Maternal urine for prenatal diagnosis—an analysis of cell-free fetal DNA in maternal urine and plasma in the third trimester

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INTRODUCTION

The detection of cell-free fetal (cff)-DNA in maternal plasma (Lo et al., 1997) generated new possibilities for noninvasive prenatal diagnosis. Cff-DNA is released into the maternal circulation as early as 32 days of gestation (Wataganara et al., 2004) and represents 3.4 and 6.2% of the total plasma DNA in early and in late pregnancy, respectively (Lo et al., 1998).

High concentrations have been found during the last weeks of pregnancy and in pregnancy-associated complications such as chromosomal aneuploidies (Lo et al., 1999a; Zhong et al., 2000; Farina et al., 2003), preeclampsia (Lo et al., 1999b; Leung et al., 2001; Zhong et al., 2001a; Farina et al., 2004) and preterm labor (Leung et al., 1998; Farina et al., 2005).

Cff-DNA is cleared very rapidly after delivery (Lo et al., 1999c) but the pathway of its clearance is still not completely understood. A renal clearance mechanism was postulated, since studies have shown that in preeclampsia, a condition with renal malfunction, fetal DNA clearance is impaired (Lau et al., 2002), and also because transplant and cancer-derived extracellular DNA is detectable in urine from organ transplant recipients (Zhong et al., 2001b) and colorectal carcinoma patients (Botezatu et al., 2000). A renal clearance of fetal DNA would have implications for prenatal diagnosis, but the data regarding the presence of cff-DNA in maternal urine are controversial. Some authors reported positive results (Botezatu et al., 2000; Al-Yatama et al., 2001; Koide et al., 2005), while some did not (Zhong et al., 2001b; Li et al., 2003; IlIanes et al., 2006).

With respect to these conflicting data we performed the present study in order to clarify the potential use of maternal urine for prenatal diagnosis. We tested urine samples collected in the third trimester of 151 pregnant women carrying male and female fetuses for the presence of Y-chromosome-specific DNA sequences using fluorescent PCR and real-time PCR. We also wanted to correlate the plasma concentration of cff-DNA and its concentration in urine.

MATERIAL AND METHODS

Ninety-six pregnant women carrying male fetuses, and 55 carrying female fetuses were recruited with informed consent. The study group included three...
twin pregnancies. Pregnancy-associated complications recorded were preeclampsia in five cases, gestational diabetes in nine cases, rhesus incompatibility and infectious diseases in 10 cases. Placentae including fetal membranes and the umbilical cord were weighed after birth. Urine and plasma samples were collected into EDTA containing tubes at a gestational age between 32 and 40 weeks. Sample collection as well as DNA isolation and amplification were performed by female staff only to avoid any Y-chromosome contamination. The gestational age was 38–40 weeks in 90% of the cases. Venous blood samples were prepared as described by Bauer et al. (2006a) and DNA was extracted from 400 µL plasma using QIAamp DNA Mini Kit (Qiagen, Valencia, Calif) according to the manufacturer’s recommendations. Urine was collected, as described by Li et al. (2003), and a second centrifugation step at 12 000 rpm at 4 °C for 10 min was introduced to avoid contamination of male sequences originating from spermatozoa. DNA was extracted from 300 µL urine in a first test series and then from 1 mL urine using the QIAamp MinElute Virus Kit (Qiagen, Valencia, Calif), which was especially designed for isolation of very low amounts of DNA. Some modifications to the original protocol were performed to obtain purer DNA which included additional washing steps, and the volume of 100% ethanol was increased. Before DNA elution, the column was dried for 7 min at room temperature to remove the remaining ethanol completely. 150 µL QIAGEN protease and 1 mL of buffer AL were incubated with 1 mL maternal urine at 56 °C.

The volume of 100% ethanol was increased to 1250 µL and an extra washing step with 650 µL Buffer AW1 (QIAGEN) was introduced. The volume of Buffer AW2 was also increased to 650 µL. Before DNA elution, the column was dried for 7 min at room temperature to remove the remaining ethanol completely.

Fluorescent PCR

PCR was performed for the first intron of the X/Y homologous gene amelogenin (AMXY) and for the sex-determining region, SRY, located on chromosome Y, in a multiplex PCR as described by Bauer et al. (2006b). PCR amplification was performed in a total volume of 25 µL, containing 5 µL DNA extracted from maternal plasma or 10 µL DNA extracted from maternal urine, 200 µmol dNTPs, 5–20 pmol of each primer (both Oswel, Southampton, UK), 1 × PCR Gold Buffer (without MgCl₂), 2.5 mmol MgCl₂ and 1.25 U AmpliTaq Gold polymerase (all Applied Biosystems Inc, Warrington, UK). PCR was performed using the following program: 95°C for 5 min, 95°C for 48 s, 60°C for 48 s, 72°C for 1 min for 33 cycles, final extension was accomplished at 72°C for 15 min. The PCR products were prepared for capillary electrophoresis and analyzed on the AbiPrism 310 Genetic Analyzer (Applied Biosystems Inc, Warrington, UK).

