Blood chimerism in a girl with Down syndrome and possible freemartin effect leading to aplasia of the Müllerian derivatives

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Cytogenetic and molecular genetic analysis in a case of sex-discordant dizygotic twins revealed blood chimerism in the girl (46,XY in blood and 47,XX, +21 in fibroblasts) caused by feto-fetal transfusion from her healthy brother. The girl presented with Down syndrome, aplasia of the uterus and the Fallopian tubes and normal female external genitalia. We propose that the lack of Müllerian structures is caused by the effect of the Müllerian inhibiting substance transferred from the male to the female twin in early pregnancy. This disorder of sex development is known as freemartin phenomenon in female cattle from sex-discordant twin pairs.

Key words: chimerism / Down syndrome / freemartinism / Müllerian derivatives aplasia

Introduction

The differentiation of the internal reproductive organs, which arise from either the presumptive male (Wolffian) or female (Müllerian) genital ducts of the bipotential embryo, is one of the pivotal events during normal mammalian embryogenesis. Expression of the sex-determining region of the Y chromosome (SRY) gene leads to the differentiation of the bipotent gonads to testes, producing two hormones essential for the normal male reproductive tract development, Müllerian inhibiting substance (MIS, also termed anti-Müllerian hormone or AMH) and testosterone. MIS, which is produced first by the Sertoli cells of the fetal testes, is responsible for the regression of the Müllerian ducts, the anlagen of the uterus, Fallopian tubes and upper vagina. The effect of MIS has been assumed in phenotypically female calves lacking the Müllerian duct derivatives in sex-discordant twin pairs, where a shared placenta allows the female fetus to be influenced by the blood products of the male fetus, the freemartin phenomenon. Here, we report on a female chimera affected by Down syndrome with normal female external genitalia, but lack of Müllerian structures from a sex-discordant twin pregnancy conceived after ovarian stimulation and intrauterine insemination. We propose that the lack of Müllerian structures is caused by the effect of the MIS transferred from the male to the female twin in early pregnancy.

Materials and Methods

Cytogenetic studies

Metaphase chromosomes from phytohaemagglutinin-stimulated peripheral blood lymphocytes or cultured skin fibroblasts were analyzed by standard GTG-banding procedures. For fluorescence in situ hybridization (FISH) on interphase nuclei, commercially available X and Y chromosome-specific DNA probes were used according to the supplier’s protocol (Kreatech Biotechnology, Amsterdam, The Netherlands).

Zygosity studies

DNA was extracted from peripheral blood lymphocytes, skin fibroblasts and buccal swabs from the girl, from buccal swabs of her brother and from peripheral blood of each parent. Zygosity studies using polymorphic markers located on chromosomes 13, 18, 21, X and Y (Table I) were performed after polymerase chain reaction with fluorochrome-labeled oligonucleotides and electrophoretic separation on an ABI310 genetic analyzer as recommended by the manufacturer (Elucigene™ QSTR Kit and Elucigene™ QSTR-XY Kit, Tepnel Molecular Diagnostics, Abingdon/Oxfordshire, UK).
Table I Results of the zygosity studies performed in the family.

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<th>Microsatellite marker</th>
<th>Father blood</th>
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<th>Brother buccal swab</th>
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Allele sizes are given in base pairs and are internally standardized.
*Minor peaks. **X chromosome allele 103 bp; Y chromosome allele 108 bp.

Immunofluorescence staining followed by FISH for human Y chromosome

Methanol-fixed specimens of buccal swab smears were stained with mouse monoclonal anti-human CD45 (leucocyte common antigen, LCA) antibodies (DakoCytomation, Glostrup, Denmark), and the detection was performed with Texas Red™-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). After this first staining, cells were permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 and 1.5% goat serum. The specimens were then stained with rabbit polyclonal anti-pan-cytokeratin antibodies. After this first staining, cells were permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 and 1.5% goat serum. After the immunofluorescence staining of the specimens, standard Y-FISH analysis was performed.

