0031-3998/11/6901-0068
PEDIATRIC RESEARCH

Vol. 69, No. 1, 2011
Printed in U.S.A.

Relationship Between Neonatal Blood Protein Concentrations and Placenta Histologic Characteristics in Extremely Low GA Newborns

JONATHAN L. HECHT, RAINA N. FICHOROVA, VANESSA F. TANG, ELIZABETH N. ALLRED, THOMAS F. MCELRATH, AND ALAN LEVITON; FOR THE ELGAN STUDY INVESTIGATORS

Department of Pathology [J.L.H.], Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215; Department of Obstetrics, Gynecology and Reproductive Biology, [R.N.F., V.F.T., T.F.M.] Brigham and Women's Hospital, Boston, Massachusetts 02115; Neuroepidemiology Unit [E.N.A., A.L.] Children's Hospital Boston, Boston, Massachusetts 02215

ABSTRACT: Amniotic fluid infection with chorioamnionitis is associated with increased risks of morbidity and mortality in children born prematurely. These risks depend on the presence of a fetal inflammatory response. We measured the concentrations of 25 proteins in the blood of 871 infants born before the 28th wk of gestation and examined their placentas for acute inflammation. Newborns who had inflammatory lesions of the placenta were much more likely than their peers ($p < 0.01$) to have elevated blood concentrations of cytokines (IL-1β, IL-6, and TNF-α), chemokines (IL-8, MIP-1β, RANTES, and I-TAC), adhesion molecules (ICAM-1, ICAM-3, and E-selectin), matrix metalloproteinases (MMP-1 and MMP-9), the angiogenic inflammatory factor VEGF and its receptor VEGF-R2, and acute phase proteins (SAA and CRP) during the first 3 d after birth. In contrast, newborns with poor placental perfusion had lower levels of inflammatory proteins ($p < 0.01$; IL-6, RANTES, ICAM-1, ICAM-3, VCAM-1, E-selectin, MMP-1, MMP-9, MPO, and VEGF). An inverse pattern was found between newborn levels of VEGF and its competitive inhibitor VEGF-R1 in both the inflamed and poorly perfused placenta categories. These results confirm the predictive value of placental histology for the presence or absence of elevated inflammatory response in newborns. (***Pediatr Res* 69: 68–73, 2011**)

Amniotic fluid infection with chorioamnionitis is associated with morbidity and mortality in children born prematurely (1,2). The risk of injury is associated with the presence of a fetal inflammatory response (3). We have previously characterized the placentas from a cohort of babies born before 28 wk gestation and shown associations between histologic inflammation and recovery of specific microorganisms (4,5), pregnancy disorders leading to preterm delivery (5), and ultrasound lesions of the brain and cerebral palsy diagnoses (6).

Pregnancy disorders have been divided into those associated with inflammation and those not associated, largely based on histology signs of inflammation in the placenta (5). This study assesses a broad range of inflammatory markers (25 in total: cytokines, chemokines, adhesion molecules, tissue remodeling factors, and acute phase proteins) in the blood of a large cohort of extremely low GA newborns (ELGANs) during the first 3 d of life to establish the significance of associations with placental inflammatory lesions that may serve as a basis for prevention and improved management of inflammation-related morbidity in preterm infants.

Received May 13, 2010; accepted August 23, 2010.

Correspondence: Jonathan L. Hecht, M.D., Ph.D., Department of Pathology, 330 Brookline Avenue, Boston, MA 02215; e-mail: jlhecht@caregroup.harvard.edu

Supported by a cooperative agreement with the National Institute of Neurological Diseases and Stroke Grant 5U01NS040069-05 and a center grant award from the National Institute of Child Health and Human Development (5P30HD018655-28).

METHODS

Population and sample collection. Placentas and blood samples were collected as part of a study designed to identify factors that increase risk for structural and functional neurologic disorders in ELGANs. The details of the population and placental collection are described elsewhere (4). Women delivering before 28 wk gestation at one of 14 participating institutions were asked to enroll in the study. The enrollment period covered years 2002–2004; 1250 mothers of 1506 infants consented (an estimated 260 mothers were missed or declined to participate); 1411 placentas were submitted for pathologic evaluation (totals refer to the number of umbilical cords; *i.e.* twins are counted as two placentas); and 871 newborns had both blood samples and placentas available for analysis. The study was conducted in accordance with human subject research guidelines and the Declaration of Helsinki and was approved by Institutional Review Boards at each participating institution.

Drops of blood were collected on Schleicher & Schuell 903 paper (Whatman International Ltd, Florham Park, NJ) on the first postnatal days (range, 1–3 d), allowed to air dry, and stored at −70°C in sealed bags with desiccators until processed. All blood was from the remainder after specimens were obtained for clinical indications.

Placental examination. The procedures for gross and microscopic examination of the placentas as well as the training and interpathologist validation have been previously described (4).

Placental signs of poor perfusion including infarcts and intervillous fibrin, fetal stem vessel thrombosis, and decidual hemorrhage and fibrin deposition were coded as present or absent. Chorionic villi were scored for subjective increase in syncytial knots; few knots are expected before the third trimester.

Inflammation of the membranes was described in detail. At the chorionic plate of the disc, acute inflammation was assigned a stage from 0 to 3 (0 is none, 1 is neutrophils collecting in subchorionic space, 2 is neutrophils into chorionic plate, and 3 is neutrophils up to amnionic epithelium). The grade of inflammation at the plate ranged from 1 to 3 (1 is 1–9 neutrophils, 2 is 10–19 neutrophils, and 3 is >20 neutrophils: all recorded at 20×). Inflammation of the free membranes (chorion/decidua) was graded from 0 to 4 (0 is none, 1 is single focus of 5–10 neutrophils, 2 is several small foci or single focus of >10 neutrophils, 3 is numerous large or confluent foci, and 4 is necrotizing).

