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BIRTH OF A NORMAL GIRL AFTER IN VITRO FERTILIZATION AND PREIMPLANTATION DIAGNOSTIC TESTING FOR CYSTIC FIBROSIS

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Abstract *Background.* Cystic fibrosis is a common, severe autosomal recessive disease caused in a majority of cases by a three-nucleotide deletion (ΔF508) in the cystic fibrosis transmembrane regulator gene. Current methods of prenatal diagnosis involve chorionic-villus sampling or amniocentesis. In vitro fertilization and diagnosis during embryonic development before implantation would allow only unaffected embryos to be selected for transfer to the uterus, thereby avoiding the need to terminate a pregnancy.

Methods. Preimplantation diagnosis of cystic fibrosis was attempted in the cases of three couples, both members of which carried the ΔF508 deletion. In vitro fertilization techniques were used to recover oocytes from each woman and fertilize them with her husband's sperm. Three days after insemination, embryos in the cleavage stage underwent biopsy and removal of one or two cells for DNA amplification and analysis.

Results. Only two oocytes from one woman were fertilized normally; DNA analysis of one of the embryos failed and cystic fibrosis was diagnosed in the other (i.e., it was homozygous for ΔF508), so neither was transferred. The oocytes of each of the other two women produced noncarrier, carrier, and affected embryos. Both couples chose to have one noncarrier embryo and one carrier embryo transferred. One woman became pregnant and gave birth to a girl free of the deletion in both chromosomes.

Conclusions. Preimplantation diagnosis of the ΔF508 deletion causing cystic fibrosis is possible through in vitro fertilization, biopsy of a cleavage-stage embryo, and amplification of DNA from single embryonic cells. This approach should be equally applicable to other single-gene diseases in which the defect has been identified. Analysis of a series of pregnancies, however, will be required to assess the method adequately. (N Engl J Med 1992;327: 905-9.)

PRENATAL diagnosis of inherited disease currently involves sampling cells of fetal origin by amniocentesis in the second trimester of pregnancy, or chorionic-villus sampling in the first trimester, followed by detection of the genetic defect by cytogenetic, biochemical, or DNA analysis. Couples at risk of having affected children face the risks associated with these sampling procedures and the difficult decision whether to continue an affected pregnancy. Some couples repeatedly terminate pregnancies in an attempt to have a normal child. Many couples would prefer that embryos be screened before any are implanted in the uterus, so that the parents could be certain before pregnancy that their offspring would be free of a specific defect.[1] In vitro fertilization (IVF) provides access to human embryos. The removal of cells by biopsy of the embryo and genetic analysis would identify unaffected embryos that could be transferred to the uterus. The fetus would be free of the disease, avoiding the possibility of the parents' deciding to terminate the pregnancy.[2,3]

Preimplantation diagnosis after IVF has other potential advantages. After superovulation, several embryos can be screened simultaneously in a single reproductive cycle, maximizing efficiency in identifying the unaffected embryos of couples at high risk. Furthermore, the transfer of two healthy embryos leads to better cumulative conception rates than those achieved through natural cycles.[4] Biopsy of one or two cells at the eight-cell stage does not affect preimplantation development adversely,[5] and several normal baby girls have been born after identification of the sex of embryos of couples at risk of having children with X-linked disease.[6]

Cystic fibrosis is the most common potentially fatal autosomal recessive disease in the white population, affecting about 1 in 2500 births; the frequency of carriers ranges from 1 in 20 to 1 in 25.[7] The disorder is caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene on chromosome 7.[8] The most common of these mutations is a specific 3-bp (base pair) deletion causing a loss of a phenylalanine

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residue at amino acid position 508 (ΔF508). Although the overall frequency of ΔF508 among cystic fibrosis chromosomes is 68 percent, it varies markedly among geographic regions and ethnic groups, ranging from 80 percent in northern European populations and 75 percent in North Americans of mixed ancestry to 40 percent in Ashkenazic Jews and 30 percent in some populations in southern and eastern Europe.[9] Using the polymerase chain reaction (PCR) with nested primers, we have amplified a normal 154-bp fragment of the CFTR gene, including the ΔF508 region, from single cells.[10] The efficiency of amplification from single cells of various types, including blastomeres, is high, with a success rate of 93 percent for amplification from single cells and a specificity of 100 percent for correct diagnosis of the genotype.[10] The biopsy of the blastomere and DNA analysis are accomplished within eight hours, allowing diagnosis and embryo transfer to be performed on the same day.

