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Cord blood-derived endothelial colony-forming cell function is disrupted in congenital diaphragmatic hernia

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1Department of Molecular Pathology, Tokyo Medical University, Tokyo, Japan; 2Department of Reproductive Biology, National Institute for Child Health and Development, Tokyo, Japan; 3Division of Neonatology, Center for Maternal-Fetal and Neonatal Medicine, National Center for Child Health and Development, Tokyo, Japan; 4Department of Human Genetics, National Institute for Child Health and Development, Tokyo, Japan; and 5Department of Systems BioMedicine, National Institute for Child Health and Development, Tokyo, Japan

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Fujinaga H, Fujinaga H, Watanabe N, Kato T, Tamano M, Terao M, Takada S, Ito Y, Umezawa A, Kuroda M. Cord blood-derived endothelial colony-forming cell function is disrupted in congenital diaphragmatic hernia. Am J Physiol Lung Cell Mol Physiol 310: L1143–L1154, 2016. First published April 29, 2016; doi:10.1152/ajplung.00357.2015.—Vascular growth is necessary for normal lung development. Although endothelial progenitor cells (EPCs) play an important role in vascularization, little is known about EPC function in congenital diaphragmatic hernia (CDH), a severe neonatal condition that is associated with pulmonary hypoplasia. We hypothesized that the function of endothelial colony-forming cells (ECFCs), a type of EPC, is impaired in CDH. Cord blood (CB) was collected from full-term CDH patients and healthy controls. We assessed CB progenitor cell populations as well as plasma vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1α (SDF1α) levels. CB ECFC clonogenicity; growth kinetics; migration; production of VEGF, SDF1α, and nitric oxide (NO); vasculogenic capacity; and mRNA expression of VEGF-A, fms-related tyrosine kinase 1 (FLT1), kinase insert domain receptor (KDR), nitric oxide synthase (NOS) 1–3, SDF1, and chemokine (C-X-C motif) receptor 4 (CXCR4) were also assessed. Compared with controls, CB ECFCs were decreased in CDH. CDH ECFCs had reduced potential for self-renewal, clonogenicity, proliferation, and migration. Their capacity for NO production was enhanced but their response to VEGF was blunted in CDH ECFCs. In vivo potential for de novo vasculogenesis was reduced in CDH ECFCs. There was no difference in CB plasma VEGF and SDF1α concentrations, VEGF and SDF1α production by ECFCs, and ECFC mRNA expression of VEGF-A, FLT1, KDR, NOS1-3, SDF1, and CXCR4 between CDH and control subjects. In conclusion, CB ECFC function is disrupted in CDH, but these changes may be caused by mechanisms other than alteration of VEGF-NO and SDF1-CXCR4 signaling.

endothelial progenitor cells; endothelial colony-forming cells; congenital diaphragmatic hernia; cord blood

While the survival rate of congenital diaphragmatic hernia (CDH) has improved over the decades due to advances in perinatal management, the mortality rate remains over 30% (21, 40, 41). Furthermore, survivors often suffer from multi-system morbidity in long-term outcome (18).

In CDH, due to the defect in the diaphragm, the abdominal viscera invade the thoracic cavity, impair lung development, and cause lung hypoplasia (25). Lung pathology demonstrated low lung volume, decreased airway generation and alveolar count, air-blood barrier thickness, decreased vascular bed, and increased vascular smooth muscles in precapillary vessels (50). The exact mechanisms of how CDH leads to pulmonary hypoplasia have not been fully elucidated.

As a developmental lung disease, bronchopulmonary dysplasia (BPD) has been intensively studied. The pathophysiology of BPD is considered to be impaired lung vascular and alveolar growth (37). In clinical settings and animal BPD models, disruption of vascular endothelial growth factor (VEGF)-nitric oxide (NO) signaling in the lungs (12, 13, 36, 42, 43) and its adverse effect on endothelial progenitor cells (EPCs), especially endothelial colony-forming cells (ECFCs), have been demonstrated (10, 12, 24). Therefore, a “vascular hypothesis,” suggesting the contribution of injured lung vascular growth during lung development to the etiology of lung hypoplasia (1), was proposed. Recently, the existence of lung-resident ECFCs and their dysfunction in BPD were suggested (3, 4). Some studies showed the relationship between decreased circulating ECFCs and the occurrence of BPD (8, 15), while others did not (52, 54). Additionally, one paper demonstrated the possibility of cord blood (CB)-derived ECFCs as a therapeutic option for BPD via paracrine effects (3), but another suggested the limitation of CB ECFC-conditioned media for that purpose (11). Therefore, the depletion and dysfunction of circulating and/or lung-resident ECFCs might be one of the possible mechanisms underlying arrested lung vascular growth in BPD (3, 14).

Lung vascular growth occurs via angiogenesis and vasculogenesis (20, 29) with the involvement of EPCs (38). Several distinct populations of cells have been reported as EPCs (5, 28, 45). By flow cytometry, circulating progenitor cells can be classified as ECFCs (CD45−CD34+AC133−CD31+), angiogenic circulating progenitor cells (CPCs) (CD45dimCD34+AC133+CD31+), and nonangiogenic CPCs (CD45dimCD34+AC133−CD31−) (22). In
Vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1 (SDF1) are among the factors involved in EPC mobilization, recruitment, homing, and differentiation in neovascularization (5, 27, 35, 47, 59, 63, 66). In the lungs of CDH patients, VEGF-A mRNA expression was decreased at the alveolar stage (60). In rat CDH models with nitrofen, lung VEGF-A protein expression was reduced (26, 55, 57). On the other hand, increased plasma VEGF was associated with the clinical severity and adverse outcomes in CDH (51). Surgically induced CDH in fetal sheep markedly reduced highly proliferative pulmonary artery endothelial cells (PAECs) and caused PAEC dysfunction (2). Additionally, antenatal treatment with simvastatin, sildenafil, or BY 41–61 decreased highly proliferative pulmonary artery endothelial cells at birth (48, 49, 60). These studies suggest that disrupted vascularization was involved in the pathophysiology of pulmonary hypoplasia in CDH. However, few studies have investigated the role of EPCs and SDF1 signaling in pulmonary hypoplasia in CDH (9, 17).

