay was not altered by LBW serum. When cultured on such matrices, it has been demonstrated that cellular reorganization is dependent on proteolytic degradation of the matrix and on cellular forces exerted on the substrate. In these cases, cell migration may not be necessary to form capillary. Moreover, matrigel contains exogenous VEGF that could by itself, interfere with the pathological effect of LBW serum and locally stimulate normal term ECFCs angiogenic capacities. Moreover, our study doesn't permit to determine if the anti-angiogenic status

of LBW neonates constitutes a physiological transitory situation or a pathological condition that could markedly affect ECFC of the neonate.

In conclusion, our results suggest that LBW-ECFCs impairment is the consequence of an inadequate fetal environment during pathological pregnancies. One explanation could be an increased senescence of fetal EPCs by the switch towards anti-angiogenic status of LBW-ECFCs induced by anti-angiogenic fetal environment. Indeed, increased senescence of fetal EPCs was demonstrated in many pathological obstetrical conditions such as pre-eclampsia, maternal diabetes and IUGR. Future studies are planned to explain the mechanisms by which LBW serum and anti-angiogenic status of LBW affect ECFCs function.

Acknowledgements

The authors thank Dr L. Arnaud (Assistance Publique-Hôpitaux de Marseille) for his help in performing immunoassays, and the staffs of the obstetric and neonatal departments for their support.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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