Pregnancy After Embryo Biopsy and Coamplification of DNA From X and Y Chromosomes

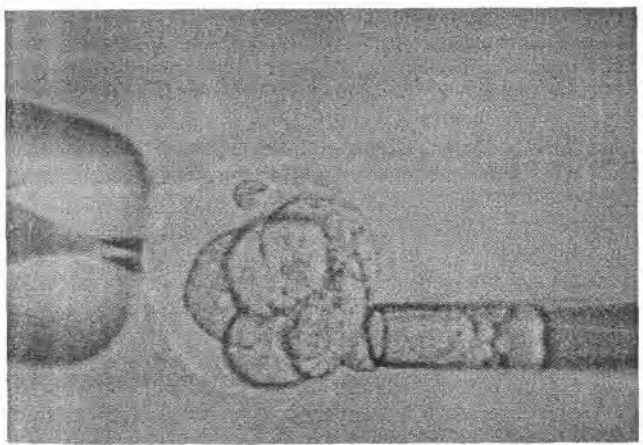

Fig 1.—The embryo biopsy procedure of an eight-cell embryo obtained after in vitro fertilization. The embryo is immobilized at the end of a holding pipette and a 15-μm hole is made in the zona pellucida with acidified Tyrode's solution.[5] A biopsy pipette is inserted through the hole and a blastomere is removed using gentle suction (magnification ×200).

Preimplantation genetic diagnosis has been suggested as an alternative to chorionic villus sampling or amniocentesis for carriers of sex-linked disorders who have a 50% risk of transmitting the disease to male offspring.[1] This allows for gender assessment of the embryo prior to implantation. The ability to remove a single blastomere from a six-cell to 10-cell human embryo without affecting further development has been demonstrated.[1,2] Handyside and coworkers[1,2] identified embryonic gender using Y-chromosome–specific DNA amplification and transferred Y-negative embryos with resultant offspring. In this system, an embryo is considered female if an amplification product is not detected. To date, fewer than 10 infants have been born following this procedure. However, one male fetus was detected after chorionic villus sampling and the pregnancy was terminated. The incorrect diagnosis was probably the result of a failed amplification or the blastomere may have been lost during processing. To diminish the likelihood of this type of inaccuracy, we used the strategy of multiplex polymerase chain reaction (PCR) by amplifying X- and Y-chromosome–specific sequences simultaneously.[3,4]

With coamplification, the X signal is absent when the assay fails or nuclear material is not present. Gender determination of multiple blastomeres of 29 biopsied monospermic embryos showed an error rate of 3.4%. Multiplex PCR was used during the current study to determine the sex of embryos from a hemophilia carrier. Two embryo biopsy cycles were performed after in vitro fertilization with subsequent transfer of X-typed embryos. The patient is now in her third trimester and carrying a singleton pregnancy with a 46,XX karyotype confirmed by amniocentesis.

Materials and Methods

Procedures were approved by the Cornell Human Investigation Committee (protocol 0890-985).

Embryo Culture and Biopsy.—Acidified Tyrode's solution was used for local digestion of the zona pellucida.[5] The blastomeres of six-cell to 10-cell embryos adjacent to the openings were removed by micromanipulation (Fig 1).[6,7] Single blastomeres were removed from the embryos for the clinical study. Two or more cells were removed and separately processed for control studies using spare embryos. Microtool-making and micromanipulation procedures have been extensively described elsewhere.[7] Nuclei were visualized with phase contrast microscopy after biopsy. Blastomeres were washed five times in serum-free culture medium, transferred to PCR tubes, and freeze-thawed in liquid nitrogen three times. Thirty microliters of sterile water was added, and the tubes were incubated at 95°C for 15 minutes after covering with paraffin oil.

PCR.—Polymerase chain reactions were performed in 50 μL of reaction buffer containing 10 mmol/L of tromethamine hydrochloride (pH 8.3), 50 mmol/L of potassium chloride, 1.5 mmol/L of Mg^{++}, 200 μmol/L each of deoxy-nucleotide-triphosphates and 2 to 5 U of *Taq* polymerase. Twenty-five picomoles of each primer was used. Following a 7-minute incubation at 96°C, 30 repeated cycles were carried out with 1 minute at 94°C, 1 minute at 55°C, and 1.5 minutes at 72°C. Ten picograms of male DNA, 10 pg of female DNA, and sterile distilled water were used as controls in each experiment.

