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Plasma vascular endothelial growth factor A and placental growth factor: novel biomarkers of pulmonary hypertension in congenital diaphragmatic hernia

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1Newborn Intensive Care, Royal Children’s Hospital, Melbourne, Australia; 2Department of Cardiology, Royal Children’s Hospital, Melbourne, Australia; 3Murdoch Childrens Research Institute, Melbourne, Australia; and 4Department of Paediatrics, University of Melbourne, Melbourne, Australia

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Patel N, Moenkemeyer F, Germano S, Cheung MM. Plasma vascular endothelial growth factor A and placental growth factor: novel biomarkers of pulmonary hypertension in congenital diaphragmatic hernia. Am J Physiol Lung Cell Mol Physiol 308: L378–L383, 2015. First published December 5, 2014; doi:10.1152/ajplung.00261.2014.—Pulmonary hypertension (PH) due to abnormal pulmonary vascular development is an important determinant of illness severity in congenital diaphragmatic hernia (CDH). Vascular endothelial growth factor A (VEGFA) and placental growth factor (PLGF) are important mediators of pulmonary vascular development in health and disease. This prospective study investigated the relationship between plasma VEGFA and PLGF and measures of pulmonary artery pressure, oxygenation, and cardiac function in CDH. A cohort of 10 infants with CDH consecutively admitted to a surgical neonatal intensive care unit (NICU) was recruited. Eighty serial plasma samples were obtained and analyzed by multiplex immunoassay to quantify VEGFA and PLGF. Concurrent assessment of pulmonary artery pressure (PAP) and cardiac function were made by echocardiography. Plasma VEGFA was higher and PLGF was lower in CDH compared with existing normative data. Combined plasma VEGFA: PLGF ratio correlated positively with measures of PAP, diastolic ventricular dysfunction, and oxygenation index. Non-survivors had a higher VEGFA:PLGF ratio than survivors at days 3–4 of life and in the second week of life. These findings suggest that increased plasma VEGFA and reduced PLGF correlate with clinical severity of pulmonary vascular disease and may be associated with adverse outcome in CDH. This potential role for combined plasma VEGFA and PLGF in CDH as disease biomarkers, pathogenic mediators, and therapeutic targets merits further investigation.

IN INFANTS AND CHILDREN with congenital diaphragmatic hernia (CDH) abnormal pulmonary vascular development is a key pathological finding and determinant of illness severity (4). Pulmonary vascular density is reduced, vessel walls overly muscularized, and vasoreactivity impaired, leading to marked elevation of pulmonary vascular resistance (PVR) (15). The resultant pulmonary hypertension (PH) and secondary cardiac dysfunction are both strongly associated with acute and long-term morbidity and mortality in CDH (10, 24, 39). Identification of simple, reliable biomarkers of PH and cardiac function in CDH would allow improved early prognostication, monitoring of disease progression, and guide therapies (12).

Vascular endothelial growth factor A (VEGFA) and placental growth factor (PLGF), members of the VEGF family, are mediators of pulmonary angiogenesis in health and pulmonary hypertensive disease and may be important in the pathogenesis of PH in CDH (1, 18).

VEGFA acts via tyrosine kinase receptors (VEGFR1 and VEGFR2) and is essential for normal embryonic vascular development (29). VEGFA expression is altered in rat models of CDH and increased in the lungs of infants with CDH at postmortem examination (6, 34), but plasma levels of VEGFA have not been investigated in CDH.

PLGF is also proangiogenic, acting directly via VEGFR1 on endothelial cells and indirectly on nonvascular cells in many organs, including the lungs (9). Lung PLGF expression is increased in animal models of PH, and in adults with non-CDH PH plasma PLGF correlates with illness severity (32, 35). PLGF has not been previously investigated in CDH.

This study assessed plasma VEGFA and PLGF as potential biomarkers and disease mediators of pulmonary vascular disease in infants with CDH by investigating their relationship with 1) echocardiographic measures of pulmonary artery pressure and cardiac function, and 2) survival.

