Fetal production of growth factors and inflammatory mediators predicts pulmonary hypertension in congenital diaphragmatic hernia

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BACKGROUND: Congenital diaphragmatic hernia (CDH) represents a spectrum of lung hypoplasia, and consequent pulmonary hypertension (PH) is an important cause of postnatal morbidity and mortality. We studied biomarkers at the maternal–fetal interface to understand factors associated with the persistence of PH.

METHODS: Maternal and cord blood samples from fetuses with CDH and unaffected controls were analyzed using a human 39plex immunoassay kit. Cellular trafficking between the mother and the fetus was quantified using quantitative real-time PCR for nonshared alleles. Biomarker profiles were then correlated with CDH severity on the basis of the degree of PH.

RESULTS: Cord blood levels of epidermal growth factor, platelet-derived growth factor, and several inflammatory mediators increased significantly as the severity of CDH increased, whereas maternal levels of growth factors and mediators decreased significantly with CDH severity. Maternal cells were increased in fetuses with severe CDH as compared with controls, with elevated levels of the CXC chemokine ligand-10 in patients with the highest trafficking.

CONCLUSION: Patients with CDH demonstrate proinflammatory and chemotactic signals in fetal blood at the time of birth. Because some of these molecules have been implicated in the development of PH, prenatal strategies targeting specific molecular pathways may be useful adjuncts to current fetal therapies.

Congenital diaphragmatic hernia (CDH) remains one of the most challenging congenital anomalies. Although most CDH patients are now diagnosed before birth, there is a wide spectrum of disease severity, which requires accurate perinatal prognostic indicators for appropriate counseling and management. For patients at the most severe end of the spectrum, *in utero* tracheal occlusion may promote lung growth and improve postnatal outcomes (1–3). However, in spite of sophisticated fetal and neonatal management, severely affected newborns have a high mortality, and those who survive often have long-term morbidities (4).

Liver position and lung-to-head ratio (LHR) (5) are widely used to measure the degree of pulmonary hypoplasia and predict prognosis in fetuses with CDH. The degree of pulmonary hypertension (PH) is associated with poor outcome in CDH (6,7) but has been more challenging to predict prenatally. Measurement of pulmonary artery Doppler indexes (8) and maternal hyperoxygenation testing (9) have had some encouraging results but require considerable expertise. A prenatal, minimally invasive biomarker to predict the degree of PH would greatly benefit patient counseling and management.

In inflammatory disease states associated with PH, such as systemic sclerosis, there is evidence that abnormal levels of growth factors and proinflammatory cytokines lead to vascular remodeling (reviewed in ref. 10). Growth factors signal through tyrosine kinase receptors to induce abnormal proliferation and promote the migration of smooth muscle cells, endothelial cells, and fibroblasts (10). Although the precise mechanisms leading from inflammation to PH continue to be defined, it is likely that vascular injury resulting from ongoing inflammation leads to pulmonary vascular remodeling (11). The possible contribution of similar pathways in the development of PH in patients with CDH has not been explored.

We performed an unbiased analysis of growth factors, cytokines, and chemokines in maternal and cord blood to understand the molecular environment contributing to the persistence of PH in CDH. We also studied the trafficking of cells between the mother and the fetus to determine whether particular cytokines/chemokines secreted in the context of CDH may alter trafficking. We report that infants with CDH with persistence of PH at 2 wk of age have elevated levels of growth factors and proinflammatory cytokines, as well as increased numbers of maternal cells in their cord blood at the time of birth. These findings indicate that molecular signals leading to

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the development of PH are present before birth and suggest that targeted therapies to block inflammation *in utero* may be beneficial in patients with severe CDH.

RESULTS

Patient Demographics

Maternal and cord blood samples were collected from 19 patients with CDH and 23 unaffected controls (**Table 1**). The only significant demographic differences between the groups were that mothers of fetuses with CDH were more likely to be nulliparous than controls ($P = 0.01$) and were more likely to deliver earlier than controls (median gestational age in weeks (interquartile range): CDH: 38.1 (37.1–38.9) vs. control: 39.0 (38.6–40.0), $P < 0.01$).

CDH Clinical Features

Seventeen of 19 hernias were left-sided (**Table 2**). The median LHR was 1.00 (interquartile range: 0.76–1.35) and the median age of LHR measurement was 27 wk (interquartile range: 24 1/7–30 wk). Nine of 14 fetuses with liver herniation into the chest had LHR <1.0. There were no survivors among three patients who received extracorporeal membrane oxygenation support. All six neonatal deaths were within the first week of life. The patient with late demise expired at 3 mo of age secondary to severe PH refractory to medical management.

Three patients with LHR <1.0 and liver herniation into the chest underwent fetal endoscopic tracheal occlusion at 27-wk gestation. Of these, one patient was delivered via cesarean section at 38 wk after the balloon was retrieved by a second fetal intervention at 32 wk. The two other patients required an *ex utero* intrapartum treatment procedure (12) at delivery for balloon retrieval at 29 and 36 wk, respectively.

Severity Classification

We applied the CDH severity classification using the degree of PH measured on echocardiogram performed at 2 wk after birth as described. Under the dichotomous classification, there were six mild and 13 moderate-to-severe patients. Under the three-level classification, there were six mild, three moderate, and 10 severe patients. All patients with prenatal liver herniation and LHR <1 exhibited "severe" PH at 2 wk. We evaluated the relationship of PH to cytokine levels and PCR for microchimerism. Results were similar regardless of which classification scheme was used; two-level results are presented for clarity.

Cytokine Profiles in Cord Blood

We used a panel of 39 cytokines to analyze whether patients with CDH have a different cytokine profile in the cord blood plasma as compared with unaffected controls. Cytokine data were available for 17 CDH patients and 22 controls. We found that patients with CDH overall had elevated levels of epidermal growth factor (EGF), eotaxin, interleukin-3 (IL-3), macrophage inflammatory protein-1β (MIP-1β), platelet-derived growth factor-AA (PDGF-AA), and IL-1α in the cord blood as compared with controls (**Table 3**).