We report here our first attempts at preimplantation diagnosis of cystic fibrosis after IVF. Three couples in which both parents carried the ΔF508 deletion were treated. One of the two women undergoing embryo transfer became pregnant and gave birth to a normal girl unaffected by cystic fibrosis and free of both parents' alleles with the deletion.

Methods

Approval for this study was given by the Royal Postgraduate Medical School Ethics Committee, the national body (Interim Licensing Authority) regulating research into human fertilization and embryology in the United Kingdom, and the institutional review board of Baylor College of Medicine.

Patients

Three English couples had each had at least one child with cystic fibrosis. Each woman was in her mid-30s and fertile and wished to minimize the risk of termination of a pregnancy affected by cystic fibrosis; one woman had had tubal ligation. The patients were fully counseled about the various reproductive options. They were warned that the chance of pregnancy after a single IVF treatment was only about one in three, that there were theoretical reasons that the risk of miscarriage might be higher than after normal conception, and that a specific genetic diagnosis could not be regarded as totally reliable. They were also advised that only five babies had been born after embryo biopsy, so that this procedure must still be regarded as experimental, and that a pregnancy might produce twins because the aim of IVF would be to transfer two embryos if possible. If pregnancy was achieved, it was recommended that the couple consider chorionic-villus sampling to confirm the preimplantation diagnosis. After repeated clinic visits involving detailed counseling and clinical evaluation, each couple entered the IVF program at Hammersmith; all were treated without charge on a National Health Service basis. IVF and embryo micromanipulation were conducted by the London team, and molecular analysis of the blastomere was performed in London by the Baylor (Houston) group.

IVF Procedures

The couples were advised not to have coitus during treatment. Superovulation was produced by administering exogenous gonadotropins, after the pituitary gland had been desensitized with a gonadotropin-releasing hormone analogue. This approach yields large numbers of eggs of consistent quality, optimizing the number of embryos available for screening. Oocytes were collected from the women on an outpatient basis; they were retrieved by the vaginal route under ultrasonic guidance.[11] Insemination with the husband's sperm and embryo culture were performed in Earle's medium supplemented with 10 percent heat-inactivated maternal serum, and handling and micromanipulation of embryos in the same medium buffered with HEPES. Fertilization was considered normal if two pronuclei were seen 18 hours after insemination. Selected embryos were transferred on the evening of the same day as the embryo biopsy and DNA analysis.

Biopsy of Cleavage-Stage Embryos

Embryo biopsy was carried out early in the morning of the third day after fertilization.[12] The embryos were immobilized on a holding pipette by means of a micromanipulator. A hole was drilled into the zona pellucida with a stream of acidified medium (pH 2.4) applied through a fine micropipette pushed up against the outer surface. A second micropipette (internal diameter, about 30 μm) was then pushed through this hole, and a cell was removed by gentle aspiration. Each cell was checked for the presence of an interphase nucleus by interference contrast microscopy; if the interphase nucleus was present, the embryo was immediately returned to culture. If a nucleus was not visible and the embryo was sufficiently advanced, a second cell was removed.

Preparation of Single Cells

Under sterile conditions in a horizontal laminar-flow cabinet, each blastomere was carefully washed twice in drops of HEPES-buffered medium supplemented with bovine serum albumin, to remove traces of maternal serum and any contaminating sperm. The blastomere was transferred to a 0.5-ml microfuge tube containing 15 μl of water, under a dissecting microscope; this allowed close observation of the cell to confirm that each had been delivered successfully to the tube. A medium blank was prepared from the final wash drop in each case. The samples were then freeze–thawed twice in liquid nitrogen, overlaid with 50 μl of mineral oil, and heated to 93°C for 30 minutes to inactivate cellular DNases and proteases and to denature DNA-associated proteins.

