Blood lymphocyte chimerism associated with IVF and monochorionic dizygous twinning: Case report


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We report on dizygotic (DZ) twins, conceived by IVF and ICSI with assisted hatching, who each had a mixture of 46,XX and 46,XY cells in blood lymphocytes. The female twin had mild genitalia abnormalities but further study revealed anatomically normal reproductive anatomy. Chromosome and fluorescence in situ hybridization studies of buccal, skin and ovarian tissue were normal, as were buccal tissue DNA studies. Fetal ultrasound and fetal membrane pathology were consistent with a monochorionic, diamniotic placenta (MCDAP). These twins thus have blood chimerism but are not chimeric in the other tissues studied. The mechanism for the chimerism could be due to either placental vascular anastamoses (after the development of the haematoblast stem cells) or due to an admixture of trophoblast cells during early blastocyst development. Such trophoblast cell admixtures would be restricted to the extraembryonic tissues so that general physical development in the fetus is normal and without somatic cell chimerism. This case in combination with others previously reported suggests that in IVF conceptions, the prevalence of blood chimerism associated with twinning, and the occurrence of DZ twinning associated with MCDAP, may be higher than previously thought.

Key words: assisted hatching/assisted reproduction techniques/blood chimerism/IVF/monochorionic placenta

Introduction

An increased rate of twin pregnancies, usually dizygotic (DZ), is a well-known complication of IVF. More recently, an increased rate for monozygotic (MZ) twinning has been appreciated (Blickstein et al., 1999; Schachter et al., 2001). Often, MZ twins will exchange blood via placental anastamoses, resulting in blood chimerism. The definition of blood chimerism means that cells are derived from at least two zygote lineages and have populated only the lympho-haematopoietic compartment. Chimerism should be distinguished from cellular mosaicism, a term used when genetically different cells have arisen from a single zygote lineage.

Since mosaicism is much more common than chimerism, detection of two different cell lines in a neonate’s blood chromosome study is generally presumed to be mosaicism, provided that the chromosomal sex is concordant. However, the possibility of chimerism is raised when both 46,XX and 46,XY cells are detected. We report unlike-sex twins, conceived by IVF and ICSI with assisted hatching, who each had a mixture of 46,XX and 46,XY cells in their blood lymphocytes.

Maternal history

The patient was a 38 year old para 0111 who conceived via IVF. Her prior pregnancies resulted in a spontaneous abortion at 4 weeks gestation and a low transverse Caesarean delivery at 29 weeks gestation due to severe pre-eclampsia. Additional surgical history included a sterilization operation, attempted tubal reanastomosis, and laparoscopic ovarian cystectomy. In addition to IVF, she was being treated with metformin for hyperinsulinaemia contributing to her secondary infertility. Glucose challenge tests were negative on two occasions during the pregnancy. Her medical and family history otherwise were unremarkable.

IVF procedure

The patient was placed on oral contraceptives prior to ovarian stimulation. After ovarian suppression had been achieved, the ovaries were then stimulated with a combination of recombinant FSH (225 IU) and hMG (75 IU) s.c. Monitoring of follicular activity was performed with a combination of serum estradiol (E2) measurements and transvaginal ultrasonography. Once the lead ovarian follicle reached
a size of 14 mm, a GnRH antagonist, cetrorelix 0.25 mg s.c., was administered daily until administration of hCG. The hCG was administered when two lead follicles were ≥18 mm, after 9 days of ovarian stimulation, and transvaginal oocyte retrieval was performed 2 days later. Thirteen oocytes were retrieved, eight were judged to be mature, and because of male factor abnormalities, ICSI was performed on these oocytes. After viewing the presence of a polar body upon removal of surrounding cumulus cells using hyalurondase enzyme, a single sperm was injected into each oocyte. Micro-injected oocytes were viewed 18 h post ICSI to determine fertilization status.

