BASIC SCIENCE: OBSTETRICS

Identification and characterization of proteins in amniotic fluid that are differentially expressed before and after antenatal corticosteroid administration

JoonHo Lee, MD; Joong Shin Park, MD, PhD; Errol R. Norwitz, MD, PhD; Byoung Jae Kim, MD; Chan-Wook Park, MD, PhD; Jong Kwan Jun, MD, PhD; Hee Chul Syn, MD, PhD

OBJECTIVE: We sought to examine changes in the intraamniotic proteomic environment after the administration of antenatal corticosteroids to women with impending preterm delivery.

STUDY DESIGN: Amniotic fluid samples were collected at the time of clinically indicated amniocentesis before and within 7 days of administration of antenatal corticosteroids for impending preterm delivery (n = 12). Proteins differentially expressed before and after corticosteroids were identified by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry. They were isolated, characterized, and quantified by fast protein liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, in-gel tryptic digestion, immunodepletion assays, enzyme-linked immunosorbent assay, and electrospray ionization tandem mass spectrometry.

RESULTS: Five protein peaks of interest were identified and characterized, all of which were significantly decreased after antenatal corticosteroid administration. These included 2 isoforms of transthyretin, albumin, prothrombin fragment 2, and lumican.

CONCLUSION: Four proteins, identified and characterized in amniotic fluid, were differentially expressed with antenatal corticosteroid administration. These data may provide additional insight into the molecular mechanisms by which antenatal corticosteroids prevent neonatal complications.

Key words: Albumin, amniotic fluid, antenatal corticosteroids, lumican, prothrombin fragment 2, surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, transthyretin

Cite this article as: Lee J, Park JS, Norwitz ER, et al. Identification and characterization of proteins in amniotic fluid that are differentially expressed before and after antenatal corticosteroid administration. Am J Obstet Gynecol 2010;202:388.e1-10.

Antenatal administration of glucocorticoids significantly reduces neonatal mortality and the incidence of respiratory distress syndrome (RDS), intraventricular hemorrhage, and necrotizing enterocolitis in neonates delivering <34 weeks' gestation.[1-3] Ever since Liggins and Howie[4,5] demonstrated that antenatal corticosteroids increase pulmonary surfactant in newborn lambs and reduces the incidence of RDS in premature infants, investigators have been trying to understand how these agents work to promote fetal lung development.[6-10] Several mechanisms have been proposed, including: (1) acceleration of type II pneumocyte maturation with upregulation of surfactant messenger RNA and protein levels and increased phospholipid synthesis; (2) increased antioxidant activity; and (3) differential regulation of pulmonary growth factors and apoptosis resulting in improved developmental structural changes.[11] Despite these observations, the precise mechanism(s) by which antenatal corticosteroids act to promote cellular differentiation and maturation within the fetal lungs and other organ systems is not well understood. For example, administration of glucocorticoids to pneumocytes in vitro promotes cellular differentiation and surfactant production, but this effect is seen also in the presence of a glucocorticoid receptor inhibitors,[12] suggesting that a nongenomic or glucocorticoid receptor–independent mechanism is involved. To improve our understanding of the effects of antenatal corticosteroids on fetal lung maturation in vivo, this study uses proteomic analysis by surface-enhanced laser desorption/ionization (SELDI) time-of-flight (TOF) mass spectrometry to examine changes in the intraamniotic proteome after clinically indicated antenatal corticosteroid administration in patients with impending preterm delivery.

SELDI-TOF mass spectrometry is a high-throughput proteomic tool for the identification of discriminatory protein

From the Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul, Korea (Drs Lee, J. S. Park, Kim, C-W. Park, Jun, and Syn), and the Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine, New Haven, CT (Dr Norwitz).

Presented orally at the 30th Annual Meeting of the Society for Maternal-Fetal Medicine, Chicago, IL, Feb. 1-6, 2010. The racing flag logo above indicates that this article was rushed to press for the benefit of the scientific community.

Received Nov. 22, 2009; revised Jan. 9, 2010; accepted Jan. 19, 2010.

Reprints: Joong Shin Park, MD, PhD, Department of Obstetrics and Gynecology, Seoul National University College of Medicine, Seoul National University Hospital, 28 Yeongeon-dong, Jongno-gu, Seoul 110-744, Korea. jsparkmd@snu.ac.kr.

This study was supported by Grant no. 04-2008-0170 from the Seoul National University Hospital Research Fund.

0002-9378/$36.00 • © 2010 Mosby, Inc. All rights reserved. • doi: 10.1016/j.ajog.2010.01.056

biomarkers in complex body tissues and fluids for specific diseases.[13,14] This same technology has been previously applied to amniotic fluid (AF) by our group and other investigators to discover discriminatory protein biomarkers for intraamniotic infection/inflammation (IAI),[15-17] fetal aneuploidy,[18] and preeclampsia.[19] This is the first reported attempt on the use of proteomic analysis to identify proteins that are differentially expressed in the AF of women before and after antenatal corticosteroid administration.