We hypothesized that the function of EPCs, especially ECFCs, in CDH patients is impaired due to disrupted VEGF-NO signaling or EPC function, improved pulmonary hypertension or pathological features of lung hypoplasia in animal CDH models (48, 49, 61). These studies suggest that disrupted vascularization was involved in the pathophysiology of pulmonary hypoplasia in CDH. However, few studies have investigated the role of EPCs and SDF1 signaling in pulmonary hypoplasia in CDH (9, 17).

MATERIALS AND METHODS

Subject Enrollment and Data Collection

This study was approved by the ethics committee of the National Center for Child Health and Development (NCCHD), Tokyo, Japan. Written informed consent was obtained from the parents whose unborn child was diagnosed with CDH (CDH group) or had no abnormal findings in their screening tests (control group). Exclusion criteria included preterm or postterm birth infants, known congenital infection and chromosomal abnormalities, as well as infants treated prenatally with fetoscopic tracheal occlusion (FETO). CB samples were obtained at birth at the NCCHD. Clinical data of infants and their mothers were collected from electronic medical records.

CB Samples and Endothelial Colony-Forming Cell Culture for Isolation

CB was collected directly from the umbilical vein using a heparinized syringe immediately after birth. All CB samples were processed within 4 h after collection. CB was diluted with PBS (Invitrogen, Carlsbad, CA), underlaid with Ficol-Paque PLUS (GE Healthcare Bio-Sciences, Uppsala, Sweden), and centrifuged at 740 g for 30 min. Then, the mononuclear cell (MNC) buffy coat was retrieved. MNCs were plated on collagen-coated culture plates (Corning, Corning, NY) at a density of up to $5 \times 10^6$ cells/cm², incubated for 14 days in EGM-2 (Lonza, Walkersville, MD) with 10% FBS (Life Technologies, Carlsbad, CA), and expanded by standard techniques. Isolated ECFCs were characterized by light microscopy, flow cytometry, single cell colony formation assay, in vitro tube formation assay and in vivo vasculogenesis assay as previously described (10, 24, 45). Low passage cells (p3 or p4) were used for each experiment.

Flow Cytometry Analysis

Measurement of circulating progenitor cell populations in CB. We measured circulating progenitor cell populations in CB using polychromatic flow cytometry (PFC). MNCs ($0.5–1.0 \times 10^7$) from CB were processed as described in the previous section were resuspended in PBS with 2% FBS. Cells were stained with the following antibodies: CD14-PE-Cy5.5 (abcam, Cambridge, UK), CD31-FITC (BD Pharmingen, San Jose, CA), CD34-PE (BD Pharmingen), CD45-Pacific Orange (Invitrogen), CD133/1 (AC133) pure (Miltenyi Biotec, Auburn, CA) conjugated by Zenon Pacific Green Mouse IgG1 Labeling Kit (Molecular Probes, Eugene, OR), glycophorin-A (R&D Systems, Minneapolis, MN) and CD41 (R&D Systems) conjugated by Pacific Blue Monoclonal Antibody Labeling Kit (Molecular Probes), and the LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Invitrogen). We used Abc Anti-Mouse Bead Kit (Molecular Probes) and ArC Amine Reactive Compensation Bead Kit (Molecular Probes) as single stained controls for appropriate multicolor compensation. Gating was established using an unstained control and fluorescence-minus-one control samples with biexponential gating (22). Cell populations were quantified as a percentage of the gated CD14-negative MNCs after erythrocytes, platelets, and dead cell exclusion as described before (8). ECFCs (CD45$^-$CD34$^+$CD133$^-$CD31$^-$), angiogenic CPCs (CD45$^-$CD34$^+$CD133$^+$CD31$^-$), and nonangiogenic CPCs (CD45$^-$CD34$^+$CD133$^+$CD31$^-$) were measured. The CPC/non-CPC ratio was calculated by dividing angiogenic CPCs by non-angiogenic CPCs. The observers who evaluated the results were blinded to the identity of the samples.

Characterization of Isolated ECFCs

Cells ($0.5–1.0 \times 10^6$) were resuspended in 80 μl of DNase Staining Buffer (PBS with 2% FBS, 80 units/ml Dornase alfa (Sigma-Aldrich, St. Louis, MO)) with 20 μl of mouse serum (Sigma-Aldrich). Cells were stained with one of following: AC133-PE (Miltenyi Biotec), CD45-FITC, CD31-FITC, CD34-PE, CD38-FITC, CD90-FITC, CD105-FITC, CD146-FITC, and the appropriate FITC-mouse IgG1, and PE-mouse IgG1, isotype controls. All antibodies except for AC133-PE were purchased from BD Pharmingen. Propidium iodide was applied to the samples to identify dead cells immediately before analysis.

Cells were analyzed using the Attune Acoustic Focusing Cytometer and the Attune Cytometric Software v1.2.5 (Applied Biosystems, Carlsbad, CA).

