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Evidence of Perturbations of the Cytokine Network in Preterm Labor

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Abstract

Objective—Intra-amniotic infection/inflammation is the only mechanism of disease with persuasive evidence of causality for spontaneous preterm labor/delivery. Previous studies about the behavior of cytokines in preterm labor have been largely based on the analysis of the behavior of each protein independently. Emerging evidence indicates that the study of biological networks can provide insight into the pathobiology of disease, and improve biomarker discovery. The goal of this study is to characterize the inflammatory-related proteins network in the amniotic fluid in patients with preterm labor.

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Materials and Methods—A retrospective cohort study was conducted, and included women with singleton pregnancies who presented with spontaneous preterm labor and intact membranes (n=135). These patients were classified according to the results of amniotic fluid culture, broad-range polymerase chain reaction coupled with electrospray ionization mass spectrometry (PCR/ESI-MS), and amniotic fluid concentration of interleukin (IL)-6 into the following groups: 1) those without intra-amniotic inflammation (n=85); 2) those with microbial-associated intra-amniotic inflammation (n=15); and 3) those with intra-amniotic inflammation without detectable bacteria (n=35). Amniotic fluid concentrations of 33 inflammatory-related proteins were determined using a multiplex bead array assay.

Results—1) Patients with preterm labor and intact membranes who had microbial-associated intra-amniotic inflammation had a higher amniotic fluid inflammatory-related protein concentration correlation than those without intra-amniotic inflammation (113 perturbed correlations). IL-1β, IL-6, MIP-1α, and IL-1α were the most connected nodes (highest degree) in this differential correlation network (degree of 20, 16, 12, and 12, respectively); 2) patients with sterile intra-amniotic inflammation had correlation patterns of inflammatory-related proteins that were both increased and decreased when compared to those without intra-amniotic inflammation (50 perturbed correlations). IL-1α, MIP-1α, and IL-1β were the most connected nodes in this differential correlation network (degrees of 12, 10, and 7, respectively); and 3) there were more coordinated inflammatory-related protein concentrations in the amniotic fluid of women with microbial-associated intra-amniotic inflammation than in those with sterile intra-amniotic inflammation (60 perturbed correlations), with IL-4 and IL-33 having the largest number of perturbed correlations (degree of 15 and 13, respectively).

Conclusion—We report for the first time an analysis of the inflammatory-related protein network in spontaneous preterm labor. Patients with preterm labor who had microbial-associated intra-amniotic inflammation had more coordinated amniotic fluid inflammatory-related proteins than either those with sterile intra-amniotic inflammation or those without intra-amniotic inflammation. The correlations were also stronger in patients with sterile intra-amniotic inflammation than in those without intra-amniotic inflammation. The findings herein could be of value in the development of biomarkers of preterm labor.

Keywords

chemokine; prematurity; biomarker; chorioamnionitis; correlation network; intra-amniotic infection; interactome; network analysis; sterile inflammation

Introduction

Preterm birth is the leading cause of neonatal morbidity and mortality worldwide [1-7], and occurs after the spontaneous onset of preterm labor in two-thirds of cases [8]. Accumulating evidence suggests that preterm parturition is a syndrome caused by multiple pathologic processes [9, 10] including intrauterine infection [9-28], vascular disease [29-32], uterine overdistension [33-38], decline in progesterone action [39-43], breakdown of maternal-fetal tolerance [44-50], decidual senescence [51-53], and other pathologic processes yet to be discovered [54-60]. Of these, intra-amniotic infection (also termed microbial-associated intra-amniotic inflammation: presence of microorganisms in the amniotic cavity and intra-

amniotic inflammation) has been causally linked to spontaneous preterm delivery [18]. Indeed, at least one of every four preterm infants is born to a mother with an intra-amniotic infection that is largely subclinical [18].

The amniotic cavity is normally sterile, but microorganisms can gain access to the lower genital tract through an ascending pathway [10, 11, 18, 61], although other pathways have been proposed as well (hematogenous dissemination from distant sites, such as the oral cavity) [62-72]. Bacteria and their products can elicit an intra-amniotic inflammatory response after they are recognized by pattern recognition receptors [24, 73-78] and induce the production of cytokines [14, 27, 79-126] and chemokines [90, 93, 95, 97, 103, 104, 106, 113, 119, 126-145] and other inflammatory mediators including prostaglandins [146-152] and proteases [100, 105, 153-172].

Although intra-amniotic inflammation has traditionally been attributed to microorganisms and their products, such as lipopolysaccharide (LPS) [173, 174], lipoteichoic acid or peptidoglycans ([175], lipoglycans [25, 176-178], or others, it has now become clear that a subgroup of patients with intra-amniotic inflammation do not have microorganisms identified by cultivation methods or molecular microbiologic techniques to identify bacteria or viruses [179-185]. We have coined the term “sterile intra-amniotic inflammation” to refer to this condition.

Previous studies about the behavior of cytokines in spontaneous labor at term and preterm labor have been based on data derived from bioassays for these molecules, and specific individual immunoassays [27, 98, 105, 182-184, 186-192]. Since biological functions are the expression of integrated and interdependent networks of cells and molecules [193-197], the study of biological networks, rather than individual cells/molecules, is considered necessary to improve the understanding of the pathophysiology of disease [193-197]. The objective of this study was to characterize the behavior of the inflammatory-related protein network in the amniotic fluid of women in preterm labor, according to the presence/absence of intra-amniotic inflammation and microorganisms in the amniotic cavity.

Materials and Methods

Study population

A cohort of women with singleton pregnancies who presented with spontaneous preterm labor and intact membranes (n=135) was selected from the clinical database and Bank of Biological Samples maintained by Wayne State University, the Detroit Medical Center, and the Perinatology Research Branch of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD). The inclusion criteria were: 1) singleton gestation; 2) transabdominal amniocentesis performed between 20 and 35 weeks of gestation prior to the rupture of the chorioamniotic membranes; 3) absence of chromosomal or structural fetal anomalies; and 4) sufficient amniotic fluid for molecular microbiologic studies. These patients were included in prior studies which provide descriptions of microbiologic studies, amniotic fluid IL-6 concentration, and high mobility group box-1 (HMGB-1) [184]. Each patient provided written informed consent, and the use of biological specimens and clinical data for research purposes was approved by the Institutional Review Boards of NICHD and Wayne State University.

