Two sibling cases of hydrops fetalis due to alloimmune anti-CD36 (Nak\textsuperscript{a}) antibody

Satoru Okajima\textsuperscript{1,4}, Kazutoshi Cho\textsuperscript{1,4}, Hitoshi Chiba\textsuperscript{2}, Hiroshi Azuma\textsuperscript{3}, Toshiko Mochizuki\textsuperscript{2}, Miki Yamaguchi\textsuperscript{3}, Shin-ichiro Sato\textsuperscript{2}, Hisami Ikeda\textsuperscript{3}, Hideto Yamada\textsuperscript{1}, Hisanori Minakami\textsuperscript{1}, Tadashi Ariga\textsuperscript{4}, Kunihiko Kobayashi\textsuperscript{4}

\textsuperscript{1}Maternity and Perinatal Care Center and \textsuperscript{2}Department of Laboratory Medicine, Hokkaido University Hospital, Hokkaido University School of Medicine, Sapporo, Hokkaido Japan
\textsuperscript{3}Hokkaido Red Cross Blood Center, Sapporo, Hokkaido, Japan
\textsuperscript{4}Department of Pediatrics, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido Japan

Summary

Two female sibling cases, who were born to a CD36 deficient mother, were presented with Coombs’ test-negative hydrops. The alloimmune anti-CD36 (Nak\textsuperscript{a}) antibody was accidentally found in the mother’s serum after an episode of anaphylactic shock with thrombocytopenia, which occurred in an individual receiving fresh frozen plasma prepared from the mother’s donated blood. The mother was then diagnosed as having type II CD36 deficiency, lacking CD36 on both platelets and monocytes, while both of her daughters were CD36 positive. Analyses of the CD36 gene revealed that the mother was a compound heterozygote for the CD36 gene mutation with a novel C→T transition at nt 1366 in exon 12, corresponding to Arg386Trp, and a known 12bp deletion at nt 1438–1449 in exon 13. On the other hand, both patients, who showed half the normal level of CD36 on platelets and monocytes, were heterozygote with one mutation at Arg386Trp. The anti-CD36 antibody in the mother seemed to be responsible for the hydrops fetalis observed in her daughters, because the IgG isolated from the mother’s serum showed suppressive effects on the CFU-E colony formation of CD34\textsuperscript{+} cells from a control donor. This is the first case report of hydrops fetalis caused by an alloimmune anti-CD36 antibody.

Keywords

Platelet immunology, gene mutations, immunity (auto-)

Introduction

CD 36 is a glycoprotein expressed in various human cells, including platelets, monocytes, erythroblasts, capillary endothelial cells, mammary epithelial cells and adipocytes \textsuperscript{(1)}. CD 36 interacts with a variety of ligands and is believed to play important roles in fatty acid metabolism. The anti-CD36 antibody has been used in the separation of fetal nucleated erythroblasts from maternal blood for the purpose of non-invasive prenatal genetic screening.

CD 36 deficiency is divided into two subgroups according to phenotype; neither platelets nor monocytes express CD 36 in type I deficiency, while in type II deficiency CD 36 is expressed only on monocytes and not on platelets. The incidences of type I and type II deficiency in the Japanese population have been reported as 1.0% and 5.8%, respectively \textsuperscript{(1)}. Platelet CD 36 deficiency (i.e., both type I and type II whole) is common in Asians and African Americans, whereas it is very rare in Caucasians \textsuperscript{(1)}.

CD36 deficiency has been recognized as possibly leading to CD36 isoimmunization and causing refractoriness to platelet transfusion. Neonatal alloimmune thrombocytopenia caused by the anti-CD36 antibody has also been reported \textsuperscript{(2)}.

Hydrops fetalis is generally classified into two groups by the results of Coombs’ test: immune and non-immune hydrops. Here we present two female sibling cases of immune hydrops fetalis with negative Coombs’ tests, who were born to a mother with CD36 deficiency having the alloimmune anti-CD36 (Nak\textsuperscript{a}) antibody. This is the first case report of hydrops fetalis caused by the alloimmune anti-CD36 antibody.

