First Unaffected Pregnancy Using Preimplantation Genetic Diagnosis for Sickle Cell Anemia

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SICKLE CELL ANEMIA IS ONE OF THE most common human autosomal recessive disorders. It is caused by a mutation substituting thymine for adenine in the sixth codon (GAG to GTG) of the gene for the β-globin chain on chromosome 11p, thereby encoding valine instead of glutamic acid in the sixth position of the globin chain. The frequency of sickle cell trait (carrier status) among the African American population at birth is about 8%, and the incidence of sickle cell anemia at birth is 0.16%, or 1 per 625 births.1 Furthermore, the widespread presence of the sickle gene in other ethnic groups has also been confirmed.2 For example, in urban centers in the United States, nearly 10% of patients with various sickling disorders identify themselves as non–African American.3 Children affected with sickle cell anemia experience recurrent episodes of pain (during sickle cell crises) and increased susceptibility to potentially life-threatening conditions, including bacterial infections, cerebrovascular accidents, and organ failure. According to US statistics collected between 1981 and 1992, there were 6.8 deaths per 1000 Af-

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there is no satisfactory treatment for the sickling condition, although blood transfusion may reduce the risk of a first stroke in children and gene therapy holds promise for a curative approach.

Early prenatal diagnosis of the disease is critical because it allows a couple to consider pregnancy termination as an option. The first DNA diagnostic procedure for prenatal purposes was reported 20 years ago. Subsequently, it was recognized that the mutation itself affected the cleavage site of a restriction enzyme, Ddel, that could recognize the DNA sequence of CTNAG (N = A, T, C, or G). While DNA from a normal allele (CTGAG) would be digested by the enzyme, DNA from an affected allele in which A is substituted by T (CTGTG) would not. The resulting differences between DNA fragment sizes can then be recognized by electrophoresis, thus forming the basis for diagnosis. With the advent of polymerase chain reaction (PCR), rapid DNA analysis methods have become available, and these techniques are now widely used for prenatal diagnosis.

An alternative and powerful diagnostic tool for identifying sickle cell status in embryos is preimplantation genetic diagnosis (PGD), which became possible nearly a decade ago. Preimplantation genetic diagnosis takes advantage of assisted reproductive techniques in conjunction with modern molecular methods. With PGD, the genetic status of an embryo can be determined before transfer into the uterus after in vitro fertilization (IVF), thus eliminating the risks of bearing a child with the disease.

While PGD for sickle cell anemia has been performed in the mouse model, routine clinical application in the human previously has not been successful. In this article, we describe our experience using PGD to determine the precise genetic status of embryos generated by assisted reproduction for a couple who are heterozygous carriers of the sickle cell mutation.

METHODS
A 34-year-old female patient had undergone 2 previous induced abortions because she was carrying fetuses affected with sickle cell anemia. Genetic diagnosis indicated that both female and male partners were carriers of the sickle cell mutation. After extensive counseling, the couple gave informed consent and elected to undergo preimplantation genetic diagnosis. The study was approved by the Weill Medical College of Cornell University (New York, NY) Institutional Review Board.

To confirm the genetic status of the couple and establish a protocol for single-copy gene amplification from single cells, blood was collected from both partners. Lymphocytes were isolated using Ficoll-Paque density gradient separation (Pharmacia Biotech Inc, Piscataway, NJ) with the protocol provided by the manufacturer. Single lymphocytes were loaded into 0.5-mL tubes containing 5 µL of lysis buffer and stored at −20°C before trial testing.