The fetal inflammatory response was gauged by inflammation in the umbilical cord, which was graded from 0 to 5 (0 is none, 1 is neutrophils within the inner third of one umbilical vessel, 2 is neutrophils within the inner third of at least 2 umbilical vessels or through the wall of one vessel, 3 is neutrophils in perivascular Wharton's jelly, 4 is inflammation extending deep into Wharton's jelly, and 5 is "Halo lesion"; ring of precipitate in Wharton's jelly encircling each vessel). Neutrophilic and eosinophilic infiltration into fetal stem vessels in the chorionic plate was also noted as present or absent.

Blood spot elution and protein analysis. All blood spots were from drops of blood that remained in the needle or syringe after specimens were obtained for clinical indications. The earliest specimen was the one analyzed. Sixty-seven percent was obtained on d 1, 33% obtained on d 2, and 0.23% on d 3. Dried blood spots were stored at −70°C in sealed bags until processed. All blood spots were processed for elution and protein analysis in a central laboratory (Laboratory of Genital Tract Biology, Brigham and Women's Hospital) using standardized operational procedures as described below. For protein elution, the frozen dried blood spots (DBS) were punched using

12-mm disposable biopsy AcuPunch (Acuderm, Inc., Fort Lauderdale, FL). The punched paper specimen was submerged in 300-μL PBS-based buffer containing 0.1% Triton $\times$100 (Sigma Chemical Co.-Aldrich, St. Louis, MO) and 0.03% Tween-20 (Fisher, Hampton, NH) vortexes for 30 s and incubated on a shaker for 1 h at 4°C. The punched paper along with the buffer was then transferred over the filter of a SpinX tube (Corning Fisher), centrifuged at 2000 $\times$ g followed by collection of the filtered eluted blood. An additional wash of the paper punch was performed in 100 μL for a final elution volume of 400 μL. The eluted blood samples were aliquoted and stored frozen at −70°C in barcoded air-tight microtubes (USA Scientific, Orlando, FL).

Proteins were measured in duplicate using the meso scale discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD). This electrochemiluminescence (ECL) detection system has been validated by comparisons with traditional ELISA (7,8). The ECL multiplex assays, measuring up to 10 proteins simultaneously, were optimized to allow detection of each biomarker within the linearity concentration range of the eluted samples. The MSD Discovery Workbench Software was used to convert relative luminescent units into protein concentrations using interpolation from several log calibrator curves. Split quality control blood pools tested on each plate showed interassay variation of <10–20% for each protein. The total protein concentration in each eluted sample was determined by BCA assay (Thermo Scientific, Rockford, IL) using a multilabel Victor 2 counter (Perkin Elmer, Boston, MA), and the measurements of each inflammatory marker were normalized to milligrams total protein.

We measured the following 25 proteins: IL-1β, IL-6, IL-6 receptor (IL-6R), TNF-α, TNF receptor-1 (TNF-R1), TNF-R2, IL-8 (CXCL8), monocyte chemotactic protein-1 (MCP-1; CCL2), MCP-4 (CCL13), macrophage inflammatory protein-1β (MIP-1β; CCL4), regulated upon activation, normal t cell expressed, and [presumably] secreted (RANTES; CCL5), IFN-inducible T cell alpha-chemoattractant (I-TAC; CXCL11), intercellular adhesion molecule-1 (ICAM-1; CD54), ICAM-3 (CD50), vascular cell adhesion molecule-1 (VCAM-1; CD106), E-selectin (CD62E), matrix metalloproteinase-1 (MMP-1), MMP-9, C-reactive protein (CRP), serum amyloid A (SAA), myeloperoxidase (MPO), VEGF, VEGF receptor-1(VEGF-R1; Flt-1), VEGF-R2 (KDR), and insulin growth factor binding protein-1 (IGFBP-1). This choice of proteins provided a broad and redundant representation of all categories of inflammatory mediators, *e.g.* cytokines and their receptors, chemokines, adhesion molecules, tissue remodeling factors, and acute phase proteins (Table 1), whereas the numbers of biomarkers in each group was determined by reliable simultaneously detection in three platforms of similar dynamic ranges.

Data analysis. We evaluated the generalized null hypothesis that the risk of a blood protein concentration in the highest quartile for GA was not associated with any histologic characteristic of the placentas.

The protein concentrations varied with GA at delivery, and therefore for the purposes of statistical analysis, the samples were divided into three groups defined by GA (23–24, 25–26, and 27 wk). We dichotomized the distribution of each protein's concentration into the highest quartile and the lower three quartiles based on the rationale that the most extreme levels of inflammatory mediators would be most biologically relevant. To control the variation of protein concentrations with GA at delivery, we dichotomized each protein's concentration separately in each of the three GA groups (23–24, 25–26, and 27 wk).

Our unit of measurement is the OR (and 99% CI) that newborns whose placenta had each histologic finding were more or less likely to have a protein measurement in the top quartile compared with newborns without that placental characteristic. We selected the 99% CI rather than the conventional 95% CI because we wanted to modify our analyses for multiple comparisons (25 proteins and 7 histologic characteristics), while not appreciably increasing the risk of a type 1 (false negative) error. Consequently, only $p < 0.01$ was considered statistically significant (9).

The ORs and 99% CIs for Tables 2 and 3 were calculated with logistic regression equations and for Table 4 were calculated with multinomial (also known as polytomous or polychotomous) logistic regression models. For each placental variable, we report the OR of having the concentration of each blood protein in the highest quartile for GA after adjustment for GA.