Cases

Case I

A Japanese girl, now 9 years old, is the first daughter of a healthy mother indicated later. At 18 weeks of gestational age, ultra-
sonography revealed that she had ascites, and the mother was admitted to our hospital at 24 weeks 3 days of gestational age. Fetal anemia (Ht 10%, Hb 3.2 g/dl) and hypoproteinemia (less than 1.0 g/dl) was revealed from umbilical cord censis. Although its cause was unknown (negative Coombs’ test, negative Parvo B19 infection, etc.), fetal therapies for emergent severe anemia, such as intraperitoneal RBC transfusion and albumin replacement, were performed. At 30 weeks 3 days of gestational age, the first patient was delivered by emergent caesarian section. She weighed 2420 g at birth with her RBC count was 120 × 10^12/µl, Hb 4.5 g/dl, Ht 13.4%, reticulocyte 62.1%, and platelet count 47 × 10^9/µl. An RBC transfusion (total 109 ml) and several bloodlettings were performed to improve her anemia and congestive heart failure. After approximately a week of supportive care regimens such as conventional mechanical ventilation, abdominocentesis, thoracentesis and intravenous infusions, generalized edema disappeared and did not recur. Her minimum body weight was 1420 g. Her maximum serum total bilirubin concentration was 14.5 mg/dl. She was discharged on her 94th day of age without any complications, and has had no sequelae to date.

**Case 2**

A Japanese girl, now 8 years old, is the second daughter of the mother of case 1. She was also noted to have ascites by ultrasonography from 25 weeks of gestational age. Since the clinical course was very similar to that of case 1, she was delivered by emergency caesarian section at a gestational age of 29 weeks 1 day. Her body weight at birth was 1920 g. Her RBC count was 99 × 10^12/µl, Hb 3.9 g/dl, Ht 13.6%, reticulocyte 130.6%, and platelet count 108 × 10^9/µl. Instead of RBC transfusion and several bloodlettings, a single-volume exchange transfusion with 200 ml RBC was performed under isovolemic conditions. After approximately a week of supportive care regimens, generalized edema had disappeared and never recurred. Her minimum body weight was 1000 g, and her maximum serum total bilirubin concentration was 12.4 mg/dl. She was discharged on the 162nd day of age without any apparent sequelae, and remains sequelae-free to date.

At this point, because of the negative Coombs’ test, we considered that both subjects were suffering from non-immune hydrops due to fetal anemia resulting from the same but unknown etiology.

**The mother of the two cases**

The mother of the two cases is Japanese, and was born to consanguineous parents (first cousins) in 1970. She had been apparently healthy and had no history of receiving blood transfusion. Five years after delivery of her second daughter, she donated blood to the Japan Red Cross Society. Fresh frozen plasma prepared from her donated blood was transfused to a female patient undergoing neurological surgery. Immediately after transfusion, she went into anaphylactic shock with her platelet counts decreasing from 200 × 10^9/µl to 20 × 10^9/µl. After a screening test for anti-platelet antibodies, the anti-CD36 (Naké) antibody was detected from the mother’s plasma. On the basis of this report, we formulated the following hypotheses for the mechanism of hydrops fetalis in the two subjects and investigated the possible etiology.

- The mother has CD36 deficiency, whereas both her daughters are CD36-positive.
- An anti-CD36 alloimmune antibody transferred from the mother is responsible for hydrops fetalis observed in her daughters.

**Materials and methods**

**Materials**

Venous blood from both daughters and their mother were obtained by informed consent.

