Placenta 34 (2013) 873–878

Contents lists available at SciVerse ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Identification of biomarkers for preterm delivery in mid-trimester amniotic fluid☆,☆☆

A. Kim [a], E.S. Lee [b], J.C. Shin [c,**,1], H.Y. Kim [d,*,1]

[a] Department of Obstetrics and Gynecology, Institute of Wonkwang Medical Science, College of Medicine, Wonkwang University, Iksan, Republic of Korea
[b] Department of Obstetrics and Gynecology, College of Medicine, SoonChunHyang University, Seoul, Republic of Korea
[c] Department of Obstetrics and Gynecology, College of Medicine, Seoul, Republic of Korea
[d] Department of Obstetrics and Gynecology, College of Medicine, Kosin University, Busan, Republic of Korea

ARTICLE INFO

Article history:
Accepted 24 June 2013

Keywords:
Preterm birth
MMP-8
IL-6
VEGF
Amniotic fluid

ABSTRACT

Objective: We investigated whether the level of vascular endothelial growth factor (VEGF) and inflammatory markers in mid-trimester amniotic fluid have predictive value for spontaneous preterm birth in singleton pregnancy.
Method: Our subjects were 72 pregnant women who were undertaken with amniocentesis from 16 to 19 weeks of gestation. 36 cases were women with preterm delivery, and other 36 cases were matched women with full-term delivery. Stored amniotic fluid was investigated after the delivery. The levels of matrix metalloproteinases-8 (MMP-8), interleukin-6 (IL-6), C-reactive protein (CRP), and VEGF were measured by enzyme-linked immunosorbent assay (ELISA) and Western blot.
Results: The levels of MMP-8 and IL-6 in preterm group were significantly higher than control group (5.76 ± 1.53 ng/ml vs 4.89 ± 1.77 ng/ml and 170.54 ± 55.69 pg/ml vs 141.92 ± 57.21 pg/ml, respectively) ($p < 0.05$). In terms of VEGF, the levels were elevated in preterm group (30.76 ± 4.06 pg/ml vs 22.36 ± 7.03 pg/ml) ($p < 0.05$).
Conclusion: This study suggests that elevated levels of IL-6 and MMP-8 in amniotic fluid at mid-trimester are predictive of preterm delivery, and that VEGF which is representative of angiogenesis can be a new and useful predictor of preterm delivery.

1. Introduction

Preterm birth is an important obstetric problem that causes considerable costs to healthcare systems. Also preterm-birth-associated perinatal morbidities, which are responsible for almost half of all long-term neurological disabilities, and neonatal mortalities reap a huge financial and emotional burden for the affected families. Preterm birth is defined as delivery that occurs before 37th weeks of gestation; it accounts for 10% of all pregnancies [1]. Over the last 25 years, there has been an increase in intensive research in this area because of a rising number of medically-indicated preterm births in singletons, and because of increased instances of preterm delivery of multiple pregnancies conceived with assisted reproductive technology (ART) [2].

Preterm delivery is responsible for a significant percentage of neonatal morbidity and mortality. The etiology of preterm birth is unknown and is likely associated with multifactorial causes, including infection stress and demographic factors. Therefore, efforts have been made to identify predictors of preterm birth, especially since some of the causes of preterm delivery can be treated [1].

Biomarkers have been defined as ''parameters, which can be measured in a biological sample, and which provide information on potential effects of that exposure" [3]. Based on the known risk factors and pathways of preterm birth, several biomarkers have been tested to see if they can predict spontaneous preterm birth. Among those risk factors evaluated to date, fetal fibronectin in cervicovaginal fluid and ultrasound assessment of cervical length have been most strongly and consistently associated with subsequent spontaneous preterm birth. The test is most accurate in predicting spontaneous preterm birth within 7–10 days of a woman presenting with symptoms [4].

☆☆ This study was supported by a grant from Kosin University College of Medicine (2012).
* Corresponding author. Tel.: +82 51 990 6226; fax: +82 51 990 3300.
** Corresponding author. Tel.: +82 2 590 2749; fax: +82 2 595 1549.
E-mail addresses: ttochil@hanmail.net (J.C. Shin), hykyale@hanmail.net (H.Y. Kim).
[1] The last two authors equally contributed to this work for correspondence.