Table 1. *Percent of children classified by GA characteristics*

Characteristic	GA (wk)		
	23–24	25–26	27
Birth weight (g)			
500–749	88	32	13
Birth weight *Z*-score			
<2	2	8	4
Number of fetuses			
One (singleton)	68	70	60
Antenatal steroids			
Full course	61	63	71
Indication for delivery			
Labor	51	44	43
Membrane rupture	19	22	22
Cervical insufficiency	5	12	18
Placenta abruption	15	13	9
Preeclampsia	9	5	3
Fetal indication	1	4	6
Placenta bacteriology			
Any organism	68	46	40
Inflammation of chorionic plate			
Stage 1	10	11	10
Stage 2	12	13	8
Stage 3	42	28	20
Inflammation of umbilical cord			
Grade 1–2	10	14	12
Grade 3	12	9	6
Grade 4–5	6	12	7
Inflammation of fetal stem vessels			
Present	31	29	20
Infarct			
Present	13	17	20
Syncytial knots			
Increased	15	17	28
Column N	169	367	262

Values are represented in percent.

RESULTS

Incidence of chorionic and umbilical cord inflammation* versus *poor perfusion of ELGAN placentas. We observed intense inflammation of the chorionic plate in 18% of placentas, of the cord in 15%, and of the chorionic plate vessels in 23%. Inflammation of the chorionic plate, membranes, and umbilical cord tended to occur preferentially in the presence of each other. Infarct and increased syncytial knots also tended to occur with one another, but these two groups of lesions occurred in the same placenta only about 10% of the time. Morphologic features associated with poor uteroplacental perfusion including infarcts, increased syncytial knots, and decidual hemorrhage suggesting abruption were seen in 15, 18, and 16% of placentas, respectively. The frequency of perfusion-related lesions was inversely related to those associated with inflammation. Younger GA favored inflammation (Table 1), especially when the inflammation was intense. A subset of 10 placentas showed isolated umbilical cord inflammation. We identified cord inflammation in 3% of placentas that had no chorionic inflammation and in 8% that did not have chorionic plate vasculitis (10).

Association between inflammatory biomarkers in ELGAN blood and placental inflammation. Although the incidence of inflammation in the placenta declined with increasing GA (23–24 wk: 85/170 = 50%; 25–26 wk: 161/367 = 44%; and 27 wk: 74/262 = 28%), the newborn protein concentrations associated with placental inflammation declined minimally with age at birth. A majority of the inflammation-related proteins adjusted by GA were elevated in newborns whose placenta had moderate to severe inflammation (Tables 2 and 3). Among these proteins were cytokines (IL-1β, IL-6, and TNF-α), cytokine receptors (IL-6R, TNF-R1, and TNF-R2),

Table 2. *ORs (99% CIs) of a concentration in the top quartile (for GA) of the protein among children whose placenta had the stage of chorionic plate inflammation compared to the infants whose placenta had no chorionic plate inflammation*

	Acute inflammation of the chorionic plate		
Protein	Stage 1	Stage 2	Stage 3
Cytokines and their receptors			
IL-1β	1.4 (0.7, 2.9)	2.2 (1.1, 4.3)*	4.4 (2.7, 7.1)*
IL-6	2.2 (1.1, 4.5)*	3.1 (1.6, 6.1)*	2.8 (1.7, 4.6)*
IL-6R	1.1 (0.5, 2.4)	1.5 (0.7, 3.1)	3.4 (2.1, 5.7)*
TNF-α	1.4 (0.7, 2.9)	1.5 (0.7, 3.0)	3.6 (2.2, 5.9)*
TNF-R1	1.3 (0.6, 2.8)	1.5 (0.7, 3.1)	3.6 (2.2, 6.0)*
TNF-R2	1.3 (0.6, 3.0)	2.4 (1.2, 5.0)*	5.9 (3.5, 9.9)*
Chemokines			
IL-8 (CXCL8)	1.2 (0.6, 2.6)	1.8 (0.9, 3.6)	3.1 (1.9, 5.1)*
MCP-1 (CCL2)	0.9 (0.4, 1.9)	0.9 (0.4, 1.8)	0.8 (0.5, 1.4)
MCP-4 (CCL13)	1.0 (0.5, 1.9)	0.7 (0.4, 1.5)	0.9 (0.5, 1.4)
MIP-1β (CCL4)	1.5 (0.7, 3.2)	2.3 (1.2, 4.6)*	3.8 (2.3, 6.3)*
RANTES (CCL5)	3.4 (1.7, 6.6)*	2.5 (1.1, 4.5)*	2.3 (1.4, 3.9)*
I-TAC (CXCL11)	1.9 (0.9, 4.0)	2.1 (1.04, 4.4)*	5.7 (3.4, 9.5)*
Adhesion molecules			
ICAM-1 (CD54)	1.4 (0.6, 3.1)	3.0 (1.5, 6.1)*	4.9 (2.9, 8.2)*
ICAM-3 (CD50)	2.5 (1.2, 5.2)*	2.7 (1.3, 5.6)*	6.2 (3.7, 11)*
VCAM-1 (CD106)	1.4 (0.7, 2.8)	1.3 (0.6, 2.6)	1.8 (1.1, 2.9)*
E-selectin (CD62E)	2.7 (1.2, 5.9)*	4.2 (2.1, 8.7)*	10 (5.8, 18)*
MMPs			
MMP-1	0.8 (0.4, 1.8)	2.0 (1.03, 3.9)*	2.2 (1.3, 3.5)*
MMP-9	2.4 (1.1, 5.2)*	2.5 (1.2, 5.3)*	6.8 (4.0, 12)*
Other indicators of inflammation			
CRP	1.8 (0.8, 3.9)	2.5 (1.2, 5.1)*	4.9 (2.9, 8.3)*
SAA	2.2 (1.00, 4.7)	4.2 (2.1, 8.4)*	5.4 (3.1, 9.2)*
MPO	1.4 (0.6, 3.0)	2.9 (1.5, 5.7)*	4.7 (2.8, 7.8)*
Growth factors, their receptors, and binding proteins			
VEGF	1.9 (0.9, 4.0)	2.9 (1.5, 5.9)*	6.2 (3.8, 10)*
VEGF-R1 (Flt-1)	0.6 (0.3, 1.2)	0.5 (0.2, 1.1)	0.5 (0.3, 0.9)†
VEGF-R2 (KDR)	2.0 (0.98, 3.9)	1.9 (0.95, 3.7)	2.8 (1.7, 4.5)*
IGFBP-1	0.7 (0.4, 1.5)	0.5 (0.3, 1.1)	0.4 (0.2, 0.6)†
N	82	88	223

Adjustment has been made for GA category (*i.e.* 23–24, 25–26, and 27 wk).
* Significantly increased risk, $p < 0.01$.
† Significantly reduced risk, $p < 0.01$.