MATERIALS AND METHODS

Setting and subjects. Infants with CDH were prospectively recruited from consecutive admissions to the Newborn Intensive Care Unit (NICU) at the Royal Children’s Hospital (RCH), Melbourne, Australia, between July 2012 and July 2013. The RCH NICU is a surgical neonatal center receiving referrals of out-born infants with CDH within the state of Victoria. Infants were managed by the attending neonatal team according to a departmental CDH guideline. This included a strategy to minimize lung injury incorporating permissive hypercapnia and early use of high-frequency oscillation and jet ventilation. Additional pulmonary vasodilators [e.g., sildenafil, inhaled nitric oxide (iNO)], cardiotropic agents (e.g., dopamine, dobutamine, milrinone, and norepinephrine), and prostaglandin E1 (to maintain ductal patency) were used based on clinical and echocardiographic assessment of cardiovascular function.

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Surgical repair of the CDH was delayed if necessary to optimize preoperative clinical status.

**Plasma samples for VEGFA and PLGF analysis.** Whole blood samples (0.4 ml) were obtained on days 1 or 2, 3, and 5 of life and every fifth day thereafter until death or discharge from NICU, and within 24 h pre- and post-diaphragmatic hernia repair. After collection samples were immediately centrifuged and plasma was stored at −70°C. Samples were obtained from arterial, capillary, or venous blood as there is good agreement between venous and arterial VEGFA (16).

Blinded post hoc analyses of VEGFA and PLGF concentrations in each plasma sample were performed using a magnetic bead-based quantitative multiplex assay (Human Angiogenesis/Growth Factor panel, HAGPIMAG-12K, Millipore, Billerica, MA), which detects VEGFA165, the commonest plasma isoform of VEGFA, and all PLGF isoforms. Assays were performed using 25-μl samples diluted 1 in 3 according to manufacturer’s instructions. Samples and standards were analyzed using the Luminex 200 instrument platform with Luminex 200 software (xPONENT version 3.1; Luminex, Austin, TX). A five-parameter logistic regression model with weighting was used to create standard curves (pg/ml) and calculate sample concentrations.

**Echocardiographic data.** Echocardiograms were performed immediately after obtaining each whole blood sample. All data were collected by the authors (N.P. and F.M.) using a GE VividI (GE Healthcare) with 6 MHz probe. Blinded analysis of echocardiographic data was performed using Echopac (GE Healthcare).

Pulmonary artery pressure (PAP) was assessed by two techniques. Estimated RV systolic pressure (RVSyste) was calculated using maximum tricuspid regurgitation velocity (TR) when present, as previously described, and assuming a right atrial pressure of 5 mmHg (33).

The ratio of right-to-left:left-to-right flow in the patent arterial duct (PDAAr:L) was also calculated by measurement of velocity time integral (VTI) of Doppler flow in the PDA (21, 26).

Ventricular function was assessed by measurement of pulse wave tissue Doppler imaging (TDI) myocardial velocities in the right ventricle (RV) free wall, interventricular septum (IVS), and left ventricle (LV) free wall, as previously described (25). In each position a systolic (S’) and early diastolic (E’) velocities were measured to quantify systolic contraction and early diastolic relaxation, respectively. Tricuspid valve diastolic inflow Doppler velocities (E and A) were obtained, and E:A ratio (TVE:A) calculated as a measure of diastolic RV function. All echo data were averaged over five consecutive cardiac cycles.

**Physiological, treatment, and outcome data.** Oxygenation index (OI) at the time of blood sampling was calculated where possible (OI = FIO2 × mean airway pressure/PaO2). Timing of surgical CDH repair, combined duration of respiratory support [DRS: ventilation and continuous positive-airway pressure (CPAP)], and length of hospital stay (LOS) were recorded.