Clinical definitions—Microbial invasion of the amniotic cavity (MIAC) was defined according to the results of AF culture and PCR/ESI-MS (Ibis® Technology - Athogen, Carlsbad, CA) [179, 180, 198, 199]. Intra-amniotic inflammation was diagnosed when the AF IL-6 concentration was ≥ 2.6 ng/ml [27, 98, 105, 181-184, 186, 187, 189, 190]. Based on the results of AF cultures, PCR/ESI-MS and AF concentrations of IL-6, patients with preterm labor with intact membranes were classified into three groups: Group 1 included those without intra-amniotic inflammation (n=85); Group 2 consisted of those with microbial-associated intra-amniotic inflammation (combination of MIAC and intra-amniotic inflammation) (n=35); and Group 3 included those with intra-amniotic inflammation without detectable microorganisms (an elevated AF IL-6 concentration without evidence of microorganisms in the amniotic cavity using both cultivation and molecular methods) (n=15). The patients with the presence of microorganism in the amniotic cavity but without intraamniotic inflammation were classified into Group 3 (no intraamniotic inflammation) since the presence of such microorganisms may represent contamination.

Spontaneous preterm labor was diagnosed by the presence of at least two regular uterine contractions every 10 minutes associated with cervical changes in patients with a gestational age between 20 and 36 6/7 weeks. Preterm delivery was defined as birth prior to the 37th week of gestation.

Multiplex determination of inflammatory-related proteins

Amniotic fluid concentrations of 33 inflammatory-related proteins were determined using a multiplex bead array assay developed by the investigators (see Table 1 for the complete list of analytes). The mediators are cytokines (chemokines are a subset of cytokines), and we also included the prototypic alarmin, HMBG-1, which is elevated in cases of sterile intra-amniotic inflammation [184] calgranulin A and C [200], which are anti-microbial peptides, and the anti-microbial protein, lactoferrin [201]. All capture antibodies were purchased from R&D Systems (Minneapolis, MN) with the exception of the capture antibodies for IL-4 and IL-10 (Biolegend, San Diego), IL-12 p70 (Becton Dickinson, New Jersey), IL-18 (eBiosciences, San Diego), Lactoferrin (Abcam, Massachusetts). Individual Luminex bead sets (Luminex, Riverside, CA) were coupled to inflammatory-related protein -specific capture antibodies according to the manufacturer's recommendations. Conjugated beads were washed and kept at 4°C until use. The standards for each analyte were purchased from R&D systems [with the exception of Calgranulin A (USBiological, Massachusetts), HGMB-1 and Lactoferrin (Abcam, Massachusetts)], and resuspended at concentrations ranging from 50 μg/ml to 8 ng/ml and diluted serially 1:3 to generate standard curves. Detection antibodies were purchased from R&D Systems as biotinylated affinity purified goat polyclonal antibodies, or from BioLegend (IL-10), Becton Dickinson (IL-12), eBiosciences (IL-18), Abcam (Lactoferrin) and ThermoFisher (HGMB-1). Biotinylated detection antibodies were used at twice the concentrations recommended for a classical ELISA. All assay procedures were performed in assay buffer containing PBS supplemented with 1% normal mouse serum (GIBCO BRL), 1% normal goat serum (GIBCO BRL), and 20 mM Tris-HCl (pH 7.4). The assays were run using 2000 beads per set of each of 33 inflammatory-related proteins measured per well in a total volume of 50μL. Samples were diluted in assay buffer and run in duplicates at two dilutions 1:2 and 1:32. A total of 50μL of each amniotic fluid sample

was added to the well and incubated overnight at 4°C in a Millipore Multiscreen plate (Millipore, Billerica, MA). The liquid was then aspirated using a BioPlex Pro II plate washer (Bio-rad, Hercules, CA), and the plates were washed twice with 200μL of assay buffer. The beads were then resuspended in 50μL of assay buffer containing biotinylated polyclonal antibodies against the measured inflammatory-related proteins for 30 minutes at room temperature. The plates were washed twice with PBS, the beads were resuspended in 50μL of assay buffer, and 50μL of a 16 μg/mL solution of streptavidin-PE (Molecular Probes, Eugene, OR) was added to each well. The plates were read on a Luminex-100 platform. For each bead set of the 33 tested, a minimum of 100 beads was collected. The median fluorescence intensity of these beads was recorded for each bead and was used for analysis with the Bioplex Manager software (version 6.1; Bio-Rad) using a 5-parameter (5P) regression algorithm. The assay characteristics are described in Table 1.

Statistical analysis

The goal of the statistical analysis was to: 1) assess the differences in analyte concentration among groups; 2) determine if pairwise analyte correlations were different among groups; and 3) build the network of significantly perturbed correlations and identify highly connected nodes and network modules.

Demographics data analysis—The Kolmogorov-Smirnov test was used to test whether the distribution of continuous variables was normal. Chi-square or Fisher's exact tests were used for comparisons of proportions. Kruskal-Wallis and the Mann-Whitney U tests were used to compare median concentrations of analytes between and among groups. Statistical analysis of demographics data was performed using SPSS 19 (IBM Corp, Armonk, NY, USA). A p value < 0.05 was considered statistically significant.

Analysis of the difference in concentrations among groups—Analyte concentration data was log (base 2) transformed to improve normality of the data distribution. To test for differential analyte concentration between groups, a linear model was fit to the analyte concentration using the group indicator (e.g. no intra-amniotic inflammation vs. sterile intra-amniotic inflammation) and gestational age as predictors. Significant p values for the group coefficient were adjusted using the Benjamini & Hochberg method over all 33 analytes to compute q-values [202]. Significance of differences in concentration was determined based on a q-value <0.1 and fold change >1.5.