ECFC Colony Assay

In cell culture for ECFC isolation from CB, colonies of ECFCs were identified as well-circumscribed monolayers of cells with cobblestone appearance and counted by visual inspection with an inverted microscope, Nikon Eclipse TS100 (Nikon, Tokyo, Japan) on day 14. The numbers of ECFC colonies were expressed as the frequency of colonies per $10^7$ plated MNCs or per milliliter of CB. The observers who evaluated the results were blinded to the identity of the samples.

Single Cell Colony Formation Assay

To assess self-renewal potential of ECFCs, a single ECFC was placed into each well of a 96-well collagen-coated culture plate using a FACSAria III (Becton Dickinson, San Jose, CA) and cultured in 200 μl of EGM-2 with 2% FBS. Media were changed every 4 days. On day 14, each well was examined for colony growth. To enumerate the number of cells per well, we counted cells by visual inspection with an inverted microscope or trypsinized the cells and counted them with a hemocytometer. Additionally, to test the ability of the clonal progeny of a single ECFC to form secondary colonies, cells in individual wells containing more than 50 cells were subcultured to a
24-well collagen-coated culture dish containing 500 µl of EGM-2 with 2% FBS. After 7 days, wells were examined for colony growth or cell confluence by visual inspection with an inverted microscope. The observers who evaluated the results were blinded to the identity of the samples.

**ECFC Growth Assay**

ECFCs were plated on six-well collagen-coated culture plates at 2.5 × 10^5 cells/well in EGM-2 with 10% FBS and incubated overnight. ECFCs were cultured in EGM-2 with 2% FBS for 6 days. Media were changed once every 2 days. Cell counts were conducted on day 0, 3, and 6 with the Vi-CELL XR (Beckmann Coulter; Pasadena, CA) and the results in triplicate were averaged for each time point.

**Migration Assay with Modified Boyden Chamber Method**

Cell chemotaxis was assessed using a “modified Boyden chamber assay” with Cultrex In Vitro Angiogenesis Assay Endothelial Cell Invasion Kit (Trevigen, Gaithersburg, MD) as described by the manufacturer. Briefly, ECFCs were seeded at 20,000 cells/well in 50 µl of EBM-2 to the top chambers coated with coating buffer. To create a positive chemotactic gradient, 150 µl of EGM-2 with 2% or 5% FBS were added to the bottom chambers. After incubation for 48 h, 150 µl of Cell Dissociation Solution/Calcine-AM were added to the bottom chambers and incubated for 1 h. The bottom chambers were read at 485-nm excitation and 520-nm emission to obtain relative fluorescence units using the Wallac 1420 ARVO SX multilabel counter (Perkin Elmer, Waltham, MA). The numbers of cells that migrated from the top chambers to the bottom ones were calculated according to the standard curve. Data were represented as percent (%) migration [i.e., the percentage of these cell numbers to those in the top chambers at the start of the experiments (20,000 cells/well)]. All experiments were done in triplicate wells.

**Scratch Wound Healing Assay**

Cell chemokinesis was assessed using a “scratch wound healing assay” (7). ECFCs were plated on 24-well collagen-coated culture plates in EGM-2 with 10% FBS at 80,000 cells/well. When cells reached 70–80% confluence, media were changed to EBM-2 with 2% FBS. After 24 h, cell monolayers were scratched with a sterile cell scraper to make wounds of equivalent sizes and media were replaced with EGM-2 with 2, 5, or 10% FBS to remove nonadherent cells. Images of the wound area were collected using Olympus CKX41 inverted microscope (Olympus; Tokyo, Japan) and were captured with Olympus DP21. Nonpopulated scratch areas were measured by ImageJ version 1.48 software (NCBI), and the percent (%) closure (percentage of the original wound area occupied at 6, 12, 18, and 24 h by cells that had migrated into the wound area) was calculated. All experiments were done in quadruplicate wells. The observers who evaluated the results were blinded to the identity of the samples.

**NO Production Assay**

ECFCs were plated at 2.5 × 10^5 cells/well into the six-well collagen-coated culture plates containing EGM-2 with 10% FBS and incubated overnight. After pretreatment in EGM-2 with 2% FBS without VEGF supplementation for 24 h, cells were incubated in HBSS (IBCO, Carlsbad, CA) either with or without 0.5 µM of 3-amino,4-aminomethyl-2',7'-difluorofluorescein diacetate (DAF-FM DA; Santa Cruz Biotechnology, Dallas, TX) for 30 min for loading and then incubated in EBM-2 either with or without 25 ng/ml of recombinant human VEGF (rhVEGF; R&D Systems) for 30 min and harvested enzymatically with 0.25% trypsin/0.02% EDTA. Fluorescence intensity was quantified by flow cytometry in the system for FITC. Intracellular NO concentration of ECFCs was expressed by differences in mean fluorescence intensity between ECFCs treated either with or without DAF-FM DA (24). All experiments were done in triplicate.

**Tube Formation Assay**

ECFCs were seeded at 20,000 cells/well in 96-well plates coated with 50 µl of Matrigel (Corning, 10 mg/ml) or growth-factor reduced Matrigel (Corning, 6 mg/ml) in EGM-2 with 2, 5, or 10% FBS. Six and 12 h after plating, images of capillary-like structures were collected using an inverted microscope. The total length of capillary-like structures per well was quantitated using the ImageJ software. Each experiment was done in triplicate (44). The observers who evaluated the results were blinded to the identity of the samples.