We next performed analyses by CDH disease severity as well as pairwise comparisons. We found that levels of EGF, IL-3, monocyte chemoattractant protein-3 (MCP-3), MIP-1β, PDGF-AA, interferon-α2 (IFN-α2), and IL-1α increased significantly across severity groups (control < mild CDH < moderate-to-severe CDH, $P < 0.05$ by Kendall's Tau-c test) (**Table 3** and **Figure 1a**). Further pairwise comparisons suggested that the most consistent differences were between control and mild CDH as compared with moderate-to-severe CDH (**Table 3**). After the Bonferroni adjustment for multiple comparisons, levels of EGF, MIP-1β, and IFN-α2 remained significantly elevated in moderate-to-severe CDH as compared with controls ($P < 0.017$). Of note, a comparison between controls and patients with mild CDH did not yield any significant differences (**Table 3**), suggesting that the biomarker profiles seen above are unique to patients with more severe CDH, as measured by the persistence of PH or demise.

Table 1. Demographics

	Control ($n = 23$)	CDH ($n = 19$)	*P* value
Maternal age (y)	28 (21–34)	31 (22–35)	0.72
First pregnancy	2 (8.7%)	8 (42.1%)	**0.01**
Labor	14 (60.9%)	14 (73.7%)	0.38
Mode of delivery			
Vaginal	10 (43.5%)	12 (63.2%)	0.20
C-section/ EXIT	13 (56.5%)	7 (36.8%)	0.20
Gestational age (weeks)	39.0 (38.6–40.0)	38.1 (37.1–38.9)	**0.001**
Male fetus	11 (47.8%)	12 (63.2%)	0.32
Fetal weight (g)	3,480 (3,275–3,850)	3,000 (2,000–3,580)[a]	**0.007**

Data presented as median (interquartile range) or *n* (%); significant *P* values are boldfaced.

CDH, congenital diaphragmatic hernia; EXIT, *ex utero* intrapartum treatment procedure.

[a]Weight in many CDH patients was estimated because patients were too unstable to weigh.

Table 2. Patients with congenital diaphragmatic hernia

CDH characteristics ($n = 19$)	Median (IQR) or *n* (%)
Left sided	17 (89.5%)
LHR	1.00 (0.76–1.35)
Liver herniation	14 (73.7%)
Prenatal tracheal occlusion	3 (15.8%)
ECMO	3 (15.8%)
Neonatal demise	6 (31.6%)
Late demise	1 (7.7%)
Repair type ($n = 13$)	
Primary	4 (30.8%)
Muscle flap	5 (38.5%)
Patch	4 (30.8%)

CDH, congenital diaphragmatic hernia; ECMO, extracorporeal membrane oxygenation; IQR, interquartile range; LHR, lung-to-head ratio.

Table 3. Cord blood cytokines—median (IQR)