On the day of preliminary trial testing, sample tubes were removed from the freezer and heated to 65°C for 10 minutes before they were placed back on ice. Five microliters of neutralization buffer was added to each tube. A nested PCR approach was used for the amplification of the region surrounding the sickle cell mutation. The primers used have been described previously. Polymerase chain reaction was performed after adding a standard mixture of all components, including 2.5 mmol of Mg²⁺, 0.2 mmol of dNTPs (containing dATP, dCTP, dGTP, and dTTP, Perkin Elmer, Foster City, Calif), 100 ng of primers, and 2 U of Taq polymerase (AmpliTaq, Perkin-Elmer). A hot start at 95°C was applied for 3 minutes to ensure complete denaturation of the template. For the PCR profile, the following parameters were used: 93°C denaturation for 30 seconds; 50°C for 40 seconds for annealing; and 72°C for 45 seconds for extension. A total of 20 amplification cycles were applied for outer primers. For the inner primer set, identical parameters were used, except that the annealing temperature was raised to 55°C and a total of 40 cycles were used.

Following the nested PCR amplification, 18 µL of amplified product was digested with the restriction enzyme Ddel (GIBCO/BRL, Rockville, Md) for 3 hours. Subsequently, 10 µL of digested product was run on a 10% acrylamide gel. On completion of electrophoresis, the gel was stained with ethidium bromide and photographed by UV transillumination. As predicted, unaffected DNA showed 3 bands (201, 90, and 74 base pairs [bp]), carrier DNA showed 4 bands (291, 201, 90, and 74 bp), and an affected homozygous sample showed only 2 bands (291 and 74 bp). Testing of single lymphocytes from the male and female subjects showed the same predicted patterns (4 bands of predicted sizes), together with an unaffected DNA control (3 bands) (FIGURE 1).

The IVF procedure has been described previously. Briefly, to ensure that several embryos would become available for DNA analysis, multiple ovarian follicular development was initiated with gonadotropin therapy. After pituitary desensitization with gonadotropin hormone–releasing hormone agonist (leuproline acetate, TAP Pharmaceutical, Chicago, Ill), ovarian stimulation was begun on day 3 of the ensuing menstrual cycle using intramuscular administration of a combination of urofollitropin (75 U of pure follicle-stimulating hormone) and menotropins (150 U of follicle-stimulating hormone and luteinizing hormone) (Serono Laboratories, Norwell, Mass). Follicular growth was monitored by daily serum estradiol levels and pelvic ultrasonograms. To induce final oocyte maturation, 3300 IU of human chorionic gonadotropin was administered when 2 follicles of 18 mm in average diameter were observed on ultrasonogram. Transvaginal oocyte retrieval was performed 35 hours later. To avoid sperm contamination and possible amplification of sperm DNA, intracytoplasmic sperm injection was used. After 16 hours of incubation, fertilization was confirmed by the identification of 2 pronuclei. Normally fertilized concepti were then transferred to droplets of human tubal fluid (made on site) supplemented with 15% ma-
tential serum under mineral oil (ER Squibb & Sons Inc, Princeton, NJ). Biopsy was performed on the morning of the third day after harvest. All embryos were maintained at 37°C in an atmosphere of 5% carbon dioxide. Cleavage rate and morphologic appearance were assessed daily.

Blastomere biopsy was carried out in the early morning, approximately 65 hours after oocyte collection. Briefly, a holding pipette was used to stabilize the embryo (at the 9 o’clock position). A hole was made at the 3 o’clock position by expelling a small amount of acidified Tyrode solution (pH, 2.35) onto the zona pellucida through a small-bore pipette. Reverse suction was applied as soon as a hole of appropriate size was created to reduce possible damage caused by exposing the blastomeres to the acidic solution. A large inner-diameter biopsy pipette replaced the pipette containing the acidified Tyrode solution. Subsequently, 1 or 2 cells were aspirated, depending on the total cell number of the embryo. Blastomeres were rinsed in biopsy medium 3 times before loading into a PCR tube containing 5 µL of lysis buffer. Tubes were processed and PCR amplification and restriction enzyme analysis were performed as described herein.

Micromanipulated embryos were further cultured in medium droplets overnight. Embryo transfer was performed in the afternoon of day 4.

Pregnancy was determined by serum β-human chorionic gonadotropin measurement on cycle days 28 and 35, followed by ultrasonographic assessment at 7 weeks’ gestation. The genetic status of the fetuses was confirmed after amniocentesis by an independent laboratory. Cultured amniocytes were also sent to our laboratory for follow-up PCR analysis.