Differential correlation analysis—The goal of this analysis was to test whether the correlation of concentrations between each possible pair of analytes (e.g. IL-1α and IL-6; IL-1α and IL-33, etc.) was different among groups, while adjusting for the effect of gestational age. Adjustment for gestational age was performed to account for differences in the duration of pregnancy at the time of amniocentesis between groups. A linear model was fit to the log transformed data of each analyte as a function of gestational age using samples in each group separately. The residuals (actual value – fitted value) were then used to compute Pearson correlations for each pair of analytes within each group of patients. Since these correlations were determined from data adjusted for a covariate (gestational age), these correlations are also called partial correlations. To test for differences in partial correlations

between groups, the partial correlations were first converted into an intermediary variable z, using Fisher's transformation. Under the null hypothesis (partial correlations are equal between groups) the standardized differences in z values between groups were assumed to follow a standard normal distribution. Significant differences in partial correlations were considered to be present when the p-value was <0.01, and the magnitude of correlation differences was at least 0.2. The rationale for using more stringent criteria is that when testing 528 differential correlations simultaneously, one would expect in average 528 × 0.05=26.4 positive differential correlation due to chance alone (false positives). When using a criteria of p<0.01, the number of false positives would be reduced to 5 (528 × 0.01). The additional requirement that the magnitude of differential correlation be >0.2 reduces even further the number of false positives, as differential correlations with higher magnitude are less likely to be observed due to chance alone

Network analysis—A network was constructed for each between-group comparison (e.g. sterile intra-amniotic inflammation vs. no intra-amniotic inflammation) by linking/connecting the analytes with a significantly different correlation between the respective groups. For each node (analyte) in the network, we calculated the *degree* and the *average absolute difference in correlations*. While the first metric gives the number of links (significantly perturbed correlations) of a given node to all others, the latter describes the typical between-groups change in correlation (regardless of direction). The network was further analyzed to identify modules (groups of analytes) so that analytes (network nodes) within modules are more connected with others within the same module than would be expected by chance [203].

Results

Demographic characteristics

The characteristics of the study population stratified by the presence or absence of microorganisms in the amniotic cavity and intra-amniotic inflammation were the subject of a detailed report in this Journal [184]. Briefly, the frequencies of sterile intra-amniotic inflammation, microbial-associated intra-amniotic inflammation, and no intra-amniotic inflammation were 26% (35/135), 11% (15/135), and 63% (85/135), respectively. The most frequent microorganisms identified in the amniotic cavity were *Ureaplasma spp.* Patients with sterile and microbial-associated intra-amniotic inflammation had significantly lower median gestational age at delivery (interquartile range: IQR) than those without intra-amniotic inflammation [25 (23-32) weeks, 26 (23-32) weeks vs. 32 (29-33) weeks; each p< 0.001] (Table 2). There was no significant difference in the median gestational age at delivery between patients with microbial-associated intra-amniotic inflammation and those with sterile intra-amniotic inflammation (p=0.6) (Table 2). The amniotic fluid inflammatory response [IL-6, white blood cell count (WBC)] was significantly greater in microbial-associated intra-amniotic inflammation than in sterile intra-amniotic inflammation [amniotic fluid IL-6 median (IQR): microbial-associated intra-amniotic inflammation of 96 (17-266) ng/ml vs. sterile intra-amniotic inflammation: 12 (5-21) ng/ml; p < 0.001; median WBC counts (IQR): 295 (2-960) cell/mm^3 vs. 3 (1-17) cell/mm^3, p=0.002] (Table 2). Our study was conducted before the publications of studies reporting that vaginal progesterone reduces

the rate of preterm delivery and neonatal morbidity, and thus, none of our patients received vaginal progesterone.

Inflammatory-related protein concentrations among subgroup of preterm labor with intact membranes

Sterile intra-amniotic inflammation vs. no intra-amniotic inflammation—The geometric mean amniotic fluid concentration for all 33 inflammatory-related analytes was higher in patients with sterile intra-amniotic inflammation than in those without intra-amniotic inflammation (fold change range 1.1-11.4). The differences were significant for 27 of the 33 analytes (q-value<0.1 and fold change >1.5; Table 3). The largest fold changes were observed for IL-6 and IL-8 (10.6 and 11.4, respectively).

Microbial-associated intra-amniotic inflammation vs. no intra-amniotic inflammation—The geometric mean amniotic fluid concentration for all 33 inflammatory-related analytes was significantly higher in patients with microbial-associated intra-amniotic inflammation than in those without intra-amniotic inflammation (q value < 0.1, and fold change > 1.5; Table 3). IL-6, IL-8, and MIP-1β had the largest magnitude of change (fold changes: 115.4, 106, and 64.8, respectively) (Table 3).

Microbial-associated intra-amniotic inflammation vs. sterile intra-amniotic inflammation—Patients with microbial-associated intra-amniotic inflammation had higher concentrations of inflammatory-related proteins than those with sterile intra-amniotic inflammation, with fold changes that ranged from 2.3-12.3 for all 33 analytes (Table 3). The fold changes for MIP-1β, MIP-1α, IL-1β, IL-6, and IL-8 were approximately 10 (Table 3).

Differential correlation and network analysis

Differences in the correlation patterns of pairs of analytes were assessed among all three groups of patients (See Figure 1A) for an illustration. Below are the detailed results of this analysis for each pairwise comparison.

Sterile intra-amniotic inflammation vs. no intra-amniotic inflammation—The inflammatory-related protein differential correlation network between the sterile intra-amniotic inflammation and no intra-amniotic inflammation groups is displayed in Figure 2A. Of the 33 analytes, 28 had at least one correlation that was significantly perturbed in patients with of sterile intra-amniotic inflammation compared to that of patients without intra-amniotic inflammation. The perturbed correlations between pairs of analytes are represented by lines in Figure 2. Among 50 perturbed correlations shown in this figure, 33 were positive (increased correlation) and 17 were negative (decreased correlation). The number of perturbed correlations (*degree*) was the largest for IL-1α, MIP-1α, and IL-1β (12, 10, and 7, respectively). The average absolute difference in correlation ranged from 0.28 (for HMGB-1) to 0.61 (Calgranulin-A) which indicates a considerable magnitude of differential correlation between groups. For example, the correlation between IL-1β and MIP-1α was −0.28 (p=0.01) in the group without intra-amniotic inflammation, but it was reversed to 0.61 (p<0.001) in the group with sterile intra-amniotic inflammation (absolute difference in correlation of 0.61-(−0.28) =0.89, p<0.001) (Supplementary File 1). Of the three modules

identified in this network, the MIP-1α and IL-1β module included analytes with larger differences in concentration and higher degree, than those in the other two modules. IL-1α and CXCL-9/MIG had the largest degree in the second and third modules, respectively.