Real-time PCR

Real-time quantitative PCR analysis was performed, as described by Zhong et al. (2001a), in a 25 µL reaction volume using a sequence detector (AB 7900 Sequence Detection System Applied Biosystems Inc, Warrington, UK). Amplification primers and fluorescent probes, designed to detect the SRY gene on the Y chromosome, were used for detecting fetal DNA in maternal urine and plasma. These primers are designed to amplify a 78 base pair (bp) fragment. For the detection of Y-chromosome-specific sequences in maternal urine the DYS-14 primer set (Zimmermann et al., 2005) was additionally used.

As a control for the amplification of total plasma DNA, all samples were subjected to a TaqMan assay for the β-globin gene on chromosome 11. The β-globin primer set used in the present study is described by Lo et al. (1998).

Statistical analysis

The data were tested for normality by the Kolmogorov–Smirnov test. Total DNA and cff-DNA were not normally distributed. Correlations between continuous variables were determined using the Spearman correlation coefficients as measures of direction and strength of associations. Associations between dichotomous and continuous variables were determined using biveral or point-biveral correlation analyses. A test consistent with P < 0.05 was considered significant. Data were evaluated using SPSS for the PC (release 13.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Male-specific DNA was detected in 31 of 96 (32.3%) maternal urine samples collected from women pregnant with male fetuses by quantitative real-time PCR using the DYS-14 assay. The six (6.3%) samples with the highest amount of fetal DNA in maternal plasma also showed a positive signal using the SRY-assay. However, the concentration of fetal DNA was too low to be quantified in all 31 positive urine samples. Therefore, the correlation between the cff-DNA concentration cleared by the kidneys and its concentration in maternal plasma could not be calculated. The fragments specific for each internal control (β-globin) were detected in all 151 urine samples, which indicates a proper DNA extraction and amplification.

To avoid male contamination by spermatozoa an additional centrifugation step of the urine samples was introduced before DNA isolation, since Y-chromosome-specific signals were detected before, most probably originating from sperms. The analysis of the corresponding plasma samples provided male-specific sequences in 90 of 96 plasma samples obtained from women carrying male fetuses using fluorescent PCR, and in all 96 plasma samples using quantitative TaqMan PCR. In 1 of 55 samples obtained from a woman carrying a female fetus,
DISCUSSION

Our results indicate that fetal DNA is cleared by the kidneys in small amounts, as Y-chromosome-specific sequences were detected in maternal urine using two different PCR systems, but the concentration was too low to be quantified. DNA isolation and amplification from urine give some difficulties. Urine is known to contain PCR inhibitors. However, the isolation and amplification of urinary DNA originating from the maternal urinary tract (Illanes et al., 2006), from transplants (Zhong et al., 2001b) or cancer (Botezatu et al., 2000; Umansky and Tomei, 2006) gave strong evidence that urine is able to preserve DNA. We successfully detected the internal control (β-globin) in all urine samples. Since virus DNA was amplified and detected in urine, we used a DNA isolation kit designed for virus isolation which allowed the detection of very small amounts of DNA. In addition, we used two sets of primers (DYS-14 and SRY) and found that DYS-14 was more sensitive, which is related to its occurrence in multiple copies on the Y-chromosome compared to the SRY-locus, which is present in only one copy.

Concerning these difficulties, the results ofcff-DNA in maternal urine are very inconclusive. Our data confirm the results published by Koide et al. (2005). For their experiments they used different sets of primers and successfully amplified a mean of 0.5 copies of male DNA in five of seven maternal urine samples (maximum 1.9 copies per 30 mL urine). They found that fetal DNA was excreted into maternal urine in fragments of 63 bp. Based on these data one could calculate that the amount of fetal DNA in maternal urine is about 10,000 times less than the amount of fetal DNA in maternal plasma. A renal DNA clearance mechanism should allow the detection of higher amounts of trans-renal DNA in urine and of a higher percentage of cleared plasma DNA, especially during the third trimester, which would explain its short half-life. Botezatu et al. (2000) and Al-Yatama et al. (2001) were also successful in detecting male-specific DNA in urine samples. Botezatu et al. (2000) amplified Y-chromosome-specific sequences in 8 of 10 urine samples obtained in the first trimester. Al-Yatama et al. (2001) detected cff-DNA in 38% of urine samples originating from women bearing male fetuses. However, they also found male DNA sequences in plasma and urine samples from women carrying female fetuses. This might be related to the contamination of female samples with male DNA. Urine is most likely contaminated by sperm.