Six of the eight oocytes fertilized normally with two observable pronuclei. All fertilized oocytes cleaved normally following a further 24 h of culture in Quin’s Cleavage Medium (Sage). Three of the six embryos were cryopreserved and stored for future use at the day 6 blastocyst stage. Due to the patient’s age, three 8-cell embryos underwent assisted hatching using acid Tyrode’s solution (Irvine Scientific). The patient elected to have only two embryos transferred. The transfer procedure was performed without difficulty using the two grade A compacting 8-cell embryos (3 days after oocyte retrieval) using a Cook softpass transfer catheter with an outer Frydman sheath.

Two weeks after the embryo transfer, the pregnancy was determined by rising hCG titres. Three weeks thereafter, two gestational sacs were confirmed by transvaginal ultrasound. From the time of embryo transfer and for a total of 8 weeks the patient received daily luteal phase support in the form of oral E2 2–4 g and 50 mg of i.m. progesterone in oil.

**Prenatal history**

She was seen for her first prenatal visit at 9 weeks gestation. At that time, an ultrasound examination was performed because of a history of vaginal spotting over the prior 2 weeks. A viable twin pregnancy, that appeared to be mono-chorionic/diamniotic, was confirmed.

At 16 weeks gestation, she underwent genetic counselling because of advanced maternal age. She declined genetic amniocentesis but requested a genetic ultrasound examination. During this ultrasound examination, the non-presenting twin was found to have diminished amniotic fluid volume, a marginal cord insertion, an echogenic intracardiac focus, and an elevated ratio of the head circumference:abdominal circumference. In addition, the fetuses appeared to have unlike monochorionic diamniotic membranes. No vascular anastamoses, infarctions or calcifications were noted. The placenta weighed 560 g and was described as being monochorionic/diamniotic.

At 27 weeks gestation, the patient presented to our Labor and Delivery unit complaining of decreased fetal movement. Non-stress tests of both fetuses were reactive. However, she was noted to have blood pressures ranging from 116–154/72–78 and 3+ proteinuria. She was admitted to the antepartum service for suspected pre-eclampsia. By the fifth hospital day, at 28 weeks gestation, her systolic blood pressure had increased to the 160 s to 180 s, and delivery by repeat Caesarean was performed without complications.

**Birth and postnatal history**

Twin A weighed 1146 g, was a phenotypic male, and had 18P scores of 9 at both 1 and 5 min. Twin B weighed 654 g, was described by the Pediatric staff present at the delivery as having ambiguous genitalia, and had Apgar scores of 6 and 7 at 1 and 5 min respectively. Both infants were admitted to the Neonatal Intensive Care Unit.

Twin B was the first to have a chromosome study performed because of the clinical appearance of ambiguous genitalia (see Figure 1). At day 2 of life, a peripheral blood study showed 46.XX (15)/46,XY (5), so chimerism was considered. An ultrasound showed presence of a uterus in the normal location, but ovaries could not be identified. For completeness, a chromosome study then was performed on the normal appearing male (twin A) at day 7 of life. Surprisingly, the peripheral blood also showed a mosaic pattern: 46.XX (21)/46,XY (6). A repeat blood study on twin A at day 19 of life showed a similar 46,XX (20)/46,XY (5) result.

The placenta weighed 560 g and was described as having a single disc. The twin A cord had a marginal, and the twin B cord had a velamentous, insertion. The twins shared a common middle membrane that was thin and easily separated, making it a likely MCDAP. The chorionic plates, other than having the velamentous insertion for twin B, were unremarkable. No vascular anastamoses, infarctions or calcifications were noted.

**Endocrine studies**

Endocrine studies performed at 5 weeks of age (34 weeks gestational age) in the phenotypic male. Twin A, revealed a baseline FSH of 1.3 mIU/ml, LH 3.5 of mIU/ml, E2 of <20 pg/ml and testosterone of 262 ng/dl. An hCG stimulation test (5000 IU/m2 x 1) was performed and studies obtained 3 days later revealed an FSH of 0.4 mIU/ml, LH of 0.2 mIU/ml, estradiol of <20 pg/ml, and testosterone of 489 ng/dl. The testosterone values confirmed the presence of functioning testicular (Leydig cell) tissue. The elevated Müllerian inhibiting factor (MIF) confirmed the presence of functional Sertoli cells. The lack of measurable E2 confirmed the absence of ovarian tissue.