Ten-microliter–amplification products were analyzed by 1.4% agarose gel electrophoresis in tromethamine hydrochloride–ethylenediaminetetraacetic acid buffer at 300 mV for 10 minutes in a Hoefer mini-gel apparatus. Homidium bromide was incorporated directly into the gel for visualization of DNA amplification products using UV light. The analysis was

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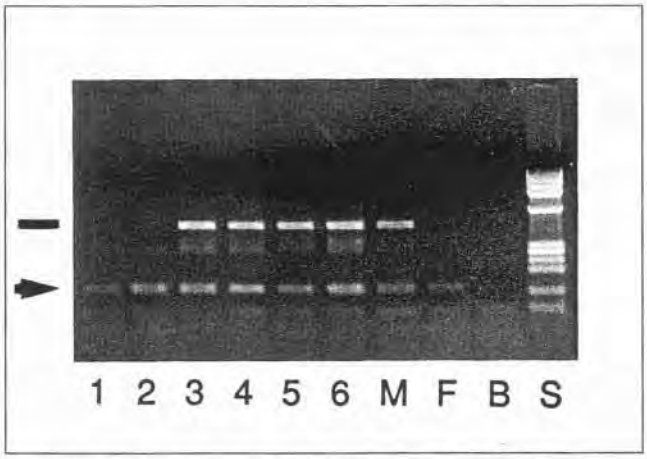

Fig 2.—Multiplex polymerase chain reaction (PCR) of DNA from blastomeres of human polyspermic embryos and genomic DNA. Agarose gel electrophoresis and visualization of DNA amplification products by homidium bromide staining and UV light. Lanes 1 and 2 contain amplification products from single blastomeres of the same polyspermic embryo using multiplex PCR. The only band visualized is the 130 base pair fragment (identified by an arrow), unique for the X chromosome. The band in lane 1 is faint and not visualized well in the photograph. This happens occasionally but identification is clear when the bands are directly visualized. Lanes 3 and 4 contain amplification products from single blastomeres of the same polyspermic embryo. In this case, the 500 base pair fragment (identified by a line) unique to the Y chromosome is visualized along with the 130 base pair fragment from the X chromosome (male karyotype). Lanes 5 and 6 contain amplification products from blastomeres of another polyspermic embryo that also has a male karyotype. The control lanes labeled M, F, and B, contain 10 pg of male DNA, 10 pg of female DNA, and water, respectively. The lane marked S contains a standard with DNA of known fragment length.

completed in less than 5 hours. The work was carried out in a clean area that was specifically allocated to single-cell DNA analysis. Samples containing more than 10 pg of DNA were never brought into this area. Surgical hats and masks were used, and surgical gloves were changed frequently to avoid contamination. Only female technicians handled the specimens after the biopsy procedures.

Case Description.—Embryos were obtained by IVF from a patient who is a carrier of hemophilia.[7,8] In this particular case, IVF was required since the patient had longstanding unexplained infertility and her husband was azoospermic; 12 prior cycles of donor sperm failed to result in pregnancy. Given this history, it is unlikely that a spontaneous pregnancy would occur with her partner. Furthermore, the couple was instructed to abstain from sexual activity during the IVF procedures in which donor sperm were again used.

Embryos were sexed and those with X chromosomes were transferred during two separate cycles. Three embryos containing only X-chromosome amplification products were transferred in the first cycle. Pregnancy did not occur. In the second cycle, four embryos were transferred, resulting in an ongoing singleton gestation.

Controlled Studies.—Eleven biopsied monospermic embryos that were not selected for transfer from the patient described above were reanalysed by PCR. These embryos either were at the blastocyst stage or had been arrested during compaction. In addition, blastomeres of eighteen 4-day-old arrested, cleaved monospermic embryos from 14 regular IVF patients were separated and processed in a double-blind fashion. Blastomeres from 18 polyspermic embryos, also from regular IVF patients, were separated and processed as well.