Detection of the ΔF508 Deletion

To amplify DNA from single cells, a protocol for nested PCR was chosen to increase the level of amplification and reduce the accumulation of nonspecific DNA products.[13] An outer set of primers was used initially to amplify a 193-bp (or 190-bp) fragment encompassing the common CTT (cytosine, thymine, thymine) trinucleotide deletion (ΔF508) of the CFTR gene. A PCR mix (35 μl) was added that had a final concentration of 10 mM TRIS-hydrochloride (pH 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.01 percent (wt/vol) gelatin, 200 μM each of four deoxynucleotide triphosphates, 0.8 μmol of each primer (1F, 5′-GACTTCACTTCTAATGATGAT-3′; 1R, 5′-CTCTTCTAGTTGGCATGC-3′), and 1 unit of *Taq* polymerase. The reaction mixtures (volume, 50 μl) were incubated at 93°C (3 minutes), followed by temperature cycling (20 rounds) at 92°C (45 seconds), 40°C (45 seconds), and 72°C (90 seconds) and a final incubation at 72°C (5 minutes). Nested amplification[14] was accomplished by removing 2 μl of the product of this reaction to fresh reaction tubes containing an inner set of primers (2F, 5′-TGGGAGAACTGGAGCCTT-3′; 2R, 5′-GCTTTGATGACGCTTCTGTAT-3′) at a final concentration of 0.8 μM each, with fresh PCR mix (as described above). The nested-PCR amplification was performed for an additional 30 cycles, with cycling at 92°C (45 seconds), 50°C (45 seconds), and 72°C (90 seconds), with a final extension at 72°C (5 minutes). Aliquots (5 μl) of amplified DNA from a single blastomere were mixed with either an equal volume of previously amplified PCR product known to be homozygous normal (154 bp–154 bp) or homozygous mutant (151 bp–151 bp), heated to 93°C for 10 minutes to denature all DNA strands, and then cooled to 65°C for 10 minutes to allow annealing of homoduplex and heteroduplex (154 bp–151 bp) strands. The samples (10 μl) were analyzed by electrophoresis through 10 per-

cent polyacrylamide at 200 V for 30 minutes and staining with ethidium bromide (200 μg per milliliter) before visualization under ultraviolet light.

Results

Each couple underwent a single IVF treatment (Table 1). The results in these fertile women (Patients 1, 2, and 3) did not differ from those expected in infertile women undergoing IVF. The quality and stage of the embryos varied at the time of biopsy early on the third day after fertilization. Several embryos had reached only the four-cell stage, and others were mostly fragmented, with a few intact cells. Altogether, 13 embryos between the four-cell and eight-cell stages underwent biopsy, and generally one blastomere (cell) was removed. A second blastomere was removed from each of three embryos at the seven-cell and eight-cell stages because no nucleus was visible in the first blastomere removed. Consequently, a total of 16 cells was available for genetic analysis.

DNA amplification across the CFTR ΔF508 locus of the single blastomere from the embryos was accomplished with nested PCR (Fig. 1). This approach greatly enhances the intensity of the appropriate DNA band on the gel while minimizing nonspecific DNA amplification at other genomic loci. Overall, DNA amplification was successful with 12 of the 16 blastomeres (75 percent), and in none of the procedures involving the 13 embryos was there any amplification in the medium blank controls run simultaneously.

A rapid approach to detection of the 3-bp deletion involved the intentional formation of DNA strand heteroduplexes and of polyacrylamide minigels that could be run in 30 minutes. A DNA heteroduplex consists of two nonidentical single strands of DNA that are similar enough to form double-stranded DNA along the majority of the nucleotide sequence. DNA heteroduplexes can be induced to form artificially among PCR products as the normal 154-bp single strands of DNA anneal by deoxynucleotide complementarity with the nearly identical 151-bp strands (if present). Under these conditions, both homoduplexes (151 bp–151 bp and 154 bp–154 bp) and heteroduplexes (151 bp–154 bp) form spontaneously, and when analyzed by polyacrylamide-gel electrophoresis, the migration of a DNA heteroduplex is retarded. Consequently, this technique highlights the presence of the 3-bp deletion by producing an intense, slower-migrating band on the gel; this observation is the basis of the assay used in this study to detect the ΔF508 mutation in a single blastomere. When PCR-amplified product from a single blastomere is mixed with previously amplified product of known genotype, homoduplexes or heteroduplexes (or both) will form and will permit the molecular diagnosis of cystic fibrosis in a cell of unknown genotype.

