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Cord Blood Endothelial Colony-Forming Cells from Newborns with Congenital Diaphragmatic Hernia

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Abstract

Endothelial colony-forming cells (ECFC) are decreased in the cord blood of preterm infants with moderate-to-severe bronchopulmonary dysplasia (BPD). We quantified ECFC from infants with congenital diaphragmatic hernia (CDH), a neonatal disorder with severe lung hypoplasia. Unlike newborns who develop BPD, those with CDH had increased and highly-proliferative cord blood ECFC.

Keywords

Endothelial Progenitor Cells; Vasculogenesis; Congenital Diaphragmatic Hernia

Congenital diaphragmatic hernia (CDH) is an early developmental anomaly that affects 1 out of every 2000-3000 newborns [1, 2] and results in significant morbidity and mortality [3]. CDH occurs during the embryonic stage of lung development when a diaphragmatic defect occurs and is associated with both marked pulmonary hypoplasia and thoracic herniation of the abdominal viscera [4]. The lungs of infants with CDH have marked alveolar simplification and decreased vascularity [5, 6]. Although the precise mechanisms remain unclear, CDH clearly results in disrupted growth of the pulmonary vasculature and high risk for severe pulmonary hypertension.

Vascular growth occurs by at least two distinct mechanisms: angiogenesis (direct extension and branching of existing vessels) and vasculogenesis (de novo formation from primitive hemangioblasts) [7]. Bone marrow-derived circulating endothelial progenitor cells (EPC) are thought to contribute to vasculogenesis in the developing lung and elsewhere [8]. Circulating EPC have been characterized as early- and late-outgrowth by primary culture methods [8-10]. Although both cell types may be pro-angiogenic [8, 11], late-outgrowth endothelial colony-forming cells (ECFC) have a typical endothelial appearance, express endothelial cell surface antigens, are highly proliferative and self-renewing in vitro, and do not express hematopoietic surface markers [12].

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ECFC are increased in umbilical cord blood as compared with adult peripheral blood [10]. However, cord blood ECFC are markedly decreased in preterm infants who develop moderate or severe bronchopulmonary dysplasia (BPD) [13, 14], the chronic lung disease associated with preterm birth [15]. The lungs of infants with both BPD and CDH share histologic features of alveolar and vascular simplification [5, 6, 16]. However, circulating ECFC levels have not been previously reported for infants with CDH. We speculated that CDH would also be associated with decreased cord blood-derived ECFC.

Methods

With approval by the Colorado Multiple Institutional Review Board and informed consent from pregnant mothers at the University Hospital Anschutz Inpatient Pavilion, cord blood was collected and processed as previously described [17]. Subjects with known HIV/HBV/HCV infection were excluded. Maternal and infant clinical data were collected until discharge and stored in a secure REDCap database [18]. Subjects with CDH were identified through the High-Risk Maternity and Newborn Program.

Mononuclear cells (MNC) were isolated from cord blood by gradient centrifugation within 24 hours. MNC were cultured on type 1 collagen in complete EGM-2 media (Lonza; 10% fetal bovine serum [FBS]). ECFC colonies were identified daily and enumerated on day 14. As previously described, immunohistochemistry, polychromatic flow cytometry (PFC), and in vitro angiogenesis assays were performed to confirm the ECFC phenotype [13].

Low-passage ECFC (p3) were cultured on collagen (2.5×10^4 cells/well) for 6 days in low-serum complete EGM-2 (2.5% FBS) and incubated in room air. Cells were counted in triplicate by hemacytometer. Fold-increase (FI) was calculated ($\text{FI} = \text{day } n \text{ count} / \text{day } 0 \text{ count}$).

Statistical analyses

We analyzed non-parametric data with Mann Whitney testing and present all results as medians with interquartile ranges (IQR). Fisher exact test was used to analyze categorical data. Analysis was performed with the Prism software package (v. 5.0a, GraphPad). Significance level was set at $\alpha = 0.05$.

Results

The characteristics of the 39 enrolled infants (6 with CDH and 33 controls) are summarized in the Table. The infants with CDH were born two weeks earlier ($p = 0.04$). Study groups were not different in terms of birth weight, length, or occipitofrontal circumference. Infants with CDH had lower Apgar scores at 1 and 5 minutes ($p < 0.01$). As expected, these infants required significantly more mechanical ventilation days ($p = 0.01$), CPAP + ventilation days ($p = 0.03$), and supplemental oxygen days ($p = 0.02$) as compared with controls.