Endocrine studies performed at 7 weeks of age (36 weeks gestational age) in the phenotypic female, Twin B, revealed a baseline FSH of 141.8 mIU/ml, LH of 42.6 mIU/ml, E2 of 40 pg/dl, and testosterone of 72 ng/dl (see Table I). The elevated gonadotrophins and presence of testosterone in the face of peripheral blood mosaicism raised concern that Twin B
might have mixed gonadal dysgenesis and premature gonadal failure or be a true hermaphrodite. An hCG stimulation test was also performed on Twin B and revealed a stimulated FSH of 137 mIU/ml, LH of 58 mIU/ml, E2 of 33 pg/ml, and testosterone of 90 ng/dl.

In addition to concern about genital development, Twin B had bilateral inguinal canal swellings with palpable tissue in the mid-canal regions. An ultrasound confirmed the bilateral inguinal hernias with no evidence of bowel herniation, but there were soft tissue areas in the canal interpreted as being possible omentum. However, an exploratory laparotomy revealed herniated, normal-appearing ovaries. A vaginoscopy examination was normal. The ovaries were repositioned, and the hernias were repaired. For further extended chromosome study, biopsies were obtained from one of the ovaries and from inguinal skin. Biopsy of one ovary was also sent for pathology and showed gonadal tissue consistent with an ovary with numerous oocytes. Both twins remained hospitalized until ~2 months of life, and their hospital course was otherwise relatively benign. Twin A (phenotypic male) subsequently developed a left-sided inguinal hernia that was repaired, and a circumcision was also performed. A sample of the foreskin was sent for extended chromosome analysis.

**Materials and methods**

**Buccal swab microsatellite study**

DNA was extracted from buccal samples from the mother, father and the twins, using the PureGene DNA extraction system (Gentra, Inc.).
Seven autosomal microsatellite markers (D10S677, D2S1394, D3S2460, D1S1665, D8S1106, D2S1391, D1S1609) were amplified by PCR and genotyped by studying banding pattern of PCR products on native high percentage polyacrylamide gels and ethidium bromide staining. X/Y genotyping was done by multiplex PCR and analysed by agarose gel electrophoresis.

**Cytogenetic studies**

Metaphase cells were harvested from stimulated peripheral blood cultures, skin fibroblast cell cultures and ovarian tissue cultures, following standard cytogenetic techniques. Chromosome spreads were G-banded by standard trypsin-Giemsa banding technique. Metaphase cells were analysed microscopically for each sample submitted. Imaging and karyotyping were performed via computer imaging techniques (Applied Imaging, Inc.).

**Fluorescence in situ hybridization (FISH) studies**

Interphase and metaphase cells obtained from the peripheral blood, skin tissue, ovarian tissue, and buccal mucosa were treated with 0.75 mol/l KCl for 30 min and fixed in 3:1 methanol/glacial acetic acid prior to application of routine in situ hybridization and detection methods utilizing the following DNA probe sequences: SRY, DXZ1, DYZ1 and DYZ3. FISH images were digitally generated utilizing CytoProbe Imaging Software (Applied Imaging, Inc.).

**Results**

**Buccal swab microsatellite study**

Five markers (D10S677, D2S1394, D3S2460, D1S1665, D8S1106) showed clear dizygosity between the twins, with no evidence of an extra parental allele in either twin (data not shown). The lower level of detection of mosaicism with this method is estimated to be at the 10–15% level. One marker (D2S1391) showed an aberrant band in one twin which was not seen in either parent and was presumed to be artefact. One marker (D1S1609) was uninformative in the family. XY PCR analysis showed female for the girl and male for the boy.

**Discussion**

A MCDAP in humans has been considered only to be associated with MZ twinning (Husby et al., 1991). However, our case and several other recent reports indicate that MCDAP can occur in DZ twinning (Kline et al., 2003; Quintero et al., 2003; Souter et al., 2003). These cases all have been reported in IVF pregnancies, and it thus appears that DZ twinning with MCDAP may be an associated risk of IVF. In MZ twins, MCDAP is known to be associated with an increased risk for fetal complications because of aberrant vascular connections leading to twin–twin transfusion syndrome and/or vascular disruptions. In the IVF-related cases of DZ twinning and MCDAP discussed here, there were no apparent malformations or disruptions, and the placenta examination showed no obvious vascular anastomoses. However, the phenomenon of placental fusion described herein would mean that this type of DZ twinning is behaving like MZ twinning, and thus it could logically be expected to confer increased risk for fetal abortion and/or birth defects.