RESULTS

Polymerase chain reaction and restriction enzyme analysis of single lymphocytes from both the female and male partners clearly indicated that each carried the sickle cell mutation. Forty-six of 48 single lymphocytes, 24 from the female and 24 from the male, were successfully amplified. As predicted, 4 bands of correct size were obtained from both partners. An example of the gel is shown in Figure 1.

During the first IVF attempt in November 1996, 18 oocytes were retrieved. Four of 6 mature oocytes were normally fertilized after single-sperm injection by intracytoplasmic sperm injection, yielding 4 embryos.

Embryo biopsy was performed on all 4 embryos on day 3 by removing a single blastomere from each conceptus. Polymerase chain reaction and restriction enzyme analysis revealed that 1 was homozygous unaffected, 2 were carriers, and 1 was homozygous affected. Transfer of 1 unaffected embryo on day 4 failed to result in a pregnancy.

The second IVF attempt was initiated in August 1997, during which 16 oocytes were retrieved. Of those, 8 were mature and underwent intracytoplasmic sperm injection. Seven concepti cleaved at least once by the following day. On the morning of the third day, 2 cells were removed from 2 embryos and 1 cell from 5 embryos. Polymerase chain reaction amplification was successful in 7 of 9 blastomeres (5 of 6 embryos). Amplification failed in 1 cell from embryo 5 and in 1 cell from embryo 6. Restriction enzyme digestion demonstrated that 4 were homozygous unaffected, 2 were carriers (Figure 2), and 1 was of unknown status due to PCR amplification failure. On the afternoon of day 4, all concepti that underwent biopsy demonstrated further cleavage. Selection of embryos for transfer was based on PGD diagnosis, growth rate, and morphology. Two unaffected embryos were of poor quality, displaying slow cleavage and multinucleation (embryo 2) or high fragmentation (embryo 4) and therefore were not suitable for transfer (Table). Because there were only 3 high-quality transferable embryos—2 unaffected (embryo 1 with 15 cells; embryo 6 with 8 cells) and 1 carrier (embryo 8 with 10 cells) (Figure 3 and the Table)—and because the patient was willing to accept a fetus of carrier status, all 3 embryos were transferred.

A twin pregnancy was confirmed by ultrasonography at 7 weeks. Amnio-

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centesis performed by an independent laboratory at 16.5 weeks revealed that neither fetus harbored the sickle cell mutation. DNA analysis of amniocytes shipped from the prenatal diagnostic laboratory to our own laboratory also showed that both fetuses were unaffected (Figure 4). The patient delivered healthy, unaffected fraternal twin girls at 39 weeks' gestation.

**COMMENT**

After natural conception, couples who carry autosomal recessive mutations risk a 25% chance of delivering an affected child, and half of the offspring may carry the mutation. Although prenatal testing is currently available, some couples have strong personal objections to aborting affected fetuses. For these couples, PGD provides a realistic alternative to prenatal testing.

Although the first pregnancy achieved by PGD for sex determination to avoid the transmission of a sex-linked disorder occurred nearly a decade ago,17 PGD for single-gene defects is still in the experimental stage because of its complexity and technical difficulties. At present, PGD is primarily applied for severe genetic disorders for which detailed genetic information is available. Normal pregnancies following a search for specific mutations have been reported for only a few genetic diseases, including cystic fibrosis22-24 and Tay-Sachs disease.25

Despite the fact that sickle cell anemia is one of the most common genetic disorders and detailed genetic information is available,1,26 unaffected pregnancies following PGD for sickle cell anemia previously have not been reported. Lack of previous success in this area presumably is due to the length of time and effort required to overcome technical difficulties inherent in these procedures, as well as lack of available research funding. Our results demonstrate that sickle cell anemia can be detected in single cells by PCR and restriction enzyme analysis and that unaffected pregnancies can be established by the transfer of embryos of known genetic makeup that have undergone biopsy.