Written informed consent for this analysis and the publication of the results was obtained from the parents.

Case report and results

The diagnosis of Down syndrome was obvious after birth in a female child of a 32-year-old healthy woman, who conceived after intrauterine insemination. The parents were referred to our institution for genetic counseling as the patient was 2 years old. Fetal ultrasound was consistent with a monochorionic diaphragmatic placenta; however, its presence was not documented after birth. A mild polyhydramnios was diagnosed during pregnancy and a Caesarean section was carried out in the 32nd week of gestation because of tocolysis-resistant contractions. The girl presented with typical morphological features of Down syndrome such as epicanthus, up-slanting palpebral fissures, macrocephaly, brachycephaly, simian crease and duodenal atresia due to a pancreas anulare. Jejuno-duodenal anastomosis was performed and the intraoperative abdominal inspection revealed the presence of gonads (macroscopic evaluation suggestive of ovarian structures) as well as the absence of uterus and Fallopian tubes. The external genitalia were unambiguously female. The patient’s twin brother is healthy and has no signs of trisomy 21.

Cytogenetic analysis of blood lymphocytes obtained from the female twin at the age of 1 week revealed a normal male karyotype (46,XY) in 10 metaphases. Microscopic evaluation of the FISH analysis from blood revealed one X- and one Y-specific signal per cell in 97 out of 100 cells and two X-specific signals per cell in 3 out of 100 cells. Because of this unexpected results, skin biopsy was performed at age of 2 months, and a 47,XX,+21 karyotype was detected in all skin fibroblast cells from the female twin.

At the age of 2 months, FSH concentration was elevated (7.2 mU/ml, normal range <1 mU/ml), whereas LH was within (0.1 mU/ml) the normal range for newborns. Inhibin B, a marker for Sertoli and granulosa cells, was not detectable (0 pg/ml). The concentration of testosterone was 0.04 ng/ml, and no increase was detectable after hCG stimulation. The concentrations of adrenal androgens including 17α-hydroxyprogesterone were in the normal range.

The results of the zygosity studies are presented in Table I. The polymorphic marker allelic pattern obtained from the buccal swab of the
boy was identical to the pattern derived from the blood sample of the girl. DNA analysis performed on fibroblasts from the girl’s skin revealed a female genome, which is different from the male genome detected in her blood. These findings demonstrate a blood chimerism in the patient. In addition, trisomy 21 due to a first meiosis non-disjunction in the mother was found in the girl.

Major peaks corresponding to the fibroblast pattern and minor peaks corresponding to the blood/brother pattern were present in DNA from buccal swabs of the girl (Fig. 1). Therefore, we sought to test if Y chromosome-positive epithelial cells are detectable in the oral mucosa. Epithelial cells were labeled via immunofluorescent staining with anti-pan-cytokeratin antibodies and leukocytes were counterstained with anti-LCA antibodies. No Y-specific signal was detected in all 72 cytokeratin-positive cells analyzed.

The HLA typing results of the family members are shown in Fig. 2. Typing results of HLA-A, -B and -DRB1 could be consistently assigned to most probable haplotypes. HLA typing of the patient using DNA from fibroblasts showed a homozygous pattern for HLA-A, -B and DRB1 (Fig. 2). HLA typing from peripheral blood DNA revealed two additional alleles, each for HLA-A and -B, and one additional allele for HLA-DRB1, which are identical to the HLA alleles/HLA haplotypes detected in the twin brother. The findings are consistent with the hypothesis of a stable blood chimerism in the patient. Although the microsatellite marker analysis showed solely marker alleles from the twin brother in the peripheral blood of the girl, HLA analysis demonstrated the presence of HLA alleles of both individuals. This discrepancy is most probably due to the higher sensitivity of the HLA typing methods, which are able to detect the girl’s HLA alleles at very low copy numbers.

