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Original Article

Detection of angiogenic factors in midtrimester amniotic fluid and the prediction of preterm birth

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ABSTRACT

Objective: We investigated whether the level of vascular endothelial growth factor (VEGF), placental growth factor (PlGF), and soluble VEGF receptor-1 (sFlt-1) in midtrimester amniotic fluid of preterm birth have different values compared with term delivery.
Materials and Methods: Our participants were 86 pregnant women who had undergone amniocentesis from 16 to 19 weeks of gestation. Forty-three cases were women with preterm delivery, and the other 43 cases were matched women with full-term delivery. Stored amniotic fluid was investigated after the delivery. The levels of VEGF, PlGF, and sFlt-1 were measured by enzyme-linked immunosorbent assay and Western blot.
Results: The levels of VEGF and PlGF in the preterm group were significantly higher than in the control group (30.48 ± 8.57 pg/mL vs. 26.06 ± 8.24 pg/mL and 28.83 ± 7.83 pg/mL vs. 25.35 ± 8.26 pg/mL, respectively) ($p = 0.017$ and 0.048, respectively). In terms of sFlt-1, the levels were decreased in the preterm group (10,478.51 ± 4012.56 pg/mL vs. 12,544.05 ± 4140.96 pg/mL) ($p = 0.021$).
Conclusion: This study explains that elevated levels of VEGF and PlGF, suggestive of angiogenesis and tendency of inflammation at midtrimester, are predictive of preterm delivery, and their availability is maximized by downregulation of sFlt-1.

Introduction

Placental angiogenesis and vasculogenesis play an important role for normal development of the fetus [1]. Altered angiogenic marker expression has been studied in obstetric complications, such as preeclampsia [2]. Increased levels of the angiogenic markers like soluble fms-like tyrosine kinase-1 [soluble vascular endothelial growth factor (VEGF) receptor-1, sFlt-1] or soluble endoglin and lower levels of placental growth factor (PlGF) may permit detection of preeclampsia before symptom onset [3,4]. The relation between these angiogenic factors and preeclampsia seems to be very strong and more predictive of risk for preeclampsia [5].

Another important complication during pregnancy is preterm birth, which is defined as delivery that occurs between 24 weeks of gestation and 37 weeks of gestation [6]. Preterm delivery is responsible for a significant percentage of neonatal morbidity and mortality. 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 [7]. Relations between angiogenic markers and preterm delivery have become important to characterize the placental aspect associated with preterm labor or the inflammatory role of angiogenic markers in pregnancy [8]. Little is known about angiogenic marker patterns in relation to preterm delivery uncomplicated by preeclampsia. Preeclampsia-like angiogenic marker changes have been reported late in pregnancy among spontaneous preterm labor cases; however, similar changes occur in term pregnancies before labor onset [8].

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Two previous reports suggest that low sFlt-1 levels may mark a subset of spontaneous preterm delivery [8,9]. We measured mid-pregnancy levels of VEGF and PlGF, as well as sFlt-1 in midtrimester amniotic fluid, and assessed their associations with preterm delivery.

Materials and methods

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. Postdelivery 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.

Patients

A total of 596 pregnant women with singleton gestations underwent amniocentesis and their samples of amniotic fluid were stored until delivery. Amniocentesis was carried out 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 596 women with available samples of amniotic fluid, the study included 86 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 ($n = 43$) and 43 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.

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 minutes at 400 rpm and stored in aliquots at −70°C until analysis at the completion of follow-up.

Enzyme-linked immunosorbent assay

Invitrogen assay kits (Carlsbad, CA, USA) were used for VEGF, PlGF, and sFlt-1. 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 VEGF, PlGF, and sFlt-1, followed by the addition of a biotinylated secondary 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 any unbound enzyme, a substrate solution was added, upon which the bound enzyme acts to produce color. The intensity of this colored product is directly proportional to the concentrations of VEGF, PlGF, and sFlt-1 present in the specimen. The coefficients of variation of intra-assay and interassay precision were 5.1–9.8% for VEGF, 8.5–10.2% for PlGF, and 5.0–5.6% for sFlt-1, respectively. The minimum detectable doses of VEGF, PlGF, and sFlt-1 were <5 pg/mL, 1 pg/mL, and 2 pg/mL, respectively.

Western blot

A total of 1–2 mL of amniotic fluid was prepared by dilution with sodium dodecyl sulfate loading buffer (Fermentas, Waltham, MA, USA), followed by boiling and cooling. The amniotic fluid samples underwent electrophoresis in a 13.5% sodium dodecyl sulfate-polyacrylamide gel (Koma Biotech, Seoul, Korea). Thereafter, proteins were electrotransferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) at 30 V for 1 hour. Nonspecific binding was blocked for 1 hour in noise-cancelling reagents (Millipore). After washing, membranes were incubated for 2 hours at room temperature with antibodies. The antibodies used (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were rabbit antihuman VEGF antibody (147) (CAT. # sc-507), goat antihuman PlGF antibody (C-20) (CAT. # sc-1880), and mouse antihuman sFlt-1 antibody (C-20) (CAT. # sc-315). A 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) tablet (Sigma-Aldrich, St Louis, MO, USA) 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 shown using Gel Doc XR+ with Image Lab software (Bio-Rad, Hercules, CA, USA).

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 the two groups were conducted using the Student t test in a normal distribution and X^2 test for univariate analysis in the categorized variables. The receiver operating characteristic (ROC) curve was applied to calculate each factor's predictive value for preterm delivery. SPSS version 18.0 (SPSS Inc., Chicago, IL, USA) was used for statistical calculations. A p value < 0.05 was considered statistically significant.

Results

A total of 43 patients delivered at <37 weeks of gestation; all spontaneous preterm labors included an intact membrane. The