Chimerism of Buccal Membrane Cells in a Monochorionic Dizygotic Twin

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ABBR EV IAT I ONS
FISH—fluorescence in situ hybridization
MCDZT—monochorionic dizygotic twin
PCR—polymerase chain reaction

Dr Fumoto was responsible for acquisition of clinical data of the twins as an attendant doctor and reviewed the manuscript; Dr Hosoi was responsible for acquisition of clinical data and informed consent as an attendant doctor and reviewed the manuscript; Dr Ohnishi wrote the draft of manuscript; Dr Hoshina was responsible for zygotic analyses as an attendant doctor; Dr Yan conducted immunofluorescence examinations; Dr Saji performed microsatellite analyses; Dr Oka conceptualized and designed the study, and all authors approved the final manuscript as submitted.

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abstract

No monochorionic dizygotic twins (MCDZTs) with cellular chimerism involving cells other than blood cells have been reported in the literature to date. Here we report a probable first case of MCDZTs with buccal cell chimerism. A 32-year-old woman conceived twins by in vitro fertilization by using 2 cryopreserved blastocysts that were transferred into her uterus. An ultrasound scan at 8 weeks’ gestation showed signs indicative of monochorionic twins. A healthy boy and a healthy girl were born, showing no sexual ambiguity. Cytogenetic analyses and microsatellite studies demonstrated chimerism in blood cells of both twins. Notably, repeated fluorescence in situ hybridization and microsatellite studies revealed chimerism in buccal cells obtained from 1 of the twins. Although the mechanism through which buccal cell chimerism was generated remains to be elucidated, ectopic differentiation of chimeric hematopoietic cells that migrated to the buccal membrane or the cellular transfer between the 2 embryos at the early stage of development might be responsible for the phenomenon. This hypothesis raises an interesting issue regarding embryonic development and cellular differentiation into organs during fetal development. Given the possibility of cryptic chimerism in various organs including gonadal tissues in MCDZTs, close observation will be required to determine whether complications develop in the course of the patients’ growth. Pediatrics 2014;133:e1097–e1100
Until recently, monochorionic twins were considered to be exclusively monozygous. However, Souter et al disconfirmed this in a 2003 report of monochorionic dizygotic twins (MCDZTs).1 After this report, many cases of MCDZTs have been reported worldwide, and it is now widely accepted that MCDZTs are not extremely rare events, particularly in pregnancy by in vitro fertilization. It is notable that blood chimerism was found in many of these twins; that is, blood cells derived from 2 distinct zygotes were present in peripheral blood of each twin. This phenomenon could be explained by the anastomosis of blood vessels of both zygotes during the fetal period. However, no MCDZTs with cellular chimerism involving cells other than blood cells have been reported in the literature to date.2–5 Here we report a probable first case of MCDZTs with buccal cell chimerism.

**PATIENT PRESENTATION**

A 32-year-old woman conceived twins by in vitro fertilization by using 2 cryopreserved blastocysts that were transferred into her uterus. These blastocysts had been produced by intracytoplasmic sperm injection into her own oocytes, which were then cultured in vitro for 4 days and frozen in liquid nitrogen 2 years before the fertilization. An ultrasound scan at 8 weeks’ gestation showed signs that were indicative of monochorionic twins, including the absence of a chorionic membrane between the fetuses (Fig 1). A subsequent scan obtained at 24 weeks’ gestation showed twins with discordant gender. The pregnancy was without complication and showed no evidence of twin-twin transfusion syndrome until 35 weeks’ gestation, at which point a healthy boy and a healthy girl were delivered by caesarian delivery. There was no evidence of sexual ambiguity (Table 1).