Table 3. *ORs (99% CIs) of a concentration in the top quartile (for GA) of the protein among children whose placenta had the grade of umbilical cord inflammation compared to the infants whose placenta had no umbilical cord inflammation*

	Acute inflammation of the umbilical cord		
Protein	Grade 1–2	Grade 3	Grade 4–5
Cytokines and their receptors			
IL-1β	2.5 (1.4, 4.6)*	2.7 (1.3, 5.3)*	5.4 (2.7, 11)*
IL-6	1.9 (1.1, 3.6)*	1.7 (0.8, 3.6)	2.7 (1.4, 5.3)*
IL-6R	2.9 (1.6, 5.2)*	2.9 (1.4, 5.8)*	2.3 (1.2, 4.7)*
TNF-α	2.2 (1.2, 4.0)*	3.2 (1.6, 6.5)*	4.7 (2.4, 9.2)*
TNF-R1	2.3 (1.2, 4.2)*	2.1 (1.01, 4.4)*	4.0 (2.0, 7.9)*
TNF-R2	3.1 (1.7, 5.7)*	4.4 (2.2, 8.9)*	5.1 (2.5, 10)*
Chemokines			
IL-8 (CXCL8)	1.8 (0.97, 3.5)	3.5 (1.7, 7.1)*	3.9 (1.9, 7.6)*
MCP-1 (CCL2)	0.9 (0.5, 1.8)	0.7 (0.3, 1.7)	0.7 (0.3, 1.6)
MCP-4 (CCL13)	0.9 (0.5, 1.8)	1.1 (0.5, 2.2)	1.0 (0.5, 2.0)
MIP-1β (CCL4)	3.0 (1.6, 5.4)*	2.8 (1.4, 5.8)*	3.2 (1.6, 6.3)*
RANTES (CCL5)	1.5 (0.8, 2.8)	2.0 (0.99, 4.1)	1.6 (0.8, 3.2)
I-TAC (CXCL11)	3.7 (2.0, 6.7)*	3.1 (1.5, 6.4)*	6.2 (3.1, 12)*
Adhesion molecules			
ICAM-1 (CD54)	2.7 (1.5, 5.0)*	3.6 (1.8, 7.4)*	5.1 (2.5, 10)*
ICAM-3 (CD50)	3.6 (2.0, 6.6)*	4.3 (2.1, 8.7)*	4.2 (2.1, 8.4)*
VCAM-1 (CD106)	1.1 (0.6, 2.0)	1.2 (0.6, 2.6)	1.9 (0.98, 3.8)
E-selectin (CD62E)	5.2 (2.8, 9.5)*	8.2 (4.0, 17)*	6.6 (3.3, 13)*
MMPs			
MMP-1	1.6 (0.9, 3.0)	1.8 (0.9, 3.8)	1.5 (0.7, 3.0)
MMP-9	3.4 (1.9, 6.3)*	5.4 (2.6, 11)*	3.9 (1.9, 7.7)*
Other indicators of inflammation			
CRP	3.6 (1.9, 6.2)*	3.4 (1.6, 7.0)*	5.7 (2.9, 11)*
SAA	4.1 (2.2, 7.6)*	4.2 (2.0, 8.5)*	4.9 (2.4, 9.7)*
MPO	3.2 (1.7, 5.7)*	3.9 (1.9, 7.9)*	3.9 (2.0, 7.8)*
Growth factors, their receptors, and binding proteins			
VEGF	3.5 (1.9, 6.4)*	4.5 (2.2, 9.1)*	4.9 (2.5, 9.8)*
VEGF-R1 (Flt-1)	0.8 (0.4, 1.4)	0.4 (0.1, 0.99)†	0.7 (0.3, 1.5)
VEGF-R2 (KDR)	2.1 (1.1, 3.9)*	3.0 (1.5, 6.0)*	2.8 (1.4, 5.6)*
IGFBP-1	0.3 (0.2, 0.8)†	0.4 (0.1, 0.9)†	0.7 (0.3, 1.4)
N	103	68	74

Adjustment has been made for GA category.
* Significantly increased risk, $p < 0.01$.
† Significantly reduced risk, $p < 0.01$.

chemokines (IL-8, MIP-1β, RANTES, and I-TAC), adhesion molecules (ICAM-1, ICAM-3, VCAM-1, and E-selectin), MMPs (MMP-1 and MMP-9), systemic inflammation markers (CRP, SAA, and MPO), an angiogenic protein (VEGF), and one of its receptors (VEGF-R2). Blood proteins showed a dose-response pattern to inflammation that was best seen with increasing stage of neutrophil infiltration at the chorionic plate. Data for moderate to severe inflammation in the membranes and vasculitis of the fetal stem vessel are not only shown but were also strongly associated with elevated blood proteins. Infants whose placenta had histologic inflammation were at reduced risk of having an elevation of two proteins with angiogenic properties (VEGF-R1 and IGFBP-1). An almost inverse pattern was characteristic of infants whose placenta had syncytial knots or infarcts (Table 4).