**Figure 1** Allelic patterns of the gender marker amelogenin (left panel) and the microsatellite marker D18S543 (right panel) in blood samples of both parents and in buccal swabs of the patient and her brother. The minor peaks in the patient corresponding to her brother’s genome are marked with arrows.

**Figure 2** Pedigree with haplotype assignment of HLA typing results of the family. HLA typing at the low-resolution level of HLA-A, -B and DRB1-locus was performed using peripheral blood DNA (pb-DNA) of the parents, fibroblast-DNA (fib-DNA) of the patient and DNA isolated from a buccal swab (bs-DNA) of her twin brother. The alleles are assigned to most probable HLA-haplotypes. HLA typing of the patient from pb-DNA, which was performed at the molecular-allele level, revealed five additional HLA-alleles (underlined), which are consistent with the HLA-alleles of the non-identical twin brother. [HLA-DRB1*1501 of the brother is set in parentheses, since it would not be ‘visible’ in the DRB1*15-positive patient. HLA-allele ambiguities with differences outside of exons 2 and 3 (HLA -A or -B) or exon 2 (HLA-DRB1) are not shown. The first two digits of the molecular allele numbers representing the serologic HLA specificities are printed in bold.]

**Discussion**

The term chimerism in an individual refers to the coexistence of cells derived from more than one genetically distinct zygote. It can be caused by iatrogenic procedures (organ transplantation or blood transfusion), feto-fetal or feto-maternal transfusion or the fusion of two separately fertilized oocytes. The last possibility (tetragametic chimerism) appears to be rather rare. We report on a female chimera affected by Down syndrome with normal female external genitalia, but lack of Mullerian structures from a sex-discordant twin pregnancy. The observed normal male karyotype in her lymphocytes can be explained by blood chimerism due to feto-fetal transfusion between the healthy twin brother as a donor and the patient as an acceptor. As shown previously, the observed polyhydramnios in the acceptor might be explained by an increased production of atrial natriuretic factor due to volume overload (Wieacker et al., 1992). Molecular genetic analysis demonstrates that the genome of the peripheral blood lymphocytes corresponds to the genome of the twin brother, a finding which explains the normal male karyotype in her blood lymphocytes. Cytogenetic and molecular genetic analyses of patient’s fibroblasts demonstrate female trisomy 21, leading to the phenotype of Down syndrome.
According to the molecular genetic results in peripheral blood, the acceptor’s lymphocyte population is nearly completely substituted by lymphocytes of the donor. Genotyping of HLA-A, -B and -DR performed on DNA from fibroblasts and peripheral blood of the patient showed the presence of additional HLA alleles in the blood. The additional alleles were completely mismatched with the girl’s own HLA alleles detected separately in her fibroblasts. Analysis of the HLA typing results of the family revealed consistently that the additional alleles stem from the twin brother. They are most likely derived from haematopoietic cells, which had engrafted during fetal life. In bone marrow transplantation, these HLA mismatches would mean an immunological incompatibility of donor and recipient giving rise to a graft rejection or a severe graft versus host disease. However, the girl has developed a stable blood chimerism without such reactions. This finding suggests that immunological tolerance during intruterine development (Ober, 1998) can be maintained for a considerable time, presumably for the rest of the individual’s life without giving rise to an immunological rejection.