**I. Studies for characterization of CD36 deficiency**

**Analysis for CD36 expression by flow cytometry**

CD36 expression on monocytes and platelets was determined by flow cytometry as previously described (1). Briefly, venous blood was taken and placed into tubes containing EDTA-2K. For CD36 expression on the cells, 25 µl of platelets (1 × 10^9/µl) or mononuclear cells (4 × 10^6/µl) were incubated with 10 µl of FITC labeled anti-CD36 monoclonal antibody FA6–152 (Immuno-tech, Marseilles, France) for 30 min at 4°C. For mononuclear cells, 10 µl of PE labeled anti-CD14 monoclonal antibody RMO52 (Immuno-tech, Marseilles, France) was added prior to incubation to identify monocytes. After washing with 10 mM EDTA-PBS, the mean fluorescence intensity of the platelets or the monocytes was evaluated by flow cytometry (FACS Calibur, Becton-Dickinson, San Jose, CA). Monocytes were analyzed under the gating condition of CD14 positive cells.

To evaluate the reactivity of the anti-CD36 alloimmune antibody in the mother, 25 µl of platelets (1 × 10^9/µl) or mononuclear cells (4 × 10^6/µl) either from the daughters, the mother or a normal control were incubated with 10 µl of the mother’s serum for 30 min at 4°C. After washing twice with 10 mM EDTA-PBS, the cells were incubated with 20 µl of 1:100 diluted PE labeled anti-human IgG (Jackson, West Grove, PA) for 30 min at 4°C.

**Mutation analysis of the CD36 gene**

**Genomic DNA**

Genomic DNA was extracted from whole EDTA-blood, using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) and subjected to sequencing for all exons. Each exon including a part of the flanking introns was amplified by PCR and directly sequenced with a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) as previously reported (1).

**cDNA sequence**

Total RNA was extracted from whole EDTA-blood, with a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany), and then subjected to reverse transcription and polymerase chain reaction (RT-PCR), using the primer set of 5′-GTATGCAAGTCCTGATGTTTC-3′ (forward; nt 1251–1271 in the exon 11) and 5′-TCAACAATGGTCCAGTCTC-3′ (reverse; nt 1462–1481 in the exon 14), with the help of a One Step RNA PCR Kit (Takara, Otsu, Japan). The obtained PCR product was directly sequenced with the 310 Genetic Analyzer.
2. CFU-E colony assay

Cytokines

IL-3 and stem cell factor (SCF) were provided from Kirin Brewery Co Ltd. (Tokyo, Japan), and erythropoietin (EPO) was from Chugai Pharmaceutical (Tokyo, Japan).

CFU-E colony assay

Peripheral blood CD34+ cells were isolated from normal control and differentiated into CFU-E according to the protocol described previously (3, 4). Briefly, peripheral blood mononuclear cells separated from theuffy coat by Ficoll-Hypaque were centrifuged with a 10% bovine serum albumin (BSA) cushion to remove platelets, and then processed using a nylon-fiber syringe system to deplete monocytes. CD34+ cells were separated by immunomagnetic beads coated with a CD34 monoclonal antibody (Nihon DINAL, Tokyo, Japan) and cultured at 37°C in 5% CO2 with Iscove’s Modified Dulbecco’s Medium (IMDM; Gibco BRL, US) supplemented with 10 ng/mL of IL-3, 10 ng/mL of SCF, 2 U/mL of EPO, 1% BSA, 50 μM of 2-mercaptoethanol (2-ME) and 30% fetal calf serum (FCS), which was designated as complete medium. After 7 days, the cultured cells were cryopreserved and stored in liquid N2 until used.

The stored cells were thawed and pre-cultured in IMDM containing 2 U/mL of EPO, 1% BSA, 50 μM of 2-ME and 30% FCS overnight and then washed with cold PBS containing 0.1% BSA. This process was critical to recover the ability of the CFU-E colony formation of the stored cells to the level equivalent to that before storage. Subsequently, the washed cells were suspended in complete medium at a concentration of 6×10^3/ml in the presence of IgG from the mother’s serum purified by protein A column, normal human IgG (Jackson ImmunoResearch Laboratory Inc, USA) or monoclonal anti-CD36 antibody, or in the absence of these antibodies. The concentration of IgG purified from the mother’s serum was adjusted to 5, 20 and 80 μg/ml and that of monoclonal anti-CD36 antibody to 2μg /ml. One ml aliquot of cell suspension was placed in the 35ml suspension culture dish in duplicate and incubated at 37°C in 5% CO2 for 7 days. Then, the number of CFU-E colonies which consisted of 8 ~ 49 cells were counted on an inverted microscope (5). Unfortunately, these experiments were performed only three times for practical reasons, so that we could not analyze the results statistically.