The highest quartile concentrations of MPO, a marker of neutrophil activation, and the acute phase proteins CRP and SAA were significantly associated with inflammation of the chorionic plate or membranes regardless of the involvement of the umbilical cord (Table 5). The 10 infants whose placentas had isolated umbilical cord inflammation were also at increased risk of elevated concentrations of these three proteins, but the increase was not statistically significant.

Association between inflammatory biomarkers in ELGAN blood and histologic signs of poor placental perfusion. In contrast to newborn with inflamed placentas, newborns with signs of poor placental perfusion expressed either no risk or significantly lower risk of elevated inflammatory proteins in their blood (Table 4). They were less likely to have higher levels of IL-6, RANTES, ICAM-1, ICAM-3, VCAM-1, E-selectin, MMP-1, MMP-9, and MPO ($p < 0.01$) if both infarcts and increased syncytial knots were observed. The

Table 4. *ORs (99% CIs) of a concentration in the top quartile (for GA) of the protein among children whose placenta had infarct, increased syncytial knots, or decidual/hemorrhage/fibrin deposition compared to children whose placenta did not have that histologic characteristic*

Protein	Infarct	Increased syncytial knots	Decidual hemorrhage/ fibrin deposition
Cytokines and their receptors			
IL-1β	0.8 (0.5, 1.4)	0.6 (0.4, 1.1)	0.8 (0.4, 1.4)
IL-6	0.6 (0.3, 1.05)	0.5 (0.3, 0.96)*	0.7 (0.4, 1.3)
IL-6R	0.7 (0.4, 1.2)	0.6 (0.4, 1.1)	1.1 (0.7, 2.0)
TNF-α	0.7 (0.4, 1.2)	0.7 (0.4, 1.2)	0.9 (0.6, 1.6)
TNF-R1	0.5 (0.3, 1.00)	0.7 (0.4, 1.2)	1.1 (0.6, 1.9)
TNF-R2	0.7 (0.4, 1.2)	0.7 (0.4, 1.2)	1.0 (0.6, 1.8)
Chemokines			
IL-8 (CXCL8)	0.6 (0.3, 1.2)	0.7 (0.4, 1.3)	1.0 (0.6, 1.7)
MCP-1 (CCL2)	1.1 (0.6, 1.9)	0.9 (0.5, 1.5)	0.7 (0.4, 1.3)
MCP-4 (CCL13)	0.9 (0.5, 1.5)	0.9 (0.5, 1.5)	1.1 (0.7, 1.9)
MIP-1β (CCL4)	0.4 (0.2, 0.9)	0.7 (0.4, 1.2)	1.0 (0.6, 1.7)
RANTES (CCL5)	0.4 (0.2, 0.7)*	0.5 (0.3, 0.9)*	1.1 (0.6, 1.8)
I-TAC (CXCL11)	0.5 (0.3, 1.01)	0.7 (0.4, 1.2)	1.0 (0.6, 1.7)
Adhesion molecules			
ICAM-1 (CD54)	0.5 (0.3, 0.99)*	0.9 (0.5, 1.5)	0.8 (0.5, 1.5)
ICAM-3 (CD50)	0.2 (0.1, 0.5)*	0.3 (0.2, 0.6)*	0.8 (0.4, 1.4)
VCAM-1 (CD106)	0.5 (0.3, 0.9)*	0.7 (0.4, 1.2)	1.2 (0.7, 2.1)
E-selectin (CD62E)	0.5 (0.2, 0.9)*	0.5 (0.3, 0.96)*	1.1 (0.6, 1.9)
MMPs			
MMP-1	0.3 (0.2, 0.7)*	0.6 (0.4, 1.1)	1.0 (0.6, 1.7)
MMP-9	0.5 (0.3, 0.99)*	0.4 (0.2, 0.7)*	0.8 (0.5, 1.5)
Other indicators of inflammation			
CRP	0.7 (0.4, 1.2)	0.9 (0.5, 1.5)	0.6 (0.3, 1.1)
SAA	0.6 (0.3, 1.04)	0.7 (0.4, 1.3)	0.7 (0.4, 1.3)
MPO	0.5 (0.3, 0.95)*	0.4 (0.2, 0.8)*	0.8 (0.4, 1.4)
Growth factors, their receptors, and binding proteins			
VEGF	0.6 (0.3, 1.1)	0.5 (0.3, 0.9)*	1.3 (0.8, 2.2)
VEGF-R1 (Flt-1)	1.3 (0.7, 2.1)	2.3 (1.4, 3.8)†	0.9 (0.5, 1.6)
VEGF-R2 (KDR)	0.5 (0.3, 0.97)*	0.8 (0.5, 1.3)	0.9 (0.5, 1.6)
IGFBP-1	2.0 (1.2, 3.3)†	2.2 (1.3, 3.5)†	1.2 (0.7, 2.1)
N	135	159	137

Adjustment has been made for GA category.
* Significantly reduced risk, $p < 0.01$.
† Significantly increased risk, $p < 0.01$.

Table 5. *ORs (99% CIs) of a concentration in the top quartile (for GA) of the protein among children whose placenta had the combination of histologic lesions relative to the risk among those whose placenta had no inflammatory lesion*

	ORs (and 95% CIs)			
Inflammation of				
Plate or membranes	Yes	Yes	No	No
Umbilical cord	Yes	No	Yes	No
CRP	5.3 (3.0, 9.6)*	3.1 (1.8, 5.5)*	2.5 (0.4, 15)	1.0
SAA	5.6 (3.1, 10)*	4.1 (2.4, 7.1)*	2.7 (0.4, 17)	1.0
MPO	4.3 (2.5, 7.6)*	2.5 (1.4, 4.2)*	2.8 (0.5, 15)	1.0
N	124	164	10	446

Adjustment has been made for GA category.
* Significantly increased risk, $p < 0.01$.

presence of decidual hemorrhage and fibrin deposition was neutral in terms of risk for elevated inflammatory proteins. VEGF was decreased only in the newborns with syncytial knots in the placenta and occurred at the background of increased VEGF-R1, again confirming the inverse relationships between these two biomarkers seen albeit in the opposite direction in the presence of inflammation. VEGF-R2 and IGFBP-1 showed also negative association with poor placental perfusion in contrast to the positive association observed with inflammation.