**Statistical analysis.** Plasma VEGFA, PLGF, and VEGFA:PLGF ratio were presented as descriptive data summarized as median (range), for paired days in the first week of life and for each week of life during admission. The relationship between plasma VEGFA/PLGF and OI/echocardiographic measures was assessed using a multilevel (mixed effects) model to account for intrasubject correlation. Comparison was made between survivors and nonsurvivors in the first 2 wk of life, using data averaged for each subject, by unpaired t-test. A P value < 0.05 was considered significant. Data analysis was performed using Stata 13 (StataCorp).

**Ethical approval.** This study protocol was submitted to, and approved by, the Research Ethics Committee of the Royal Children’s Hospital, Melbourne. Prospective parental informed consent was obtained in all cases.

**RESULTS**

**Demographic and outcome data.** Eighty plasma samples from 10 infants were obtained for VEGFA/PLGF analysis with paired echocardiographic data. Demographic and outcome data are provided in Table 1. Median gestation was 38.2 (range 37.0–40.0) wk and mean birth weight 3.24 (2.47–3.75) kg. CDH was left-sided in 9 infants and right-sided in 1 infant (Table 1, subject 8).

Six infants survived to NICU discharge (median 24 days, range 16–52 days). Two infants died without surgical repair, one from severe PH (subject 10) at 7 days of age, and one from severe PH combined with respiratory insufficiency at 9 days (subject 8). Two infants died after surgical repair from severe PH and cardiac dysfunction at 160 days (subject 7) and 72 days (subject 9).

Seven infants received high-frequency oscillation ventilation (HFOV), six received iNO, and nine received PGE1. Nonsurvivors received all of these therapies. In each nonsurviving case extracorporeal membrane oxygenation (ECMO) therapy was considered by the treating clinical team. However, in the absence of sufficient reversibility of pulmonary hypertension/respiratory insufficiency to be compatible with survival, ECMO was not felt to be appropriate. Median duration of respiratory support (ventilation and CPAP hours) was 242 (range 73–476) h in the survivors, and 959 (176–3,225) h in the nonsurvivors.

**Plasma VEGFA and PLGF during NICU admission.** Median VEGFA concentration on day 1 of life was 1,767 (range 16–5,363) pg/ml, and when averaged over the first 10 days, allowing comparison with previous studies, was 1,624 (range 458–4,221) pg/ml. PLGF concentration on day 1 was 2.4 (0.9–26.3) pg/ml.

**Correlation with measures of PAP, oxygenation, and cardiac function.** VEGFA demonstrated significant negative correlation with TVE:A and LV E’. Conversely, PLGF demonstrated...
VEGFA and PLGF in survivors and nonsurviving infants. VEGFA and PLGF levels, and VEGFA:PLGF ratio in surviving and nonsurviving infants, are presented in Fig. 1 and Table 3. VEGFA:PLGF ratio was significantly elevated in nonsurvivors compared with survivors at days 3–4. Average weekly VEGFA:PLGF ratio was also elevated in nonsurviving infants, which was statistically significant in weeks 2 and 3 of life.

DISCUSSION

This study observed a pattern of elevated plasma VEGFA and reduced plasma PLGF in infants with CDH, which correlated with measures of pulmonary hypertension, cardiac function, and oxygenation, and distinguished survivors from nonsurvivors in the first weeks of life. While limited by sample size, reflecting the relative rarity of this condition, these novel findings identify a previously unreported potential role for VEGFA and PLGF as mediators of disease pathogenesis, clinical biomarkers, and therapeutic targets in CDH.

Plasma VEGF and PLGF have not to our knowledge been measured in CDH before. In the absence of a control group comparison can be made with existing normative data. Plasma VEGFA in CDH was 10 times higher than previously reported levels in healthy term newborns both on day 1 (cord blood) and in the first 10 days of life (19, 37). Furthermore, plasma PLGF levels on day 1 in the CDH cohort were lower than levels previously reported in cord blood from normal newborns (22, 37). Accepting the limitations of comparing cord plasma and newborn plasma levels, there appeared to be a consistent pattern of elevated VEGFA and reduced PLGF in CDH. In view of this pattern we additionally calculated a combined, previously unreported, VEGFA:PLGF ratio for inclusion in all subsequent analyses.

