BIOPSY OF HUMAN PREIMPLANTATION EMBRYOS AND SEXING BY DNA AMPLIFICATION

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Summary A single cell was removed, through a hole made in the zona pellucida, from each of 30 human embryos at the 6–10 cell cleavage stage three days after in-vitro fertilisation. A normal proportion of the embryos (37%) developed to the blastocyst stage by day six in culture and 6 hatched from the zona. Each male embryo was sexed from the DNA by amplification of a repeated sequence specific for the Y chromosome. In 15 embryos with the normal two pronuclei the sex was determined also by in-situ hybridisation with a Y specific probe or fluorescent chromosome staining to detect metaphase Y chromosomes; the results of Y specific amplification were confirmed. This approach may be valuable for couples at risk of transmitting X-linked disease.

Introduction

THE detection of inherited diseases in very early preimplantation embryos would allow the selection and transfer of only healthy zygotes to the uterus. After preimplantation diagnosis, couples with a high risk of a genetically defective baby could embark on a pregnancy knowing that it was free from a specific serious inherited disorder. These parents would avoid the dilemma posed by prenatal diagnosis later in gestation—whether or not to abort a much wanted but affected fetus.[1] In certain X-linked inherited disorders for which a specific diagnosis is not yet available, the sex of the fetus may currently be ascertained after chorion villus sampling or amniocentesis. Male pregnancies are terminated, though half of these are unaffected. Although preimplantation diagnosis of sex would not allow the birth of a healthy boy, it would prevent unnecessary termination of an established pregnancy. To date, only entire human preimplantation embryos have been successfully sexed.[2,3] This required the destruction of the whole zygote, so is not directly clinically applicable. Moreover, the use of a tritiated Y specific probe for in situ hybridisation in these studies required a long period for diagnosis. In practice, even if only part of the embryo was analysed, the embryos would need to be frozen until diagnosis was available. Consequently, uterine transfer would have to be undertaken in a subsequent menstrual cycle. This complex process would probably increase the risk of damage to the embryo and reduce its chance of successful development.

The advent of the polymerase chain reaction (PCR) to amplify known sequences of DNA has great potential.[4] It is now possible to sex the fetus by amplification of a repeated sequence unique to the Y chromosome.[5] Moreover, the technique is rapid and its use to amplify the DNA from a single cell[6] paves the way for DNA diagnosis in the human preimplantation embryo after in-vitro fertilisation (IVF).

We report here our experience of non-destructive sexing of early human embryos.

Materials and Methods

Human Embryo Biopsy and Culture

After approval of the project by the ethics committee of the Royal Postgraduate Medical School and by the Voluntary Licensing Authority for Human In Vitro Fertilisation and Embryology, patients were approached at least one month before proposed IVF for tubal infertility to ascertain whether they would consider donating "surplus" embryos, if available as a consequence of their treatment, for studies aimed at better diagnosis of genetic disease. After induction of multiple ovulation with a gonadotropin releasing hormone analogue and human menopausal gonadotropins, oocyte collection was timed according to stringent endocrinological and ultrasound criteria.[7] Mature oocytes were harvested by ultrasonographically directed or laparoscopic methods, preincubated for about six hours, and inseminated (day 0). 15–18 hours later the oocytes were checked for the development of pronuclei, to confirm normal fertilisation. Culture was in 1 ml T6 medium[8] (or Earle's minimal essential medium) containing 10% maternal serum in 5% CO_2, 5% O_2, and 90% N_2. On day 2, each embryo was examined and up to 3 normally cleaving embryos with optimum morphology were selected for transfer. After written confirmation of the couple's informed consent, any remaining "surplus" embryos were cultured for a further day before biopsy.