**In Vivo Vasculogenesis Assay**

Transplantation of ECFCs. Cellularized gel implants were transplanted into mice as previously described (31, 32, 56, 65). ECFCs were suspended in a solution composed of EGM-2, 10% FBS, 1.5 mg/ml rat tail collagen I (BD Biosciences), 100 ng/ml human fibronectin (Millipore, Billerica, MA), 1.5 mg/ml sodium bicarbonate (Sigma-Aldrich), and 25 mM HEPES (Lonza) at 2 × 10^6 cells/ml. Then, 1 ml of the cell suspension was transferred into the 24-well culture plate, incubated at 37°C for 30 min for polymerization, and then covered with EGM-2 with 2% FBS and incubated overnight. The following day, gels with ECFCs from a newborn were implanted into the flank of three 8-wk-old NOD/SCID mice (CLEA, Tokyo, Japan). The right flank of each mouse was implanted with ECFCs from controls, and the left flank with CDH ECFCs. Fourteen days after transplantation, grafts were harvested and analyzed for vessel formation by immunohistochemistry. All experiments were approved by the Institutional Animal Care and Use Committee of the National Research Institute for Child Health and Development.

**Immunohistochemistry**

Paraffin-embedded tissue sections of 7-µm thickness were deparaffinized and immersed in the Target Retrieval Solution (Dako, Carpenteria, CA) for 15 min at 121°C at 15 psi above atmospheric pressure. Slides were incubated at room temperature with anti-human CD31 (clone JC70A; Dako) for 30 min followed by 30-min incubation with EnVision + Dual Link, Single Regents (Dako) and then developed with DAB solution (Dako) for 5 min. Isotype-matched nonbinding antibody (Dako) was used to control for nonspecific reactivity. Tissue sections were counterstained with hematoxylin and eosin. Slides were dehydrated and mounted using Mount-Quick (Daido Sangyo, Tokyo, Japan). Enumeration of vessels perfused and stained positively for anti-human CD31 was performed by visual inspection using Olympus BX51 upright microscope and Olympus DP71. The observers who evaluated the results were blinded to the identity of the samples.

**ELISA**

VEGF and SDF-1α concentrations in CB plasma were measured with the Quantikine ELISA Kits (R&D Systems) as described by the manufacturer. In addition, we compared VEGF and SDF-1α protein production by ECFCs between both study groups. Briefly, ECFCs were plated in a six-well collagen-coated culture plate at 0.6 × 10^5 cells/well in EGM-2 with 10% FBS. On the next day, media were changed to EBM-2 with 2% FBS to exclude the effects of exogenous VEGF or other growth factors and minimize the effects of FBS. After 24-h incubation, cell culture supernatants were collected and VEGF and SDF-1α protein concentrations were measured with the Quantikine ELISA Kits.
**RT-PCR and Quantitative Real-Time PCR**

Total RNA extraction from ECFCs was performed using RNesay Plus Mini (Qiagen, Venio, the Netherlands). The cDNA was synthesized by RT-PCR with Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative PCR was performed on the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems).

Reaction products of VEGF-A, fms-related tyrosine kinase 1 (FLT1), kinase insert domain receptor (KDR), NO synthase (NOS) 1, NOS2, NOS3, SDF1, CXCR4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by Thunderbird SYBR qPCR Mix (Toyobo Life Science, Osaka, Japan) with 0.3 μmol/l of gene specific primers as described by the manufacturer. Each experiment was done in triplicate. Results were normalized to GAPDH, and relative mRNA expression to those of ECFCs from a particular participant in the control group was analyzed with the 2^−ΔΔCt method.

The gene specific primer sequences were as follows: VEGF-A: forward 5'-AGGGCAATCATACTACAGGAATG-3', reverse 5'-AGGGTCTCGATTGGATGGCA-3'; FLT1: forward 5'-GAAAACGCATAATCTGGGACAGT-3', reverse 5'-GCGTGGTGTTGCTTATTTGGA-3'; KDR: forward 5'-GTGATCGAAATGACACTGGAG-3', reverse 5'-CATGTTGGTCACTACAGAAGCA-3'; NOS1: forward 5'-TTCCCTCTCGCAAAGATTGTG-3', reverse 5'-AAGTGCTAGTGTTGTCGATCT-3'; NOS2: forward 5'-TTCCATAGTGCAACCATGCGAAG-3', reverse 5'-GCCATCACGCCACAGTTTC-3'; NO3: forward 5'-GAGTTGCAATGCTGATCT-3', reverse 5'-TTCCATAGTGCAACCATGCGAAG-3'; SDF1: forward 5'-ATTCTCAGACTTAATGGTGCC-3', reverse 5'-ACACTGTTTCTGTCGATCT-3'; CXCR4: forward 5'-ACTACACAGGAAATGGGTGG-3', reverse 5'-CCACATTGCCAGTTAGAAGA-3'; and GAPDH: forward 5'-ACAACCTTTGGTATCGTGGAAGG-3', reverse 5'-GCCATCACGCCACAGTTTC-3'.

**Statistics**

Statistical analysis was performed with the Prism software package version 4 (GraphPad, La Jolla, CA). For analyses of participants' clinical characteristics, data were presented as count (relative frequency, %), mean ± SD, or median (range). Fisher’s exact test, unpaired t-test, or Mann-Whitney’s U-test were conducted. For analyses of experiments with CB and ECFC function assays, data are presented as mean ± SE, and paired or unpaired t-tests were conducted. All statistical tests were two-sided. P < 0.05 was considered significant.

**RESULTS**

**CB Samples**

Figure 1 shows the number of newborns who were screened and included in this study, as well as the number of CB samples and ECFCs available for each analysis.

Briefly, we obtained CB samples from 10 newborns in each group and analyzed the data of 5–10 samples from each group in each experiment.