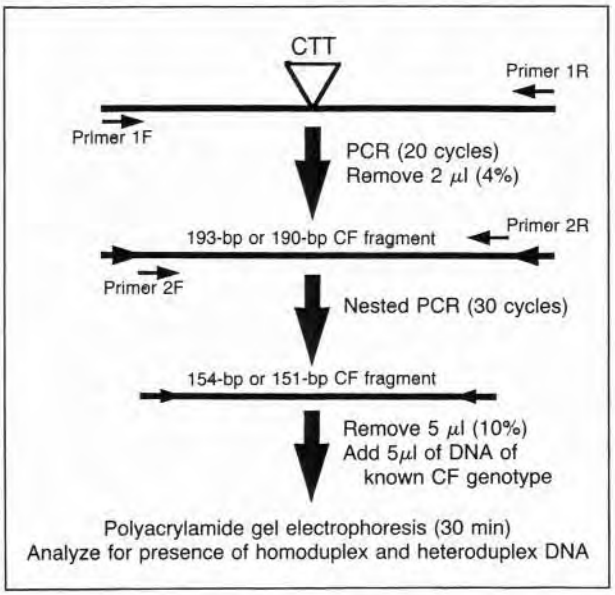

Figure 1. Strategy of Nested DNA Amplification at the ΔF508 Locus (Trinucleotide Deletion CTT).

PCR amplification of DNA from single cells is most efficiently accomplished by 20 thermocycles with the use of an outer set of primers (1F and 1R), followed by a second round of 30 cycles with a nested (internal) pair of primers (2F and 2R). Amplification from normal homoallelic DNA generates a 154-bp product, whereas homoallelic ΔF508 chromosomes produce a 151-bp fragment. CF denotes cystic fibrosis.

PCR amplification was successful in the blastomeres of all of the five embryos from Patient 1; single-cell analysis showed that two embryos were homozygous (affected, Δ:Δ), two were heterozygous (carrier, N:Δ), and one did not carry the ΔF508 deletion (normal, N:N) (Table 1). In one of the heterozygous embryos, the diagnosis was confirmed independently by analysis of a second cell. Patient 1 and her husband chose to have one noncarrier embryo and one carrier embryo transferred since these were the best morphologically; no pregnancy ensued. Amplification was unsuccessful with one embryo from Patient 3, and the other embryo was shown to be affected (Δ:Δ); there-

Table 1. IVF, Preimplantation Diagnosis, and Outcome in Three Women at Risk for Transmitting Cystic Fibrosis.

Patient No.	Oocytes Recovered	Embryos Fertilized Normally*	Embryos Examined†	Blastomere Diagnosis‡			Embryos Transferred	Outcome of Implantation
				NN	NΔ	ΔΔ		
1	19	6	5	1	2	2	2	No pregnancy
2	11	6	6	2	1	2	2	Singleton birth
3	7	2	2	0	0	1	0	—

*Fertilization rates (38 percent) seem lower than average because only normally cleaving embryos were considered for biopsy.

†Embryos were examined after biopsy on the third day after fertilization.

‡NN denotes a homozygous normal embryo, NΔ a heterozygous carrier embryo, and ΔΔ a homozygous affected embryo (a diagnosis of cystic fibrosis).

fore, none were transferred. Finally, DNA amplification and diagnosis were successful with five of the six embryos from Patient 2; analysis revealed two noncarrier embryos (N:N), one heterozygote carrier (N:Δ), and two affected embryos (Δ:Δ).

The gel analysis of the six blastomeres from Patient 2 is shown in Figure 2. The single cells isolated from the second and fifth embryos contained a prominent retarded heteroduplex, represented in each case by the band in the second lane produced when the PCR product from the cell was mixed with amplified DNA known to be homozygous (Δ:Δ) for the deletion. The heteroduplex band was not seen when amplified DNA from a normal subject (N:N) was added. Since heteroduplexes form only when DNA containing the deletion is added, this indicated that cells 2 and 5 (and the corresponding embryos) did not carry the cystic fibrosis mutation. Single cells 1 and 6 displayed a heteroduplex band only in the lanes where homozygous normal (N:N) DNA was added, indicating that these cells were homozygous affected (Δ:Δ). Single cell 4 produced heteroduplex bands in both lanes, indicating that the corresponding embryo was heterozygous (N:Δ) for cystic fibrosis. Finally, PCR amplification of cell 3 failed, since only the exogenously added DNA was seen on the gel; in this case no diagnosis could be made. After Patient 2 and her husband were consulted, a homozygous normal embryo (the second embryo obtained) at the five-cell stage and a heterozygous embryo (the fourth embryo) at the four-cell stage were transferred. By day 14, serum human chorionic gonadotropin levels indicated that Patient 2 was pregnant, and subsequent ultrasonography confirmed the presence of a single fetal sac and heart.