Embryos with 6–10 cells by day 3 were studied. After transfer to microdrops of Hepes buffered medium under oil, biopsies were taken with a pair of Leitz micromanipulators under a stereo dissecting microscope. Each embryo was immobilised with a flame-polished holding pipette held in one micromanipulator. A drilling pipette (diameter 10–20 μm) containing acid Tyrode's solution (pH 2·5)[9,10] and a biopsy pipette (diameter 30–50 μm) containing medium were stabilised in a double holder system in the other micromanipulator. The drilling pipette was brought in contact with the zona and a hole was made by expulsion of the acidified medium. The pipette was withdrawn and the biopsy pipette was pushed through the hole in the zona and a single blastomere was removed. The procedure took 5–10 minutes and the embryo was then promptly returned to culture. Morphological development was assessed each day, and on day 6 the embryos were prepared for cytological analysis.

DNA Amplification by PCR

Oligonucleotides encompassing 149 base pairs (bp) of a 3·4 kilobase repeat sequence on the Y chromosome have been described.[5,11] These were synthesised on an Applied Biosystems 380A synthesiser and purified by polyacrylamide gel electrophoresis, elution, phenolchloroform extraction, and ethanol precipitation. Since contamination of the reaction with extraneous Y sequences is a potential hazard in 10^{10}-fold amplification, all buffers were passed through a 0·2 μm filter ('Acrodisc'). The oligonucleotides and sheared salmon sperm DNA were subjected to digestion with the restriction enzyme EcoR1 and then boiled to destroy the enzyme. This step prevents amplification of any contaminating Y sequence since the target sequence contains an EcoR1 site. All manipulations were performed under class II containment to reduce further the risk of contamination. Without these stringent precautions we frequently observed an amplified DNA band in buffer controls and female DNA samples identical to that seen with male derived DNA. Presumably this contamination is derived from airborne skin cells or fragmented DNA. It is therefore important to include appropriate negative controls and to observe a strict anti-contamination protocol. Single blastomeres removed from each embryo were washed three times in the biopsy medium under sterile conditions, placed in 90 μl of reaction buffer, and stored at −20°C. The buffer contained: "tris" HCl pH 8·3 10 mmol/l, KCl 50 mmol/l, $MgCl_2$ 1·5 mmol/l, gelatin 0·01%, and 200 μm each of dATP, dGTP, dCTP, and dTTP. Immediately before amplification, the cells were incubated at 94°C for 30 min to ensure complete lysis and inactivation of nucleases. 1 μg of each oligonucleotide, 10 ng of sheared salmon sperm DNA, and 2·5 U of thermostable DNA polymerase (Taq polymerase; Cetus) were added and the mixture was incubated at 94°C for a further 150 s. This was followed by 60 cycles of incubation at 65°C for 90 s

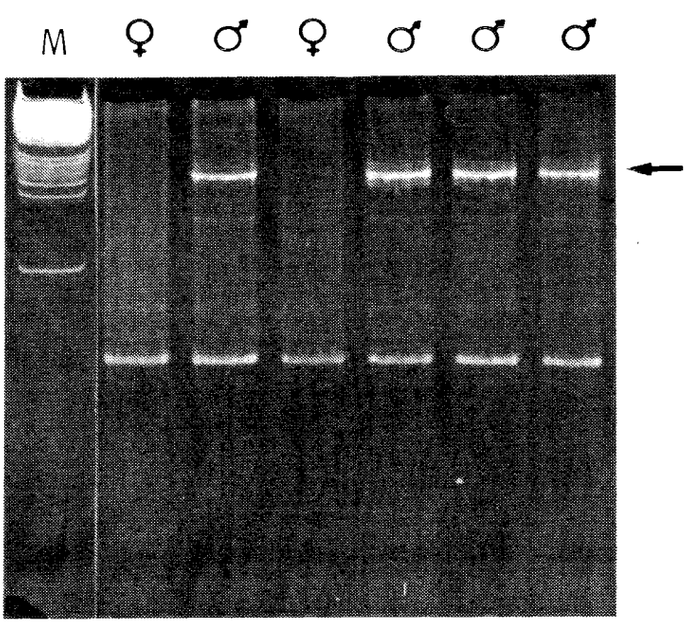
alternating with 94°C for 30 s in a Perkin Elmer Cetus Thermal Cycler (Perkin Elmer, Beaconsfield, UK). 10 µl of the reaction product was electrophoresed on a 12% polyacrylamide gel, stained with ethidium bromide, and inspected under ultraviolet light. A cell was judged male if the 149 bp fragment of the Y specific repeat was visible, and female if the band was absent or very faint. The whole procedure, from receipt of the cell to direct visualisation of amplified DNA, took approximately 5 h.