	Control ($n = 22$)		All CDH ($n = 17$)		Mild CDH ($n = 6$)		Moderate-to-severe CDH ($n = 11$)		Control vs. all CDH[a]	Mild vs. moderate-to-severe[b]	Control vs. moderate-to-severe[b]	Control vs. mild[b]	Tau test[c]	Tau coefficient
Chemokines and growth factors (pg/ml)														
EGF	203	(92.1–373)	500	(228–694)	270	(100–385)	638	(359–717)	**0.008**	0.035	**0.001**	0.614	**0.002**	**0.450**
Eotaxin	64.8	(34.8–96.5)	105	(62.2–120)	114	(75.8–128)	81.5	(55.1–113)	**0.041**	0.547	0.109	0.093	0.074	0.260
FGF-2	113	(82.0–153)	118	(71.9–179)	124	(71.9–135)	91.5	(55.8–194)	0.977	0.920	0.939	0.867	0.999	0.000
Flt-3 ligand	1.6	(1.6–20.5)	1.6	(1.6–15.1)	1.6	(1.6–1.6)	9.0	(1.6–35.1)	0.948	0.129	0.471	0.311	0.640	0.061
Fractalkine	83.8	(57.2–102)	86.1	(65.5–112)	69.5	(42.1–98.6)	105	(83.0–144)	0.650	0.088	0.158	0.240	0.376	0.130
GRO (CXCL-1)	515	(273–682)	351	(220–784)	227	(161–351)	586	(285–1,237)	0.497	0.070	0.516	0.022	0.881	–0.024
IL-3	3.8	(1.6–7.4)	8.1	(5.3–17.2)	11.3	(1.6–16.6)	7.8	(5.3–22.9)	**0.043**	0.880	0.068	0.183	**0.048**	**0.286**
IP-10 (CXCL-10)	184	(133–243)	175	(140–279)	153	(110–279)	175	(140–482)	0.713	0.421	0.401	0.614	0.576	0.083
MCP-1	167	(121–337)	162	(96.8–177)	113	(78.4–162)	165	(128–204)	0.308	0.269	0.789	0.104	0.504	–0.099
MCP-3	10.5	(1.6–16.0)	14.3	(8.0–18.7)	10.0	(6.3–14.3)	16.4	(9.9–30.0)	0.100	0.088	0.026	1.000	**0.042**	**0.296**
MDC	2,361	(2,026–3,047)	2,285	(1,387–3,057)	2,415	(784–2,809)	2,285	(1,387–3,916)	0.910	0.421	0.731	0.780	0.754	0.047
MIP-1α	18.1	(10.6–35.8)	23.4	(17.8–39.7)	22.4	(18.5–33.4)	31.1	(14.7–71.9)	0.380	0.688	0.302	0.823	0.347	0.138
MIP-1β	73.4	(52.7–111)	95.1	(73.0–240)	88.9	(72.7–95.5)	127	(73.0–300)	**0.034**	0.159	**0.016**	0.502	**0.016**	**0.351**
TGF-α	11.2	(5.9–16.0)	10.8	(8.5–14.2)	11.0	(7.7–14.2)	10.8	(8.5–25.6)	0.854	0.763	0.775	0.955	0.806	0.037
VEGF	123	(56.9–295)	161	(75.3–344)	131	(52.6–344)	161	(75.3–378)	0.412	0.615	0.268	1.000	0.361	0.134
PDGF-AA	2,713	(2,013–4,405)	4,565	(3,518–5,562)	4,391	(1,317–5,583)	4,565	(3,518–5,562)	**0.041**	0.688	0.030	0.401	**0.040**	0.300
PDGF-AB/BB	38,789	(25,585–78,740)	56,547	(42,511–66,468)	58,988	(17,736–66,072)	56,547	(42,511–73,913)	0.445	0.547	0.268	0.911	0.376	0.130
RANTES	113,551	(74,513–141,000)	83,936	(36,526–119,603)	72,560	(28,271–124,460)	83,936	(36,526–119,603)	0.126	0.763	0.285	0.146	0.169	–0.201
Inflammatory mediators (pg/ml)														
G-CSF	26.9	(8.1–61.4)	13.0	(6.2–26.4)	8.2	(6.2–9.9)	14.8	(4.8–35.2)	0.148	0.228	0.400	0.104	0.294	–0.154
GM-CSF	22.4	(16.0–33.4)	25.2	(20.6–36.4)	20.7	(18.3–22.6)	26.8	(22.4–62.0)	0.552	0.088	0.252	0.614	0.307	0.150
IFN-α2	57.9	(43.2–73.4)	92.8	(45.6–135)	48.2	(41.2–80.3)	129	(75.4–144)	0.067	0.021	**0.005**	0.614	**0.017**	**0.347**
IFN-ϒ	4.9	(4.4–6.1)	4.5	(3.0–6.0)	3.5	(2.6–5.1)	5.9	(4.1–10.5)	0.590	0.087	0.606	0.068	0.967	–0.008
IL-1α	1.6	(1.6–3.7)	10.0	(1.6–29.5)	2.9	(1.6–18.0)	12.9	(1.6–53.2)	**0.030**	0.466	0.026	0.301	**0.023**	**0.312**
IL-1β	9.3	(1.6–35.1)	15.7	(3.5–49.6)	10.0	(1.6–41.9)	29.7	(3.5–62.8)	0.376	0.419	0.249	0.955	0.290	0.154
IL-1rα	75.8	(34.5–229)	118	(65.8–217)	135	(101–177)	94.7	(61.1–289)	0.412	0.999	0.468	0.576	0.437	0.114
IL-6	1.6	(1.6–8.7)	4.6	(1.6–33.1)	1.6	(1.2–2.0)	22.1	(4.3–61.4)	0.436	0.034	0.074	0.268	0.187	0.189
IL-8	30.3	(7.7–421)	45.7	(21.3–147)	35.6	(21.3–126)	129	(20.8–255)	0.445	0.366	0.303	1.000	0.333	0.142
IL-9	1.6	(1.6–2.8)	5.4	(1.6–14.9)	2.6	(1.3–15.7)	6.9	(1.6–14.9)	0.100	0.578	0.045	0.705	0.082	0.247
IL-10	4.0	(1.6–9.3)	10.2	(1.6–16.8)	2.6	(1.6–16.8)	11.0	(1.6–28.8)	0.502	0.614	0.232	0.671	0.441	0.112
IL-12 (p40)	40.9	(29.3–52.5)	40.5	(16.3–67.5)	33.2	(21.3–99.9)	45.5	(15.4–60.5)	0.887	0.546	0.939	0.867	0.775	–0.043
IL-12 (p70)	3.6	(1.8–5.0)	2.6	(2.3–6.5)	2.4	(2.0–2.5)	3.6	(2.6–10.2)	0.876	0.070	0.411	0.130	0.743	0.049
sIL-2Rα	158	(1.6–269)	211	(84.6–408)	225	(8.5–319)	211	(84.6–505)	0.191	0.393	0.167	0.573	0.139	0.215
TNF-α	14.8	(11.8–16.5)	15.5	(11.0–21.5)	12.9	(9.6–15.0)	18.3	(11.0–25.5)	0.388	0.035	0.061	0.300	0.164	0.203
sCD40L	24,315	(13,530–32,544)	23,502	(11,568–42,598)	17,775	(10,252–23,502)	30,964	(11,568–48,564)	0.821	0.269	0.567	0.198	0.946	0.012

Data not shown for IL-2, IL-4, IL-5, IL-7, IL-13, IL-15, IL-17, and TNF-β. Cytokines with significant *P* values are boldfaced.

CDH, congenital diaphragmatic hernia; EGF, epidermal growth factor; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO (CXCL-1), CXC chemokine ligand-1; IL, interleukin; IP-10 (CXCL-10), CXC chemokine ligand-10; IQR, interquartile range; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated and normal T cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

[a]Wilcoxon's rank-sum test. [b]Wilcoxon's rank-sum test with Bonferroni-adjusted $\alpha = 0.017$. [c]Kendall's tau-c test between control, mild CDH, and moderate-to-severe CDH groups.