**Clinical characteristics of the study newborns**

Among the 20 newborns enrolled, there was no significant difference in maternal, prenatal, and perinatal factors (maternal age, complications and smoking, chorioamnionitis, premature rupture of membrane, prenatal corticosteroids,
delivery mode, meconium staining, gestational age, birth weight, small for gestational age, sex, and so on) between both groups, except for Apgar score. In each experiment, the comparison of clinical characteristics of the participants between both groups yielded the same results as described above. The CDH group had a lower Apgar score at 1 and 5 min than that of the control group [4 (1–8) vs. 8 (7–9); \( P < 0.001 \) and 4 (1–9) vs. 9 (8–9); \( P < 0.001 \), respectively], because in our hospital CDH patients were intubated and mechanically ventilated with muscle relaxant immediately after birth. However, umbilical artery pH that correlated with severity of perinatal asphyxia was not significantly different. All CDH were left-sided.

**Characterization of ECFCs**

Isolated ECFCs developed a cobblestone-like morphology (Fig. 2A). On flow cytometry, these cells were positive for CD31, CD105, and CD146 (endothelial cell markers), weakly positive for CD34, but negative for CD133, CD38, CD45, and CD90. Because in our hospital CDH patients were intubated and mechanically ventilated with muscle relaxant immediately after birth. However, umbilical artery pH that correlated with severity of perinatal asphyxia was not significantly different. All CDH were left-sided.

**Fig. 2. Characterization of ECFCs.** A: images of typical ECFCs. ECFCs had a cobblestone-like appearance. Scale bars = 500 and 200 \( \mu \)m, respectively. B: flow cytometry of ECFCs. ECFCs express endothelial cell surface markers. Each single-stain result (black) was compared with the corresponding isotype control (gray). ECFCs were strongly positive for CD31, CD105, and CD146, weakly positive for CD34, but negative for CD133, CD38, CD45, and CD90. C: representative image of in vitro tube formation assay. ECFCs formed capillary-like structures. Scale bars = 500 \( \mu \)m. D: representative image of in vivo vasculogenesis assay. ECFCs formed chimeric vessels stained positively for anti-human CD31 (brown) and perfused with mouse erythrocytes. Scale bars = 50 \( \mu \)m.
**ECFCs Are Reduced in CB of CDH Patients**

In PFC of CB, the ECFC population in CB from CDH patients was 80% lower than that from controls (0.0020 ± 0.0005 vs. 0.0098 ± 0.0020%; \( P = 0.008 \); Table 1). There was no significant difference in other CPC populations and the CPC/non-CPC ratio.

In addition, the numbers of ECFC colonies yielded on day 14 in ECFC culture for isolation from CB were enumerated. There was no significant difference in the number of MNCs in CB \([3.8 ± 0.8 \text{ vs. } 4.1 ± 0.7 (\times 10^7 \text{ MNCs/ml of CB}; P = 0.824)\]. The colony counts of CDH ECFCs per \(10^7\) MNCs and that per ml of CB were 72 and 76% lower than those of controls respectively \((293 ± 4.0 \text{ colonies/10}^{7} \text{ MNCs, } P = 0.015; \text{Fig. 3A} ; 1.6 ± 0.7 \text{ vs. } 6.7 ± 1.4 \text{ colonies/ml of CB, } P = 0.005; \text{Fig. 3B})\).

**ECFC Self-Renewal, Clonogenic, and Proliferative Potentials Are Impaired in CDH Patients**

In a single cell colony formation assay, ECFCs are classified into three categories according to their proliferative potential; high proliferative potential-endothelial colony-forming cell (HPP-ECFC), which forms a colony that contains >2,000 cells; low proliferative potential-endothelial colony-forming cell (LPP-ECFC), which forms a colony that has 51 to 2,000 cells; and endothelial cell (EC) cluster which forms a colony composed of 2 to 50 cells \((31, 33, 34)\).

Although the percentage of single cells undergoing at least 1 cell division was not significantly different between both groups \((55.7 ± 9.1 \text{ vs. } 41.7 ± 6.5%; P = 0.260)\), the percentages of HPP-ECFCs and EC clusters among these cells were 92% lower and 320% higher in CDH, respectively, compared with those of control \((\text{HPP-ECFC}: 2.8 ± 2.8 \text{ vs. } 35.9 ± 9.8\%; P = 0.032; \text{EC cluster}: 52.1 ± 9.2 \text{ vs. } 12.4 ± 2.3\%; P = 0.009; \text{Fig. 4A})\).

The average number of cell progeny derived from a single cell was 85% smaller in CDH than in control \((293 ± 155 \text{ vs. } 1,943 ± 344 \text{ cells/colony or cluster}, P = 0.001; \text{Fig. 4B})\). Additionally, the percentage of single cell progeny forming a secondary colony was also 77% smaller in CDH than in control \((16.5 ± 6.8 \text{ vs. } 70.4 ± 9.8\% ; P = 0.001; \text{Fig. 4C})\).

Moreover, on day 0 of the ECFC growth assay, there was no significant difference in ECFC counts between both groups \([0.023 ± 0.005 \text{ vs. } 0.018 ± 0.001 (\times 10^6 \text{ cells/well}), P = 0.412; \text{Fig. 5}])\). On day 6, the number of CDH ECFCs was 61% smaller than that of control \((0.200 ± 0.072 \text{ vs. } 0.517 ± 0.084 (\times 10^6 \text{ cells/well}), P = 0.018; \text{Fig. 5})\).

