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Regular Article

Activation of coagulation in amniotic fluid during normal human pregnancy

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ABSTRACT

Introduction: Amniotic fluid (AF) is an important medium for fetal development which exhibits high procoagulant activities; however, the role of these procoagulants during pregnancy has not been elucidated and might be associated with pregnancy complications. The current study aimed to evaluate factor X (FX) activation and its association with tissue factor (TF), tissue factor pathway inhibitor (TFPI) and coagulation activation markers in AF during normal human pregnancy.
Methods: Activation of FX and concentration of TF, free TFPI, D-dimer and prothrombin fragments (F1+2) were evaluated in AF samples obtained for chromosome analysis from 91 women with normal pregnancy: 65 samples were taken from patients at 16–20 weeks of gestation, 9 samples were drawn at 21–30 weeks and 17 samples—after 30 weeks of gestation.
Results: Activation of FX in AF significantly increased during normal pregnancy (from 65 ± 41 to 205 ± 80 equivalent RVV ng/mg total protein, $P < 0.0001$). TF and TFPI levels in AF also rose with gestational age. In contrast, the AF concentration of D-dimer and F1+2, markers of coagulation activation significantly decreased when expressed per mg total protein. Levels of free TFPI correlated with TF ($r = 0.5$, $P < 0.001$), and were 8-fold higher than those of TF during pregnancy.
Conclusion: High levels of TFPI might be associated with the inhibition of procoagulant activity in amniotic fluid during normal pregnancy, which may account for the rarity of clinical amniotic fluid embolism.
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Introduction

Amniotic fluid (AF) is a complex substance containing nutrients and growth factors involved in fetal growth and development. AF also provides mechanical cushioning and antimicrobial effectors that protect the fetus. AF analysis allows assessment of fetal maturity and prenatal diagnosis of fetal diseases [1].

During embryogenesis, the AF volume increases faster than the embryonic size. Water and solvents present in AF and originating from maternal plasma, are transferred through fetal membranes by hydrostatic and osmotic forces. As the placenta and fetal vessels develop, the placenta acquires a major role in the transport of water and solutes from maternal plasma to the fetus and AF [1].

In addition to nutrients and growth factors, AF contains coagulation factors which exert procoagulant, anticoagulant and fibrinolytic activities [2–6] but their role in fetal development has not yet been completely determined.

Tissue factor (TF), the main in-vivo coagulation initiator and a direct FX activator, is present in AF [6–10] and might have an impact on the initiation of disseminated intravascular coagulation (DIC) associated with amniotic fluid embolism [8,10,11]. Recently, tissue factor pathway inhibitor (TFPI) was also found in AF [8]. It is not yet clear if the presence of TF and its natural inhibitor in AF is related to coagulation per se or it is mainly associated with embryonic development [12–17].

Since amniotic fluid appears to be an important medium for fetal development which exhibits high procoagulant activity, information regarding the balance between FX activator, TF and TFPI in AF and their association with pregnancy complications could be most valuable.

The present study aims to evaluate FX activation and its association with TF, TFPI and coagulation activation markers in AF during normal human pregnancy.

Methods

Patient characteristics: 120 healthy pregnant women who referred to the Rambam Health Care Campus for genetic amniocentesis were enrolled in the study.

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Inclusion citeria

Prenatal diagnosis was indicated for one of the following reasons:

1. Advanced maternal age (over 35 years at the last menstrual period).
2. Abnormal result of biochemical serum screening for fetal chromosome anomalies.
3. Markers for chromosome anomalies observed on fetal sonography.
4. Request for genetic amniocentesis without specific medical indication.

Exclusion criteria

1. Age below 18 years or above 40 years.
2. Known systemic disease, e.g., diabetes mellitus, hypertension, hypothyroidism, hyperthyroidism, renal disease, etc.
3. Use of anticoagulant therapy in the present pregnancy.
4. Multifetal pregnancy.

Clinical evaluation

Pregnancy outcomes were validated in follow-up telephone conversations or in review of computerized medical records, about 6 months after AF sampling. Gestational age at delivery, neonatal weight, and occurrence of vascular complications of pregnancy were recorded. These complications included gestational diabetes, hypertention, pre-eclampsia (PET), premature separation of the placents (abruption) and intrauterine fetal growth restriction (IUGR).

Amniocentesis was performed with continuous ultrasonic guidance and in aseptic conditions. After preparation of the abdominal skin and draping, a #21 gauge needle was inserted into the amniotic sac, avoiding fetal parts and transplacental passage. 30 ml of clear amniotic fluid were drawn and sent to the genetic lab for evaluation of fetal karyotype and AF alpha-fetoprotein level. Six ml of clear amniotic fluid were collected for coagulation studies. Patients with bloody taps were not included in the study.

Of 120 patients constituting the original study group, 29 were eventually excluded: 15 patients with gestational vascular complications, 8 with fetal chromosome anomalies and 6 with pregnancy termination performed for other indications.

