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Kinetics of *SRY* gene appearance in maternal serum: detection by real time PCR in early pregnancy after assisted reproductive technique

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BACKGROUND: Fetal DNA circulating in maternal serum offers a possibility for non-invasive prenatal diagnosis but its kinetics during very early pregnancy is still unclear. In order to clarify this point, the studies on the kinetics of fetal DNA appearance in maternal serum were conducted on patients undergoing assisted reproduction. METHODS: Using a quantitative real time PCR assay, the presence of *SRY* gene sequences was evaluated in the serum of patients at the onset of pregnancy. RESULTS: Twenty-seven patients were originally studied but first trimester abortion occurred in five cases. Among the 22 ongoing pregnancies, ten were found to bear at least one male fetus and all sera from these women gave positive results for *SRY* gene detection. The *SRY* gene was found to be detectable as soon as day 18 after embryo transfer in one case and it had been found in the other nine patients by day 37. CONCLUSIONS: Fetal DNA is found in maternal serum even before the fetal circulation is established, which is highly suggestive that it is released, at least in part, from the trophoblast. Detection of fetal DNA in maternal serum very early in pregnancy may have clinical implications such as with the management of pregnant women carrying a fetus at risk for congenital adrenal hyperplasia.

Key words: assisted reproduction/fetal DNA/maternal serum/pregnancy

Introduction

Since the first demonstration by Lo *et al*. (1997), cell-free fetal DNA is known to be present in maternal blood from pregnant women and hence represents an important avenue for non-invasive prenatal diagnosis. It has recently been demonstrated that fetal DNA can be used as a reliable tool for both fetal Rh status and for fetal sex determination (Costa *et al*., 2002a;b). Therefore, fetal DNA circulating in maternal blood offers the possibility of a non-invasive approach to prenatal diagnosis of women at risk for RhD allo-immunization or for a fetus with an X-linked genetic disease. This approach may yield a high level of sensitivity and specificity as soon as the eighth week of gestation.

However, the origin of fetal DNA circulating in maternal serum is still unclear even though many hypotheses have been proposed. Fetal cells are rare in maternal blood (about one per ml) (Bianchi, 1995) and thus cannot be the sole source of the high amount of fetal DNA. Some of those cells are apoptotic (van Wijk *et al*., 2000), hence circulating DNA might be derived from these apoptotic cells. The placenta is thought to be a probable source of fetal DNA as the concentration of fetal DNA increases as pregnancy progresses. In addition, a positive correlation exists between maternal β-hCG level (which is secreted by trophoblastic cells) and the amount of circulating fetal DNA. Passive diffusion of fetal DNA through the placental barrier remains a possibility.

If fetal DNA in maternal blood is to be used routinely for non-invasive prenatal diagnosis, it is important to explore the kinetics of its appearance in the maternal serum. To date, only two studies have focused on the timing of fetal DNA appearance. Thomas *et al*. (1994) have amplified Y chromosome-specific sequences at day 19 post-conception (p.c.) in two samples but they examined whole maternal blood using qualitative conventional PCR. Lo *et al*. (1998) have detected *SRY* sequences in maternal plasma at week 7 of gestation in a small series of patients using real time PCR. Since improved sensitivity can be obtained by using a newly described method (Costa and Ernault, 2002), a study was conducted to determine the kinetics of appearance of this fetal DNA in maternal serum. This study was focused on the very beginning of pregnancy after assisted reproduction, thus providing a precise knowledge of time of conception.

Materials and methods

Patients and samples

Twenty-seven pregnant women were recruited from the Unité d'Assistance Médicale à la Procréation, Baudelocque, Hôpital

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Table I. Outcome of pregnancies (positive hCG: $n = 27$)

Pregnancy	Ultrasound pregnancy diagnosis ($n = 26$)	Ultrasound ongoing pregnancy >12 weeks gestation ($n = 22$)	Ongoing second trimester ($n = 21$)	Deliveries >28 weeks gestation ($n = 20$)	Male infants ($n = 11$)
γ	22	20	20	20	8
γγ	3	1	0	0	2[a]
γγγ	1	1	1	1	1

[a]Two male fetuses still-born at 21 weeks gestation.
γ = singleton pregnancy; γγ = twin pregnancy; γγγ = triplet pregnancy.