Microbial-associated intra-amniotic inflammation vs. no intra-amniotic inflammation—The network of inflammatory-related protein differential correlations between patients with microbial-associated intra-amniotic inflammation and those without intra-amniotic inflammation is shown in Figure 2B. Of the 33 analytes, 31 had at least one correlation that was significantly perturbed in microbial-associated intra-amniotic inflammation, compared to no intra-amniotic inflammation. Similar to the comparison between sterile intra-amniotic inflammation and no intra-amniotic inflammation, IL-1β and MIP-1α belonged to the same module, while IL-1α belonged to a different module. However, in contrast to the comparison between the group with sterile intra-amniotic inflammation and the group without intra-amniotic inflammation, the degree of IL-1β was the largest in this network (degree=20). The total number of perturbed correlations (n=113) in this comparison (microbial-associated intra-amniotic inflammation vs. no intra-amniotic inflammation) was larger than in the previous comparison (n=50; sterile intra-amniotic inflammation vs. no intra-amniotic inflammation; Figure 2A). All perturbed correlations were increased in this comparison (as denoted by the red lines in Figure 2B), while this was only the case for some of the perturbed correlations in the contrast described in Figure 2A.

Microbial-associated intra-amniotic inflammation vs. sterile intra-amniotic inflammation—There were 60 perturbed (all increased) correlations in microbial-associated intra-amniotic inflammation compared to sterile intra-amniotic inflammation (Figure 2C). IL-4 and IL-33 had the largest number of disrupted correlations (degrees are 15 and 13, respectively), each of these two analytes belonging to a different module.

Since the frequency with which patients received glucocorticoids in the group with sterile intra-amniotic inflammation was lower than in the other two groups (see Table 1), we determined whether steroid administration could have been a confounder in the differential expression and differential correlation analyses. Since no significant association was found between analyte concentration and steroid administration in any of the three groups, we concluded that steroid administration was not a confounding factor in these analyses.

Discussion

Principal findings of the study

1) Patients with preterm labor and intact membranes who had microbial-associated intra-amniotic inflammation had a higher amniotic fluid inflammatory-related protein concentration correlation than those without intra-amniotic inflammation. IL-1β, IL-6, MIP-1α, and IL-1α were highly connected nodes (highest degree) in this differential correlation network; 2) patients with sterile intra-amniotic inflammation had correlation patterns of inflammatory-related proteins that were both increased and decreased when compared to those without intra-amniotic inflammation. IL-1α, MIP-1α, and IL-1β were the most connected nodes in this differential correlation network; and 3) there were more coordinated inflammatory-related protein concentrations in the amniotic fluid of women

with microbial-associated intra-amniotic inflammation than in those with sterile intra-amniotic inflammation. IL-4 and IL-33 had the largest number of perturbed correlations with other inflammatory-related proteins in the differential correlation network. These observations provide evidence that the inflammatory-related protein network behaves differently in women with preterm labor according to the presence or absence of intra-amniotic inflammation and/or microorganisms.

The study of protein networks in health and disease

Cytokines are organized in complex and redundant networks. Therefore their study requires a global analysis of the entire network rather than a catalogue of changes in concentrations of individual cytokines. Such a network analysis was developed in the current study for cytokine network in preterm labor with intact membranes. In biology, networks can be used to represent knowledge about genes and gene products by providing a flexible and detailed description of biological functions and gene interactions. For instance, the Gene Ontology [204] vocabulary assigns genes and gene products to molecular functions, biological processes and cellular components, and can be depicted as a graph in which the set of genes annotated to a certain term (node) is a subset of those annotated to its parent nodes. Similarly, the Kyoto Encyclopedia of Genes and Genomes (KEGG) [205] maintains manually-drawn pathway maps representing knowledge on the molecular interaction networks for metabolism, cellular processes, human disease, etc. The advantage of representing such knowledge in graph format, rather than as lists of gene products, is to allow for improved interpretation of expression changes from omics studies. For example, some pathway analysis methods based on observed gene expression changes between a disease and control group would treat differential expression of interconnected genes in the pathway as more relevant in terms of the relationship to the disease than the differential expression of non-connected genes.

The study of biological networks is now emerging as an important discipline: network medicine, due to the availability of omics data [206]. RNA and protein expression profiles across samples are often highly correlated and their pairwise relations can be conveniently described using a network representation. The term “interactome” is often used to describe all physical interactions among cellular components. By necessity, knowledge of the interactome is incomplete at this time; however, there is evidence that disease-disease relationships can be uncovered by exploring an incomplete interactome [197]. Molecules with similar expression patterns may form complexes, belong to the same pathways, or participate in regulatory and signaling circuits. A “guilt-by-association” approach can be used to guide the typical differential expression analysis task by not relying exclusively on gene level differential expression statistics, but also considering the evidence from neighboring genes in the co-expression network [207]. Similarly, outcome prediction from high-dimensional gene expression data can benefit from network analyses by replacing each highly connected network module with a module representative meta-molecule, hence reducing redundancy and improving prediction performance [206, 208].

The study of co-expression networks of molecules profiled with low- or high-throughput technologies has been proposed as a complementary approach to the simple description of

differential abundance/expression analyses between conditions and clinical states [209-211]. Information extracted from correlation network analysis has been shown to improve disease classification [212-217] and identify potential therapeutic targets [218-223]. For instance, assessing the differences in network organization between patients with breast cancer who were alive after follow-up versus those who died from disease allowed identification of key hub genes (highly connected nodes) whose predictive performance was better than the one derived from commercially available genomic breast cancer diagnostic tests [216]. Moreover, candidate oncogenes have emerged from studies of gene regulatory networks [215]. Studies of the cytokine co-expression network in plasma has provided new insights into the pathophysiology of chronic fatigue syndrome by discovering distinct cytokine communities or modules recognizable as pre-programmed immune functional components in this disease [210]. The current repositories of biological pathways have been derived from research conducted on adult subjects or in cultured cells, and hence are not specific to the biology of pregnancy or parturition. Therefore the discovery of functionally-related network modules allows expanding upon existing repositories of biological pathways (e.g. KEGG, Reactome [224]), enabling determination of the connection between different diseases and conditions.