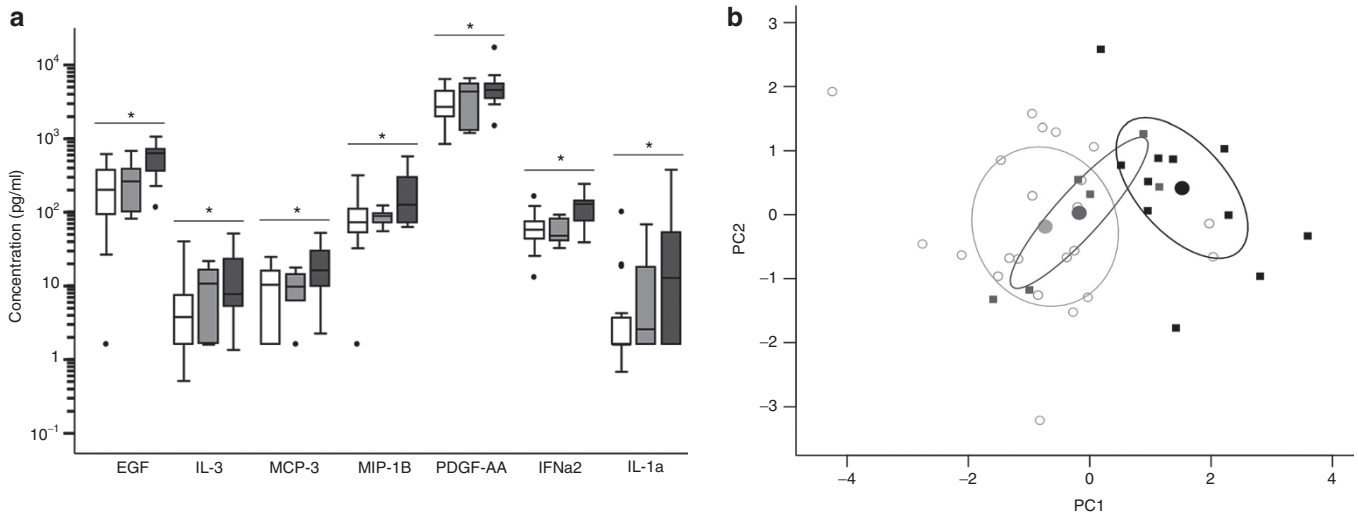

Figure 1. Fetal biomarkers in congenital diaphragmatic hernia (CDH). (**a**) Levels of growth factors, chemokines, and inflammatory mediators in cord blood of unaffected controls (white bars), patients with mild CDH (light gray bars), and patients with moderate-to-severe CDH (dark gray bars). The horizontal line represents the median for each group. *$P < 0.05$ with Kendall's Tau-c test for trend across groups. Control: $n = 22$, mild CDH: $n = 6$, moderate-to-severe CDH: $n = 11$. EGF, epidermal growth factor; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor. (**b**) Supervised principal component (PC) analysis of biomarkers in cord blood. Biomarkers found to be significantly correlated with severity using Kendall's Tau-c test were entered into a supervised PC analysis. PC1 comprised 35.4% of the total variability and PC2 comprised 19.3%. Open circles: control; dark gray squares: mild CDH; black squares: moderate-to-severe CDH. The central point of each cluster is represented by filled circles (light gray: control; dark gray: mild CDH; black: moderate-to-severe CDH).

Cytokine levels were also assessed with supervised principal component analysis, which resulted in the reduction of EGF, IL-3, MCP-3, MIP-1β, PDGF-AA, IFN-α2, and IL-1α into two principal components (PCs) that together described 54.7% of the total variance of the cord blood cytokines. IL-3, MCP-3, MIP-1β, IFN-α2, and IL-1α contributed the most to PC1, whereas EGF and PDGF-AA contributed the most to PC2. The score plot (**Figure 1b**) shows clustering of patients into the three severity groups (control, mild CDH, and moderate-to-severe CDH), with separation of control and moderate-to-severe patients, and with mild CDH found in between the two clusters. Ordinal logistic regression was performed to investigate whether these two principal components were significant predictors of severity. PC1 was significantly associated with higher odds of being classified as more severe (odds ratio = 3.67, 95% confidence interval = 1.74–7.73, $P = 0.001$), whereas PC2 was not (odds ratio = 1.37, 95% confidence interval = 0.67–2.76, $P = 0.381$). Therefore, an increase in biomarkers related to PC1 is associated with a greater likelihood of increased CDH severity.

Cytokine Profiles in Maternal Blood

In maternal plasma, we first compared all controls to all mothers with CDH fetuses and found decreased levels of fibroblast growth factor-2 (FGF-2), macrophage-derived chemokine and vascular endothelial growth factor (VEGF) in mothers carrying fetuses with CDH (**Table 4**). We also found that maternal levels of FGF-2, macrophage-derived chemokine, and VEGF decreased across groups with increasing severity of CDH (control > mild CDH > moderate-to-severe CDH, $P < 0.02$ by Kendall's Tau-c test) (**Table 4** and **Figure 2a**). In pairwise comparisons, FGF-2 was significantly decreased in mothers carrying fetuses with moderate-to-severe CDH as compared with controls ($P = 0.014$).

We performed a supervised principal component analysis for maternal cytokines, which resulted in reduction of FGF-2, macrophage-derived chemokine and VEGF biomarkers into two principal components that explained 90.3% of the total variance. VEGF contributed the most to PC1, whereas FGF-2 and macrophage-derived chemokine contributed the most to PC2. The three severity groups are less differentiated in the maternal score plot (**Figure 2b**); however, with ordinal logistic regression, PC1 was found to be significantly associated with a decrease in the odds of carrying a fetus with a moderate-to-severe CDH (odds ratio = 0.41, 95% confidence interval = 0.22–0.75, $P = 0.001$).

Maternal–Fetal Cellular Trafficking

Given the increased levels of several chemokines in the cord blood of patients with CDH, we next examined whether there was increased trafficking of maternal cells into the fetuses in this setting. PCR testing was performed for 18 controls and 19 patients with CDH. Two patients with CDH and one control had no informative (nonshared) allele for the detection of maternal cells in fetal blood ("maternal microchimerism" (MMc)), and two patients with CDH had no informative allele for the detection of fetal cells in maternal blood ("fetal microchimerism" (FMc)). We first analyzed MMc levels by amplifying for nonshared maternal alleles in fetal blood. This analysis showed that there is a range of trafficking in unaffected term infants, as has been reported (13). Among CDH patients, the median (interquartile range) levels of MMc (expressed as

Table 4. Maternal blood cytokines—median (IQR)