DISCUSSION

We have shown an association between placental inflammation and a fetal inflammatory response that is consistent with previous studies of term and preterm births (11). Previous work has shown similar associations, most commonly for IL-6 and IL-8, (12,13) as well as for IL-1β, TNF-α and MMP-9, and other chemokines, regulatory proteins, and growth factors (14–16). The range of biomarkers analyzed in this study is one of the largest simultaneously assessed in published reports and represents all categories of inflammatory mediators.

Placental inflammatory infiltrates include both maternal and fetal contributions. Chorionic plate infiltration is viewed as histologic evidence of a maternal response, (17,18) whereas inflammation of umbilical cord vessels and fetal stem vessels in the chorionic plate, so-called fetal vasculitis, are the histologic hallmarks of a fetal inflammatory response (4,11,13,19). Neonatal morbidity has tended to be better predicted by fetal vasculitis than by maternal inflammation at the chorionic plate (11,19).

However, we describe a strong association between histologic markers of both maternal and fetal inflammation and systemic inflammatory response in the newborn. The intensity of placental inflammation is thought to evolve in a sequence, developing from chorionic plate inflammation to fetal vasculitis at the chorionic plate to cord inflammation (18). The intensity may reflect the duration and extent of infection (20). This sequence implies that maternal response precedes fetal response. Consistent with this model, we observe that most cord inflammation occurs in combination with high-grade/stage plate inflammation. In addition, we see a dose-response relationship between maternal fetal plate inflammation and blood proteins (Tables 2 and 3).

Despite the model, an accelerated fetal response may sometimes occur. A subset of placentas showed isolated umbilical cord inflammation. Similar findings have been observed in 5 to 8% of preterm and 17% of term placentas (21). Infants with cord-only inflammation tended to have elevated concentrations of blood acute phase reactants. Although the maternal response is generally thought to precede the fetal response, this subset of cases suggests that an accelerated fetal response is possible. We have previously reported that some microorganisms including *Actinomyces* sp., group B, group D, and alpha-hemolytic *Streptococci* are more likely to promote fetal vasculitis than high-grade chorionic plate inflammation (4).

This study also shows that the inflammatory response is not merely a local neutrophil-mediated process in the placenta but is systemic. The fetus is exposed to intraamniotic infection at three interfaces: the subamniotic tissue of the placental disc

and cord, in the lungs, and in the GI tract through oral intake of amniotic fluid. With placental histologic inflammation, we see increased levels of circulating factors that reflect neutrophil activation (MPO), and endothelial activation allowing chemotaxis and leukocyte migration (*e.g.* MMP-9, E-selectin, VCAM-1, ICAM-1, and ICAM-3). We also see a systemic response in the acute phase reactants produced by the liver (SAA and CRP). In sepsis, circulating blood cells and vascular cells produce TNF-α and IL-1β leading to activation of nuclear factor-kappa B (NF-κB) and subsequent production of IL-6, IL-8, and IFN-γ, which mediate systemic effects including acute-phase reactants such as CRP and SAA as well as MMP-9. TNF-α and IL-1β can also induce premature labor (22).

Some of the vulnerability of the brain, lung, bowel, and eye in ELGANs has been attributed to their propensity to respond to inflammatory stimuli more vigorously than infants born at term (12). Consequently, our finding of such strong links between circulating proteins and evidence of a putative stimulus raises the possibility that these circulating proteins might be intermediates between the inflammatory stimulus and organ damage.

Because our study used whole blood lysates, our analyses are based on measuring both the soluble and cell-bound forms of membrane receptors (*e.g.* E-selectin, VCAM-1, ICAM-1, ICAM-3, TNF-R1 and 2, IL-6R, and VEGF-R1 and 2). Circulating forms of VCAM-1, E-selectin, and ICAM-1 have been detected in plasma and are elevated during systemic inflammatory conditions and on endothelial cells. The origins of circulating VCAM-1, E-selectin, and ICAM-1 are unclear, but they may arise from shedding or proteolytic cleavage from endothelial cells (23–25). Similarly, other membranous proteins (*e.g.* TNF-α receptors) undergo shedding through the actions of MMPs and thus increased levels of these receptors may reflect MMP up-regulation in addition to up-regulation of the specific receptor genes (26).

The concentrations of a few protein biomarkers (*e.g.* IGFBP-1 and VEGF-R1) decreased with placental inflammation and increased with syncytial knots and infarcts, which are presumably histologic indicators of vascular insufficiency. The concentrations of IGFBP-1, one of the binding proteins that control serum levels of IGF, VEGF-R1, and the soluble fms-like tyrosine kinase-1, (27) are abnormally high in women who have preeclampsia, a syndrome associated with poor placental vascularization and fetal growth restriction. *In vitro* IGFBP-1 can be induced by chronic hypoxia (28). Thus, an increased level might be expected with histologic characteristics attributed to vascular insufficiency. The low levels of IGFBP-1 seen with histologic inflammation may be explained by increased degradation by MMP-9 and other MMPs that were not measured in this study since this phenomenon has been observed in inflamed amniotic fluid (29).

Newborns whose placenta had infarcts and increased knots were unlikely to show increased concentrations of inflammatory proteins. This is consistent with previous observations that vascular and inflammatory characteristics of the placenta tend not to occur together (5,30).