In conclusion, network analysis can be used to: 1) prioritize differentially expressed molecules by identifying those with very different correlation environments between conditions, and 2) characterize functionally relevant modules of such molecules. Moreover, the disruption of normal correlations between cytokine may be a pathological factor itself, apart from the changes in concentration of individual cytokines that can be negligible.

Inflammatory-related protein network connectivity in microbial-associated intra-amniotic inflammation

Patients with preterm labor and intact membranes who had microbial-associated intra-amniotic inflammation had increased correlations of inflammatory-related proteins in the amniotic cavity when compared to either those without intra-amniotic inflammation or with sterile intra-amniotic inflammation. Previous studies of protein network analysis show that tracking local network changes can be useful in prioritizing candidate proteins, compared to traditional differential expression analysis on individual protein [182-184]. In the current study, amniotic fluid IL-1β, IL-6, MIP-1α, and IL-1α had the strongest change in expression coordination with other inflammatory proteins, while IL-6, IL-8, and MIP-1β were the top ranked proteins when the absolute concentrations among groups were examined. Therefore, the information obtained from a simple comparison of mean concentrations is different from that derived from the differential correlation analysis.

IL-1β [225-228], IL-6 [229-231], MIP-1α [232-238], and IL-1α [239, 240] are potent pro-inflammatory cytokines which can be upregulated by microbial and non-microbial products. Such proteins are produced by different cells such as macrophages, lymphocytes, neutrophils, dendritic cells, etc. Upregulation of expression occurs during leukocyte recruitment, production of prostaglandins and matrix degrading enzymes, all of which have been implicated in the mechanisms of labor [18, 24, 241]. IL-1β was the first cytokine discovered to play a role in preterm labor associated with intra-amniotic infection [79, 82, 242]. We and other investigators

have reported that amniotic fluid concentrations of IL-1β [79, 81, 94, 109, 243, 244], IL-6 [14, 86-88, 94, 95, 109, 189, 244, 245], MIP-1α [113, 138, 140, 246], and IL-1α [81, 83, 242, 247] were significantly higher in women with preterm labor who had intra-amniotic infection than in those who did not.

The cytokine network is complex, and the nature of the interactions among its members depends on many factors, such as the cell types, disease states (normal vs. sterile inflammation vs. infection), duration of the insult, and experimental models (*in vitro* vs *in vivo*) [248]. For example, IL-1β can upregulate the gene expression of TNFα, IL-1β, IL-6, MIP-1α, and IL-8, while it down-regulates TGF-β1 gene expression [227, 240, 249]. Our observations show that the inflammatory-related protein network connectivity in women with microbial-associated intra-amniotic inflammation is denser and coordinated than in those with sterile inflammation or without intra-amniotic inflammation. We argue that network analysis provides deeper insight into understanding the pathophysiological mechanisms of intra-amniotic infection/inflammation in preterm labor, as well as identifying potentially relevant modules of cytokines that correspond to distinct disease pathways in preterm labor. Also, in practice, such an approach may help to minimize the number of individual cytokines measured in order to characterize pathologic states, since some elements of the network may have key roles in the regulation of the entire network/modules.

Inflammatory-related protein network connectivity in sterile intra-amniotic inflammation

We have previously reported that sterile intra-amniotic inflammation was present in a subset of patients with preterm labor with intact membranes [182, 184], preterm prelabor rupture of membranes [181], a sonographic short cervix [183], and clinical chorioamnionitis at term [185]. Sterile intra-amniotic inflammation is clinically significant because it is a risk factor for impending preterm delivery and neonatal morbidity [183, 184].

Inducers of sterile intra-amniotic inflammation in preterm labor remain to be determined. “Danger signals” resulting from cellular stress or necrotic cells may engage damage-associated molecular patterns (DAMPs), which can, in turn, activate signals such as the receptor for advanced glycation end products (RAGE) and stimulate an intra-amniotic inflammatory response [186, 250, 251]. The concentrations of the prototypic alarmin, high mobility group box-1 (HMGB-1) [184], is higher in patients with sterile intra-amniotic inflammation than in those without intra-amniotic inflammation. Moreover, among women with sterile inflammation, those with a concentration of HMGB-1 > 8.55 ng/mL have a shorter interval to delivery than those with lower concentrations of this alarmin [184], suggesting that alarmins are involved in this condition. Indeed, IL-1α, an alarmin previously reported in amniotic fluid, can induce labor in pregnant animals [83, 242, 247]. A role for the inflammasome in parturition and preterm labor has been recently proposed [252-254].

In the current study, amniotic fluid IL-6 and IL-8 were the top two ranked inflammatory-related proteins in the differential expression analysis. Yet, in the differential network analysis, amniotic fluid IL-1α, MIP-1α, and IL-1β are the main cytokines involved in sterile intra-amniotic inflammation. Interestingly, the amniotic fluid IL-1α concentration changed only moderately (fold change=1.7; q-value=0.06). IL-1α is a dual-function cytokine constitutively expressed in resting cells under homeostatic condition [239, 255-257]. This

cytokine plays a major role in sterile inflammation, as it exclusively secreted during necrosis as an alarm signal and is part of the danger-associated molecular patterns (DAMPs) model [239, 258-262]. The administration of IL-1α to pregnant animals can induce preterm labor and delivery, an effect that is abrogated by pre-treatment with the IL-1 natural receptor antagonist [83]. The finding that IL-1α is a key cytokine derived from network analysis in patients with sterile intra-amniotic inflammation supports the concept that top ranked proteins derived from network connectivity analysis are informative in premature labor.