Results

The sensitivity of the assay system was analyzed either by comparing groups of blastomeres from monospermic embryos (n=18) or by assessing biopsied male embryos from the patient described here and comparing them to single blastomeres (n=11). Forty-eight blastomeres from 18 polyspermic embryos were also analyzed. At least two sibling blastomeres per embryo were studied. Amplification products of 500 and 130 base pairs were obtained from the Y chromosome and the X chromosome, respectively.[6,8] Both X- and Y-chromosome–specific amplification products were obtained from the male control, only the X-specific amplification products were obtained from the female control, and no products were obtained from the water control. The assays of blastomeres from biopsied monospermic embryos (n=29) revealed that gender was incorrectly assessed in one case (3.4%).

Figure 2 demonstrates multiplex PCR on blastomeres from human polyspermic embryos. Amplification products were not identified in two tubes (not shown). This would not necessarily produce an error in preimplantation diagnosis since such an embryo would not be transferred. Two blastomeres from the same embryo had Y-amplification products exclusively. This would also not result in embryo transfer. However, it suggests that selective failed amplification can occur. Two blastomeres from the same embryo were found to have a Y product in one tube and no Y product in the other despite the presence of X products in both tubes. Two blastomeres from these embryos were sexed in a later experiment. Both X and Y products were detected, suggesting that this represented a selective failed amplification of the Y band. Alternatively, since polyspermic embryos are fertilized by more than one sperm and since some have been found to be mosaic for ploidy, it is possible that cells from the same polyspermic embryo would have different results by this method.[9,10] Thus, the maximum error rate of sexing polyspermic embryos by this method was one in 18. Twelve of the polyspermic embryos had X and Y chromosomes and six had only X chromosomes, which is approximately the expected sex ratio from embryos fertilized by more than one sperm.

In the first IVF cycle, 10 six-cell to 10-cell embryos underwent biopsy; four were XY, four were XX, and in two the cells were lost. Amplification was not obtained in three water-blank or in three culture-medium controls. Three presumed female embryos were transferred later in the same day but the patient failed to become pregnant. In the second IVF cycle, 12 six-cell to 10-cell embryos underwent biopsy. Four contained only X chromosomes and five contained X and Y chromosomes. From three of the embryos, either the blastomeres were lost or the signal was weak. Four presumed female embryos were transferred later in the same day and the patient became pregnant. Amniocentesis at 16 weeks' gestation confirmed a 46,XX karyotype.

Comment

The current results confirm that it is possible to correctly diagnose the sex of an embryo by removing a single blastomere from an embryo and subjecting it to simultaneous amplification of X- and Y-chromosome–specific DNA by PCR. The results extend those of others who obtained offspring after biopsy of cleaved human embryos using a single Y primer.[1] While it would be preferable to determine directly whether an embryo is affected with hemophilia, currently this is not possible by embryo biopsy and PCR analysis. In this patient, sexing the embryo was the best alternative for preventing the transmission of this disease. In theory, preimplantation diagnosis could be applied to any genetic disease in which the responsible gene is known by modifying existing assays to detect gene mutations or linked markers in single cells. This would provide an alternative for couples desirous of preventing the birth of an affected child, but unwilling to consider pregnancy termination.

Polymerase chain reaction with both X- and Y-chromosome–

specific primer pairs applied to a single human blastomere provides gender analysis in less than 5 hours. Reamplification and multistep amplification are not necessary. However, it is imperative that anticontamination policies be strictly enforced while using this procedure. Furthermore, negative controls must always be included in these analyses.

In our hands, an error was detected only once when cells from 29 monospermic embryos were compared. Evaluations of 18 polyspermic embryos revealed also a low error rate of 6%. It has been found that the incidence of mosaicism for ploidy is elevated among polyspermic embryos.[10] Though it has been suggested that this could reduce their value for preimplantation genetic diagnosis, ploidy is essentially not determined during XY multiplex PCR. An amplification product of exclusively X, for instance, could be representative of several genetically different embryos including the haploid X, the diploid XO, the diploid XX, and the triploid XXX. Though gender determination should not be confused with assessment of ploidy, it is advisable to determine the accuracy of XY PCR with monospermic rather than polyspermic embryos. Ultimately, the accuracy of this technique will await its successful use in a large number of couples at risk for transmitting a sex-linked disorder.

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