	Control ($n = 22$)		All CDH ($n = 18$)		Mild CDH ($n = 6$)		Moderate-to-severe CDH ($n = 12$)		Control vs. All CDH[a]	Mild vs. moderate-to-severe[b]	Control vs. moderate-to-severe[b]	Control vs. mild[b]	Tau test[c]	Tau coefficient
Growth factors and chemokines (pg/ml)														
EGF	53.3	(19.1–144)	62.3	(24.4–90.6)	62.7	(24.3–89.3)	62.3	(34.7–160)	0.828	0.512	0.665	0.823	0.705	0.056
Eotaxin	63.7	(42.3–83.3)	46.1	(31.5–64.7)	36.5	(31.5–48.9)	50.3	(31.7–68.5)	0.174	0.454	0.331	0.198	0.279	–0.158
FGF-2	87.7	(54.6–139)	42.3	(23.7–94.9)	67.7	(31.4–130)	38.3	(23.2–78.0)	**0.018**	0.454	**0.014**	0.287	**0.014**	**–0.356**
Flt-3 ligand	1.6	(1.6–13.7)	1.6	(1.6–8.8)	1.6	(1.6–8.8)	1.6	(1.6–5.3)	0.247	0.906	0.303	0.745	0.260	–0.143
Fractalkine	87.3	(49.8–111)	65.8	(24.0–86.5)	75.8	(67.3–82.3)	43.1	(19.7–92.8)	0.082	0.349	0.077	0.401	0.055	–0.278
GRO (CXCL-1)	299	(147–844)	302	(173–590)	215	(80.5–453)	350.7	(177–671)	0.664	0.303	0.829	0.218	0.907	–0.019
IL-3	1.6	(1.6–1.6)	1.6	(1.6–3.4)	1.6	(1.6–1.6)	1.6	(1.6–3.8)	0.601	0.780	0.541	0.916	0.572	0.069
IP-10 (CXCL-10)	286	(176–407)	310	(192–501)	170	(142–274)	369.1	(303–512)	0.550	0.061	0.097	0.179	0.279	0.158
MCP-1	122	(86.4–189)	140	(95.8–310)	146	(75.5–310)	133.5	(98.7–284)	0.828	0.851	0.801	0.955	0.886	0.023
MCP-3	11.4	(3.3–15.8)	12.9	(7.9–17.9)	15.1	(7.9–17.9)	12.2	(7.6–20.3)	0.377	0.963	0.449	0.519	0.410	0.120
MDC	834	(567–980)	532	(379–704)	474	(323–568)	534.0	(392–787)	**0.004**	0.512	0.028	**0.014**	**0.011**	**–0.368**
MIP-1α	16.2	(1.6–46.3)	10.5	(1.6–15.0)	7.2	(1.6–13.8)	12.1	(1.6–15.1)	0.124	0.705	0.203	0.234	0.174	–0.195
MIP-1β	42.1	(29.0–96.1)	39.8	(31.2–55.2)	54.3	(25.6–91.8)	36.3	(31.7–46.1)	0.913	0.303	0.614	0.576	0.705	–0.056
TGFα	11.9	(7.0–28.7)	12.4	(9.2–17.5)	10.7	(9.1–12.3)	14.6	(10.4–24.4)	0.724	0.160	0.407	0.576	0.473	0.105
VEGF	60.9	(42.6–86.2)	32.7	(7.6–47.8)	32.0	(10.8–43.5)	32.7	(1.6–75.2)	**0.009**	0.925	0.061	**0.014**	**0.014**	**–0.356**
PDGF-AA	1,931	(1,042–2,784)	1,589	(1,267–2,869)	1,589	(880–2,127)	1,575	(1,313–3,210)	0.910	0.399	0.773	0.454	0.924	0.016
PDGF-AB/BB	36,237	(16,649–78,132)	31,357	(22,955–55,653)	31,357	(16,252–39,709)	32,290	(23,839–60,122)	0.865	0.598	0.885	0.533	0.989	–0.004
RANTES	80,305	(41,946–137,830)	41,484	(37,907–67,696)	38,303	(30,878–38,957)	58,660	(39,695–80,603)	0.113	0.035	0.449	0.029	0.332	–0.142
Inflammatory mediators(pg/ml)														
G-CSF	21.8	(8.1–48.5)	30.7	(1.7–46.6)	21.3	(9.0–69.4)	36.4	(1.6–44.6)	0.765	0.925	0.773	0.867	0.764	–0.045
GM-CSF	33.3	(20.2–51.1)	28.1	(10.7–39.1)	25.4	(20.7–36.0)	28.1	(10.0–48.9)	0.158	0.779	0.280	0.218	0.205	–0.184
IFN-α2	27.1	(21.1–51.4)	34.5	(22.1–41.2)	26.6	(18.9–34.2)	35.9	(27.5–42.2)	0.654	0.190	0.482	0.867	0.433	0.114
IFN-ϒ	6.8	(5.1–13.4)	4.9	(3.0–8.0)	4.4	(3.8–6.0)	5.2	(2.5–8.7)	0.115	0.999	0.207	0.198	0.133	–0.278
IL-1α	3.5	(1.6–17.3)	15.3	(1.6–28.0)	7.0	(1.6–18.5)	17.8	(1.6–31.1)	0.224	0.504	0.187	0.637	0.179	0.188
IL-1β	6.7	(1.6–43.1)	8.6	(1.6–21.6)	8.6	(1.6–25.5)	8.6	(1.6–20.8)	0.580	0.772	0.770	0.495	0.661	–0.064
IL-1rα	47.5	(19.7–185)	118	(15.4–161)	80.5	(14.6–118)	128	(37.1–171)	0.407	0.512	0.358	0.779	0.334	0.141
IL-6	5.1	(1.6–11.0)	9.6	(1.9–26.5)	10.5	(2.0–26.5)	7.3	(1.7–26.9)	0.200	0.888	0.316	0.269	0.235	0.171
IL-8	27.6	(7.5–163)	19.5	(5.1–78.8)	17.9	(8.2–45.7)	23.2	(4.8–90.6)	0.568	0.999	0.540	0.823	0.593	–0.079
IL-9	1.6	(1.6–1.6)	1.6	(1.6–2.4)	1.6	(1.6–1.6)	1.6	(1.6–2.5)	0.581	0.472	0.353	0.744	0.470	0.094
IL-10	3.4	(1.6–9.8)	1.6	(1.6–14.3)	1.6	(1.6–2.3)	6.2	(1.6–18.4)	0.809	0.368	0.679	0.255	0.999	0.002
IL-12 (p40)	14.9	(1.6–37.7)	1.6	(1.6–53.7)	1.6	(1.6–21.3)	21.1	(1.6–57.0)	0.909	0.537	0.654	0.351	0.967	0.008
IL-12 (p70)	4.7	(1.6–13.7)	2.9	(1.6–8.2)	2.9	(2.3–19.8)	3.2	(1.4–7.7)	0.549	0.303	0.330	0.779	0.396	–0.124
sIL-2Rα	1.6	(1.6–1.6)	1.6	(1.6–1.6)	2.7	(1.6–27.5)	1.6	(1.6–1.6)	0.795	0.062	0.266	0.343	0.453	–0.081
TNF-α	7.2	(5.4–11.3)	5.8	(4.6–7.6)	6.3	(4.8–9.1)	5.8	(4.5–7.5)	0.109	0.454	0.105	0.433	0.083	–0.251
sCD40L	12,885	(6,443–27,625)	11,475	(6,747–15,551)	12,222	(8,877–14,548)	10,604	(5,697–18,695)	0.462	0.833	0.540	0.574	0.520	–0.095

Data not shown for IL-2, IL-4, IL-5, IL-7, IL-13, IL-15, IL-17, and TNF-β. Cytokines with significant *P* values are boldfaced.