Cytological Analysis

Detailed methodology is described elsewhere[12] and is summarised below. After morphological assessment on day 6, biopsied embryos were transferred to microdrops of medium containing 0·05 µg/ml colchicine ('Colcemid', Sigma) and incubated for 5–12 h. The colchicine treated embryos were spread on microscope slides for examination of the nuclei by a modification of Dyban's method for mouse embryos.[13] Embryos were incubated in a hypotonic solution of 1% (w/v) sodium citrate and the zona pellucida was removed with acid Tyrode's solution. Each embryo was fixed in methanol/acetic acid (3/1) in a precooled watch glass until cleared. The fixed embryo was placed on a precooled microscope slide and extra drops of fixative were added from the side as necessary to promote adequate spreading of the nuclei. The position of the embryo was then marked, the slide was stained with 10% (w/v) Giemsa solution, and the numbers of interphase and metaphase stage nuclei were counted. After destaining, those with metaphases were stained with spermidine-bis-acridine[14,15] and were analysed for the presence of fluorescent Y chromosomes. Finally, the embryos were sexed by in-situ hybridisation to the probe pHY2·1 which recognises a sequence repeated 2000 times on the Y chromosome and fewer than 200 times on the autosomes.[16] The probe was biotinylated by nick translation and was detected with streptavidin linked alkaline phosphatase (BRL Blugene kit, catalogue no 8279SA) as previously described.[17,18]

Results

Biopsy material was taken from 38 embryos—30 "normal" embryos (ie, possessing two pronuclei); 5 polyspermic embryos with three or more pronuclei; 3 parthenogenetic embryos with either one or no pronucleus. 3 normal embryos were fixed immediately after biopsy; 10 of the remaining 27 (37%) developed into blastocysts and 6 (22%) had hatched from the zona by day 6. The mean cell number in these blastocysts was 35·6 (range 18–74). Of the abnormal zygotes, 1 of the 5 polyspermic embryos developed to the hatching blastocyst stage, and 2 of 3 parthenote embryos developed to the cavitating morula stage.



Amplification product electrophoresed on polyacrylamide and displayed under UV illumination.

A strong 149 bp band is present in male cells (arrow). The constant band at approximately 40 bp represents "primer dimer". Oligonucleotides (23 and 25 bp) are also faintly visible

COMPARISON OF DNA AMPLIFICATION, IN SITU HYBRIDISATION, AND FLUORESCENT STAINING FOR SEXING PREIMPLANTATION EMBRYOS

DNA amplification	In-situ hybridisation	Fluorescent staining
Male		
1	M	M
2	NA	NA
3	M	M
4	M	NA
5	NA	NA
6	M	M
Female		
1	NA	NA
2	F	NA
3	F	NA
4	F	NA
5	F	F
6	F	NA
7	F	F
8	F	F
9	F	F
10	F	F
11	F	NA
12	F	F
Polyspermic		
2 F	NA	NA
3 M	F	NA
4 M	NA	NA
5 M	F	NA
Parthenogenetic		
1 F	F	F
2 F	F	F
3 F	NA	NA

NA = not analysed.

Single cells from 25 embryos were sexed by PCR (figure); in the early stages of the study 13 were lost because of technical problems. 4 males and 11 females sexed by PCR were confirmed as correctly sexed by other methods. The 3 parthenote embryos were sexed by PCR as female. 2 polyspermic embryos sexed as male by PCR showed no hybridisation with the Y probe (table).

