Luteal phase serum cell-free DNA as a marker of failed pregnancy after assisted reproductive technology

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Purpose: DNA-damaging factors have been reported in patients that failed to achieve pregnancy after assisted reproductive technologies (ART). The hypothesis was that increased circulating cell-free DNA released by damaged cells could predict unfavorable conditions leading to failed ART treatment. The objective was to compare the relative concentrations of cell-free DNA in the luteal phase sera of nonpregnant versus pregnant patients.

Methods: Frozen-thawed sera (30 IVF cases) were obtained 1 week after embryo transfer. There were 16 pregnant and 14 nonpregnant cases and controls consisting of male sera ($n = 8$ cases). Modified isocratic capillary electrophoresis was performed and the images analyzed for cell-free DNA.

Results: Circulating cell-free DNA were identified in the sera of all patients. The serum concentrations of high (12 kb) and low (1 kb) molecular weight cell-free DNA were similar for both nonpregnant and pregnant patients. Male control sera had higher cell-free DNA concentrations compared with females. Evaluation of sera from a control case showed no fluctuations in cell-free DNA concentrations throughout specific days of the menstrual cycle.

Conclusions: The results do not support the use of the luteal phase cell-free DNA concentration as a marker for failed pregnancies. The equal concentrations of high and low molecular weight cell-free DNA and ladder band-like gel patterns suggested cell apoptosis as the source of DNA.

KEY WORDS: Cell-free nucleic acids; capillary electrophoresis; assisted reproductive technologies; noninvasive diagnosis; luteal phase serum.

INTRODUCTION

Embryotoxic factors have been detected in the sera of patients with recurrent spontaneous abortions (RSA) or endometriosis (1–10). About 24% (8,10) to 70% (2) of the sera of RSA patients and 0% (9) to 100% (7) of the endometriosis patients were

found to be embryotoxic. Recently, this laboratory (11) and others (12) demonstrated the presence of DNA-damaging embryotoxic factors in patients that failed to achieve pregnancy and embryo implantation after assisted reproductive technologies (ART). This finding raised the possibility that the toxic factors might also damage body cells leading to the release of cell-free DNA into the circulation. Hence, it would be appropriate to investigate the presence of cell-free DNA in patients as a marker of failed embryonic implantation.

Circulating extracellular DNA have been detected in both healthy and sick individuals (13) and are present in large concentrations in cancer patients due to apoptosis (14–16) and active secretion (17).

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Circulating fetal DNA have also been reported in the maternal circulation during pregnancy (18–19). Thus, the existence of cell-free DNA has been proven but its association with pregnancy status after ART treatment has not been established. The objective of this study was to determine and compare the relative concentration of cell-free DNA in the sera of nonpregnant versus pregnant patients after ART treatment. The capillary electrophoresis (CE) method (20–22) was modified to provide an inexpensive and rapid assay for cell-free DNA based on items commonly available in the ART laboratory.

MATERIALS AND METHODS

Preparation of Each Serum

The sera used were from discarded specimens ($N = 30$ cases) that had been used for laboratory testing in preparation for the in vitro fertilization (IVF) procedures carried out over 2 years ago. The patient population for this study consisted of randomly selected 30 IVF patients that had the ICSI procedure and were in the less than 35 years age category. The mean age was 30.7 ± 0.6 (mean $\pm$ S.E.M.). The primary diagnosis for the patients varied and so the data were not categorized by diagnosis due to the small sample size. Both, biochemical (2 cases) and clinical pregnancies were grouped. The sera used in the assays were obtained one week after embryo transfer and relevant for the context of the study related to early embryotoxicity. The project has previously been reviewed and approved by the Institutional Review Board.

The ART procedures were the same as the established protocols (23). Briefly, patients scheduled for the in vitro fertilization (IVF) procedure at the Center for Fertility and In Vitro Fertilization were given routine examinations. Ovulation induction was achieved in the female patients by administering recombinant follicle stimulating hormone (average of 4 ampules in split doses daily, 75 I.U. per ampule) for about 9 to 12 days followed by a 10,000 units of human chorionic gonadotropin trigger on the tenth day where the estradiol was over 500 pg/ml and the largest follicular diameter was over 18 mm. Patients on leuprolide acetate cycles followed a similar induction protocol except 0.5 mg leuprolide acetate was administered beginning on day 21 of the previous cycle and ending on the last day of stimulation (23).

The culture medium used for all protocols was G1.2 medium (Vitrolife Fertility Systems,

Englewood, CO). At the time of the IVF procedure, the sperm cells were washed by standard wash procedures and used in the intracytoplasmic sperm injection (ICSI) procedure. Day 3 embryos were transcervically returned to the uterine lumen of the originating patients. Each patient was monitored for evidence of clinical pregnancy. The criteria for clinical pregnancy were both an elevated serum human chorionic gonadotropin (hCG) concentration as well as the ultrasound detection of uterine sac(s) each with a beating heart. There were 16 pregnant and 14 nonpregnant cases and all assays were carried out in replicates. Separate controls consisting of male sera ($n = 8$ cases) or electrophoresis buffer were also included in each assay.

