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Cluster analysis of placental inflammatory proteins can distinguish preeclampsia from preterm labor and premature membrane rapture in singleton deliveries less than 28 weeks gestation

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Abstract

Problem—Inflammation within the preterm placenta is common and leads to adverse outcomes for premature infants. The risks of complications are different between iatrogenic (e.g. preeclampsia) and spontaneous (e.g. preterm labor and membrane rapture) causes of preterm delivery, suggesting different underlying biology contributes to these placental conditions.

Method of Study—Thirty preterm singleton placentas from the following groups were analyzed: 1) severe preeclampsia (PE), 2) preterm premature membrane rupture (pPROM) and 3) preterm labor (PL). Proinflammatory and anti-inflammatory cytokines, adhesion and angiogenic molecules were measured in placental lysates using a multiplex assay. *K*-means cluster analysis was used to generate patterns of protein level intensity.

Results—Three cluster patterns were apparent. Placentas from PE had high levels of VEGF combined with low levels of acute inflammatory proteins (IL-1β, IL-18, IL-6, TNF-α), low IL-1 RA and high TGF-β. PL and pPROM had higher anti-inflammatory IL-1 RA and thrombomodulin combined with lower VEGF, regardless of proinflammatory cytokines and adhesion molecules. Half of the PL and pPROM cases had clusters of heightened inflammatory responses (lower TGF-β clustered with higher intensity of inflammatory mediators).

Conclusions—Discriminating protein patterns were elucidated and may serve as a foundation from which to understand the biological mechanisms underlying these pregnancy complications.

Keywords

inflammation; placenta; preterm labor; preterm premature membrane rupture; preterm delivery

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Introduction

Inflammation within the preterm placenta is both common [1] and associated with an increased risk of adverse outcomes among premature infants [2-7]. However, the incidence of intrauterine inflammation and the risk of associated neonatal morbidity is different between the iatrogenic (preeclampsia) and spontaneous (preterm labor and membrane rupture) causes of preterm delivery [8-10]. Preeclampsia (PE) is characterized by wider systemic rather than focal intrauterine inflammation, [11] whereas preterm labor (PL) and preterm premature membrane rupture (pPROM) are characterized by inflammatory events believed to begin within the intrauterine environment [12, 13]. We hypothesized that the inflammatory protein profile in preterm placenta would be distinct for each of these three pregnancy complications. Fifteen inflammatory proteins were measured in the preterm placenta samples and *K*-means cluster analysis was used to determine differences in intensity profiles across these 15 analytes.

Methods

Samples

Placentas from 30 consecutive singleton deliveries for the indications of PE, pPROM, or PL between 23 0/7 and 27 6/7 weeks' gestation were sampled. The estimated date of delivery was calculated with first trimester ultrasound dates given priority over a firm last menstrual period and confirming second trimester ultrasound. This study was approved by the institutional review board of Brigham and Woman's Hospital (Boston, MA) and all participants provided written, informed consent.

Definition of placental/pregnancy conditions

We defined PL as the presence of painful regular uterine contractions with cervical dilatation in the setting of intact fetal membranes. pPROM was defined as a positive vaginal pool, ferning, and an elevated vaginal pH prior to the onset of regular uterine contractions. Magnesium sulfate and nifedipine were the tocolytics employed in this population. Indomethacin is not frequently used as a tocolytic in our center and was not administered to any of the patients in this analysis. We recorded the administration of antibiotics for the indication of suspected clinical chorioamnionitis. We defined preeclampsia as a primary presentation of maternal hypertension (two blood pressure elevations six or more hours apart of at least 140/90 mmHg) with an accompanying proteinuria (at least 300 mg in 24 hours). We understand that delivery in this early gestational age range implies the diagnosis of severe rather than mild PE. Patients manifesting progressive hypertension resistant to medical therapy, central neurologic symptoms such as headache or visual changes, elevation of the transaminases or a decline in the platelet count to below 100,000 were diagnosed with severe preeclampsia and delivered for maternal indications. Patients with the primary presentation of hemolysis, elevated liver enzymes and low platelets (HELLP) syndrome were excluded from this analysis. None of the patients suffered eclamptic events. Pregnancies complicated by other initiators of preterm delivery including cervical insufficiency, placental abruption, or non-reassuring fetal status, were not considered in this analysis.

Collection of placenta samples and measurement of analytes

Within 20 minutes of delivery, each placenta was examined under sterile conditions to insure that the amnion was adherent and the chorion was not exposed. The midpoint of the longest distance between the cord insertion and the edge of the placental disk was identified. After the amnion was peeled away, the chorion and underlying tissue were tented with sterile forceps, cut and removed. This method of tenting ensured that the biopsy did not

proceed through to the underlying villous surface and that the sample was thereby not contaminated with maternal blood. The sample was placed in a sterile cryovial for flash freezing and storage at −80°C until use.