MATERIALS AND METHODS

Study design

Samples of AF were collected at Seoul National University Hospital, Korea, at the time of clinically indicated amniocentesis to document fetal lung maturity and/or exclude IAI both prior to and within 7 days of administration of antenatal corticosteroids for impending preterm delivery for maternal or fetal indications (n = 12). The research protocol was approved by the institutional review board at Seoul National University Hospital, Korea, and written consent was obtained from all patients. Inclusion criteria included: (1) singleton gestation; (2) well-dated pregnancy by first-trimester ultrasound or firm menstrual dating consistent with a second-trimester ultrasound; (3) clinically indicated amniocentesis to document fetal lung maturity or exclude IAI because of impending preterm delivery; (4) gestational age at initial amniocentesis <34 weeks; (5) no antenatal corticosteroids prior to initial amniocentesis; and (6) a follow-up amniocentesis performed to exclude IAI or document fetal lung maturity within 2-7 days of antenatal corticosteroid administration or sampling of AF at the time of indicated cesarean delivery within the same time period. An aliquot of each of the AF samples was sent immediately for biochemical and microbiologic examination to exclude IAI, including aerobic and anaerobic culture and white blood cell count. The remainders of the AF samples were stored at −80°C until further analysis. Patients were excluded from the study if there was clinical evidence of IAI or if either of their AF samples showed evidence of IAI (eg, positive culture or white blood cell count $>20/mm^3$).[20]

After initial amniocentesis, all subjects received a full course of antenatal corticosteroids (dexamethasone, 6 mg intramuscularly every 12 hours for 4 doses) and were managed according to standardized clinical protocols. The timing and route of delivery was dictated by standard obstetric indications. Maternal/fetal demographic information, laboratory results, and clinical outcomes were abstracted from the medical records.

AF proteomic profiling using SELDI-TOF mass spectrometry

Before AF samples were analyzed by SELDI-TOF mass spectrometry (ProteinChip system model series 4000; Ciphergen Biosystems, Fremont, CA), the ProteinChip Array (Ciphergen Biosystems) was calibrated as follows: superoxide dismutase (double charged) 7795.7 Da, skeletal myoglobin 8475.7 Da, superoxide dismutase 15,591.4 Da, myoglobin 16,951.51 Da, β-lactoglobulin A 18,363.34 Da, peroxidase 43,240.0 Da, and albumin 66,410.0 Da.

After each spot of the Q10 ProteinChip Array was equilibrated, 2 μL of each AF sample was loaded with 8 μL of binding buffer into each spot and incubated in a humidity chamber for 1 hour at room temperature. The Q10 ProteinChip Array was selected because previous studies in our laboratory have shown that it gives the most discriminatory AF proteomic profile.[19] After washing and air drying, 1 μL of a saturated solution of 5 mg of sinapinic acid in 50% acetonitrile/0.5% trifluoroacetic acid was loaded to each spot. The ProteinChip arrays were then analyzed by a ProteinChip Reader using ProteinChip software (Ciphergen Express Client 3.0). After the peak calibration, peak alignment, and normalization of each proteomic profile obtained from the analysis, AF samples taken before antenatal corticosteroid administration were matched to and compared with the AF samples taken from the same patient after corticosteroid administration. In this way, patients served as their own controls. The proteomic "fingerprint" (peaks of interest) that was able to discriminate the AF profiles before and after antenatal corticosteroid administration was then identified.

Purification and isolation of the protein peaks of interest

AF samples were purified by centrifugation and by passage through a 0.45-μm syringe filter. Filtered AF was then loaded onto a fast protein liquid chromatography (FPLC) column (1.0-mL HiTrap Q; GE Healthcare Corp, Waukesha, WI). FPLC elution fractions were separated and concentrated. Thereafter, 1 μL of each FPLC fraction was loaded onto spots on Au ProteinChips. The prepared ProteinChips were read using the ProteinChip Reader as described above. The FPLC fractions containing the proteins of interest were then collected separately and concentrated.

To isolate the proteins of interest, 80 μL of concentrated FPLC fractions containing each of the peaks of interest were mixed with sample buffer and boiled. The mixture was electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel, which was stained with Coomasie blue-250. The gel band corresponding to the molecular weight of the protein of interest was excised, washed, and dehydrated by incubation with 100% acetonitrile.

The elution method was performed to confirm that the protein contained within the dehydrated SDS-PAGE gel was the same as the protein of interest identified by SELDI-TOF mass spectrometry. The dehydrated gel was then incubated with elution solution and sonicated. After the prepared solution was applied to a NP20 chip (Ciphergen Biosystems) and the mass/charge intensity obtained, the gel band whose mass/charge intensity was identical to that of the peak of interest was selected for further identification and characterization.

Identification of the proteins of interest by in-gel tryptic digestion and ESI-tandem mass spectrometry

Excised pieces of SDS-PAGE gel containing the proteins of interest were subjected to overnight in-gel trypsin digestion at 37°C using the method of Jensen et al.[21] The gel pieces incubated with the