VEGF promotes blood vessel formation and endothelial maintenance when bound to the second of its circulating receptors, VEGF-R2 (KDR/Flk-1). VEGF is up-regulated by proinflammatory activation downstream from IL-1β and TNF-α signaling (31). In keeping with this, we found that VEGF concentrations were elevated in newborns whose placenta had both maternal and fetal inflammation and lower in newborns whose placenta did not have inflammation but did have syncytial knots and or infarcts.

The first VEGF receptor, VEGF-R1 (Flt-1), seems to function as a competitive inhibitor, minimizing the physiologic capability of VEGF bound to it. VEGF-R1 in the maternal circulation, likely produced by placental trophoblast, is elevated in preeclampsia (12). VEGF-R1 in the newborn has not been extensively reported. We were not surprised to see elevated concentrations of d 1 VEGF-R1 in newborns whose placenta had infarcts and increased syncytial knots since both these histologic lesions are common in preeclampsia. However, decreased VEGF-R1 associated with placental inflammation was a new finding and may be a result of physiologic degradation of VEGF-R1 by matrix metaloproteinases. This is a normal regulatory function of MMP that increases the bioavailability of VEGF for endothelial cells (32). It is possible that inflammation in the placenta or systemic activation of MMP may have a similar but nonlocalized effect.

The fact that we did not find any significant relationship between decidual hemorrhage (*i.e.* abruption) and any protein in the newborn's blood might truly indicate no relationship. However, abruption is seen among preterm deliveries associated with both severe chorioamnionitis (33) and preeclampsia (34). Perhaps this heterogeneity diminished our ability to identify a relationship between inflammation and decidual hemorrhage.

The major strengths of our study are the large number of proteins uniformly measured at one time, the large number of infants born before the 28th wk of gestation, selection of our sample on the basis of GA rather than birth weight, and recording of all histologic findings in a uniform manner after efforts to reduce observer variability. A limitation of our study is the small number of newborns who had isolated umbilical cord inflammation. We were also, as in all observational studies, unable to distinguish between causation and association as explanations for what we found.

In summary, we found a strong inflammatory signal in the blood of newborns delivered before the 28th wk of gestation whose placenta had moderately severe inflammation of the chorionic plate alone, severe inflammation of both chorionic plate and umbilical cord inflammation, or severe inflammation of just umbilical cord alone. Our findings suggest a need for placental examination in all ELGAN with emphasis on a detailed description of the pattern of inflammation since the presence of placental inflammation predicted increased ORs of newborn inflammatory response within the first 3 d of life regardless of GA. Histologic placental inflammation, especially when of high stage in the chorionic plate or causing fetal vasculitis, should be regarded as a fetal inflammatory response. This information may be useful in stratifying ELGAN for studies of intervention.

Acknowledgments. We gratefully acknowledge the contributions of our subjects and their families, as well as those of their colleagues.