0143-4004/$ – see front matter © 2013 The Authors. Published by Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.placenta.2013.06.306

An inflammatory response and microbial invasion of the amniotic cavity are generally regarded as a pathologic state associated with preterm labor [2]. The molecular mechanisms by which intrauterine inflammation causes preterm labor are not well understood, although a number of cytokines, chemokines, and inflammatory mediators have been implicated. And inflammatory cells have been known to secret a plethora of proangiogenic factors including VEGF [5]. Until now, no other biomarkers have been consistently found to be useful in clinical settings to predict spontaneous preterm birth in asymptomatic women, especially at mid-trimester [6]. In this reason, the study aimed to identify and determine the accuracy of matrix metalloproteinases-8 (MMP-8), interleukin-6 (IL-6), and C-reactive protein (CRP), for inflammatory markers and vascular endothelial growth factor (VEGF) for an angiogenic marker in mid-trimester amniotic fluid for the prediction of spontaneous preterm birth in women with singleton pregnancies.

2. Materials and methods

2.1. Study design

The research was designed to be a prospective study. Collection of amniotic fluid samples from January 2009 to June 2012 and clinical data were approved by the Institutional Review Board of Kosin Medical Center. All patients gave written informed consent, in accordance with the Helsinki criteria. After excluding fetal aneuploidies, anomalies, and cases who experienced pregnancy loss within 30 days of amniocentesis, we enrolled and stored the samples of amniotic fluid for later analysis. Post-delivery patient obstetric data were reviewed, and the clinical outcomes were obtained. Gestational age was determined based on the last menstrual period and the first trimester obstetric ultrasound evaluation (crown rump length at 7–9 weeks). Preterm delivery was defined as birth before 37 weeks of gestation.

2.2. Patients

A total of 526 pregnant women with singleton gestations were carried out amniocentesis and their samples of amniotic fluid were stored until delivery. Amniocentesis was performed for proper clinical indications (advanced maternal age, abnormal quad/triple test, family history of chromosomal abnormalities, suspected fetal anomalies or viral infection, and maternal request) at 16–19 weeks of gestation. Among 526 women with available samples of amniotic fluid, the study included 72 women for study objects. Patients were invited to donate amniotic fluid for research purposes. The clinical outcome was obtained by chart review. Inclusion criteria were uneventful pregnancy course before the procedure, absence of congenital fetal malformations, absence of clinical signs of infection, normal volume of amniotic fluid as assessed by ultrasound, and healthy pregnant woman without chronic or medical disease. Any preterm delivery associated with an obstetrical complication, such as hypertensive disorders in pregnancy, obstetrical hemorrhage, fetal growth restriction, or premature rupture of the membrane, was excluded from the amniotic fluid analysis.

We retrieved samples from every case known to have resulted in delivery before 37 weeks of gestation ($n = 36$) and 36 control samples from women who delivered at $\geq$37 weeks of gestation. The control samples were matched with the preterm group at a 1:1 ratio from sampling until testing (storage time). Matches were based on maternal age, gestational age (weeks) at the time of amniocentesis, and the indication for the procedure.

2.3. Collection of amniotic fluid and storage

Transabdominal amniocentesis was performed with a 21-gauge needle under ultrasound guidance to evaluate the position of the fetus. Amniotic fluid was first taken for further diagnostic testing, depending on the indication of the invasive procedure. Afterward, 5 ml from a total volume of 20 ml of amniotic fluid was collected for research purposes. Samples were transported immediately to the laboratory in a capped sterile syringe; amniotic fluid samples were then centrifuged for 10 min at 400rpm and stored in aliquots at −70 °C until analysis at the completion of follow-up.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Invitrogen assay kits (Carlsbad, CA, USA) were used for measuring matrix MMP-8, IL-6, CRP, and VEGF. These kits are based on the solid phase sandwich enzyme-linked immunosorbent assay (ELISA) method. During the first incubation, samples were pipetted into wells coated with antibodies specific for human MMP-8, IL-6, CRP, and VEGF, followed by the addition of a biotinylated second antibody. During the first incubation, the human antigen bound simultaneously to the immobilized (capture) antibody on one site and to the solution phase-biotinylated antibody on a second site.