The chimerism in the twins that we report may be limited to blood lymphocytes, suggesting that the mechanism for the chimerism is due either to placental vascular anastomoses (after the development of the haematoblast stem cells) or to an admixture of trophoblast cells during early blastocyst development. These trophoblast cell admixtures apparently were restricted to the extraembryonic tissues, since we found no evidence of chimerism by chromosomal and FISH study of buccal, skin and ovary cells and by DNA genotyping of buccal cells. Indeed, there is evidence in IVF-related DZ twins with MCDAP, using in situ hybridization with X and Y

| Table II. Chromosome and fluorescence in situ hybridization (FISH) results of various tissues studied in both twins |
|-----------------|-----------------|-----------------|
| Study type      | Twin A, boy     | Twin B, girl    |
| Blood SRY FISH | Not performed | SRY + in XY cells |
| Blood karyotype (study 2) | 46,XX[20]/46,XY[5] | Not performed |
| Buccal mucosa XX/XY FISH | Xcen(DXZ1 × 1), Ycen(DYZ3 × 1)[499]/Xcen (DXZ1 × 2)[1] | Xcen(DXZ1 × 2)[500] |
| Skin karyotype  | 46,XY[20] | 46,XX[20] |
| Skin XX/XY FISH | Xcen(DXZ1 × 1), Ycen(DYZ3 × 1)[598]/Xcen (DXZ1 × 2)[2] | Xcen(DXZ1 × 2)[500] |
| Ovarian tissue karyotype | N/A | 46,XX[20] |
| Ovarian tissue XX/XY FISH | N/A | Xcen(DXZ1 × 2)[500] |

*Within normal control data range for false positives in buccal mucosa controls. False positives (wrong sex) in controls may be attributed to cross-hybridization and/or failure of hybridization, ‘noise spots’, and/or operator contamination.

**Buccal swab, skin fibroblast and ovary chromosome and FISH studies**

Results are summarized in Table II and demonstrate that the chromosome mosaicism observed in lymphocytes was not present in buccal swab cells, skin biopsy cells or ovarian tissue. Study of these tissues showed normal male and female karyotypes (or FISH results) congruent with the phenotypic examination of genitalia.
probes, for a mixture of cells in the lining of the amnion (Souter et al., 2003).

There are several reasons to explain the mild clitoromegaly in the phenotypic female twin. The administration of progesterone is usually well tolerated by the mother during the first trimester. It has previously been reported to cause clitoromegaly in female infants. As such, this mechanism could cause viral anomalies that might be considered part of the spectrum of pseudohermaphroditism. The mother of these twins was given i.m. progesterone for the first 8 weeks of her pregnancy. A variation of normal is another possibility, since a prominent clitoris is often present in premature infants. Although virilization in the female has been reported in cattle with chimeric male and female DZ twins (e.g. freemartinism) (Moore, 1966), it has not been previously reported in humans.

Repair of inguinal hernias allowed an opportunity for gross inspection of both ovaries and biopsy of one ovary, that showed a normal 46,XX result and normal histology. Vaginoscopy and ultrasound of the uterus were normal, indicating that the Müllarian duct derivatives developed normally. It should be noted that the left ovary, which was not biopsied, did have a large ovarian cyst which could result from the elevated gonadotrophins. The finding of the unmeasurable MIF along with the tissue chromosome analyses support the hypothesis that she only expressed chimerism in her blood. However, we are still not able to rule out a low level of chimerism in ovarian tissue.

Gonadotrophin levels in premature infants have previously been reported to be markedly elevated. Premature females, in particular, have been reported with peak FSH levels up to 270 mIU/ml and peak LH levels up to 89 mIU/ml (Shinkawa et al., 1983). These levels have been shown in premature infants to peak up to 10 weeks after birth (Tapanainen et al., 1981). The elevated testosterone values found in Twin B strongly suggest the presence of testicular tissue. Nevertheless, it is hard to reconcile with the normal, albeit one-sided, ovarian biopsy.