Patient 2 subsequently delivered a healthy girl weighing 2.7 kg (7 lb 3 oz). When the infant was evaluated by a pediatrician at four weeks of age, her physical examination and development were normal. Testing at an independent laboratory demonstrated that neither chromosome allele had the deletion.

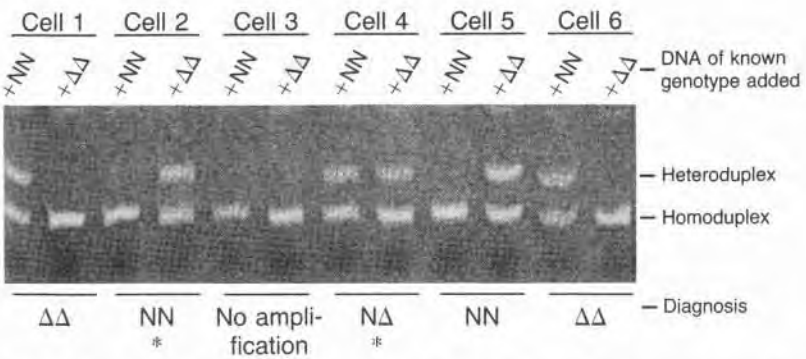

Figure 2. Diagnosis of Cystic Fibrosis by Gel Analysis of Single Blastomeres from Six Cleavage-Stage Embryos.

Heteroduplex (151 bp–154 bp) bands are formed when exogenously amplified DNA of known cystic fibrosis ΔF508 allelic composition is added to the unknown amplification product. The asterisks denote the cells from embryos that were later transferred to the uterus; implantation of the second embryo produced an unaffected girl.

NN denotes a homozygous normal embryo, NΔ a heterozygous carrier embryo, and ΔΔ a homozygous affected embryo (with a diagnosis of cystic fibrosis).

Discussion

After IVF and removal of one or two cells from each embryo, we identified noncarrier, carrier, and affected embryos by nested-PCR amplification of DNA in a fragment including the site of the predominant ΔF508 deletion of the CFTR gene causing cystic fibrosis. Biopsies were performed in 11 embryos from Patients 1 and 2, and 6 of these were unaffected (noncarrier or carrier status), allowing 2 embryos per patient to be chosen for transfer. We consider this to be optimal since pregnancy rates are doubled when two embryos are transferred,[13] but in the event of a twin pregnancy separate samples of the chorionic villi can be recovered from each conceptus. Careful management of IVF is important at all stages, particularly during follicular stimulation, to optimize the number and quality of oocytes recovered and fertilized.

The value of preimplantation diagnosis as an alternative to other conventional prenatal diagnostic methods depends largely on pregnancy rates. The establishment of a pregnancy in one of the two women with transferred embryos is encouraging. A more extensive series of patients will be necessary to assess the pregnancy rates in fertile women. The two embryos transferred into Patient 1 were in the eight-cell stage at biopsy, and in each case two cells had to be removed because the first cell had no visible nucleus. Although this patient did not become pregnant, pregnancies have been established in other women with such embryos (unpublished data). The two embryos transferred to Patient 2 were a homozygous normal embryo in the five-cell stage and a heterozygous carrier embryo in the four-cell stage from which a single cell had been removed. That the child of Patient 2 was shown at birth to be a noncarrier of cystic fibrosis indicates that the five-cell embryo resulted in this pregnancy. This suggests that although embryos in early stages may be sensitive to micromanipulation,[15] this sensitivity does not prevent the establishment of viable pregnancies.

Extensive preliminary work has demonstrated that nested amplification of DNA from single cells is highly efficient with various types of cells, including those from cleavage-stage embryos, and has always accurately identified the genotype of the cells.[10] However, a number of pregnancies must be studied to assess the incidence of misdiagnosis. This is particularly true of an autosomal recessive disease such as cystic fibrosis since, like the two