For processing, each sample was removed from its vial, weighed, and mixed with Tissue Protein Extraction Reagent (TPER) (Pierce, Rockford, IL) on dry ice in a 1:10 ratio by weight and sonicated. The sample was then centrifuged at 10,000 rpm at 4°C for 10 minutes and the supernatant was collected and re-centrifuged for an additional 10 minutes, after which it was snap frozen and stored at −80°C. The sample was later thawed and tested with the Meso Scale Discovery (MSD) multiplex microarrays using the Sector Imager 2400 (Meso Scale Discovery, Gaithersburg, MD). In each lysate, the total protein level was determined by BCA assay (Pierce) and the expression levels of each of the analytes normalized to mg total protein.

The 15 chosen analytes represent components of the inflammatory response. The proteins (and their functions) included: interleukin (IL)-1β, IL-6, tumor necrosis factor α (TNF-α), IL-18 (primary acute inflammatory response to stress or microbial ligands); IL-8 (neutrophil and monocyte chemotaxis downstream from acute cytokine or microbial ligands activation); P-selectin, E-selectin, vascular cell adhesion molecule (VCAM)-1, inter-cellular adhesion molecule (ICAM)-1, and ICAM-2 (endothelial adhesion molecule response downstream from acute cytokine or toll-like receptor activation); transforming growth factor β (TGF-β), IL-10, IL-1 RA, and thrombomodulin (anti-inflammatory modulation); and vascular endothelial growth factor (VEGF, angiogenesis). Consistent with standard technique, after adjusting for total protein concentration, we transformed the concentrations to Z-scores (mean = 0, and deviations above and below expressed as standard deviation units) [14, 15].

Statistical analysis

The non-parametric Kruskal-Wallis test and the Chi-square test were used to examine for differences between sample characteristics. Clustering analysis was performed using the TIGR-Multi Experiment Viewer software [16]. A *K*-means procedure was employed using Euclidian distances [16, 17]. The *K*-means procedure performs unsupervised clustering (i.e. the samples are grouped solely by their expression profile and without reference to clinical or histologic background). As the *K*-means procedure can be sensitive to random changes in the initiation parameters, we present consensus clusters that represent the expression profiles grouped together in at least 80% of 10,000 separate runs. One-way ANOVA was used to test for differences in expression Z-scores between the clusters. We present the P-values adjusted for multiple comparisons using the Bonferroni correction.

Results

Among the 30 placentas tested in this study, 9 (30%) were from pregnancies complicated by PE, 11 (37%) by PL, and 10 (33%) by pPROM. The median gestational age for all deliveries in the sample was 26 weeks with a non-significant tendency for PL to deliver earlier (Table 1). Consistent with our institutional policy, all patients were administered antenatal steroids. There were no differences between the groups with respect to the rate of steroid completion (Table 1). Similarly, the interval between diagnosis and delivery and the interval between steroid administration and delivery was not significantly different for each of the delivery indications (Table 1). Both babies and placentas were significantly smaller in the setting of PE than those with either PL or pPROM. All women with PE had a cesarean delivery, compared with 64% of those whose pregnancies were complicated by PL and 70% of those with pPROM. Exposure to antenatal antibiotics and tocolytics also differed by delivery indication. Patients delivering in the setting of PL or pPROM were significantly more likely to have received antenatal antibiotics (Table 1). The rate of antibiotic exposure did not

significantly differ between PL and pPROM (p=0.423). Similarly, exposure to tocolytics was significantly more likely among the PL categories (Table 1). There was a significant difference between the rate of tocolytic use comparing the PL and pPROM categories (p=0.001). The sex ratio of babies was 1:1 for the sample. For reference, the median and range of each analyte concentration is presented in Appendix A.

After application of the *K*-means clustering procedure, the specimens cluster into three analyte expression profiles. The *K*-means algorithm clusters the cases of preeclampsia together, suggesting a common PE expression profile that is distinct from that of PL or pPROM (Table 2). This 'preeclampsia' pattern is notable for the relatively increased VEGF, P-selectin and TGF-β and relatively low levels of the other analytes. Unlike the preeclampsia cluster, a second pattern, which we call the 'high-inflammation' pattern (Table 2), was not synonymous with a single cause of preterm delivery but rather this second cluster included placentas from both PL (63%) and pPROM (37%) pregnancies. This pattern is characterized by very high levels of the acute inflammatory, chemokine, vascular, and inflammation modulating proteins. The *K*-means procedure also identified a third protein pattern that included placentas complicated by PL (40%) and pPROM (60%). This pattern was characterized by relatively low levels of the acute inflammatory and chemokine molecules and TGF-β. We therefore called this third pattern the 'low inflammation' cluster (Table 2). Both the high- and low-inflammation patterns share reduced expression levels of the angiogenic marker VEGF.