Capillary Electrophoresis

Each serum specimen (50 μ L) was aspirated into a single glass micro-hematocrit capillary tube (blue band plain, internal diameter 1.2 mm, wall 0.2 mm, VWR Scientific Inc., San Francisco, CA). Care was taken to avoid trapped air bubbles. Each capillary tube was held horizontal in such a manner such that the serum was just about to dribble out of one end of the capillary tube. This end of the tube was used to pierce a thick block of agarose gel.

The agarose (2.4%) was previously prepared by mixing 2.4 g agarose (Catalog No. 15510-019, Life Technologies, GIBCO BRL, Grand Island, NY) in 100 mL of $1 \times$ TAE solution (Tris-acetate-ethylenediaminetetraacetic acid, 0.04 M Tris-acetate with 0.001 M EDTA) and heating until dissolved. The heated agarose was poured into a 50 mL glass beaker and permitted to solidify upon cooling. The solidified agarose was tapped out of the beaker to form a block of thick agarose gel and stored submerged in $1 \times$ TAE solution at 4°C until utilized.

Each capillary tube of serum containing the agarose plug at one end was sealed at the other end with electrical conducting gel (Liquasonic, Chester Labs, Inc., Cincinnati, Ohio). This was achieved by filling a 3 mL syringe and needle unit (Becton-Dickinson, Franklin Lakes, NJ) with the conducting gel, placing the needle tip into the open end of the capillary tube until just below the serum meniscus and injecting the conducting gel while slowly removing the syringe and needle unit out of the tube. The capillary tubes were placed submerged at the center of a horizontal gel electrophoresis tank containing TAE solution. The agarose plug end of the tubes were placed closest to the positive (anode) end. A

Table I. Capillary Electrophoresis Analyses of Circulating Cell-Free DNA in Luteal Phase Sera (Day 19) from Pregnant Versus Nonpregnant Patients after Assisted Reproductive Technologies Procedures (Mean Pixels $\pm$ S.E.M.)

Parameter	N	Mean intensity of high molecular weight (12 kb) DNA fragments	Mean intensity of low molecular weight (1 kb) DNA fragments
Negative control	8	26.8 $\pm$ 5.1	21.9 $\pm$ 2.4
Not pregnant	28	83.9 $\pm$ 4.7	57.9 $\pm$ 3.4
Pregnant	32	82.4 $\pm$ 2.6	60.3 $\pm$ 2.8
Male control	16	109.6 $\pm$ 7.2*	71.1 $\pm$ 5.3*

* $p < 0.05$.

constant voltage of 100 volts was applied for 0.4 h (24 min).

After electrophoresis, the agarose plug in each tube was expelled using a plastic stylus or plunger on to glass slides. The agarose plug was held in place using a drop of instant glue while making sure that the agarose plug end exposed to serum was not glued. After 5 min of drying, the slides were stained with 1:10,000 diluted SYBR Green stock fluorescent stain (Molecular Probes Inc., Eugene, OR) for 5 min and carefully rinsed through several changes of water.

Each slide was examined using an ultra-violet epi-fluorescent microscope at 50 $\times$ magnification. The fluorescent images were captured by placing an inexpensive QuickCam Pro camera (Logitech Inc., Fremont, CA) over the microscope eyepiece and saving the 640 $\times$ 480 pixels images to hard-drive storage. Then the images were converted to grey-scale and the pixel intensities analyzed using Paint Shop Pro 6 software (Jasc Software, Inc., Eden Prairie, MN). Higher pixel intensities reflected greater concentrations of cell-free DNA in the agarose plug. For accuracy, the mean pixel intensity of each image was obtained after subtracting the background intensity. Variation between capillary electrophoresis runs on different occasions were tested and adjusted by including a known control serum specimen in each run. A 1 Kb DNA ladder marker (Invitrogen, Carlsbad, CA) was included to determine the cell-free DNA fragment sizes.

Statistical Analysis

The pixel intensities were measured at 4 random points located at the extreme end of the agarose column exposed to serum (high molecular weight fragments; 12,000-bp) and another 4 points at 1 mm from that end of the agarose-serum junction (low molecular weight fragments; 1000-bp). The data were recorded as mean pixel intensity $\pm$ standard error

of the mean (S.E.M.). Data on the mean pixel intensity for sera of each group were compared using ANOVA and significance tested using the Student's t-test statistic. A difference with $p < 0.05$ was considered significant.