CDH, congenital diaphragmatic hernia; EGF, epidermal growth factor; FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO (CXCL-1), CXC chemokine ligand-1; IL, interleukin; IP-10 (CXCL-10), CXC chemokine ligand-10; IQR, interquartile range; MCP, monocyte chemoattractant protein; MDC, macrophage-derived chemokine; MIP, macrophage inflammatory protein; PDGF, platelet-derived growth factor; RANTES, regulated and normal T cell expressed and secreted; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

[a]Wilcoxon's rank-sum test. [b]Wilcoxon's rank-sum test with Bonferroni-adjusted $\alpha = 0.017$. [c]Kendall's tau-c test between control, mild CDH, and moderate-to-severe CDH groups.

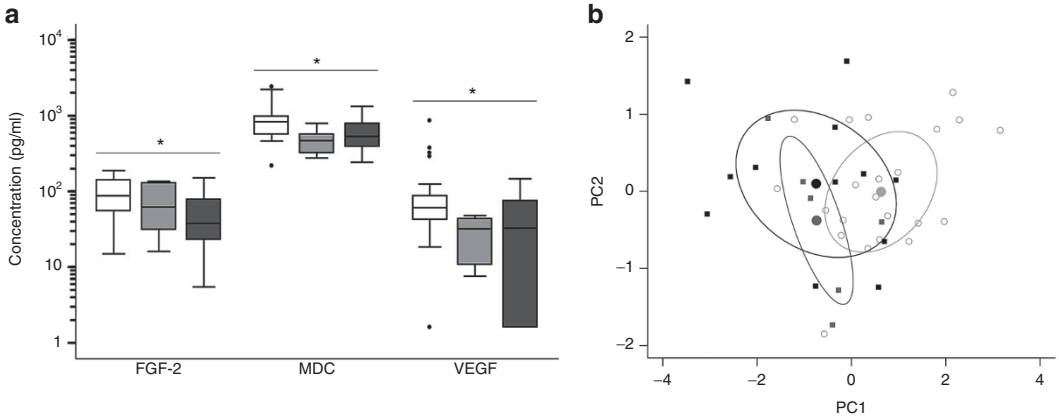

Figure 2. Maternal biomarkers in congenital diaphragmatic hernia (CDH). (**a**) Levels of growth factors and chemokines in maternal blood in unaffected controls (white bars), patients with mild CDH (light gray bars), and patients with moderate-to-severe CDH (dark gray bars). The horizontal line represents the median for each group. *$P < 0.02$ with Kendall's Tau-c test for trend across groups. Control: $n = 22$, mild CDH: $n = 6$, moderate-to-severe CDH: $n = 12$. FGF, fibroblast growth factor; MDC, macrophage-derived chemokine; VEGF, vascular endothelial growth factor. (**b**) Supervised principal component analysis of biomarkers in maternal blood. Biomarkers found to be significantly correlated with severity using Kendall's Tau-c test were entered into a supervised principal component (PC) analysis. PC1 comprised 35.4% of the total variability and PC2 comprised 19.3%. Open circles: control; dark gray squares: mild CDH; black squares: moderate-to-severe CDH. The central point of each cluster is represented by filled circles (light gray: control; dark gray: mild CDH; black: moderate-to-severe CDH).

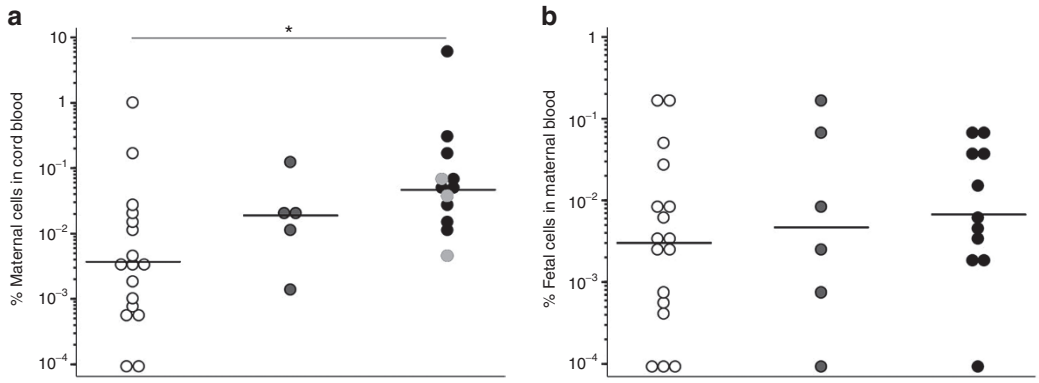

Figure 3. Maternal–fetal cellular trafficking. Percentage of (**a**) maternal cells in cord blood and of (**b**) fetal cells in maternal blood in unaffected controls (white circles) and patients with congenital diaphragmatic hernia (CDH) stratified by disease severity (mild CDH: middle column, gray circles; moderate-to-severe CDH: third column, black circles). The horizontal line represents the median for each group. Samples with no microchimerism detected were graphed as 10^{-4} (the lower limit of detection) to allow visualization on the log scale. Control $n = 17$; mild CDH $n = 5$ in **a**, $n = 6$ in **b**; moderate-to-severe CDH $n = 12$ in **a**, $n = 11$ in **b**. *$P < 0.002$ with Kendall's Tau-c for trend across groups; $P < 0.003$ between control and moderate-to-severe CDH by Wilcoxon's rank-sum. Light gray circles in the moderate-to-severe group in **a** represent patients who underwent tracheal occlusion.