By comparing the mean Z-scores for each cluster, we examined quantitative differences between the three clusters (Table 2). The high-inflammation pattern differs prominently from the other two clusters with much higher expression levels of all inflammatory cytokines, IL-8, E-selectin, and IL1-ra. The preeclampsia cluster displays high levels of TGF-β and VEGF, but low levels of almost all other proteins, especially thrombomodulin.

To test if mode of delivery might confound the clustering procedure, analyte expression values were compared by mode of delivery for the PL and pPROM placentas (Table 3). The expression values were similar in both Cesarean section and vaginal deliveries suggesting that expression levels are unrelated to the mode of delivery. Additionally, we sought insight into whether differences in antibiotic or tocolytic exposure might lay behind the distinction high- and low-inflammation clusters. Neither antibiotics (p=0.423) nor tocolytics (p=0.688) were more likely to be used in either of these two clusters. Similarly, antenatal steroid exposure did not differ between the clusters (p=0.723).

Discussion

This study is the first to examine the utility of the *K*-means algorithm with a smaller-dimensionality multiplexed protein dataset, including selected cytokines and other inflammatory molecules of well-known biological function, to generate discriminative placental molecular patterns of preterm delivery. The need for cluster analysis and pattern recognition arises in a variety of disciplines [18]. The *K*-means procedure is one of the most robust clustering algorithms available [19]. *K*-means allows the unsupervised (i.e. without reference to prior biologic or clinical knowledge) clustering of expression patterns into similar sub-groups. This clustering algorithm has found wide application in gene expression analysis, where thousands of genes can be queried simultaneously [20].

We have contributed important observations regarding the inflammatory analyte profiles in extremely preterm placentas by identifying three patterns of protein expression within a set of placentas delivered between 23 and 28 weeks gestation. Our findings demonstrate that PE placentas have an expression profile that is unique to this cause of preterm delivery and

distinct from that of the PL and pPROM placentas. However, the expression profiles of the PL and pPROM placentas are not distinct from each other and do not segregate according to clinical presentation. Rather, these latter two causes of preterm delivery were represented almost equally in the two expression profiles that differed markedly in the level of inflammatory and chemokine analyte expression. That PL and pPROM share protein profiles is to some degree understandable given that they are both forms of spontaneous preterm birth. However, the different patterns of inflammatory involvement suggest that within PL and pPROM cases additional factors may result in inflammation being a more prominent feature of spontaneous preterm delivery in one class of births than is the case in another class of births. It would be interesting to explore factors associated with these different inflammatory profiles in PL and pPROM in a study with a larger sample size and expanded set of analytes.

Exposure to antenatal steroids did not differ by cause of delivery, but antibiotic and tocolytic exposure did. We cannot exclude the possibility that differential administration of tocolytics or antibiotics might influence our results. We believe that the magnitude of this influence is likely to be minimal as magnesium sulfate and nifedipine, the tocolytics used in our center, are not believe to act on the inflammatory cascade. Similarly, we believe the potential differential effect of antenatal antibiotics on our results is minimal as the high- and low-inflammatory clusters did not differ in their antibiotic exposure yet did differ in their degree of inflammation. However, as noted, we have limited ability to quantify these possible effects and appropriate caution is warranted in interpretation.

Consideration should also be given to the single, uniform biopsy technique that was employed in this study. There is regional heterogeneity in protein expression across the placental disk [21-23]; therefore, a larger set of biopsies from each placenta might be more representative of the whole placenta. However, given the restricted size of many of the placentas in the very early gestational age, a single biopsy that could be obtained in a uniform fashion on all study placentas may provide better standardization than attempting multiple and potentially non-standard biopsies. Moreover, variation in cytokine expression has not been shown to be related to the circumferential position with the placental disk. [21] In this study the depth of the biopsy was uniformly applied to each placenta. This sampling procedure is less likely to introduce a significant systematic bias in the observed differences in protein expression. In fact, due to the conservative nature of the K-means cluster analysis, the results of this study may under represent true differences in protein expression associated with the three different classes of preterm delivery.

Preeclampsia is viewed as a systemic maternal inflammatory disease rather than localized placental inflammatory condition [11, 24, 25], yet the placenta is likely to play a causal role in this systemic inflammation [26, 27]. In agreement, the results from this study showed limited acute inflammation within the placenta at the time of preterm delivery for PE. This observation is consistent with placental histology where the stroma of PE placentas do not display the same inflammatory signs as placentas delivered in the setting of PL or pPROM [28].