Network analysis of sterile vs. microbial-associated intra-amniotic inflammation

The differential correlation network analysis between microbial-associated and sterile intra-amniotic inflammation identified IL-4 and IL-33 as highly connected nodes (highest degree). Similarly to IL-1α, IL-33 is a "dual-function" cytokine with both a nuclear factor and an "alarmin" activity [263, 264]. It is released predominantly during cell injury and can activate cells of both the innate and adaptive immune system (i.e. T cells, B cells, macrophages, mast cells) on a manner that is context dependent [263-266]. IL-33 is the ligand for ST2 [267]. Upon binding to ST2L, IL-33 is capable to produce Th2 associated cytokines [267-271]. We have previously reported that the median amniotic fluid soluble ST2 concentration and mRNA expression of ST2 in chorioamniotic membranes were lower in preterm labor with intra-amniotic infection and acute histologic chorioamnionitis than in those without these conditions, respectively [272]. Moreover, umbilical cord sST2 concentrations are 6.7 fold higher in neonates with FIRS than in those without FIRS [273]. These observations combined with the results from the current study suggest that IL-33 plays a role in microbial-associated intra-amniotic inflammation.

IL-4 is a multifunctional pleiotropic cytokine [274, 275], and mediates the Th2 immune response [274-282]. It is known to be important for the regulation of cell proliferation, apoptosis and gene expression in various cells such as lymphocytes, macrophages and endothelial cells [274-279, 281, 282]. It has been reported that amniotic fluid [283] and maternal plasma IL-4 concentrations [284] are significantly higher in patients with preterm labor and chorioamnionitis than in those without these complications. The role of IL-4 in preterm labor is incompletely understood, and our findings justify further studies.

Conclusion

We report for the first time the analysis of the inflammatory-related protein network in the amniotic fluid of patients with spontaneous preterm labor and intact membranes. Patients with microbial-associated intra-amniotic inflammation had a more coordinated amniotic fluid inflammatory-related protein network than either those with sterile intra-amniotic inflammation or absent intra-amniotic inflammation. The inflammatory-related protein network was also denser in patients with sterile intra-amniotic inflammation than in those without intra-amniotic inflammation. IL-1α was the top-ranked protein derived from this approach, and is involved in sterile intra-amniotic inflammation. Our findings support the concept that the analysis of correlation patterns provides an alternative method to prioritize candidate proteins as disease biomarkers, and improves the understanding of disease mechanisms. A perturbation of the relationship among cytokines/inflammatory related

proteins in normal pregnancy may be an important factor in complications of pregnancy, even if the absolute changes in concentrations of particular cytokines/inflammatory related proteins are small. Future studies are required to determine whether candidate proteins derived from this approach can improve diagnostic/classification performance in the preterm labor syndrome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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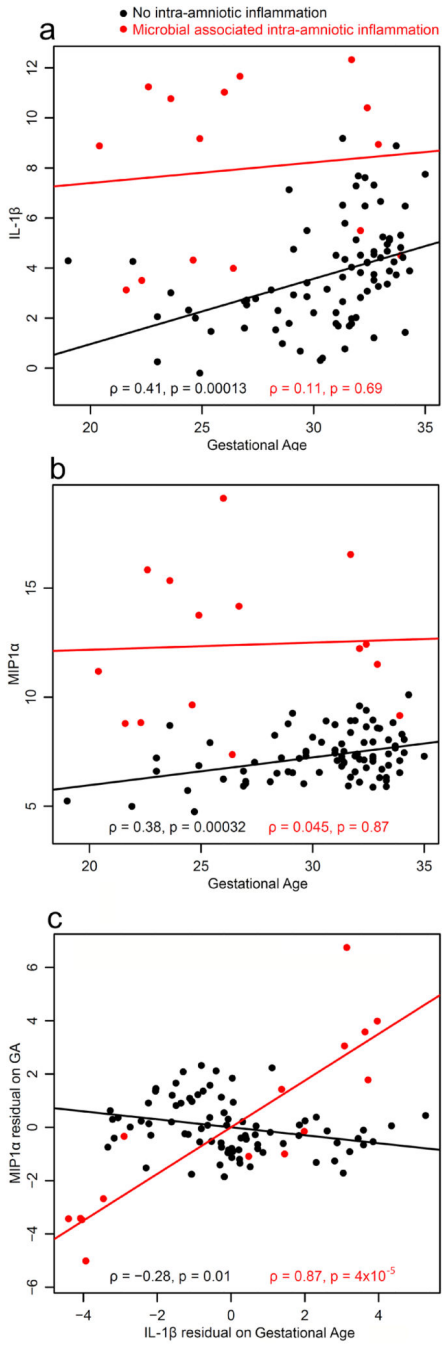

Figure 1.

Differential correlation analysis. The figure shows $\log_2$ concentration (pg/ml) of IL-1β (left panel) and MIP1α (middle panel) as a function of gestational age at amniocentesis in patients with microbial-associated intra-amniotic inflammation (red) and those without intra-amniotic inflammation (black). A linear model was fit to the $\log_2$ concentration of each analyte as a function of gestational age in each group and residuals were used to compute partial correlations between analytes (right panel). The partial correlation of residuals was positive and significant in the microbial-associated intra-amniotic inflammation group but

negative and significant in patients without intra-amniotic inflammation, resulting in a significant differential correlation between groups.