Discussion

These preliminary observations suggest that human preimplantation embryos produced by IVF can be accurately sexed. Biopsy and PCR sexing was accomplished within about 8 h; thus selected female embryos could be transferred to the uterine environment on the day of diagnosis. Embryo transfer after clinical IVF is routinely done on day 2; however, limited experience here and in other centres suggests that pregnancy rates are similar when transfer is on day 3 (transfer on days 4–6 gives very low pregnancy rates[19]). Our strategy of early biopsy and immediate diagnosis avoids the need to transfer blastocysts or to employ cryopreservation—a procedure that would undoubtedly cause loss of some embryos.

All 15 normally fertilised embryos were correctly sexed by means of DNA amplification. In only 2 other embryos was the sex not verified by the other methods. Both of these were abnormal polyspermic embryos and both were identified as male by PCR but failed to hybridise with the Y specific probe in situ. The most probable explanation is that

polyspermic embryos are often mosaic with some multinucleate cells.[20,21] Whatever the explanation, false-positive diagnosis in an abnormal embryo is clinically irrelevant.

Is the biopsy technique itself harmless? After biopsy we have observed normal development to the blastocyst stage in 37%—ie, about the same proportion as that of unmanipulated embryos.[22] These results have to be treated with caution, however, since good morphological development does not necessarily indicate potential to implant and develop successfully. The mean of 35·6 cells in each blastocyst indicates that the embryos had undergone three further cleavage divisions and in many the cells were in active division. Further work is needed to ascertain whether this cell complement is comparable with that at a similar developmental stage without biopsy. In this study, biopsy was undertaken when the blastomeres are thought to be totipotential. Loss of blastomeres is commonly observed after human IVF at this stage of development;[23] there is no evidence that this can lead to fetal abnormality, though potential for implantation may be affected.[24]

PCR has been applied to the prenatal diagnosis of sickle cell anaemia and other inherited single gene defects on small numbers of cells.[5,24] More recently, PCR has been used to amplify unique sequence DNA from a single diploid cell and a single spermatozoon.[6] In these studies amplified DNA was detected after PCR of diluted DNA or single cells or sperm by dot blot analysis with labelled allele specific oligonucleotides[5,6,25]—a procedure that increases analysis time by a minimum of 6 hours. By choosing to amplify a highly repeated sequence of the Y chromosome (800–1500 copies) we were able to visualise the PCR product directly on ethidium bromide stained polyacrylamide gel. If single copy sequences can be amplified by PCR sufficiently for direct visualisation, then the technique developed for sexing in X-linked disorders could be applied for detecting any single gene disorder, with same-day transfer. Such techniques should be contemplated only in IVF centres with skills in molecular genetics and the special manipulative techniques.

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ANORECTAL VARICES, HAEMORRHOIDS, AND PORTAL HYPERTENSION

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Summary In a prospective study of 100 consecutive patients with cirrhosis, 44% had anorectal varices. The prevalence of anorectal varices rose with progression of portal hypertension; it was 19% in cirrhotic patients without portal hypertension compared with 59% in those who had bled from oesophageal varices. There was no evidence that endoscopic sclerotherapy directly increased the prevalence of anorectal varices. Haemorrhoids occurred independently of anorectal varices and their presence was unrelated to the degree of portal hypertension. These data provide further evidence that haemorrhoids and anorectal varices are separate and distinct entities. However, both can bleed and careful examination is essential to prevent misdiagnosis and inappropriate treatment.

Introduction

THE aetiology of, and relation between, anorectal varices and haemorrhoids are the subject of much debate.[1,2] Anorectal varices are portalsystemic collaterals which develop in patients with portal hypertension and can occur

A H HANDYSIDE AND OTHERS. REFERENCES—*continued*

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