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Soluble Fas and Fas-ligand levels in mid-trimester amniotic fluid and their associations with severe small for gestational age fetuses: a prospective observational study

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ABSTRACT

We aimed to determine the second-trimester amniotic fluid (AF) levels of soluble Fas (sFas) and Fas-ligand (FasL) and investigate their association with fetal growth. Therefore, sFas and FasL levels were measured by enzyme immunoassay in the AF of 21 small for gestational age (SGA), 13 large for gestational age (LGA), and 44 appropriate for gestational age (AGA) fetuses of pregnant women who underwent amniocentesis at between 15 and 22 weeks gestation. Our study results showed that sFas and FasL levels were detectable in AF. sFAS median (25th–75th centile) levels were 3.8 (2.8–4.6) ng/ml in SGA, 3.6 (3.1–4.5) ng/ml in AGA, and 4.0 (3.1–4.4) ng/ml in LGA. FasL median (25th–75th centile) levels were 26.0 (20.3–32.7) pg/ml in SGA, 22.7 (18.4–28.5) pg/ml in AGA, and 21.5 (15.8–30.9) pg/ml in LGA. The differences were not statistically significant. Nevertheless, statistically significant differentiation of FasL levels existed when SGA fetuses in the extremes of distribution ($\leq$5th, $\leq$2.5th centile) were considered. This is the first study presenting sFas and FasL concentrations in early second-trimester amniotic fluid in AGA, SGA, and LGA fetuses. We found indications that severe and very severe SGA fetuses ($\leq$5th and $\leq$2.5th centile) have high levels of FasL in the amniotic fluid. This finding probably reflects the increased rate of apoptosis that is assumed to exist in cases of extreme growth restriction.

1. Introduction

Amniotic fluid provides a dynamic environment for the fetus; it is formed of fetal urine and lung excretions, and it contains molecules and factors that facilitate fetal growth (Malamitsi-Puchner et al., 2005, 2006). Small for gestational age (SGA) generally refers to a fetal weight below the 10th centile for gestational age (Vrachnis et al., 2006) and fetuses are considered large for gestational age (LGA) if their weight is greater than the 90th centile for gestational age (Alexander et al., 1996). If their weight is between the 10th and 90th centile for gestational age then the fetuses are considered appropriate for gestational age (AGA).

Small for gestational age fetuses are considered to be at a higher risk of perinatal and later life complications (Botsis et al., 2006; Vrachnis et al., 2010). The underlying mechanism(s) that lead(s) to an SGA fetus remains undetermined, while reliable measures for prevention have not as yet been established. This raises the urgent need for the

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identification of biomarkers that will help to better understand the mechanisms leading to this condition so as to develop effective strategies for early detection, prevention and treatment of the condition (McMillen and Robinson, 2005; Wu et al., 2006).

Fas (CD95) is a 45-kDa cell surface receptor of the tumor necrosis factor (TNF)/nerve growth factor family (Nagata and Goldstein, 1995). It is expressed as a type I membrane protein in many tissues and cells such as the heart, lung, liver, kidney, and ovary (Watanabe-Fukunaga et al., 1992). Fas-ligand (FasL) is also a member of the TNF family and is a type II membrane protein predominantly expressed in activated cytotoxic T-lymphocytes, natural killer cells, and neutrophils (Iwama et al., 2000). The FasL expressed on the surface of these cells binds to the Fas receptor of the target cells, which results in downstream activation of a cascade of intracellular proteolytic enzymes, eventually leading to apoptosis (Nagata, 1994; Nagata and Goldstein, 1995). The soluble form of FasL, whose soluble molecule consists of the extracellular region of FasL, is cleaved from membrane FasL by matrix metalloproteinase (Kayagaki et al., 1995) and then binds to Fas receptor on target cells to induce apoptosis (Tanaka et al., 1995). Although the relationship between the membrane and soluble forms of Fas/FasL (sFas/sFasL) is still under investigation, it has been suggested that sFas has a suppressive effect on Fas/FasL-mediated apoptosis (Nagata and Goldstein, 1995), whereas sFasL may have both an augmentative and a diminutive effect (Iwama et al., 2000).