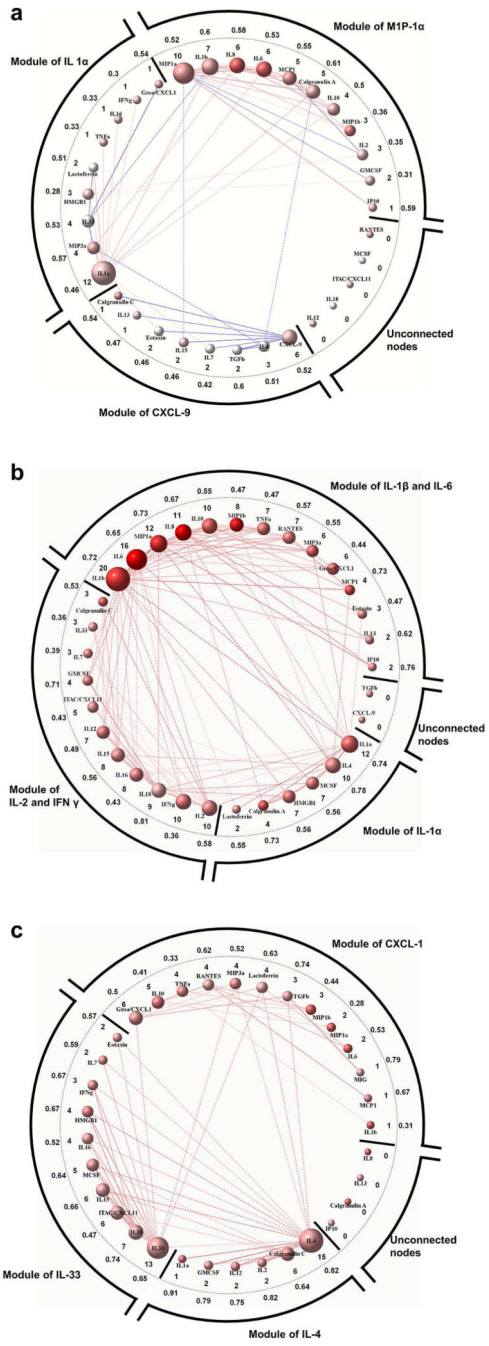

Figure 2.

Network of perturbed inflammatory related protein concentration correlations between groups of preterm labor with intact membranes. Each node (sphere) represents one of the 33 analytes, with a link (line) between two nodes representing a significantly perturbed correlation. The node color represents the direction of concentration change (red=increased; blue=decreased; white=no change in the first group compared to the second/reference group of the comparison). The color of links gives the direction of correlation change (red = increased correlation; blue = decreased correlation) while the type of line denotes the nature

of the link (solid line= within module link; dashed line= cross-module link). Thick radial lines separate the modules as well as the set of unconnected nodes, as labeled in the Figure. The numbers inside/outside the dotted black circle represent the node degree/average absolute difference in correlations.

A: Network of perturbed inflammatory-related protein concentration correlations between sterile intra-amniotic inflammation and no intra-amniotic inflammation.

B: Network of perturbed inflammatory-related protein concentration correlations between microbial-associated intra-amniotic inflammation and no intra-amniotic inflammation.

C: Network of perturbed inflammatory-related protein concentration correlations between microbial-associated intra-amniotic inflammation and sterile intra-amniotic inflammation.

Table 1

Analytes and their detection ranges

Analyte	Lower limit of detection (pg/mL)
IL-1α	0.98
IL-1β	0.88
IL-2	3.49
IL-4	30
IL-6	0.37
IL-7	0.37
IL-8	0.95
IL-10	0.37
IL-12	0.37
IL-13	0.37
IL-15	0.37
IL-16	20
IL-18	0.34
IL-33	88
Calgranulin A	25.4
Calgranulin C	1128
Eotaxin	0.27
GM-CSF	0.27
GRO-α	72.7
HMGB-1	6000
IFN-γ	6.60
IP-10	237
I-TAC	8.50
Lactoferrin	6.39
M-CSF	0.99
MCP-1	2.81
MIG or CXCL-9	64.2

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Analyte	Lower limit of detection (pg/mL)
MIP-1α	10.661
MIP-1β	10.661
MIP-3α	5513
RANTES	2.758
TGF-β	7.422
TNF-α	0.818

IFN-γ: interferon gamma; IL: interleukin; IP-10: interferon gamma-induced protein 10; GM-CSF: granulocyte macrophage colony-stimulating factor; MCSF: macrophage colony-stimulating factor, Gro-α/CXCL1: C-X-C motif ligand 1; HMGB-1: high-mobility group protein 1; ITAC/ CXCL11: Interferon-inducible T-cell alpha chemoattractant/C-X-C motif ligand 11; MDC: macrophage-derived chemokine; MIP: macrophage inflammatory protein; MIG: Monokine induced by gamma interferon; MCP: monocyte chemoattractant protein-1; RANTES: regulated on activation, normal T cell expressed and secreted; TNF: tumor necrosis factor, TARC: thymus and activation-regulated chemokine

Table 2

Clinical and demographic characteristics of the study population.

	No intra-amniotic inflammation (n=85)	Sterile intra-amniotic inflammation (n=35)	p value (no intra-amniotic inflammation vs. sterile intra-amniotic inflammation)	Microbial-associated intra-amniotic inflammation (n=15)	p value (no intra-amniotic inflammation vs. microbial associated intra-amniotic inflammation)	p value (sterile intra-amniotic inflammation vs. microbial associated intra-amniotic inflammation)
Maternal age (years)	23 (20 – 26)	23 (20 – 26.2)	0.8	24 (20 – 30)	0.7	0.4
BMI (kg/m^2)	23 (20 – 29)	23 (20 – 32)	0.6	27 (23 – 37)	0.04	0.2
Frequency of sonographic short cervix	16.5% (14/85)	11.8% (10/85)	0.04	20% (3/15)	0.35	0.9
Antenatal corticosteroid administration	45% (37/82)*	20% (7/35)	0.012	46.7% (7/15)	1	0.06
Gestational age at amniocentesis (weeks)	32 (29 – 33)	25 (23 – 32)	<0.001	26 (23 – 32)	0.006	0.83
AF white blood cells (cells/mm^3)	1 (0 – 5)	3 (1 – 17)	0.007	295 (2 – 960)	<0.001	0.018
AF glucose (mg/dL)	29 (24 – 34)	22 (18 – 28)	0.001	11 (10 – 20)	<0.001	0.002
AF interleukin-6 (ng/mL)	0.8 (0.5 – 1.1)	12 (5 – 21)	<0.001	96 (17 – 266)	<0.001	<0.001
Gestational age at delivery (weeks)	36 (34 – 38)	27 (24 – 32)	<0.001	26 (24 – 33)	<0.001	0.64
Composite neonatal morbidity	11% (9)	68% (24)	<0.001	67% (10)	<0.001	1.0
Acute placental inflammation¥	22.5% (18/80)	61% (19/31)	<0.001	79% (11/14)	<0.001	0.14
Acute histologic chorioamnionitis	21% (17/80)	58% (18/31)	<0.001	79% (11/14)	<0.001	0.04
Funisitis	13% (10/80)	29% (9/31)	0.06	57% (8/14)	<0.001	0.26

Data presented as median (interquartile) and percentage and (n); AF: amniotic fluid; BMI: body mass index. Acute placental inflammation: acute histologic chorioamnionitis and/or acute funisitis, Composite neonatal morbidity: the presence of respiratory distress syndrome, bronchopulmonary dysplasia, grade III or IV intraventricular hemorrhage, periventricular leukomalacia, proven neonatal sepsis, and necrotizing enterocolitis or perinatal mortality.