The protocol used in this study was initially developed in the mouse model by Sheardown et al.18 In their investigation, 4 tandem copies of the human β-globin gene were detected in transgenic mouse embryos. In humans, β-globin is a single-copy gene. Under clinical PGD circumstances, single-cell PCR requires an extremely sensitive protocol. In this study, we initially tested the protocol on single lymphocytes isolated from each member of the couple at risk. Lysis buffer, reported to be better for single-cell PCR,19 was used in-

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**Table.** Detailed Information on Fertilization, Embryo Morphology, Biopsy, PGD Results, Intrauterine Transfer, and Implantation*<sup>†</sup>

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<th>Embryo No.</th>
<th>No. of Blastomeres Fragmentation Before Biopsy†</th>
<th>No. of Blastomeres Removed</th>
<th>PGD Results</th>
<th>No. of Blastomeres Fragmentation on Day 4</th>
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*PGD indicates preimplantation genetic diagnosis; ellipses, data not applicable.†Cytoplasmic fragmentation occurs as cells make and break contact during cleavage. The percentage of total fragmentation is defined as the percentage of total surface area covered with cytoplasmic fragments.‡Oocyte not fertilized.
stead of the freeze-thaw method. Using the modified protocol, successful amplification was achieved in 46 (96%) of 48 single lymphocytes tested. This provided invaluable preliminary technical experience prior to executing the PCR-PGD technique in the clinical setting. However, PCR failure occurred in 1 of the 9 cells undergoing biopsy. Diagnostic failure in PCR-PGD could be due to a number of factors, including the absence of a nucleus, loss of the blastomere during handling, and blastomere mosaicism. While the current technique is apparently adequate, further modification and enhancement, such as the use of fluorescent PCR, may further improve the accuracy and efficiency of this method.

We performed biopsies on day 3 and intrauterine transfer on day 4 because the biopsy, PCR, enzyme digestion, and electrophoresis could not be completed within 10 hours. This change provided additional valuable hours for accurate diagnosis. Apparently, the extended in vitro culture did not compromise embryo viability. Because both the morphology and genetic status of each of the transferred embryos were known, it is worth examining the characteristics of each embryo. On the morning of day 3, embryo 6 (Table) was composed of 7 blastomeres, 2 of which were removed for testing. This accounts for more than one fourth of the embryo volume. At the time of intrauterine transfer on day 4, the conceptus had reached the 8-cell stage. This embryo implanted, as indicated by its genetic status (βA/βA). It is known from animal models that biopsy does not decrease implantation and subsequent live birth rates and that embryo biopsy does not necessarily impair subsequent in vitro development in humans. This particular case unequivocally shows that the removal of 2 blastomeres from a 7-cell conceptus did not compromise its developmental potential. Furthermore, our results confirm that day-3 biopsy and day-4 transfer is indeed a feasible approach for PGD. Recent progress in developing culture media and/or human autologous endometrial cocol-ture may even further enhance embryo viability, thus increasing the pregnancy rate after PGD.

In summary, this is the first unaffected pregnancy and delivery after successful PGD for sickle cell anemia. Our results demonstrate that PGD for the detection of sickle cell anemia is a powerful diagnostic tool for carrier couples who desire a healthy child but wish to avoid the difficult decision of whether to abort an affected fetus. The procedure, successfully used in this case, may also be applied to other monogenic disorders and further supports the notion that PGD is destined to be an integral part of medical therapy.

Figure 3. Embryo Morphology Prior to Transfer on Day 4

Figure 4. Polymerase Chain Reaction and Restriction Analysis From Amniocytes From the 2 Fetuses, Showing 2 Unaffected DNA Patterns

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aspect of assisted reproductive technology. Given the current methods and relatively high cost of the procedure, it is unlikely that PGD will totally replace prenatal testing. However, it is conceivable that with further refinements, PGD will certainly become an invaluable and powerful diagnostic modality.

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