It has been shown that one out of four pregnancies with monochorionic twins develops a twin–twin transfusion syndrome (Sperling et al., 2007). A monochorionic diamniotic placenta in humans has been considered to be associated with monozygotic twinning only (Husby et al., 1991). However, several reports on pregnancies conceived by assisted reproduction indicate that a monochorionic placenta can occur in dizygotic twins as well (Quintero et al., 2003; Souter et al., 2003; Williams et al., 2004), and blood chimerism as a consequence is probably more common than previously considered (van Dijk et al., 1996; Kuhl-Burmeister et al., 2000). It can be expected that the occurrence of this phenomenon will become more frequent in the view of an increased use of assisted reproductive procedures leading to a higher twin rate. Although the presence of a monochorionic diamniotic placenta was not well documented after birth, our case gives further evidence that feto-fetal transfusion might take place between dizygotic twins and demonstrates that in such a situation, a stable engraftment of HLA-mismatched haematopoietic cells in the recipient might occur. This could result in a blood chimerism where haematopoietic cell lines are in part or, as shown in this case, almost completely replaced by cells of the donor. Another case of blood chimerism in an adult person with a complete replacement of the recipient’s lymphocytes has been previously described (Sudic et al., 2001). Such an event has two important implications: (i) it can lead to false interpretation of diverse genetic analyses, e.g. in paternity testing of offspring or it can lead to erroneous assignment of monozygosity in sex-discordant twins and (ii) the lack of Müllerian structures in our patient could be misdiagnosed as an androgen insensitivity because of the male karyotype in blood. Another potential consequence of the blood chimerism is that the risk of leukemia, a typical complication of the Down syndrome, should not be significantly elevated in our patient, since a successful ‘bone marrow transplantation’ occurred during pregnancy.

It has been shown that bone marrow stem cells can differentiate into mature cells of various organs. Human allogenic transplant studies have demonstrated the presence of donor markers in several cell types, including gastrointestinal, endometrial, lung and skin epithelial cells, hepatocytes and endothelial cells (Körbling et al., 2002; Okanoto et al., 2002; Mattsson et al., 2004; Jiang et al., 2004; Taylor, 2004). In our case, we did not detect any epithelial cell of bone marrow origin in the buccal mucosa, so that the visualized male alleles of polymorphic markers could be assigned to white blood cells in the buccal swabs. However, the number of analyzed cells is rather low to allow for complete exclusion of donor-derived epithelial cells.

Although tetragametic chimerism or embryo amalgamation can lead to ovotestes in the case of sex-discordant zygotes (Strain et al., 1998), such disorder of sex development is not expected and has not been described in cases of sex-discordant blood chimerism (Sudic et al., 2001; Ekelund et al., 2001; Souter et al., 2003; Williams et al., 2004). It is therefore remarkable that our patient lacks the Müllerian structures such as the uterus or Fallopian tubes. The cellular mechanisms underlying the differentiation of Müllerian duct formation is only partially understood (for a review, see Orvis and Behringer, 2007). Regression of Müllerian structures is caused by the action of MIS produced by the Sertoli cells of testes in the early development of the male embryo. In this case, the gonads are not expected to be testes, because XY cells are restricted to peripheral blood and bone marrow. Moreover, the macroscopic evaluation of the gonads was suggestive for ovarian structures. Since no histological analysis was performed, we cannot exclude morphological alteration of the ovaries such as structures resembling seminiferous tubules and loss of germ cells, as has been seen in transgenic mice chronically expressing human MIS (Behringer et al., 1990). However, the absence of inhibin B and the elevated concentration of FSH suggest that follicle structures are compromised in this patient. We hypothesize that MIS produced by the testes of the twin brother had reached sufficient levels in the patient during the early embryonic development by feto-fetal anastomoses, leading to a regression of Müllerian structures. Serum levels of MIS in males are high in pregnancy and reach a peak in the period from 6 months to 2 years of age (Teixeira and Donahoe, 1996). On the other hand, animal model experiments have indicated that the half-life of exogenous MIS is ~4–12 h (Sriraman et al., 2001). Moreover, it has been shown that the MIS values in freemartins sharply decrease within the first fortnight (Rota et al., 2002). We therefore did not consider determination of the MIS serum level at the age of 2 years in our patient to be helpful to confirm this hypothesis. Regression of Müllerian structures in females from sex-discordant twin pregnancies caused by MIS action is known as freemartin phenomenon found in cattle and occasionally in other mammals (reviewed by Padula, 2005). The patient reported here could represent the first case of freemartinism described in humans.

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References

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