¥Placenta acute inflammation was calculated over a total of 125 specimens.

*Data were not available in 3 patients

Table 3

Amniotic fluid inflammatory-related protein concentrations in subgroups of patients with preterm labor and intact membranes

	Sterile intra-amniotic inflammation vs. No intra-amniotic inflammation			**Microbial associated intra-amniotic inflammation vs. No intra-amniotic inflammation**			**Microbial associated intra-amniotic inflammation vs. Sterile intra-amniotic inflammation**		
Proteins	**Fold Change**	**p-value**	**q-value**	**Fold Change**	**p-value**	**q-value**	**Fold Change**	**p-value**	**q-value**
IL-8	11.4	0.000	0.000	106.0	0.000	0.000	9.3	0.000	0.000
IL-6	10.6	0.000	0.000	115.4	0.000	0.000	10.9	0.000	0.000
MIP1-β	5.3	0.000	0.000	64.8	0.000	0.000	12.3	0.000	0.000
MCP-1	3.8	0.000	0.000	18.5	0.000	0.000	4.8	0.000	0.000
MIP1-α	3.4	0.000	0.000	39.8	0.000	0.000	11.6	0.000	0.000
Calgranulin C	3.1	0.000	0.000	12.0	0.000	0.000	3.9	0.000	0.000
IL-1β	2.8	0.002	0.006	30.6	0.000	0.000	11.1	0.000	0.000
RANTES	2.5	0.001	0.002	6.6	0.000	0.000	2.6	0.010	0.010
MIP3-α	2.5	0.000	0.000	10.9	0.000	0.000	4.4	0.000	0.000
Gro-α/CXCL1	2.4	0.000	0.000	8.7	0.000	0.000	3.6	0.000	0.000
Calgranulin A	2.1	0.003	0.007	15.2	0.000	0.000	7.2	0.000	0.000
IL-10	2.1	0.001	0.004	12.9	0.000	0.000	6.3	0.000	0.000
MIG	1.8	0.003	0.007	4.6	0.000	0.000	2.5	0.001	0.001
ITAC/CXCL11	1.8	0.000	0.001	4.6	0.000	0.000	2.6	0.000	0.000
IP-10/CXCL-10	1.8	0.020	0.028	4.0	0.000	0.000	2.3	0.017	0.017
IL-1α	1.7	0.047	0.060	11.0	0.000	0.000	6.6	0.000	0.000
IL-12	1.7	0.010	0.019	6.6	0.000	0.000	3.9	0.000	0.000
TNF-α	1.7	0.008	0.016	7.7	0.000	0.000	4.6	0.000	0.000
IL-16	1.6	0.003	0.007	5.3	0.000	0.000	3.2	0.000	0.000
IL-2	1.6	0.037	0.051	5.3	0.000	0.000	3.3	0.000	0.000
IL-13	1.6	0.013	0.023	4.6	0.000	0.000	2.9	0.000	0.000
IL-15	1.6	0.014	0.023	4.4	0.000	0.000	2.8	0.000	0.000
GMCSF	1.5	0.058	0.068	5.3	0.000	0.000	3.4	0.000	0.000
IFN-γ	1.5	0.013	0.023	6.1	0.000	0.000	4.0	0.000	0.000
HMGB-1	1.5	0.072	0.082	7.4	0.000	0.000	4.9	0.000	0.000

	Sterile intra-amniotic inflammation vs. No intra-amniotic inflammation			Microbial associated intra-amniotic inflammation vs. No intra-amniotic inflammation			Microbial associated intra-amniotic inflammation vs. Sterile intra-amniotic inflammation		
Proteins	**Fold Change**	**p-value**	**q-value**	**Fold Change**	**p-value**	**q-value**	**Fold Change**	**p-value**	**q-value**
TGF-β	1.5	0.016	0.024	4.2	0.000	0.000	2.8	0.000	0.000
MCSF	1.5	0.050	0.061	5.6	0.000	0.000	3.8	0.000	0.000
IL-4	1.5	0.155	0.165	5.1	0.000	0.000	3.5	0.001	0.001
Eotaxin	1.4	0.014	0.023	4.3	0.000	0.000	3.0	0.000	0.000
Lactoferrin	1.4	0.039	0.051	3.5	0.000	0.000	2.4	0.000	0.000
IL-7	1.4	0.076	0.084	5.0	0.000	0.000	3.6	0.000	0.000
IL-33	1.1	0.629	0.649	3.5	0.001	0.001	3.1	0.003	0.003
IL-18	1.1	0.698	0.698	2.5	0.000	0.000	2.4	0.000	0.000

IFN-γ: interferon gamma; IL: interleukin; IP-10: interferon gamma-induced protein 10; GM-CSF: granulocyte macrophage colony-stimulating factor; MCSF: macrophage colony-stimulating factor, Gro-α/ CXCL1: C-X-C motif ligand 1; HMGB-1: high-mobility group protein 1; ITAC/ CXCL11: Interferon-inducible T-cell alpha chemoattractant/C-X-C motif ligand 11; MDC: macrophage-derived chemokine; MIP: macrophage inflammatory protein; MIG: Monokine induced by gamma interferon; MCP: monocyte chemoattractant protein-1; RANTES: regulated on activation, normal T cell expressed and secreted; TNF: tumor necrosis factor, TARC: thymus and activation-regulated chemokine