### Table 1. Circulating progenitor cell populations in cord blood

<table>
<thead>
<tr>
<th>Cell Populations</th>
<th>Control ((n = 7))</th>
<th>CDH ((n = 6))</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECFCs(^{a}) %(^{b})</td>
<td>0.0098 ± 0.0020</td>
<td>0.0020 ± 0.0005</td>
<td>0.008</td>
</tr>
<tr>
<td>Angiogenic CPCs(^{c}) %(^{b})</td>
<td>0.0018 ± 0.0005</td>
<td>0.0012 ± 0.0004</td>
<td>0.325</td>
</tr>
<tr>
<td>Nonangiogenic CPCs(^{d}) %(^{b})</td>
<td>0.0322 ± 0.0113</td>
<td>0.0130 ± 0.0085</td>
<td>0.215</td>
</tr>
<tr>
<td>CPC/non-CPC ratio(^{e})</td>
<td>0.1057 ± 0.0434</td>
<td>0.4930 ± 0.1942</td>
<td>0.109</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SE. ECFCs, endothelial colony-forming cells; CPCs, circulating progenitor cells; CDH, congenital diaphragmatic hernia; \(\text{CD}45^{+}\text{-CD34}^{+}\text{AC133}^{+}\text{CD31}^{+}\) cell; \(\text{CD}45^{+}\text{-CD34}^{+}\text{AC133}^{+}\text{CD31}^{+}\) cells; \(\text{angiogenic CPCs/nonangiogenic CPCs})\.

**ECFC Migration Is Impaired in CDH Patients**

As for ECFC chemotaxis assessed by modified Boyden chamber assay, ECFC count that migrated toward EGM-2 with 2% FBS from EBM-2 in 48 h was 89% smaller in the CDH group than in the control group \((\%\text{migration}: 5.4 ± 2.6 \text{ vs. } 50.6 ± 18.0\% , P = 0.023; \text{Fig. 6A})\). The result of migration toward EGM-2 with 5% FBS was the same (data not shown).

As for ECFC chemokinesis assessed by scratch wound healing assay, the area refilled by CDH ECFCs was 38% smaller than that by controls 18 h after wounding in EGM-2 with 2% FBS (% closure: 24.6 ± 4.4 vs. 39.7 ± 1.5\%, \(P = 0.017; \text{Fig. 6, B and C})\). The results at other time points and in other concentrations of FBS were the same (data not shown).

**Capacity of ECFCs for NO Production Is Enhanced but Response to VEGF Stimulation Is Blunted in CDH Patients**

To assess ECFC capacity for NO production, intracellular NO concentrations of ECFCs in basal and VEGF-stimulated conditions were measured with DAF-FM DA. NO concentrations of ECFCs from all controls, as well as those from all CDH patients but one, were increased by VEGF stimulation. Although the increase by VEGF stimulation in NO concentration of ECFCs from controls was statistically significant, that from CDH patients was not. (VEGF-stimulated condition vs. basal condition; CDH; \(P = 0.114\) and control, \(P = 0.010\).
DISRUPTED ECFC FUNCTION IN CDH

Fig. 4. ECFC self-renewal and clonogenic capacity is impaired in CDH patients. A: the percentage of high proliferative potential (HPP)-ECFCs was significantly lower and that of EC clusters was significantly higher in CDH than control in a single cell colony formation assay (asterisk; \( p = 0.032 \), double asterisk; \( p = 0.009 \)). B: average number of cell progeny derived from a single cell was significantly smaller in CDH than control (double asterisk; \( p = 0.001 \)). C: percentage of single cell progeny forming a secondary colony was also significantly smaller in CDH than control (double asterisk; \( p = 0.001 \)). Bars represent mean ± SE. Unpaired t-test was used to analyze statistical significance (\( n = 5 \); control group, \( n = 6 \); CDH group).

Fig. 5. ECFC growth is impaired in CDH patients. On day 0 of the growth assay, there was no difference in ECFC counts between both groups. On day 6, the number of CDH ECFCs was significantly smaller than that of controls (asterisk; \( p = 0.018 \)). Graph represents mean ± SE. Unpaired t-test was used to analyze statistical significance (\( n = 5 \); control group, \( n = 6 \); CDH group).

respectively; Fig. 7). Also, the increase in NO concentration in CDH ECFCs was not statistically significant when the result of ECFCs from one patient without an increase in NO concentration by VEGF was excluded from statistical analysis. On the other hand, NO concentrations of CDH ECFCs in basal and VEGF-stimulated conditions were 146 and 129% higher, respectively, compared with those of the control (basal condition: \( p = 0.047 \); VEGF-stimulated condition: \( p = 0.048 \), respectively; Fig. 7).

Capacity of ECFCs to Form Capillary-Like Structures in In Vitro Tube Formation Assay Is Not Significantly Different Between CDH Patients and Controls

In the ECFC tube formation assay with the growth-factor reduced Matrigel, there was no significant difference between both groups in the total length of capillary-like structures (data not shown).

The Potential for In Vivo Vasculogenesis Is Reduced in ECFCs from CDH Patients

Since the capacity for de novo vasculogenesis in vivo is one of the most important criteria and functional parameters for ECFCs (30, 44, 53), we compared the in vivo ability of ECFCs from both groups to form vascular structures in xenotransplantation to mice, although there was no difference in an in vitro tube formation assay. The number of chimeric vessels perfused with mouse erythrocytes in the transplanted graft of the CDH group was 85% smaller than that of the control group (16.9 ± 3.5 vs. 113.2 ± 18.4 vessels/mm²; \( p = 0.007 \); Fig. 8, A and B).

Plasma VEGF and SDF1α Concentrations of CB Are Not Significantly Different Between CDH Patients and Controls

To investigate the difference in ECFC exposure to VEGF and SDF1α in fetal circulation, CB plasma VEGF and SDF1α concentrations were measured by ELISA. VEGF concentration of CDH patients tended to be higher than that of controls, but the difference was not statistically significant (60.9 ± 25.7 vs. 27.2 ± 12.7 pg/ml, \( p = 0.261 \)). There was no difference between groups in SDF1α concentration (572.4 ± 17.8 vs. 536.8 ± 22.8 pg/ml, \( p = 0.235 \)).

