OBSTETRICS

Evidence of perturbations of the cytokine network in preterm labor

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OBJECTIVE: Intraamniotic inflammation/infection 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 biologic networks can provide insight into the pathobiology of disease and improve biomarker discovery. The goal of this study was to characterize the inflammatory-related protein network in the amniotic fluid of patients with preterm labor.

STUDY DESIGN: A retrospective cohort study was conducted that included women with singleton pregnancies who had 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, and amniotic fluid concentration of interleukin (IL)-6 into the following groups: (1) those without intraamniotic inflammation (n = 85), (2) those with microbial-associated intraamniotic inflammation (n = 15), and (3) those with intraamniotic inflammation without detectable bacteria (n = 35). Amniotic fluid concentrations of 33 inflammatory-related proteins were determined with the use of a multiplex bead array assay.

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

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

Key words: chemokine, chorioamnionitis, correlation network, intraamniotic infection, interactome, network analysis, sterile inflammation

Cite this article as: Romero R, Grivel J-C, Tarca AL, et al. Evidence of perturbations of the cytokine network in preterm labor. Am J Obstet Gynecol 2015;213:836.e1-18.

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Received May 28, 2015; revised June 26, 2015; accepted July 21, 2015.

Supported, in part, by the Perinatology Research Branch, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services (NICHD/NIH), and in part, with federal funds from NICHD/NIH, under contract number HHSN275201300006C.

The authors report no conflict of interest.

Presented in poster format at the Mott Center Scientific Retreat, Detroit, MI, April 28-29, 2015.

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0002-9378/$36.00 • Published by Elsevier Inc. • http://dx.doi.org/10.1016/j.ajog.2015.07.037

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 pathological processes[9,10] that include 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 pathological processes yet to be discovered.[54-60] Of these, intraamniotic infection (also termed *microbial-associated intraamniotic inflammation*: the presence of microorganisms in the amniotic cavity and intraamniotic inflammation) has been causally linked to spontaneous preterm delivery.[18] Indeed, at least 1 of every 4 preterm infants is born to a mother with an intraamniotic 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 elicit an intraamniotic 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] as well as other inflammatory mediators that include prostaglandins[146-152] and proteases.[100,105,153-172]

Although intraamniotic inflammation has been traditionally attributed to microorganisms and their products (such as lipopolysaccharide,[173,174] lipoteichoic acid or peptidoglycans,[175] lipoglycans,[25,176-178] or others), it has now become clear that a subgroup of patients with intraamniotic inflammation does not have microorganisms (bacteria or viruses) identifiable by cultivation methods or molecular microbiologic techniques.[179-185] We have coined the term *sterile intraamniotic 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 from other specific individual immunoassays.[27,98,105,182-184,186-192] Because biologic functions are the expression of integrated and interdependent networks of cells and molecules,[193-197] the study of biologic networks, rather than individual cells/molecules, is considered necessary to improve the understanding of the pathophysiology of the 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 intraamniotic inflammation and microorganisms in the amniotic cavity.

Materials and Methods

Study population

A cohort of women (n = 135) with singleton pregnancies who experienced spontaneous preterm labor and intact membranes 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. The inclusive criteria were as follows: (1) singleton gestation, (2) transabdominal amniocentesis performed between 20 and 35 weeks of gestation before 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 previous studies that provided descriptions of microbiologic studies, amniotic fluid interleukin (IL)-6 concentration, and high mobility group box-1 (HMGB-1).[184] Each patient provided written informed consent; the use of biologic specimens and clinical data for research purposes was approved by the Institutional Review Boards of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development and Wayne State University.

Clinical definitions

Microbial invasion of the amniotic cavity was defined according to the results of amniotic fluid culture and polymerase chain reaction/electrospray ionization-mass spectrometry (PCR-ESI/MS; Ibis Biosciences, Carlsbad, CA).[179,180,198,199] Intraamniotic inflammation was diagnosed when the amniotic fluid IL-6 concentration was $\geq$2.6 ng/mL.[27,98,105,181-184,186,187,189,190] Based on the results of amniotic fluid cultures, PCR-ESI/MS, and amniotic fluid concentrations of IL-6, patients with preterm labor and intact membranes were classified into three groups: group 1 included those without intraamniotic inflammation (n = 85); group 2 comprised those with microbial-associated intraamniotic inflammation (a combination of microbial invasion of the amniotic cavity and intraamniotic inflammation; n = 35), and group 3 included those with intraamniotic inflammation without detectable microorganisms (an elevated amniotic fluid IL-6 concentration without evidence of microorganisms in the amniotic cavity by cultivation and molecular microbiologic methods; n = 15). Those patients with the presence of microorganisms in the amniotic cavity, but without intraamniotic inflammation, were classified into group 3 (no intraamniotic inflammation) as 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 gestational ages of 20 to 36 6/7 weeks. *Preterm delivery* was defined as birth before 37 weeks 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 (Table 1). The mediators are cytokines (chemokines are a subset of cytokines); we also included the prototypic alarmin, HMGB-1, that is elevated in cases of sterile intraamniotic