The study was approved by the local institutional Review Board (IRB, Helsinki committee). Explanations regarding the study were provided during the genetic counseling session before the procedure and all patients signed the informed consent form, approved by the IRB.

Amniotic fluid sample handling

AF samples were collected in 3.2% sodium citrated tubes and were centrifuged twice at 1500 g for 15 min (each centrifuge) to remove any cellular elements. D-dimer measurement was performed in fresh AF samples, while for all the other assays, AF samples were frozen at -70 ± 5 °C. Prior to testing, frozen AF samples were thawed in a water bath at 37 ± 0.5 °C.

Coagulation parameters

Direct FX activation

AF samples of 30 µl each were incubated with 0.02 U human FX (Hyphen BioMed, Neuville sur Oise, France) and 10 mM $CaCl_2$ for 30 min at 37 ± 0.5 °C, then chromogenic substrate S-2765 (Chromogenix, Instrumentation Laboratory, Milano, Italy) was added to a final concentration of 0.4 mM for additional incubation period of 30 min at 37 ± 0.5 °C. FX activation was determined at 405 nm on the FLx808 microplate reader (BioTek instruments Inc. VT, USA). Calibration curves of Russell's viper venom (RVV) (Diagnostica Stago, Asnieres sur Seine, France) were run in every test, using the same procedure. FX activation was calculated for each AF sample from the RVV calibration curve which was tested at the same time, and the activity level was expressed as an equivalent to RVV ng/ml.

D-dimer level was tested on the STA-R analyzer using STA-LIATEST® D-DI kit (Diagnostica Stago, Asnieres sur Seine, France).

Prothrombin fragment F 1 ± 2 concentration was measured by an enzyme immunoassay (ELISA) using Enzygnost* F $1 + 2$ (monoclonal), (Dade Behring, Marburg, Germany) according to the manufacturer's instructions.

For the measurement of TF and free TFPI, AF samples were prediluted 1:20 with the dilution buffer, provided in each kit, prior to the assay performance. For determination of the total protein concentration, AF samples were prediluted 1:20 with sterile water for irrigation (Teva Medical, Israel).

Free TFPI was assessed by ELISA using Asserachrom® free TFPI kit (Diagnostica Stago, Asnieres sur Seine, France).

Tissue factor (TF) level was analyzed by IMUBIND Tissue Factor ELISA (American Diagnostica, Stamford, CT).

Total protein concentration, expressed in mg/ml, was evaluated by the Bradford method. Concentration of all coagulation parameters was presented both per volume (ml) and per protein (mg total protein) to obtain comprehensive information regarding their variations in amniotic fluid during pregnancy.

Statistical analysis

AF samples were divided into 3 groups, according to the week of gestation at amniocentesis. Data were evaluated using the SPSS software, version 17 (SPSS Inc. Chicago, IL, USA). Differences in clinical characteristics and soluble coagulation parameters of AF were determined by non-parametric Kruskal Wallis tests with Dunn's post test or Anova with Post-hoc test whenever was needed. Correlations between plasma parameter levels and week of gestation were checked with Pearson Correlation analysis. To perform GLM linear modeling with gestation as a factor, the two groups at advanced gestational age (21–30 and >30 weeks of gestation) were combined into one group. In addition, multivariate analyses – Logistic regression were performed. $P < 0.05$ was considered significant.

Results

Patient characteristics did not differ among the 3 study groups (Table 1).

FX activation in AF significantly increased during pregnancy (from 65 ± 41 to 205 ± 80 equivalent RVV ng/mg protein, $P < 0.0001$) (Table 2 and Fig. 1). FX activation levels, expressed both in RVV ng/ml and RVV ng/mg total protein, correlated with the week of gestation ($r = 0.55$ and $r = 0.67$, respectively; $P < 0.0001$) (Fig. 2).

Total protein concentration in AF significantly decreased after 30 weeks of gestation, while levels of TF and TFPI expressed in ng/mg total protein significantly increased (Table 2, Fig. 1).

A significant correlation was demonstrated between levels of TF and free TFPI antigen ($r = 0.5$, $P < 0.001$). However, free TFPI levels were found to be about 8-fold higher than those of TF ($P = 0.023$).

Table 1
Clinical characteristics of the study groups.

	Gestational week at amniocentesis		
	16-20	21-30	>30
Number	65	9	17
Maternal age (y)	35.2 ± 3.0	31.3 ± 7.0	33.3 ± 6.0
Week of delivery	39.3 ± 1.3	39.9 ± 1.4	38.65 ± 1.7
Newborn weight (gr)	3393 ± 431	3527 ± 516	3256 ± 429
No. of previous gestations	2.45 ± 1.54	1.63 ± 1.6	1.56 ± 1.03
Nulliparous	4 (6%)	3 (38%)	3 (19%)

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