It is possible that the elevated testosterone levels in the female were spurious. A recent report comparing standard testosterone assay methods to liquid chromatography tandem mass spectrometry found the percentage difference between the methods to be between −80% and +40% (Wang et al., 2004), with larger errors in samples with the lowest values. The undetectable MIF of <1.5 ng/ml confirms that Twin B did not have any functioning Sertoli cells. If the testosterone values were excluded as likely spurious, all of Twin B’s laboratory findings could be supported by the hypothesis that she had a normally functioning premature female endocrine axis. Nevertheless, functional testicular tissue cannot be definitively excluded at this point and will need to be closely followed.

IVF is associated with an increased rate of DZ and MZ twinning, but it is unclear if any laboratory or procedural factor primarily is responsible for this increase (Blickstein et al., 1999; Abusheikha et al., 2000; Schachter et al., 2001; Tarlatzis et al., 2002; Unger et al., 2004). Theoretically, the assisted hatching used in our case might increase the risk for embryo amalgamation or splitting but this procedure was not used in the cases of twins with MCDAP and blood chimerism described by others (Kline, 2003; Souter et al., 2003). In view of the oocyte/embryo micromanipulations used in this case (ICSI and assisted hatching), there are various scenarios that may help explain the ultimate outcome. Assisted reproductive technologies have already been associated with an increase of MZ twin pregnancies, either through the use of assisted hatching techniques or by extended culture (day 5 blastocyst stage transfers) (Abusheikha et al., 2000). In MZ twinning, a division event (splitting of the inner cell mass) has taken place, whereas chimerism is a fusion event. Dissolving a hole in the embryo’s zonae pellucida (assisted hatching) can have the effect of releasing the developing embryo early into the environment. Two such embryos transferred together into the uterus in close proximity to each other may have both a temporal and spatial potential to fuse or at least to attach to one another prior to implantation, thus accounting for an incidence of DZ chimerism or partial chimerism.

In our particular incident, two separate sacs were observed at 6 weeks of gestation with nothing unusual noted. This implies a later fusing of the implanting placentas due to the proximity of the implantation sites alone. In addition, since no tissue chimerism was found (only blood elements), this strongly suggests that the chimerism took place through a sharing of placental vessels and subsequent shunting of hematopoietic stem cells. In any case, assisted reproductive technology procedures are responsible for higher incidences of overall twinning events and therefore special care should be taken as early as possible to avoid missing any abnormality of pregnancy, including twin–twin transfusion syndrome.

A large cohort study of unlike-sex twins, born by IVF, found similar, good clinical outcomes compared to those born from normal pregnancies, although the subgroup of IVF/ICSI twins carried a higher risk for admittance to a neonatal intensive care unit (Pinborg et al., 2004). It is not known if the chimerism observed in these cases will persist into adulthood, although the cases reported by Kline et al. (2003) first were detected at 1 year of age, so it seems that long-term persistence is to be expected. MZ twins are well known to have blood line chimerism that can persist into adulthood (Tippett, 1983) and the occurrence of blood chimerism in apparent DZ twin children is significant, when sensitive methods are used (van Dijk et al., 1996). Sex chromosome abnormalities, of course, would not be detected in MZ blood chimeras, since all would be of like sex. Although MZ twins essentially have the same genotype, postzygotic differences in the genetic or epigenetic constitution do occur and these changes could be chimeric in such MZ twins. This area of genetic inquiry, however, has received little attention in MZ twin studies, so its clinical significance is unknown.

In addition to the MCDAP cases referred to in this paper, there is a report of blood chimerism in DZ triplets born after IVF, so the prevalence of blood chimerism associated with IVF-related multiple births may be higher than previously thought (Kuhl-Burmeister et al., 2000). Our study also suggests that, in cases where a chromosome study demonstrates the presence of both a male and female karyotype,
buccal interphase FISH studies can be performed to help differentiate systemic from isolated blood chimerism.

References


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