the percentage of maternal cells in cord blood) were significantly higher than in controls (control: 0.004 (0.0008–0.015) vs. CDH: 0.037 (0.013–0.061), $P < 0.004$ by Wilcoxon's rank-sum). The levels of MMc tended to increase across severity groups (Kendall's Tau-c coefficient: 0.506, $P < 0.002$) and were significantly higher in the moderate-to-severe group as compared with controls, whereas there was no difference between mild CDH and controls (Figure 3a; moderate-to-severe CDH: 0.047 (0.020–0.102), mild CDH: 0.019 (0.011–0.02); $P < 0.003$ between moderate-to-severe CDH and control; $P = 0.2$ between mild CDH and control by Wilcoxon's rank-sum). These findings are consistent with the cytokine analysis results in which the main differences were those between controls and patients with moderate-to-severe CDH and not between controls and patients with mild CDH. The levels of MMc in the three patients who underwent fetal tracheal occlusion were similar to that seen in the other patients with CDH (0.004, 0.037, 0.06; depicted as light gray circles in Figure 3a).

We next quantified levels of FMc by amplifying for non-shared fetal alleles in maternal blood. There were no significant differences between median FMc levels between patients with CDH and unaffected controls (control: 0.003 (0.0006–0.0091) vs. CDH: 0.007 (0.002–0.038), $P = 0.36$ by Wilcoxon's rank-sum). No significant differences were seen when patients with CDH were stratified into severity groups (Figure 3b; mild CDH: 0.006 (0.0007–0.077), moderate-to-severe CDH: 0.0067 (0.002–0.038), P = not significant with Kendall's Tau-c test).

We next asked whether patients with the highest levels of MMc expressed any cytokines or chemokines that may be implicated in the trafficking of maternal cells across the placenta. We compared the cytokine profiles from cord blood of CDH patients with high levels of trafficking (MMc level >80th

percentile, $n = 4$) to those with lower levels of trafficking (MMc level ≤80th percentile, $n = 13$). Consistent with our hypothesis, patients with high MMc had significantly elevated levels of CXC chemokine ligand-10 as compared with those with lower trafficking (726.6 (263.3–1468.8) vs. 173.4 (129.0–271.6), $P = 0.027$).

DISCUSSION

This is the first study to directly examine the fetal environment for the presence of biomarkers that may correlate with the persistence of PH in CDH. We found abnormal levels of several growth factors and cytokines that have been implicated in the development of PH in other diseases, suggesting that the fetal milieu contains critical molecular signals that lead to vascular changes resulting in PH, even before respiratory effort has begun. Therefore, prenatal therapies to block key events in these molecular pathways may be beneficial in fetuses with CDH.

Our study suggests similarities between the pathophysiology of PH in CDH and other diseases. For example, growth factors such as EGF and PDGF have been implicated in the development of PH in adults (10) and were increased in cord blood of patients with CDH. Although our study does not establish a causal role for these factors in the onset of PH in CDH, it is interesting to note that blockade of EGF and PDGF has been successful in animal models of PH. For example, EGF blockade may be a useful strategy to treat monocrotaline-induced PH (14). PDGF blockade has also proven beneficial in animal models (15), and may be used clinically in select patients (16).

Inflammatory cytokines implicated in the development of PH in adults are also increased in fetuses with CDH. For example, IFN-α, given to treat hepatitis C, reportedly causes irreversible PH in some patients (17), and was increased in our cohort of patients with moderate-to-severe CDH. Increased levels of IL-1, IL-6, and tumor necrosis factor-α have been described in patients with primary PH (18,19), and our study indicated some increases in these cytokines with severe CDH.

Conversely, several cytokines and/or chemokines described in the pathogenesis of PH in other disease settings, such as fractalkine (20), were not increased in our CDH patients. In addition, we did not find elevated levels of anti-inflammatory cytokines such as IL-10 in patients with CDH, as has been reported in adults with idiopathic PH (19). This discrepancy may be because our assay could not detect small changes in IL-10 because the baseline levels are low, or because of the inability of the fetus to compensate, unlike adults with PH.

We also analyzed maternal plasma in an effort to define a biomarker that may be followed noninvasively and found significantly decreased levels of FGF-2 in mothers of fetuses with moderate-to-severe CDH. FGF-2 has been implicated in the pathogenesis of PH, and its blockade may ameliorate disease in an experimental model (21); we speculate that our findings may indicate a compensatory mechanism.

A recent study examined the levels of various cytokines in the blood of neonates with CDH, drawn shortly after birth and longitudinally over 4 d (22). The authors detected elevated levels of several proinflammatory cytokines in infants with CDH as compared with unaffected controls, although blood from patients with CDH was drawn after the initial resuscitation, whereas control blood was obtained from the umbilical cord. The authors also compared patients who did or did not require extracorporeal membrane oxygenation and found that levels of IL-8, IL-10, and MIP-1α were increased in neonates with more severe CDH. Our study adds to these observations by directly comparing both maternal and cord blood levels before any respiratory effort or mechanical ventilation, and by including other analytes known to be involved in PH, such as EGF, PDGF, and FGF. In addition, we have directly correlated cytokine levels with the degree of PH measured on echocardiogram to show that the differences between controls and patients with CDH are most pronounced in patients with moderate-to-severe PH and that patients with mild CDH exhibit little inflammation. Cellular trafficking between the mother and the fetus has been described in normal pregnancies (23,24), and may be a mechanism for the induction of maternal–fetal tolerance (25). The observation that patients with more severe CDH have higher levels of MMc as compared with controls is intriguing and suggests that molecular signals present in some CDH patients may lead to the recruitment of maternal cells or to the proliferation of maternal cells that have already crossed into the fetus. The finding of increased levels of CXC chemokine ligand-10 in patients with the highest levels of trafficking supports this hypothesis. It has been reported that endothelial progenitor cells are recruited from the bone marrow to the pulmonary vasculature in animal models of PH (26), and similar signals may lead to the recruitment of maternal cells in patients with CDH. Of note, we did not find elevated MMc after fetal intervention, contrary to what we have seen in our mouse model of fetal intervention (27) or after open fetal surgery for spina bifida (28). It is possible that the minimally invasive nature of the current approach to tracheal occlusion does not lead to increased trafficking, or signals leading to increased trafficking are different between patients with spina bifida and those with CDH.