Pathologic examination showed a monochorionic, diamniotic placenta (Fig 2) with the presence of anastomosis of the placental vessels as revealed by a milk test. Evaluation of the twins at 7 months of age showed normal external genitalia with the presence of gender-concordant gonads in both twins by ultrasonography. Endocrine function tests revealed normal and gender-appropriate levels of gonadal hormones in the serum of both twins. The following zygotism analyses were performed after informed consent was obtained from the parents. Cytogenetic analyses of blood lymphocytes obtained when the twins were 5 days old showed a 46,XY[9]/46,XX[11] karyotype in the boy and a 46,XY[2]/46,XX[28] karyotype in the girl. Repeated karyotyping at 3 months of age revealed a 46,XY[11]/46,XX[19] profile in the boy and a 46,XY[1]/46,XX[29] profile in the girl. Notably, fluorescence in situ hybridization (FISH) by using the DXZ1 probe specific for an X chromosome and the DYZ3 probe specific for a Y chromosome revealed that the buccal membrane cells obtained from the boy at 5 weeks of age showed a 46,XY[94]/46,XX[6] profile in 100 cells viewed at interphase, whereas buccal membrane cells obtained from the girl had a normal 46,XX karyotype. Repeated FISH analysis of buccal cells obtained from the boy at 2 and 11 months of age exhibited chimeric karyotypes (46,XY[95]/46,XX[5] and 46,XY[98]/46,XX[2], respectively). No blood cells were visible in the buccal cells obtained from the boy on microscopic morphologic examination. In addition, buccal samples were negative for CD45 by immunofluorescence using anti-CD45 antibody sc-25590 (Santa Cruz Biotechnology, Santa Cruz, CA), indicating the absence of significant contamination by blood cells (data not shown).

DNA was extracted from the lymphocytes of the twins (at 5 days and 3 months), buccal cells of the twins (at 2 months of age), and lymphocytes of the parents. Zygotic studies on lymphocytes and buccal cells of the twins by polymerase chain reaction (PCR) by using 4 microsatellite markers (D10S2325, D14S608, amelogenin, and D8S1179) were performed as previously

**TABLE 1** Characteristics of the Twins at Birth

<table>
<thead>
<tr>
<th></th>
<th>Twin 1</th>
<th>Twin 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotypic gender</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>2548</td>
<td>1880</td>
</tr>
<tr>
<td>Body height, cm</td>
<td>49.4</td>
<td>43.3</td>
</tr>
<tr>
<td>Apgar score (1 min, 5 min)</td>
<td>7, 8</td>
<td>8, 9</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>139</td>
<td>141</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>37.8</td>
<td>44.1</td>
</tr>
<tr>
<td>Blood type, ABO and RhD</td>
<td>A+</td>
<td>A+</td>
</tr>
</tbody>
</table>

FIGURE 1 Fetal ultrasound scan obtained at 8 weeks’ gestation. The absence of a white chorionic membrane between the fetuses indicates monochorionic twins.
described. Preliminary analyses involving 17 polymorphic microsatellite markers on the parental DNA by PCR revealed these 4 markers that can be used for the purpose of judging zygosity. The microsatellite analysis on blood lymphocytes of the twins demonstrated an admixture of microsatellite types from both twins. Putative 46,XX-cell-specific PCR products were clearly visible in the lymphocytes of the boy, whereas 46,XY-cell-specific PCR products were faintly observed in the lymphocytes of the girl. By analysis of the buccal membranes, putative 46,XX-cell-specific PCR products were faintly visible in the DNA of the buccal cells of the boy, whereas no chimeric results were observed for the buccal cells of the girl (Fig 3). These results were consistent with the karyotyping of the lymphocytes and FISH analysis of the buccal cells of the twins. 

DISCUSSION

To our knowledge, this is the first reported case of MCDZTs that exhibited chimerism in somatic cells other than blood cells. Repeated FISH and microsatellite analyses confirmed the presence of chimerism in buccal cells of the male twin. Microscopic and immunofluorescence examinations excluded the possibility of blood cell contamination in the buccal membrane. Although various tissues other than blood derived from MCDZTs have been investigated in previous studies, none of these tissues demonstrated chimerism. Previous reports thus concluded that chimerism is confined to blood cells and that clinical consequences of chimerism in other tissues would be minimal.