Table 2. Correlation (r) between VEGFA/PLGF, oxygenation index, and echocardiographic measures of pulmonary artery pressure and cardiac function

<table>
<thead>
<tr>
<th></th>
<th>OI (n = 52)</th>
<th>RVSP_est (n = 71)</th>
<th>TV_e:A (n = 78)</th>
<th>TDI Diastolic Velocities (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA (pg/ml)</td>
<td>1e⁻⁰³</td>
<td>3e⁻⁰³</td>
<td>0.02</td>
<td>RV E &lt;ɪ&gt; İVS E &lt;ɪ&gt; LV E</td>
</tr>
<tr>
<td>PLGF (pg/ml)</td>
<td>-0.29</td>
<td>0.12</td>
<td>-0.87</td>
<td>0.004</td>
</tr>
<tr>
<td>VEGFA:PLGF ratio</td>
<td>0.21</td>
<td>0.03</td>
<td>0.36</td>
<td>0.71</td>
</tr>
</tbody>
</table>

OI, oxygenation index; RVSP_est, RV systolic pressure estimated from maximal tricuspid regurgitation velocity; TV_e:A, tricuspid valve diastolic inflow ratio; TDI, tissue Doppler imaging; PLGF, placental growth factor; IVS, interventricular septum; RV and LV, right and left ventricle, respectively; E’, early diastolic velocity. Values in bold indicate significant correlation, P < 0.05.
Abnormal pulmonary vascular development is a key pathological finding in CDH. Elevated PVR and PAP lead to impaired oxygenation and secondary cardiac dysfunction, notably diastolic dysfunction in the right ventricle (25). Increased PAP and diastolic dysfunction are in turn associated with adverse outcomes in CDH including mortality, supporting their central role in disease pathophysiology and severity (10, 24, 39).

We now observed that worsening pulmonary hypertension and diastolic dysfunction in CDH were also associated with increased plasma VEGF and conversely reduced PLGF. Accordingly, the VEGFA:PLGF ratio demonstrated a consistent and universal positive correlation with severity of pulmonary hypertension, myocardial diastolic function, and oxygenation, assessed by OI.

These findings support a direct relationship between elevated VEGFA, reduced PLGF, and severity of pulmonary vascular disease in CDH. Consistent with this relationship, we observed a pattern of elevated VEGFA and reduced PLGF in those infants with CDH who died as a result of severe intracardiac PH, compared with survivors. This pattern was most obvious in the first weeks of life, which might reflect the importance of the first weeks as a critical period in VEGF-mediated pulmonary vascular development.

Cardiorespiratory therapies and surgery may theoretically have confounded our results by directly or indirectly affecting VEGF and PLGF levels. Unfortunately it was beyond the scope of this study to control for these. Also of note, VEGFA concentration increased dramatically in a subject who had a large left-to-right shunt through a PDA.

Our data add to existing studies investigating the roles of VEGFs in pulmonary hypertensive diseases including CDH, although with variable findings.

In hypoxic rat models of PH, VEGF expression and circulating VEGF are increased (8, 32), whereas in a fetal sheep model of chronic PH VEGF expression was reduced (13). In both rat and sheep models inhibition of VEGFRs leads to severe PH (13, 20, 36).

In human infants with acute persistent pulmonary hypertension of the newborn (PPHN), not due to CDH, plasma VEGF is reduced (19). Conversely, in older children with chronic idiopathic and secondary PH (non-CDH) Duncan et al. (11) observed elevated plasma VEGFA, which correlated with one-year survival, consistent with our observation of increased VEGFA in CDH.