Cochin, Paris. Pregnancies were obtained by intrauterine insemination (IUI) ($n = 13$), classical IVF ($n = 5$) or by ICSI ($n = 9$). Replaced embryos were either fresh ($n = 10$) or frozen ($n = 4$) for IVF and ICSI procedures. Most patients were nulliparous, except three who had at least one child; all previous children were male.

The start date of pregnancy (day 0 p.c.) was considered to be the day of IUI, or the day of oocyte retrieval before IVF or ICSI, or 2 days before the day of replacement of frozen embryos (if they had been previously frozen at day 2 after fertilization). The first sample of blood used for pregnancy diagnosis was drawn around day 14 after the assisted reproductive technique.

Maternal blood was obtained (after signed consent) once a week, during the 5 weeks following pregnancy diagnosis. The mean gestational age (g.a.) was 20.2 days p.c. (range 15–24) at the first blood sampling (BS1), 27.1 days p.c. (22–31) at BS2, 34.2 days p.c. (29–38) at BS3, 41.2 days p.c. (36–45) at BS4, and 48.1 days p.c. (43–52) at BS5. Five ml of blood were collected into Vacutainer SST® tubes (Becton Dickinson, France). Immediately after clotting, serum was obtained after centrifugation at 4°C for 10 min at 3000 *g*. If not treated immediately, it was frozen at –80°C.

Pregnancy evolution and the number of ongoing embryos were unknown at the time of blood sampling. Blood sampling was stopped in the event of natural pregnancy loss. Pregnancy was confirmed by ultrasonography between 6 and 8 weeks gestation (mean g.a. 39.8 days p.c., range 20–45).

Quantitative real time PCR for SRY gene in maternal serum

As a tracer for DNA extraction and amplification steps, a small amount (250 pg) of mouse DNA (Sigma, France) was added to each patient's sample (1 ml of serum) immediately prior to DNA extraction. Total DNA was extracted by using an automated procedure on a closed system as previously described (Costa and Ernault, 2002) thus avoiding cross-contamination between samples as well as contamination from the operator. The DNA was eluted in 50 ml of elution buffer, of which 10 ml were used for the PCR reaction. Amplification was carried out in a LightCycler® instrument (Roche Biochemicals, France) as previously described (Costa *et al*., 2001). Briefly, the PCR reaction was set up in a final volume of 20 µl using the Fast DNA Master Hybridization Probes Kit (Roche Biochemicals), with 0.5 µmol/l of each primer, 0.25 µmol/l of each probe, 1.25 IU of uracil DNA glycosylase (UDG) (Biolabs, France), 4.5 mmol/l of magnesium chloride, and 10 µl of extracted DNA. After an initial 1 min incubation at 50°C to allow UDG to cleave putative contaminant PCR products from previous reactions, a first denaturation step of 8 min at 95°C was started. Amplification was then performed for 50 cycles of denaturation (95°C, 10 s, ramping rate 20°C/s), annealing (56°C, 10 s, ramping rate 20°C/s), and extension (72°C, 15 s, ramping rate 2°C/s). Continuous fluorescence was monitored at the annealing step for each sample. During each run, a small amount of male DNA (10 pg) was used as a positive control while the DNA extraction elution buffer was used as a negative control.

Table II. *SRY* gene detection in maternal serum

	n	Negative	Positive
All patients	27	17	10
Ongoing pregnancies >12 weeks gestation	22	12	10
First trimester miscarriages	5	5	0
Multiple pregnancies			
γγ→γ	2	0	2
γγ	1	0	1
γγγ	1	0	1
Male-bearing pregnancies	10	0	10

In the case of a negative result for *SRY* gene amplification, the presence and correct amplification of the tracer mouse DNA in the extracted maternal serum DNA was checked. The same extracted serum DNA used for *SRY* gene amplification was assayed in a second PCR targeted at the mouse galactose-1-phosphate uridyltransferase (*GALT*) gene. This step made it possible to distinguish a true negative from a false one coming from deficient DNA extraction process or PCR inhibitors in the eluted DNA.