Figure 1: Flow cytometric analyses of CD36 on the platelets (a-d) and the monocytes (e-h), Mother (a, e), 1st daughter (b, f), 2nd daughter (c, g), and CD 36 positive control (d, h); CD36s on platelets are illustrated as a histogram and those of monocytes are illustrated as a cytogram, and monocytes are analyzed under the condition of the CD14 positive gating region (upper half region of each profile). Note that the mother’s platelets and monocytes both lacked reactivity with the monoclonal anti-CD36 antibody, and both her daughters were CD36 positive, although the intensity of their CD36 expression on platelets and monocytes appeared half that of the normal control. b: Flow cytometric analyses of the mother’s serum on the platelets and the monocytes. Note that the reactivity of the mother’s serum to platelets and monocytes was quite similar to that of the monoclonal anti-CD36 antibody.
Results

1. Studies for characterization of CD36 deficiency

Analysis of CD36 expression by flow cytometry
The mother was confirmed as having type I CD36 deficiency, since her platelets and monocytes both lacked reactivity with monoclonal anti-CD36 antibody. Both her daughters were CD36 positive, although the intensity of their CD36 expression on platelets and monocytes appeared to be half the normal level compared with the normal control (Fig. 1a, and Table 1). The reactivity of the mother’s serum to platelets and monocytes was quite similar to that of monoclonal anti-CD36 antibody (Fig. 1b, Table 1).

![Figure 2: The cDNA sequence of exon 12 and 13 of the CD36 gene.](image)

Table 1: Flow cytometric analysis of CD36 on platelets and monocytes (mean fluorescence intensity).

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Platelet</th>
<th>Monocyte</th>
<th>Platelet</th>
<th>Monocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td>5.09</td>
<td>42.93</td>
<td>15.23</td>
<td>393.35</td>
</tr>
<tr>
<td>1st Daughter</td>
<td>92.07</td>
<td>369.64</td>
<td>96.55</td>
<td>607.59</td>
</tr>
<tr>
<td>2nd Daughter</td>
<td>98.66</td>
<td>395.64</td>
<td>159.65</td>
<td>921.47</td>
</tr>
<tr>
<td>control (CD36+)</td>
<td>241.58</td>
<td>1258.16</td>
<td>210.95</td>
<td>2039.99</td>
</tr>
</tbody>
</table>

![Table 2: Results of the CFU-E colony assay (colony counts).](image)

Table 2: Results of the CFU-E colony assay (colony counts).

<table>
<thead>
<tr>
<th>Control</th>
<th>Monoclonal Ab</th>
<th>5 µg / ml</th>
<th>20 µg / ml</th>
<th>50 µg / ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>IgG</td>
<td>IgG</td>
<td>IgG</td>
</tr>
<tr>
<td>Exp. 1</td>
<td>141</td>
<td>74</td>
<td>118</td>
<td>67</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>44</td>
<td>28</td>
<td>49</td>
<td>31</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>115</td>
<td>110</td>
<td>121</td>
<td>118</td>
</tr>
</tbody>
</table>

Analysis of the CD36 gene mutation
The genomic DNA and cDNA analyses revealed that the mother was a compound heterozygote with a novel C→T transition at nt 1366 in exon 12, corresponding to Arg386Trp, and a known 12bp deletion at nt 1438–1449 in exon 13 (6). Both her daughters were heterozygote with one mutation of Arg386Trp (Fig. 2). No other mutation was detected in any exons of the CD36 gene or any exon-intron junctions of the subjects.