REFERENCES

1. Hillier SL, Martius J, Krohn M, Kiviat N, Holmes KK, Eschenbach DA 1988 A case-control study of chorioamnionic infection and histologic chorioamnionitis in prematurity. N Engl J Med 319:972–978
2. Stoll BJ, Hansen NI, Adams-Chapman I, Fanaroff AA, Hintz SR, Vohr B, Higgins RD 2004 Neurodevelopmental and growth impairment among extremely low-birthweight infants with neonatal infection. JAMA 292:2357–2365
3. van Hoeven KH, Anyaegbunam A, Hochster H, Whitty JE, Distant J, Crawford C, Factor SM 1996 Clinical significance of increasing histologic severity of acute inflammation in the fetal membranes and umbilical cord. Pediatr Pathol Lab Med 16:731–744
4. Hecht JL, Onderdonk A, Delaney M, Allred EN, Kliman HJ, Zambrano E, Pflueger SM, Livasy CA, Bhan I, Leviton A; ELGAN Study Investigators 2008 Characterization of chorioamnionitis in 2nd-trimester C-section placentas and correlation with microorganism recovery from subamniotic tissues. Pediatr Dev Pathol 11:15–22
5. McElrath TF, Hecht JL, Dammann O, Boggess K, Onderdonk A, Markenson G, Harper M, Delpapa E, Allred EN, Leviton A 2008 Pregnancy disorders that lead to delivery before the 28th week of gestation: an epidemiologic approach to classification. Am J Epidemiol 168:980–989
6. Leviton A, Allred EN, Kuban KC, Hecht JL, Onderdonk AB, O'Shea TM, Paneth N 2010 Microbiologic and histologic characteristics of the extremely preterm infant's placenta predict white matter damage and later cerebral palsy. The ELGAN study. Pediatr Res 67:95–101
7. Fichorova RN, Trifonova RT, Gilbert RO, Costello CE, Hayes GR, Lucas JJ, Singh BN 2006 Trichomonas vaginalis lipophosphoglycan triggers a selective upregulation of cytokines by human female reproductive tract epithelial cells. Infect Immun 74:5773–5779
8. Fichorova RN, Richardson-Harman N, Alfano M, Belec L, Carbonneil C, Chen S, Cosentino L, Curtis K, Dezzutti CS, Donoval B, Doncel GF, Donaghay M, Grivel JC, Guzman E, Hayes M, Herold B, Hillier S, Lackman-Smith C, Landay A, Margolis L, Mayer KH, Pasicznyk JM, Pallansch-Cokonis M, Poli G, Reichelderfer P, Roberts P, Rodriguez I, Saidi H, Sassi RR, Shattock R, Cummins JE Jr 2008 Biological and technical variables affecting immunoassay recovery of cytokines from human serum and simulated vaginal fluid: a multicenter study. Anal Chem 80:4741–4751
9. Rothman KJ 1990 No adjustments are needed for multiple comparisons. Epidemiology 1:43–46
10. Hecht JL, Allred EN, Kliman HJ, Zambrano E, Doss BJ, Husain A, Pflueger SM, Chang CH, Livasy CA, Roberts D, Bhan I, Ross DW, Senagore PK, Leviton A 2008 Histological characteristics of singleton placentas delivered before the 28th week of gestation. Pathology 40:372–376
11. Pacora P, Chaiworapongsa T, Maymon E, Kim YM, Gomez R, Yoon BH, Ghezzi F, Berry SM, Qureshi F, Jacques SM, Kim JC, Kadar N, Romero R 2002 Funisitis and chorionic vasculitis: the histological counterpart of the fetal inflammatory response syndrome. J Matern Fetal Neonatal Med 11:18–25
12. Mestan K, Yu Y, Thorsen P, Skogstrand K, Matoba N, Liu X, Kumar R, Hougaard DM, Gupta M, Pearson C, Ortiz K, Bauchner H, Wang X 2009 Cord blood biomarkers of the fetal inflammatory response. J Matern Fetal Neonatal Med 22:379–387
13. Yoon BH, Romero R, Park JS, Kim M, Oh SY, Kim CJ, Jun JK 2000 The relationship among inflammatory lesions of the umbilical cord (funisitis), umbilical cord plasma interleukin 6 concentration, amniotic fluid infection, and neonatal sepsis. Am J Obstet Gynecol 183:1124–1129
14. Weatherstone KB, Rich EA 1989 Tumor necrosis factor/cachectin and interleukin-1 secretion by cord blood monocytes from premature and term neonates. Pediatr Res 25:342–346
15. Dammann O, Phillips TM, Allred EN, O'Shea TM, Paneth N, Van Marter LJ, Bose C, Ehrenkranz RA, Bednarek FJ, Naples M, Leviton A 2001 Mediators of fetal inflammation in extremely low gestational age newborns. Cytokine 13:234–239
16. Romero R, Gomez R, Galasso M, Mazor M, Berry SM, Quintero RA, Cotton DB 1994 The natural interleukin-1 receptor antagonist in the fetal, maternal, and amniotic fluid compartments: the effect of gestational age, fetal gender, and intrauterine infection. Am J Obstet Gynecol 171:912–921
17. Døllner H, Vatten L, Halgunset J, Rahimipoor S, Austgulen R 2002 Histologic chorioamnionitis and umbilical serum levels of pro-inflammatory cytokines and cytokine inhibitors. BJOG 109:534–539
18. Redline RW 2006 Inflammatory responses in the placenta and umbilical cord. Semin Fetal Neonatal Med 11:296–301
19. Salafia CM, Sherer DM, Spong CY, Lencki S, Eglinton GS, Parkash V, Marley E, Lage JM 1997 Fetal but not maternal serum cytokine levels correlate with histologic acute placental inflammation. Am J Perinatol 14:419–422
20. Dammann O, Allred EN, Leviton A, Shen-Schwarz S, Heller D, Genest DR, Collins MH 2004 Fetal vasculitis in preterm newborns: interrelationships, modifiers, and antecedents. Placenta 25:788–796
21. Lee SE, Romero R, Kim CJ, Shim SS, Yoon BH 2006 Funisitis in term pregnancy is associated with microbial invasion of the amniotic cavity and intra-amniotic inflammation. J Matern Fetal Neonatal Med 19:693–697
22. Romero R, Espinoza J, Goncalves LF, Kusanovic JP, Friel L, Hassan S 2007 The role of inflammation and infection in preterm birth. Semin Reprod Med 25:21–39
23. Gearing AJ, Newman W 1993 Circulating adhesion molecules in disease. Immunol Today 14:506–512
24. Pigott R, Dillon LP, Hemingway IH, Gearing AJ 1992 Soluble forms of E-selectin, ICAM-1 and VCAM-1 are present in the supernatants of cytokine activated cultured endothelial cells. Biochem Biophys Res Commun 187:584–589
25. Rothlein R, Mainolfi EA, Czajkowski M, Marlin SD 1991 A form of circulating ICAM-1 in human serum. J Immunol 147:3788–3793
26. Porteu F, Nathan C 1990 Shedding of tumor necrosis factor receptors by activated human neutrophils. J Exp Med 172:599–607
27. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA 2003 Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 111:649–658
28. Fowler DJ, Nicolaides KH, Miell JP 2000 Insulin-like growth factor binding protein-1 (IGFBP-1): a multifunctional role in the human female reproductive tract. Hum Reprod Update 6:495–504
29. Lee SE, Han BD, Park IS, Romero R, Yoon BH 2008 Evidence supporting proteolytic cleavage of insulin-like growth factor binding protein-1 (IGFBP-1) protein in amniotic fluid. J Perinat Med 36:316–323
30. Hansen AR, Collins MH, Genest D, Heller D, Shen-Schwarz S, Banagon P, Allred EN, Leviton A 2000 Very low birthweight placenta: clustering of morphologic characteristics. Pediatr Dev Pathol 3:431–438
31. Hayashi T, Matsuoka K, Saitoh M, Takeda S, Kimura M 2006 Influence of alpha-tumor necrosis factor and beta-interleukin-1 on production of angiogenetic factors and thymidine phosphorylase activity in immortalized human decidual fibroblasts in vitro. J Obstet Gynaecol Res 32:15–22
32. Ito TK, Ishii G, Saito S, Yano K, Hoshino A, Suzuki T, Ochiai A 2009 Degradation of soluble VEGF receptor-1 by MMP-7 allows VEGF access to endothelial cells. Blood 113:2363–2369
33. Nath CA, Ananth CV, Smulian JC, Shen-Schwarz S, Kaminsky L 2007 Histologic evidence of inflammation and risk of placental abruption. Am J Obstet Gynecol 197:319.e1–319.e6
34. Darby MJ, Caritis SN, Shen-Schwarz S 1989 Placental abruption in the preterm gestation: an association with chorioamnionitis. Obstet Gynecol 74:88–92