Gene Expression of VEGF-A, FLT1, KDR, NOS1-3, SDF1, and CXCR4 Is Not Significantly Different Between ECFCs of CDH Patients and Controls

To elucidate the relationship of VEGF-NO signaling and SDF1-CXCR4 signaling with these differences in ECFC functions between CDH patients and controls, mRNA expression of ECFCs for several angiogenic genes was compared by quantitative real-time PCR. The difference in relative mRNA expression of all of these genes between both groups was not statistically significant (VEGF-A: 5.04 ± 0.82 vs. 0.87 ± 0.54 according to the man-

VEGF and SDF1α Production In Vitro Is Not Different in ECFCs from CDH Patients and Controls

Because there was a trend for higher VEGF-A and SDF1 mRNA expression in CDH ECFCs, we measured VEGF-A and SDF1α protein concentrations in culture media after 24-h incubation of ECFCs from both groups. VEGF concentrations in culture supernatants of all ECFCs were lower than the minimum detectable limit (<5.0 pg/ml according to the man-
DISCUSSION

In the present study, we demonstrated that CB-derived ECFC function was disrupted in CDH. The disruption might be caused by mechanisms other than alteration of VEGF-NO and SDF1-CXCR4 signaling. This study had three main findings. First, ECFCs were decreased in the CB of CDH patients. Second, ECFCs derived from the CB of CDH patients had reduced in vitro potential for self-renewal, clonogenicity, proliferation, and migration and decreased in vivo capacity for de novo vasculogenesis. Their capacity for NO production was...
enhanced but response to VEGF was blunted. Third, altered exposure to VEGF and SDF1α, together with gene expression of VEGF-A, FLT1, KDR, NOS1-3, SDF1, and CXCR4, was not involved in these ECFC functional changes.

Notably, this is the first report that demonstrates detailed functions of ECFCs in CDH. Our findings suggest that pulmonary hypoplasia in CDH may potentially be related to impaired ECFC functions and ECFC may be a new therapeutic target for CDH. As noted in studies of EPCs in adult vascular diseases (6, 16, 23, 58, 62), CB ECFCs may potentially serve as a biomarker to predict disease severity or as a therapeutic option in the management of CDH.

In this study, ECFC colonies from CB were decreased and ECFC growth was impaired in CDH compared with controls. However, in an earlier study, Baker et al. (9) showed contrary results. Although we cannot precisely explain why the results of the two studies differ, it could be attributed to the differences in the study populations and methods between the two studies. First, CDH patients in the study of Baker et al. (n = 6) were of lower gestational age (GA; 37.1 ± 2.6 wk in the Baker study vs. 38.4 ± 0.9 wk in ours) and included two premature infants (GA: 33 and 34 wk; personal communication with Dr. C. D. Baker). The control group (n = 33) was larger than the one in our study and, on average, had fewer CB ECFCs. Baker et al. (10) also reported in a separate paper that preterm infants have increased CB ECFCs with increased proliferation compared with term infants. Therefore, GA and sample size may explain some of the differences between the two studies. Hara et al. (26) found that the developmental pattern of lung VEGF-A mRNA expression was inhibited in a rat CDH model with nitrogen but was recovered by tracheal occlusion. Cloutier et al. (19) demonstrated that tracheal occlusion stimulated distal capillary growth as well as expression of specific angio- genic genes in fetal mouse lung. Both our study and that of Baker et al. excluded patients who underwent FETO (personal communication with Dr. C. D. Baker) as they hypothesized that FETO may modify lung vascular growth and EPC function in CDH. Additionally, the impaired proliferative and self-renewal potential of ECFCs in CDH was demonstrated in our study not only by growth assay but also by single cell colony formation assay, which was not done in the study by Baker et al. even though it is the most rigorous test for the proliferative and clonogenic potential of progenitor cells (30, 44, 53). There were also several minor methodological differences, including the duration between the acquisition of CB and ECFC isolation (within 24 h in the Baker study vs. within 4 h at our center). The studies were completed at two different geographical locations (Denver, CO, altitude 1,600 m vs. Tokyo, Japan, sea level). The concentration of FBS differed between the growth assays (2.5% and 2% serum, respectively). However, we cannot explain how these methodological differences would cause opposing results. Finally, all CDH patients in the Baker study were of white or Hispanic origin (personal communication with Dr. C. D. Baker), while those in the present study were Asian. However, the effect of race or ethnicity on ECFC number and function has not been described. Therefore, further studies in similar conditions with larger sample sizes are needed to confirm the results.

We showed that the capacity of ECFCs for NO production was enhanced in CDH patients overall but the response to VEGF stimulation was blunted. Additionally, the result of in vitro tube formation assay of CDH ECFCs was not different when compared with that of controls. These were unexpected. Especially, we expected impaired ECFC capacity for in vitro tube formation, which is perhaps the most relevant to the in vivo capacity of angiogenesis, in CDH.