2. CFU-E colony assay
IgG from the mother exhibited some suppression of CFU-E colony formation to the same level as the monoclonal anti-CD36 antibody in all three different concentrations (Table 2). No significant suppression was observed with the equivalent level of control IgG.

Discussion
Here we report the first sibling cases of hydrops fetalis caused by a maternal alloimmune anti-CD36 antibody. Mutation analysis revealed that the mother was a compound heterozygote of the CD36 gene mutation (including a novel mutation of Arg386Trp) with phenotype of type I CD36 deficiency. The two daughters, whose levels of CD36 on platelets and monocytes were half those of normal levels, were heterozygote with one mutation at Arg386Trp. This finding is consistent with the fact that Arg386Trp is a mutation responsible for CD36 deficiency, not a polymorphic substitution. In fact, a C→T transition at nt 1366 in exon 12 of the CD36 gene has not been reported in JSNP database (7).

Because of negative Coombs’ test and no recurrence of anemia after the therapy, we first suspected that the two daughters were suffering from non-immune hemolytic anemia or congenital defects in fetal red blood cell synthesis. Therefore, the sequences of the Hb-γ gene of the sisters were studied, but we did not find any abnormalities (results not shown).

Fortunately, an accidental episode of anaphylactic shock in an individual who received fresh frozen plasma from the mother’s donated blood led us to the correct diagnosis. There have been several case reports (up to 15 cases) of alloimmune thrombocytopenia caused by the anti-CD36 antibody (2) including a case of neonatal anemia which probably resulted from bleeding with alloimmune thrombocytopenia (8). However, no cases of hydrops fetalis due to maternal anti-CD36 antibody have been reported. If there had been extraordinary high titer or high potential for complement activation of the antibody in our case, those findings might have provided some explanations for the differences of severity. Further investigations are needed in these points.

Regarding the daughters in our study, it seems very likely that the maternal anti-CD36 antibody was related to the observed hydrops fetalis. Erythroblasts are known to be CD36-positive, whereas mature erythrocytes are CD36-negative (9). Therefore, it is understandable that the Coombs’ test was negative in both sisters. However, the precise mechanism of severe fetal anemia (hydrops fetalis) remains to be determined. The results of the CFU-E colony assay indicated that anti-CD36 antibodies affected the erythropoiesis in vitro. Thus, the anti-CD36 antibody
would have effects on the growth and the differentiation of erythroblasts in the daughters, both of whom showed extreme reticulocytosis at birth for unknown reasons. These findings are very different from that of erythroblastic synartesis, an autoimmune dyserythropoiesis, whose auto-antigen was unknown but a molecule on the membrane of erythroblasts has been proposed and one candidate antigen was CD36 (10). This autoimmune dyserythropoiesis exhibits severe anemia with reticulocytopenia. The Coombs’ test was positive in patients with erythroblastic synartesis. In addition, a bone marrow smear from case 1 did not show abnormal aggregation of erythroblasts (data not shown), which was a characteristic feature of erythroblastic synartesis. Thus, we concluded that the mechanism for anemia in erythroblastic synartesis seemed close to but different from our cases. One possibility is that the mechanism of severe anemia in our cases might depend on hemolysis from complement activation in vivo, not on the direct blockage of erythropoiesis by antibodies that we showed by CFU-E colony assay in vitro. Another possibility is that gene expression of CD36 in erythroblasts might have a large deviation and fewer-expressed erythroblasts could pass through the “gate” of the anti-CD36 antibody. Further investigations are also needed to clarify these aspects.

Although the hydrops of our cases seemed to result from immunogenic anemia of erythroblasts, this immune process could not be detected by routine immunological methods such as the Coombs’ test. It is of note that a negative Coombs’ test could not exclude the possibility of peculiar immune hydrops fetalis like our cases. There should be a significant number of cases of hydrops due to the same mechanism among those of unknown etiology because CD36 deficiency is not rare in some ethnic groups. We propose that examination of maternal CD36 expression on platelets or monocytes, or evaluations of anti-CD36 antibody in maternal serum should be considered in screening for hydrops fetalis.

References