The other two profiles, labeled here as ‘high-inflammation’ and ‘low-inflammation,’ do not coincide with the clinical disorders that initiated the preterm delivery. Rather, each of these patterns occurred with about the same frequency in both PL and pPROM placentas. The high inflammation profile was marked by increased expression of the acute inflammatory and chemokine proteins. The endothelial and inflammatory-modulator markers also appeared to be expressed at relatively increased levels, although in a less consistent fashion. Conversely, the decreased levels of the acute inflammatory, chemokine, and endothelial markers characterized the low inflammation profile. Regardless of their inflammation

pattern, PL and pPROM pregnancies featured a low expression of the angiogenic mediator VEGF. These observations support the view that although PL and pPROM have distinct clinical presentations, they may share common inflammatory etiologies. Although speculative, these results are also consistent with the hypothesis that the inflammatory nexus leading to spontaneous preterm birth may be intrauterine in some cases versus systemic in others.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Clinical characteristics by cause of preterm delivery

	Pre-eclampsia (n=9)	Preterm Labor (n=11)	pPROM (n=10)	P -value
Median gestation * (weeks)	26 (24, 27)	25 (24, 27)	26 (25, 27)	0.42
Median diagnosis-delivery interval * (hours)	96 (50, 168)	84 (53, 88)	92 (24, 210)	0.66
Median steroid-birth interval* (hours)	49 (24, 120)	53 (12, 61)	46 (24, 162)	0.83
Median birth wt * (grams)	640 (600, 690)	880 (720, 1235)	863 (652, 1049)	0.03
Median placental wt * (grams)	160 (120, 165)	230 (175, 280)	205 (190, 245)	0.03
Complete steroid course (%)	77	55	70	0.53
Exposure to any antibiotics (%)	22	82	90	0.003
Exposure to any tocolytics (%)	0	100	20	0.001
Cesarean (%)	100	64	70	0.14
Male gender (%)	22	55	70	0.12

*(interquartile range: 25^{th}, 75^{th} percentiles)

Table 2

Mean Z-score of protein level by cluster

		Clusters				
			Preeclampsia	**High- inflammation**	**Low-inflammation**	**Adjusted Bonferroni correction**
Pro-inflammatory	IL-1β	−0.63	1.87	−0.31	<0.0001	<0.0001
	IL-6	−0.66	1.64	−0.29	<0.0001	0.0002
	TNF-α	−0.38	1.8	−0.41	<0.0001	0.0003
	IL-18	−0.89	0.95	0.36	0.002	0.016
	IL-8	−0.47	1.91	−0.47	<0.0001	<0.0001
Anti-inflammatory	IL-1 RA	−0.84	1.95	−0.04	<0.0001	0.0003
Vascular Endothelial	E-selectin	−0.69	1.51	0.05	0.0003	0.003
	P-selectin	0.49	−0.8	0.08	0.115	0.231
	VCAM-1	−0.6	0.47	0.29	0.119	0.119
	ICAM-1	−0.66	−0.09	0.34	0.104	0.311
	ICAM-3	0.09	−0.98	0.13	0.088	0.353
Vascular Angiogenic	VEGF	1.15	−0.46	−0.56	<0.0001	0.0007
Immunomodulatory	IL-10	−0.05	−1.04	0.44	0.022	0.114
	TGF-β	0.86	−0.42	−0.51	0.002	0.014
	Thrombomodulin	−1.25	0.41	0.56	<0.0001	0.0002

*One way ANOVA

Table 3

Mean Z-score of analyte expression by mode of delivery

Group	Analyte	Z score by delivery mode		P-Value*
		Vaginal	Cesarean section	
Pro-inflammatory	IL-1β	0.15	0.10	0.902
	IL-6	0.13	0.33	0.679
	TNF-α	0.11	−0.01	0.793
	IL-18	0.52	−0.15	0.134
	IL-8	0.03	0.11	0.877
Anti-inflammatory	IL-1 RA	0.38	0.58	0.580
Vascular Endothelial	E-selectin	0.27	0.37	0.843
	P-selectin	−0.22	0.16	0.323
	VCAM-1	0.32	0.24	0.841
	ICAM-1	0.36	−0.01	0.438
	ICAM-3	−0.08	0.25	0.495
	VEGF	−0.45	−0.60	0.633
Vascular Angiogenic	IL-10	0.29	−0.31	0.195
Immunomodulatory	TGF-β	−0.49	0.11	0.209
	Thrombomodulin	0.43	0.51	0.839

* T-test; the adjusted P value by Bonferroni multiple comparison test was $p = 1$ for all markers.