The results of the present case are inconsistent with these studies, raising the possibility that tissues other than blood could be chimeric in MCDZTs. Such cryptic chimerism generating genetic differences in the affected tissues may cause organ dysfunction, particularly gonadal dysfunction, in gender-discordant chimeras. Notably, freemartin cattle that demonstrate chimerism of gender chromosomes as a consequence of placental anastomosis are known to have congenital abnormalities that can cause infertility. To date, no freemartin-like abnormalities have been associated with blood cell chimerism in human MCDZTs. However, gonadal dysfunction may develop during the course of growth if cellular chimerism is present in the tissues associated with gonadal development, in addition to the effect of possible hormone transfer through the vascular anastomoses. Additional cases are needed to determine whether complications involving gonadal dysfunction can develop in human MCDZTs with cellular chimerism.

The present case also provides insight into the mechanism of generation of chimeras in MCDZTs. Although blood chimerism is usually considered a consequence of placental vascular anastomosis, buccal cell chimerism cannot be explained by vascular anastomosis. The most probable explanation for the generation of buccal cell chimerism would be the ectopic differentiation of chimeric hematopoietic cells that have migrated to the buccal membrane. Hematopoietic stem cells target injured tissues, including buccal

**FIGURE 2**
A hematoxylin-and-eosin-stained section of the intertwine membranes that separated the 2 gestational sacs. The absence of an intervening chorionic membrane between the 2 amniotic membranes indicates a monochorionic, diamniotic placenta.

**FIGURE 3**
Zygotic studies on lymphocytes and buccal cells of the twins by microsatellite analysis. Three representative results (D10S2325, D14S608, and amelogenin) are included. Putative 46,XX-cell-specific PCR products (arrows) were clearly visible in the lymphocytes of the boy, whereas 46,XY-cell-specific PCR products were faintly observed in the lymphocytes of the girl. Putative 46,XX-cell-specific PCR products were also faintly visible in DNA derived from the buccal cells of the boy, whereas 46,XY-cell-specific PCR products were absent in DNA derived from the buccal cells of the girl. PB, peripheral blood.
epithelial cells, after allogeneic hematopoietic stem cell transplantation. Our case may have incurred an injury at the buccal membrane in utero that promoted blood cell homing and the resultant chimerism. If this is the case, then ectopic homing and differentiation of hematopoietic cells to other tissues may occur more generally than is typically expected, at least during fetal development. This hypothesis also raises an interesting issue regarding embryonic development and cellular differentiation into organs during fetal development.

Another possibility for our observation is that the buccal chimera was generated at an early stage of embryogenesis. Souter et al speculated that the fusion of the blastocyst membranes of the 2 embryos, leading to a fused chorion, might be a possible mechanism of generating MCDZTs. Miura et al and Assaf et al hypothesized that the fusion of outer cell masses at the morula stage between 2 eggs might be a cause of outer cell masses at the morula stage between 2 eggs might be a cause of monochorionic dizygotes in artificial fertilization. In either case, 2 fetuses might reside within close proximity when a single blastocyst with double inner masses is generated. In the present case, some cells derived from the 46,XX fetus could have migrated to the ectoderm of the 46,XY fetus when 2 inner cell masses are within close proximity, which later might have developed into the buccal membrane. The discrepancy between the present case and previously reported cases regarding the presence of non-blood cell chimerism may be explained by different timing and sites of the contact of the 2 fetuses. If this is the case, then any organs could become chimeric with various degrees of admixture of the 2 cell types in MCDZTs, depending on the timing and the sites of the contact of the 2 fetuses.

CONCLUSIONS

We have demonstrated that, contrary to previous reports, tissues other than blood cells can be chimeric in MCDZTs. Whatever the cause of chimerism might be, long-term follow-up is required to evaluate possible complications due to cellular chimerism, particularly gonadal dysfunction, in MCDZTs. Additional identification of cases and precise examination regarding non-blood cell chimerism will help us gain a better understanding of the mechanism of generation of MCDZTs.

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