The main strengths of our analysis are the study of both maternal and cord blood in patients with CDH and our unbiased approach of testing for multiple cytokines without predicting which ones may be changed on the basis of published data. We are also aware of several weaknesses in our study. The mean gestational age in patients with CDH was lower than that for our controls, which may be one confounding factor in our analysis. The ideal control group would be age-matched unaffected controls, but patients born at 36 wk usually have other abnormalities prompting delivery, such as infection or maternal comorbidity, and therefore would not be an appropriate comparison group. In addition, our hospital referral patterns led to a CDH cohort with fewer patients with mild CDH. Finally, longitudinal assessments of biomarkers in maternal blood or amniotic fluid may lead to the identification of prognostic factors before 24 wk, when such information may inform the course of prenatal care, and such a study may be designed in the future.

Tracheal occlusion is currently the mainstay of fetal intervention, but is likely to be more effective for treating pulmonary hypoplasia than for treating PH (29). Our finding that prenatal inflammatory signals correlate with the severity of postnatal PH suggests that prenatal strategies to block molecular pathways may be beneficial in severe CDH, similar to the targeted therapies currently being developed for PH in other settings (30). Animal studies of prenatal medical therapies to address PH in CDH have shown encouraging results. For example, prenatal steroids improved pulmonary vascular remodeling in the lamb model of CDH (31), and antenatal sildenafil had beneficial effects on lung microvascular development in a rat model of CDH (32). Our results, in conjunction with other reports describing increased EGF in patients with CDH (33), point to EGF inhibition as another potential therapeutic target. PDGF was recently reported to reduce pulmonary vascular remodeling in a rat model of CDH (34) and was used clinically in one patient with CDH (35). However, potential toxicities such as impairment in alveolar development owing to inhibition of PDGF receptor signaling must be considered.

This is the first study to demonstrate an association between maternal and cord blood biomarker profiles and the persistence of PH in CDH. Our results lend insight into the fetal onset of PH and suggest that prenatal therapies to block particular molecular pathways may be useful in prenatally diagnosed CDH.

METHODS

Subjects

Mothers carrying unaffected fetuses or those with CDH were prospectively enrolled (August 2009–June 2011). Inclusion and exclusion criteria for all healthy control patients were term pregnancies without preterm labor, preeclampsia, or fetal congenital anomalies. All consenting patients with fetal CDH were prospectively enrolled. Matched maternal and cord blood samples were obtained at birth and only patients with a matched pair of samples were included in the study. Written informed consent was obtained under University of California, San Francisco Institutional Review Board approval (10-00350). All patients with CDH were evaluated by fetal ultrasound and echocardiogram. Fetal tracheal occlusion was performed in three patients with severe CDH (liver herniated into the thorax and LHR <1.0). All neonates with CDH were managed as previously described (7). Surgical repair was performed after the patient was stabilized and repair type was dependent on the size of the diaphragm defect and surgeon preference.

Postnatal Classification of Severity in Infants With CDH

We have previously shown that the severity of PH at 2 wk of age (on the basis of an estimate of pulmonary artery pressure (P_{PA}) from the echocardiogram) is associated with poor neonatal outcome (7). A single cardiologist (A.J.M.-G.), blinded to patient condition, reviewed echocardiograms performed at 2 wk after birth and classified P_{PA} relative to systemic blood pressure (7). In a three-level classification system, infants were classified as "mild CDH" if there was no or mild PH (</3 systemic pressure), "moderate CDH" if there was moderate PH (≥2/3 systemic pressure) and "severe CDH" if there was severe PH (systemic-to-suprasystemic pressure) or demise before 2 wk. Alternatively, in a dichotomous classification system, infants were classified as "mild CDH" vs. "moderate-to-severe CDH" (≥2/3 systemic pressure or demise).

Sample Processing

Cord blood samples were collected at the time of birth and maternal blood was collected within 24 h of delivery and processed within 36 h. Whole blood and plasma were stored separately for the analysis of cellular trafficking and cytokines, respectively. Blood obtained from patients delivering in Detroit (11/23 controls) was shipped on ice on the day of delivery by overnight mail and processed immediately upon arrival, such that the timing and method of processing of all samples was identical.

Cytokine Assay

Cytokine profiles in the maternal and cord blood plasma samples were assayed using the standard-sensitivity Millipex Map kit (Millipore, Billerica, MA) as previously reported (36). Samples were acquired and analyzed on a Labscan 100 analyzer (Luminex, Austin, TX) using Bio-Plex manager 6.0 software (Bio-Rad, Hercules, CA). Standard curves were run in duplicate wells and each run included internal controls. Cytokines were excluded from the tables and figures if their levels were below 10 pg/ml for at least 80% of both maternal and cord blood samples.

Quantitative PCR

MMc and FMc were quantified from whole blood by researchers blinded to patient groups using a quantitative reverse transcription-PCR assay to amplify nonshared human leukocyte antigen-DR or insertion–deletion alleles between the mother and the fetus (37). This assay has a lower limit of detection between 0.001 and 0.0001% (37) and has been used previously to quantify maternal blood in fetal samples (25,28). Microchimerism levels in two CDH patients who underwent fetal intervention and seven controls have been reported as control data in the context of an analysis of the effects of open fetal surgery on trafficking (28).

Statistical Analysis

Wilcoxon's rank-sum test with Bonferroni-adjusted P values for multiple comparisons was used where appropriate. χ^2 tests were used to assess the differences in proportions between groups. Kendall's Tau-c statistic was calculated to test the magnitude and direction of increases or decreases in cytokine levels or microchimerism across groups. Cytokine levels were also assessed by supervised principal component analysis, which uses only a subset of cytokines most associated with the severity of outcome ($P < 0.05$ by Kendall's Tau-c) for reducing the dimensionality of the data. The principal components identified were then entered as predictors of severity in an ordinal logistic regression model. A P value <0.05 was considered statistically significant. All statistical analyses were performed using Stata version 12 (Stata, College Station, TX) or R software (Vienna, Austria).

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