Because the amount of fetal DNA in the maternal serum is expected to be very low during the first trimester—resulting in sample variability—four reactions were performed on each serum. Qualitative results are thus expressed as the number of positive reactions out of these four. For those samples that were positive for the presence of *SRY* gene, quantitative analyses were performed using a standard curve established with serial dilutions of male DNA and results expressed as fetal DNA copies/ml of maternal serum. Results were considered as positive when at least two out of the four experiments give a signal for the *SRY* gene; the mean copy number was that derived from these experiments.

Results

Fetal sex determination

Among the 27 patients studied, five miscarried before the end of week 6 of gestation, in which case fetal sex could not be confirmed by later ultrasonography or karyotyping as no embryo was seen. One ongoing pregnancy was twin, one was triplet. For all ongoing pregnancies, fetal sex was later confirmed by ultrasonography during the second trimester of pregnancy ($n = 21$), from cytogenetic results after amniocentesis ($n = 5$), and in one case of fetal loss at 22 weeks gestation in the twin pregnancy, from pathological examination of the fetus. Fetal sex was confirmed after delivery in 22 patients and 25 fetuses (23 live-birth). Fetal sex prediction based on *SRY* gene PCR assay on maternal serum was in complete concordance with its determination by standard procedures. Among

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Table III. Quantitative analysis of fetal DNA in maternal serum of patients carrying male embryos

Patient no.	Blood sample, mean gestational age (range)				
	BS1 20.2 days p.c. (15–24)	BS2 27.2 days p.c. (22–29)	BS3 34.2 days p.c. (29–38)	BS4 41.2 days p.c. (36–45)	BS5 48.2 days p.c. (43–52)
2	Negative	10.9	19.9	ND	ND
3	2.4	4	6.6	10.1	11.3
4	4.2	10.2	11.7	15.1	11.3
7	Negative	Negative	12.9	6.4	22.5
8	Negative	14	7	9	11.2
9	Negative	Negative	7.2	ND	9.2
19	Negative	9.9	20.5	30.5	52.8
20	Negative	1.2	16	13.7	12
24	Negative	5	10.1	9.6	18

Positive results are expressed as copies of fetal DNA per ml of maternal serum.
BS = blood sample; p.c. = post-conception; ND = not done.

these 22 ongoing pregnancies, 10 were found to bear at least one male fetus and all sera from these 10 women gave a positive result for *SRY* gene detection. All sera from women carrying a female fetus (n = 12) gave negative results. Both multiple pregnancies showed positive *SRY* gene detection, as the twin pregnancy was bearing two boys and the triplet one boy. Of note, none of the first-trimester-aborting pregnancies showed a positive *SRY* gene signal, even during the first weeks of persisting trophoblastic growth. Table I and Table II show the pregnancy outcome and the results obtained among all tested samples.

Timing of appearance of fetal DNA in maternal serum and quantitative assessment

Kinetic results are presented in Table III. For the 10 patients who carried at least one male fetus, the *SRY* gene was found to be detectable as soon as day 18 after embryo transfer for one patient (patient 5). At the time of the first blood sample (BS1), *SRY* sequences were detected in three patients (33%). The rate for positive results was 80 and 100% at BS2 and BS3 respectively. Presence of fetal DNA in maternal serum can thus be systematically demonstrated from day 37 after embryo transfer.

Quantitative results showed that the amount of fetal DNA increases slightly during the first weeks of pregnancy from about four copies (mean value) at day 20 p.c., to 25 copies (mean value) per ml of maternal serum at day 48 p.c. This latter value is in good agreement with fetal DNA concentrations determined by others during the first trimester of pregnancy (Lo, 1998; Honda, 2002). Patients 5 and 19 clearly exhibit different kinetics of appearance of fetal DNA in their sera as well as the highest amount of fetal DNA.

Discussion

The aim of this study was to determine the kinetics of appearance of the *SRY* gene in maternal serum during early pregnancy after the assisted reproductive technique. As the amount of fetal DNA was expected to be very low, a highly sensitive but safe procedure was used (Costa and Ernault, 2002). This procedure includes automated extraction of fetal DNA in a closed system associated with real time PCR. Therefore, this nearly complete automated procedure avoids false positive results due to either sample contamination or carry-over by PCR products. Using this PCR assay, 27 pregnant women recruited from the fertility treatment centre were tested in a blind prospective study. As described, the determination of fetal sex using these techniques was in perfect concordance with the fetal sex assessed later using standard procedures (Costa *et al*., 2002a). The first positive signal for *SRY* gene was detected at 18 days p.c. and all patients bearing a male fetus gave a positive signal at day 37 p.c. (Table III).