After washing, streptavidin-peroxidase (enzyme) was added, which binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and wash to remove all the unbound enzyme, a substrate solution was added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentrations of MMP-8, IL-6, CRP, and VEGF present in the specimen.

The coefficients of variation (CV) of intra-assay and inter-assay precision were 8.5–10.2% for MMP-8, 5.0–5.6% for IL-6, 6.0–9.9% for CRP, and 5.1–9.8% for VEGF, respectively. The minimum detectable doses of MMP-8, IL-6, CRP, and VEGF were < 6 pg/ml, 2 pg/ml, 10 pg/ml, and 5 pg/ml, respectively.

2.5. Western blot

A total of 1–2 mL of amniotic fluid was prepared by dilution with sodium dodecyl sulfate (SDS) loading buffer (Fermentas, Waltham, MA, USA), followed by boiling and cooling. The AF samples underwent electrophoresis in a 13.5% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) (Koma Biotech, Seoul, Korea). Thereafter, proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) at 30 V for 1 h. Nonspecific binding was blocked for 1 h in noise-cancelling reagents (Millipore). After washing, membranes were incubated for 2 h at room temperature with antibodies. The antibodies used (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were goat anti-human MMP-8 antibody (M-20), mouse anti-human IL-6 antibody (E-4), rabbit anti-human CRP antibody (c-12), and rabbit anti-human VEGF antibody (147). A BCIP/NBT tablet (Sigma–Aldrich, St Louis, Missouri) dissolved in distilled water was used as growth substrate. Chemiluminescence analysis was conducted with Luminata Crescendo Western HRP substrate (Millipore) and autoradiography film (Agfa-Gevaert, Mortsel, Belgium), according to the manufacturer's instructions. The experiment was replicated three times. Bands produced from the Western blot were showed using Gel Doc™ XR+ with Image Lab software (Bio-Rad, Hercules, CA, USA).

2.6. Statistical analyses

Results are expressed as mean and standard deviation according to the distribution of data. Kolmogorov–Smirnov's test was used to evaluate the normality of the distribution of the continuous data. Comparisons between two groups were conducted using Student's *t*-test in a normal distribution. Univariate analyses were performed using X^2 test and Fisher's exact test to evaluate the association of preterm birth with factors categorized. The receiver operating characteristic (ROC) curve was applied to calculate each factor's predictive value for preterm delivery. SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for statistical calculations. $P < 0.05$ was considered statistically significant.

3. Results

Thirty-six patients delivered at <37 weeks gestation; all spontaneous preterm labors included an intact membrane. The included cases were classified into the preterm group. The matched control group included 36 healthy pregnancies with full-term delivery. Table 1 shows the clinical and demographic characteristics of the study samples. There were no significant differences between the preterm delivery group and controls with respect to the mother's

Table 1
Clinical and demographic characterizations of the subjects.

Clinical characteristics	Preterm delivery ($n = 36$)	Term delivery ($n = 36$)	*p*-value
Maternal age (years)	34.8 ± 4.3	33.2 ± 4.3	0.855[a]
BMI (kg/m^2)	21.7 ± 2.5	21.2 ± 0.3	0.862[a]
Gravidity	1.08 ± 0.33	0.93 ± 0.46	0.156[a]
Gestational age at sampling (weeks)	18.3 ± 0.2	18.0 ± 0.2	0.673[a]
Sex (male/female)	19/17	16/20	0.638[b]
Indication of amniocentesis			
Advanced maternal age	2	2	0.938[c]
Abnormal quad/triple test	29	30	
History of fetal anomaly	5	4	

Results are expressed as mean ± SD. BMI, body mass index.
[a] Student's *t*-test.
[b] X^2 test.
[c] Fisher's exact test.