Although growth factors contained in medium or growth-factor reduced Matrigel might obscure the difference in ECFC capacity for in vitro tube formation between both groups, these findings might suggest that some ECFC functions (mobilization, proliferation, and migration, etc.) are impaired while others (such as NO production) are enhanced (partially) to compensate for the impairment in CDH. As a result, when the same numbers of ECFCs from both study groups participate in an in vitro tube formation assay, there might be no difference in developing capillary-like structures. However, compared with control, the capacity of CDH ECFCs for vascular formation was reduced in vivo due to vascular degeneration. Because the capacity of de novo vasculogenesis in vivo is one of the most important criteria and functional parameters for ECFCs, it might be reasonable to consider that the in vivo potential of CB ECFCs to form vascular network is impaired in CDH. The reason why there were different results between in vitro and in vivo tests to evaluate vascular formation could be attributed to the differences in methodology, such as the duration of experiments, which was much longer in the latter experiment (14 days) compared with that of the former (6 to 12 h). Hence, the latter experiment might have better reflected the differences in the effect of the proliferative potential of ECFCs on vascular formation in both groups. In summary, because of inhibited mobilization, migration, and proliferation, fewer ECFCs might be involved in vascular growth, resulting in fewer vessels formed in the developing lung of CDH patients.

VEGF and SDF1 are involved in the roles of EPCs, including ECFCs, in neovascularization (5, 27, 35, 47, 59, 63, 66). VEGF plays a role via the activation of endothelial NOS (eNOS: a synonym of NOS3) (24, 47, 63). Therefore, we focused on VEGF-NO signaling and SDF1-CXCR4 signaling to reveal the mechanisms of ECFC functional changes in CDH. There were no significant differences in CB plasma VEGF and SDF1α concentration, in vitro VEGF and SDF1α protein production by ECFCs, and gene expression of ECFCs for VEGF-A, FLT1, KDR, NOS3, SDF1, and CXCR4 between CDH patients and controls. These results suggest that ECFC functional changes in CDH might be caused by mechanisms other than altered exposure to VEGF and SDF1α and alteration of gene expression of VEGF-NO and SDF1-CXCR4 signaling. It would be necessary to investigate not only the other aspects of VEGF-NO or SDF1-CXCR4 signaling but also other signal pathways in CDH ECFCs to elucidate the mechanisms of ECFC functional changes in CDH.

With regard to ECFC capacity for NO production, since NOS3 mRNA expression was unaltered but NO production was increased in CDH ECFCs, we also measured mRNA for NOS1 and NOS2 in ECFCs. There were no significant differences in these gene expressions between ECFCs from both groups. Therefore, the possible mechanisms could be an increase in enzyme activity of NO, substrate, or cofactor levels in CDH ECFCs. As for the blunted increase of NO production in CDH ECFCs in response to VEGF-stimulation, there might be two possibilities: 1) the reactivity to VEGF with increasing NO production was impaired in CDH ECFCs, and 2) there was
no room to increase NO production because NO production had already reached a near-maximum level by such possible mechanisms as described above.

ECFCs are thought to reside throughout the vascular endothelium contributing to vascular integrity and be mobilized into a circulating pool of EPCs and involved in neovascularogenesis (33, 64). A recent study suggested the existence of lung-resident ECFCs and their impaired functions in BPD (3, 4). The depletion and dysfunction of circulating and/or lung-resident ECFCs might be one of the possible mechanisms underlying arrested lung vascular growth in BPD (3, 8, 14, 15), and several study have investigated the possibility of CB ECFCs as a therapeutic option for BPD (3, 11). Also, in adult chronic obstructive pulmonary disease, the number and function of ECFCs from peripheral blood were negatively correlated with the severity of pulmonary hypertension (46). Similarly, in our study, circulating ECFCs were decreased and their cell functions were disrupted in CDH. Although we evaluated only circulating ECFCs but not lung ECFCs, similar underlying mechanisms for decreased lung vascular growth might be involved in lung hypoplasia in CDH. Further studies are needed to establish a link between the depletion and dysfunction of circulating and/or lung-resident ECFCs and the developmental lung disease like BPD in preterm infants and lung hypoplasia in CDH.

Our study has several limitations. In this study, only circulating ECFC functions were examined. Hence, the results may not reflect lung ECFC functions in CDH. Our study excluded CDH patients who underwent FETO. In our hospital, CDH patients who are expected to be the most severe cases, that is classified as liver-up with more than half of the stomach herniated into the right chest in their fetal diagnosis (39), will be candidates for FETO. No patients involved in this study met these criteria. Therefore, some of the results may not apply to the most severe cases of CDH. Furthermore, except for mRNA expression of ECFCs for several angiogenic genes and their exposure to VEGF or SDF1α, the more precise mechanisms for altered ECFC functions in CDH were not investigated. Basically, we used EGM-2 with FBS in ECFC functional assays. As EGM-2 with FBS contains various growth factors (EGF, FGFβ, IGF1, VEGF, hydrocortisone, and so on), experiments that include a period of serum starvation and growth factor omission might be helpful to reveal the important signaling pathways involved in the mechanisms of altered ECFC functions in CDH. Finally, the sample size was small. Therefore, important differences might be missed, for example, altered VEGF exposure as one of the possible mechanisms for ECFC functional change in CDH. However, this is an inherent limitation, as CDH is not a common disease and estimated to occur in ~1 in every 3,500 live births (40). Therefore, further study is necessary to gain a deeper understanding of the roles of ECFCs in lung development in CDH and their potential as a new biomarker and/or therapeutic target for CDH.

In conclusion, our results showed that circulating ECFCs were reduced in CDH and CDH ECFCs had impaired in vitro potential for self-renewal, clonogenicity, proliferation and migration and in vivo capacity for de novo vasculogenesis. Additionally, the capacity of ECFCs for NO production was enhanced while response to VEGF was blunted, indicating that circulating ECFC functions are disrupted in CDH. However, these changes might be caused by mechanisms other than altered exposure to circulating VEGF and SDF1 and alteration of gene expression of VEGF-NO and SDF1-CXCR4 signaling.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