Considering CDH specifically, in the nitrofen rat model of CDH three studies observed reduced VEGF expression in the pulmonary vasculature (6, 7, 28). However, conversely Oue et al. (30) observed increased VEGF protein and mRNA expression in CDH rat lung.

The only prior study of VEGF signaling in human infants with CDH, by Shehata et al. (34), observed increased VEGF immunoreactivity in post-mortem pulmonary vascular endothelium and smooth muscle cells. This finding is consistent with our own, apparently suggesting a role for increased VEGFA in CDH pathogenesis.

PLGF has been less extensively studied in PH disease and not previously in CDH specifically. PLGF expression is increased in hypoxic rat models of PH (23, 32), and in adults with PH due to sickle cell disease and systemic sclerosis (23, 27, 32, 35). In infants with bronchopulmonary dysplasia (BPD), which may be associated with pulmonary hypertension, PLGF levels in cord blood at birth also correlate with later disease severity (38). These findings contrast our observation in PH due to CDH of reduced PLGF levels inversely related to illness severity.

What are the possible explanations for these variable and sometimes contradictory findings? As well as interspecies variation the role of VEGFs may vary with disease mechanism and time course. For example, VEGFs might demonstrate a different profile and role in acute PH states due to impaired pulmonary vasodilatation, such as PPHN, compared with chronic PH states involving structural changes in pulmonary vasculature, such as in severe CDH or idiopathic PH. Another important consideration is that the severity of pulmonary vascular disease, in any disease state, may not be related to absolute levels of a single growth factor alone but instead to disease-specific variations in relative proportions of VEGF isoforms and receptor subtypes (14).

Consistent with this hypothesis, we observed that combined VEGFA:PLGF ratio demonstrated greater correlation with measures of disease severity than absolute levels of VEGFA or PLGF alone. This combined assessment of VEGFA and PLGF has a molecular basis due to known synergism (32); PLGF can displace VEGF from VEGFR1, indirectly activate VEGFR2 by transphosphorylation, and form PLGF/VEGF heterodimers to activate VEGFR1/2 heterodimers (3, 5).

Many additional questions remain unanswered: Are VEGF and PLGF primary disease mediators of PH in CDH or part of a secondary compensatory response, or both? In which tissues are they expressed? Do they have direct effects on the heart and pulmonary vasculature? Are they modulated by exogenous factors, including oxygen and other therapies?

Further investigation is required to answer these questions and to investigate two direct clinical applications. The first is the potential use of plasma VEGFA and PLGF as disease biomarkers in CDH. We have demonstrated the feasibility of serial measurement from small blood volumes in and observed a relationship with hemodynamic status and potentially outcome in CDH. If these findings are reproducible and validated...
laboratory or point-of-care assays can be developed then plasma VEGFA and PLGF may potentially be used to stratify severity, serially assess disease stage, and guide targeted therapy (2, 12). We additionally hypothesize that use of the VEGFA:PLGF ratio could optimize the sensitivity of these biomarkers.

A second potential clinical application is development of new CDH treatments. Morbidity and mortality due to PH in CDH remain significant despite advances in intensive care (17). Anti-VEGF therapies are established in cancers and eye disease to inhibit disease-related angiogenesis (31). Improved understanding of the complexity of VEGFA and PLGF signaling may similarly lead to new VEGF-modulating therapies in CDH (31).

In conclusion, our findings identify a combined relationship between VEGFA and PLGF and illness severity and outcome in PH in CDH. Further studies are now indicated to better understand the role of these factors in CDH pathogenesis, as clinical biomarkers and potentially as new therapeutic targets.

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GRANTS

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: N.P., F.M., and M.M.C. conception and design of research; N.P., F.M., and S.G. performed experiments; N.P., F.M., and S.G. analyzed data; N.P., F.M., S.G., and M.M.C. interpreted results of experiments; N.P. and F.M. prepared figures; N.P. and F.M. drafted manuscript; N.P., F.M., and S.G. laboratory analysis and Dr. E. Aspinall for statistical expertise.

REFERENCES