We found that a crucial step in handling urine samples was a careful centrifugation before DNA extraction. At the beginning of our analysis we also had some positive urine samples collected from women pregnant with female fetuses.

Illanes et al. (2006) published negative results, as did Zhong et al. (2001b) and Li et al. (2003). In contrast to our approach they used a different DNA extraction system.

We suggest that the level of fetal DNA in maternal urine is also dependent on the women’s water intake and renal function. A concentrated urine sample obtained in the morning may contain higher concentrations of PCR inhibitors. Another limiting factor in the detection of cff-DNA in maternal urine might be the fragment size of excreted DNA. Koide et al. (2005) found fetal DNA in maternal urine in 63 bp fragments. Urine samples from patients with colon and pancreatic tumors contained DNA fragments with a size of 150–200 bp (Umansky and Tomei, 2006), which is indicative that the kidneys are able to excrete larger DNA fragments.

In our corresponding plasma samples cff-DNA was found in 90 of 96 samples using fluorescent PCR, and in all 96 samples using quantitative real-time PCR. This difference is caused by the lower sensitivity of fluorescent PCR. As a consequence, the samplesnegated by fluorescent PCR showed only low levels of fetal DNA by real-time PCR.
Although the fetal DNA level was very low, one plasma sample obtained from a woman pregnant with a female fetus was positive for male DNA using both analysis methods. The origin of this positive result could not be clarified, even after several repetitions of the DNA extraction and amplification procedure. The medical history of this woman did not show any organ transplantation or blood transfusion. Since fetal DNA does not persist after a previous pregnancy (Smid et al., 2003a), we presume that the woman had a vanishing male twin in the early stage of her pregnancy but this was not proven by a DNA analysis of placental tissue. Since her urine sample was also positive, a false positive result can be excluded. Another explanation is that the samples were taken from a woman carrying a male fetus and were given the wrong identification.

Cff-DNA showed a correlation with gestational age, a finding that was also published by Wataganara et al. (2004) for the first, and by Lo et al. (1998) for the third trimester. The time elapsed from blood collection to birth expressed in days was inversely correlated with cff-DNA levels. Since the origin of cff-DNA in maternal plasma is linked to apoptosis of trophoblast cells (Bischoff et al., 2005), our finding might reflect the terminal condition of the placenta near to time of birth. We also studied maternal plasma from three dichorionic twin pregnancies consisting of one pair of males and two mixed pairs. Compared to single pregnancies, no increase in cff-DNA was observed. This is in contrast to the data published by Smid et al. (2003b), which showed a significant increase of cff-DNA in twin pregnancies with two male fetuses. In contrast to twin pregnancies with a mixed couple, an increase in cff-DNA would have been expected for the pregnancy with two male fetuses, but the cff-DNA level was extremely low with 5.2 GE/mL. In an ongoing study we are investigating maternal plasma from twin pregnancies. We have found that the cff-DNA levels are lower in twin pregnancies compared to single pregnancies matched for gestational age (unpublished data). This finding could be related to the increase in plasma volume which might dilute the DNA levels.

The difference of cff-DNA levels between normal and complicated pregnancies did not reach statistical difference, but it was significant for total DNA levels. An explanation for this finding is that this group was very heterogeneous including only five pregnancies complicated by preeclampsia, which is known to exhibit high cff-DNA levels (Lo et al., 1999b; Leung et al., 2001; Zhong et al., 2001a; Farina et al., 2004), but 19 pregnancies with gestational diabetes, rhesus incompatibility and infectious diseases. The high levels of total DNA in this group may be explained by the activation of the immune system in the course of rhesus incompatibility and infection with a rapid turnover of inflammatory cells. High levels of β-globin DNA were also found in preeclampsia (Sekizawa et al., 2004). Total DNA levels showed also a correlation with placental weight, which was not found for cff-DNA. Our data on cff-DNA in correlation with placental weight are in agreement with the results published by Wataganara et al. (2005), which did not show a correlation between cff-DNA and placental volume.

Our data confirm that cff-DNA is cleared by the kidneys in detectable amounts, but due to its low concentration or problematic detection, maternal urine seems inappropriate for noninvasive prenatal diagnosis. We conclude that further studies are needed using different DNA isolation and amplification protocols to elucidate the renal mechanism of fetal DNA excretion.

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