The presumed sources of fetal DNA in maternal circulation are fetal cells, trophoblast and/or via the passive diffusion of DNA. During the early stage of implantation, erosion of the maternal tissues occurs under the lytic influence of the syncytiotrophoblast derived from the cytotrophoblast. From 12 days p.c., this invasive activity causes disintegration of the maternal endometrial vessel walls. At this time no sample was studied but all samples studied before 18 days p.c. were negative for the presence of *SRY* gene. The first real maternal circulation of the placenta occurs later with deeper invasion of the endometrium and erosion of the spiral arteries (Benirschke and Kaufmann, 2000). Between days 18 and 20 p.c., the first fetal capillaries can be observed in the mesenchyme of the placental secondary villi. Haemangioblastic progenitor cells, separating from the mesenchyme, differentiate in capillaries and haematopoietic stem cells. These cells stay trapped in the so-called tertiary villi until a complete fetoplacental circulation is established at ~28–30 days p.c. Fetal and maternal blood are always separated by the placental barrier, which might be permeable to fetal DNA, and are in close contact as soon as fetal intravillous circulation is established (Demir *et al*. 1989). At day 28 p.c., most patients (80%) have detectable amounts of fetal DNA in their serum (Table III). This result means that fetal DNA can be found in maternal blood even before the fetal circulation is established, which suggests strongly that fetal DNA is released from the trophoblast.

Detection profile analysis of both twin (patient 5) and triplet (patient 3) pregnancies as well as the two vanishing twin

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pregnancies (patients 19 and 8) in this study give more evidence about the mechanism of fetal DNA flow into the maternal compartment.

Patient 3 carried one boy and two girls. Her serum showed the initial detection of fetal DNA at day 22 p.c. and the amount of *SRY* copies was similar to other single male pregnancies as expected. Patient 5 carried two boys and her serum showed the earliest (day 18 p.c.) positive detection of the *SRY* gene in this study as well as unusual kinetics in the amount of fetal DNA. When compared with other patients, fetal DNA was circulating in the maternal serum at higher amounts in this twin-male pregnancy. Surprisingly, a nearly identical result was observed for patient 19 which began as a twin pregnancy, with two ongoing embryos at the first ultrasonography (day 30 p.c.) but a vanishing twin at the second (day 50 p.c.). Although no evidence could be found that the vanishing twin was a male embryo, it seems reasonable to hypothesize that continuous release of DNA by this second embryo may have represented the source of the notably high amount of fetal DNA observed in maternal serum. Conversely, for patient 8, although the first ultrasonography showed a twin pregnancy but with a single ongoing embryo, the fact that the level of fetal DNA was not increased suggests that the remaining yolk-sac was from a female one. Similarly, it can be hypothesized that the absence of SRY sequences in maternal serum of all five non-ongoing pregnancies, even though the trophoblast was present and visible by ultrasound, was probably due to the presence of a female embryo, though the possibility of extremely low fetal DNA amounts cannot be excluded.

In conclusion, this study demonstrates that free fetal DNA is detectable in maternal serum from as soon as day 18 p.c. in twin pregnancies and from day 22 in singleton pregnancies. The source of this fetal DNA is likely to be from the trophoblast as it appears before fetal circulation has been established. Therefore, fetal sex determination can be achieved very early during pregnancy by maternal serum analysis. In the clinical setting, patients carrying a fetus at risk of congenital adrenal hyperplasia could benefit from this early determination as suggested by Rijnders *et al*. (2001). This group has recently shown that accurate fetal sex determination can occur at 8 $^{3/7}$ weeks while the present results show that it is possible at 7 $^{1/7}$ weeks, even before differentiation of the indifferent genitalia occurs (8–12 weeks). However, it should be kept in mind that fetal DNA can leak into the maternal circulation from a persistent trophoblast, leading to false positive results due to the presence of male tissue. Therefore, careful ultrasonographic examination must be included in the management of such patients before performing fetal DNA analysis with the maternal serum.

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