RESULTS

Circulating cell-free DNA were identified in the sera of all patients (Table I). The concentration of high molecular weight cell-free DNA (12 kb) in the sera of patients that did not conceive after in vitro fertilization with intracytoplasmic sperm injection (ART cycles) were similar to the patients that conceived. The concentration of low molecular weight cell-free DNA (1 kb) were also similar for both groups of female patients. The sera of male controls showed significantly higher concentrations of both high and low molecular weight cell-free DNA when compared with the female cases. This was consistent with previous reports of cell-free DNA in males (24). The negative controls exhibited a low level of background fluorescence.

An analysis of the relative concentration of circulating cell-free DNA over the course of several days in a single patient undergoing hormonal stimulation for the oocyte retrieval procedure showed no statistically significant variation in the concentration of serum cell-free DNA (Table II). The results were similar for both high and low molecular weight cell-free DNA. The analysis was carried out based on a patient that did not conceive to avoid confounding changes resulting from a conception cycle.

DISCUSSION

The results showed an insignificant difference in the serum concentrations of cell-free DNA in nonpregnant versus pregnant patients after ART

Table II. Lack of Significant Variation in Circulating Serum Cell-Free DNA Throughout the Stimulated Cycle of a Nonpregnant Control Case (Mean $\pm$ S.E.M.)

Cycle day	N	Mean intensity of high molecular weight DNA fragments	Mean intensity of low molecular weight DNA fragments
-9	2	76.9 $\pm$ 0.0	57.6 $\pm$ 3.0
0	2	78.2 $\pm$ 1.8	50.5 $\pm$ 3.0
4	2	85.6 $\pm$ 1.8	53.3 $\pm$ 7.0
5	2	86.9 $\pm$ 7.0	62.6 $\pm$ 10.1
7	2	65.1 $\pm$ 4.4	61.9 $\pm$ 5.0
9	2	60.8 $\pm$ 3.5	42.0 $\pm$ 9.1
10	2	66.4 $\pm$ 0.9	44.8 $\pm$ 1.0
19	2	77.5 $\pm$ 6.1	53.3 $\pm$ 3.0

treatment. This suggested that the previously reported embryotoxic serum factors (11–12) were not associated with cellular processes that generated the cell-free DNA. Furthermore, the finding of a lack of difference suggested that cell-free DNA concentrations at the 1-week post transfer period could not be used as a marker for failed pregnancies (16). Note that the present study focused on cell-free DNA of damaged cells originating from maternal tissues and not from the transferred embryos. Interestingly, the similar concentrations of high and low molecular weight DNA fragments and the ladder or band-like appearance in the majority of gels in both groups of patients suggested cellular apoptosis rather than secretion as the source of the cell-free DNA. The similar concentrations of high and low molecular weight DNA fragments in the ART sera was considered a good sign since a predominance of low molecular weight cell-free DNA has been linked to cancer (24).

The most exciting finding was the detection of cell-free DNA in the frozen-thawed sera using the capillary electrophoresis assay without the necessity for amplification by polymerase chain reaction (PCR). Bypassing the PCR step conferred the advantage of directly evaluating the cell-free DNA derived from the entire genome without variations due to artefacts and inefficiencies in phenol-chloroform extraction and PCR amplification steps, and the limited primer targeted gene regions such as the *K-ras* oncogene (25–26). However, the standard capillary electrophoresis assay possesses certain advantages such as continuous flow through analysis and direct nucleic acids detection set up on the capillary tube (20–22). The modified assay was based on two different techniques that optimized the volume-to-surface area ratio to focus the cell-free DNA on to a small area and the use of next-generation sensitive fluorochromes such as the SYBR Green stain. The result

was the capacity to detect small quantities of DNA such as that found in serum and plasma.

A concern was adequate sensitivity of the assay to detect the cell-free DNA. Several different fluorochromes were tested and the optimal selected. In addition, we were reassured by the results showing significantly more cell-free DNA in male sera compared with female sera, consistent with reports from other research groups (24). A study (27) pointed to finding more cell-free DNA in the serum in contrast to plasma due to lysis of white blood cells during centrifugation in the extraction step. The present study compared relative rather than absolute concentrations avoiding the white blood cell confounding factor.

Interestingly, data based on a control case demonstrated a lack of fluctuation of cell-free DNA throughout specific days of a stimulated cycle of a nonpregnant patient (Table II). This suggested that the concentration of circulating of cell-free DNA was not influenced by daily reproductive hormone level changes. Note that this study involved comparisons of relative concentrations of cell-free DNA and circumvented confounding *in vivo* factors such as chelation, endonuclease activity and uptake into cells (28). Although a difference in cell-free DNA concentrations was not detected in one week post-transfer sera, nevertheless, the presence of cell-free DNA in ART patients is interesting and deserves further studies.

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