Since the Fas/FasL signaling system may promote apoptosis, which is an essential physiological process for the normal development of an embryo (Kaponis et al., 2008), we sought to examine the levels of sFas, with its suppressive effect on apoptosis, and FasL, which promotes apoptosis in the amniotic fluid of second-trimester fetuses. We also explored the association between sFas and FasL concentrations in SGA/LGA fetuses, taking into account that amniotic fluid composition seems to be similar to fetal plasma during the first half of pregnancy (Underwood and Gilbert, 2005). It might therefore be concluded that sFas and FasL amniotic fluid concentrations reflect the sFas/FasL concentrations in the fetal plasma of normal (AGA) and abnormal (SGA/LGA) fetuses. Our study is the first to our knowledge to determine any detectable concentrations of sFas and FasL in early second-trimester amniotic fluid samples and to investigate any potential differences among the SGA, AGA, and LGA fetuses.

2. Materials and methods

The study group consisted of 300 pregnant women who underwent an equal number of amniocenteses early in the second trimester of their pregnancy. Routine mid-trimester amniocentesis was carried out between 15 and 22 gestational weeks, for various indications, which included advanced maternal age, abnormal nuchal translucency screening, past history of genetic disorder and identification of fetal abnormality on the second-trimester ultrasound screening. Twin pregnancies or pregnancies with major congenital anomalies were excluded. The study was approved by the Ethics Committee of our teaching hospital and patients' informed consent was obtained.

All patients were Caucasians. A questionnaire including maternal age, weight, height, parity, cigarette smoking, medical and obstetrical–gynecological history was completed before each procedure. Gestational age was calculated by the first day of the last menstrual period and confirmed by the crown rump length of the embryos, as determined in the first trimester ultrasound examination. The duration of pregnancy, mode of delivery, neonatal birth weight/gender, and the outcome were also recorded.

The amniotic fluid samples were collected in pyrogen-free tubes and immediately centrifuged, and were kept frozen at -80° until the determination of sFas and FasL levels.

In order to allocate the centile of each fetus at delivery, a gestation-related optimal weight (GROW) computer generated program was used (Gardosi and Francis, 2009). Fetuses below the 10th customized centile were characterized as SGA and those above the 90th customized centile as LGA. Our study sample consisted of 21 SGA fetuses and 13 LGA fetuses, which were matched for gestational age, sex, maternal height, and weight and compared with 44 AGA fetuses that comprised the control group.

We additionally compared the concentrations of sFas and FasL in AGA fetuses with those in fetuses with more extreme somatometric characteristics, namely severe SGA/LGA fetuses as defined by the $\leq$5th and $\geq$95th centiles respectively, and very severe SGA/LGA fetuses as defined by the $\leq$2.5th and $\geq$97.5th centiles respectively.

The determination of sFas and FasL was performed using the commercial enzyme immunoassay (ELISA) kits Quantikine Human sFas immunoassay and Quantikine Human Fas Ligand/TNFSF6 immunoassay respectively (R&D Systems Inc., Minneapolis, MN, USA). The minimum detectable concentration for sFas was 20 pg/mL and for FasL it was 2.7 pg/mL. The precision of the sFas kit, as estimated with %CV, ranged from 2.9% to 6.7% and for the FasL it ranged from 4.1% to 8.8%.

Mainly because of the small sample sizes within each group, the distribution of the measured variables and the mothers' characteristics deviate from the normality assumption. We used the Kruskal–Wallis test for comparison of the concentrations of substances among the three groups. Mean and standard deviations for quantitative variables are presented, as well as the number and percentages for qualitative variables. We also applied logistic regression to investigate the risk of SGA vs AGA and LGA vs AGA associated with sFas and FasL concentrations. To control for possible confounding factors, we adjusted the models for maternal age (continuously, in years), Body Mass Index (BMI) (categorically <25, 25–29, 30+ kg/m^2), duration of gestation (continuously, in weeks), gender of offspring (boy vs girl), smoking during pregnancy (yes vs no, for the association between SGA and AGA), and parity (parous vs nulliparous). Significance was set at a p level of <0.05.

3. Results

Table 1 presents the descriptive characteristics of mothers and fetuses. There were no statistically significant