Cord blood from infants with CDH yielded 1.5 times as many ECFC colonies on day 14 as cord blood from control infants (10.4, IQR 5.7-16.0 vs. 6.8, IQR 1.5-8.0; $p = 0.01$; Figure, A). ECFC were successfully isolated from the cord blood of all six of the infants with CDH.

The growth of the six ECFC cell lines from infants with CDH was compared with the growth of 15 control ECFC cell lines whose growth was measured during the months before the CDH samples were collected. When compared with control ECFC, CDH ECFC demonstrated robust growth. On day six of the growth assay, the median fold increase of CDH ECFC was 21.2 (IQR 12.1-32.5) as compared with a fold increase of 9.5 (IQR 7.0-22.4) for control ECFC ($p < 0.05$; Figure, B).

Discussion

We report that contrary to our expectations, ECFC are increased in the cord blood of infants with CDH. We further found that CDH ECFC proliferate significantly more rapidly than control ECFC.

Impaired angiogenesis has been described in infants with CDH during the later stages of lung development [19]. The vascular endothelial growth factor-nitric oxide (VEGF-NO) signaling axis, an important pro-angiogenic signaling pathway in endothelial cells, is disrupted in the lungs of human infants with CDH, as well as in experimental models [19, 20]. Aside from a putative role in vasculogenesis, bone marrow-derived EPC contribute to vessel repair after injury and are increased in response to cytokines such as VEGF [21]. We speculate that differences in cord blood ECFC levels from preterm infants who subsequently develop BPD and term infants with CDH may reflect differences in maturation and the intrauterine environment. An increase in circulating ECFC may be a response to local impairment of vascular growth that occurs by mechanisms distinct from preterm infants at risk for BPD. Consistent with previous studies, we found that Apgar scores were decreased in infants with CDH [22], but whether this reflects additional mechanisms of ECFC impairment is unknown.

A limitation of our data is that although circulating ECFC are increased in number and proliferate more rapidly than controls, the angiogenic function of CDH ECFC may be impaired and has not yet been studied. The small number of infants with CDH enrolled in our study is a further limitation and there is considerable variation in ECFC number in the six CDH subjects. Nonetheless, cord blood ECFC levels are clearly not decreased in CDH infants as reported in infants with moderate-to-severe BPD [13, 14]. Further study is needed to further understand whether ECFC contribute to lung growth in CDH or may potentially modulate clinical outcomes of CDH infants.

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A, Cord blood-derived ECFC colonies in infants with CDH (n=6) compared with controls (n=33). The cord blood of infants with CDH had increased ECFC levels (per 10^7 MNC plated; $p = 0.01$). B, Comparison of growth of CDH ECFC (n=6) versus controls (n=15). On

day 6, the fold increase in growth of CDH ECFC was greater than that of control ECFC ($p < 0.05$).

Table

Clinical characteristics.

Characteristic	Full-term Controls	Congenital Diaphragmatic Hernia	p-value
Subjects (n)	33	6	
Males/females	17/16	4/2	0.67
Gestational age (weeks)	39.2±0.9	37.1±2.6	0.04
Birth weight (g)	3261±532	2722±836	0.13
Length (cm)	49.0±2.9	47.7±3.2	0.42
Occipitofrontal circumference (cm)	34.1±1.5	34.1±2.4	0.99
Maternal smoking	6.1 (2)	0 (0)	NS
Preeclampsia	0 (0)	16.7 (1)	0.16
Diabetes	15.2 (5)	0 (0)	0.57
Cesarean delivery	39.4 (13)	0 (0)	0.08
Maternal antibiotics	57.6 (19)	16.7 (1)	0.09
Chorioamnionitis	0 (0)	0 (0)	NS
Intrauterine growth restriction	9.1 (3)	16.7 (1)	0.50
Postnatal sepsis	0, 0	0, 0	NS
Apgar - 1 min	8 (7-8)	5.5 (4-7)	<0.01
Apgar - 5 min	9 (9-9)	8 (6.5-9)	<0.01
Ventilator days	0 (0-0)	14.0 (4.8-21.8)	0.01
CPAP days	0 (0-0)	0 (0-3.5)	0.36
CPAP + ventilator days	0 (0-0)	14.0 (4.8-25.3)	0.03
Total supplemental oxygen days	0 (0-0)	32.0 (21.8-70.0)	0.02

Data are presented as mean±SD, n (%), or median (interquartile range). Bold indicates statistical significance.

Definitions of abbreviations: n = number of subjects within a study group with the given condition; CPAP = continuous positive airway pressure