Somatic mosaicisms of chromosome 1 at two different stages of ontogenetic development detected by Rh blood group discrepancies

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ABSTRACT

Spontaneous Rh blood group changes are a striking sign, reported to occur mainly in patients with hematologic disorders. Upon routine blood grouping, 2 unrelated individuals showed unexplained mixed red cell phenotype regarding the highly immunogenic c antigen (RH4), clinically relevant for blood transfusion and fetomaternal incompatibility. About half of their red cells were c-positive, whereas the other half were c-negative. These apparently hematologically healthy females had no history of transfusion or transplantation, and they tested negative for chimerism. Genotyping of flanking chromosome 1 microsatellites in blood, finger nails, hair, leukocyte subpopulations, and erythroid progenitor cells showed partial loss of heterozygosity encompassing the RHD/RHCE loci, spanning a 1p region of 26.7 or 42.4 Mb, respectively. Remarkably, in one case this was detected in all investigated tissues, whereas in the other, exclusively myeloid cells showed loss of heterozygosity. Both carried the RhD-positive haplotypes CDe and the RhD-negative haplotype cde. RHD/RHCE genotypes of single erythroid colonies and dual-color fluorescent in situ hybridization analyses indicated loss of the cde haplotype and duplication of the CDe haplotype in the altered cell line. Accordingly, red cell C antigen (RH2) levels of both propositae were higher than those of heterozygous controls. Taken together, the Rhc phenotype splitting appeared to be caused by deletion of a part of 1p followed by duplication of homologous stretches of the sister chromosome. In one case, this phenomenon was confined to myeloid stem cells, while in the other, a pluripotent stem cell line was affected, demonstrating somatic mosaicism at different stages of ontogenesis.

Introduction

Antigens of the Rh blood group system are very immunogenic and routinely typed in pretransfusion testing and prenatal investigations, as antibodies against these structures may elicit hemolytic transfusion reactions or hemolytic disease of the fetus and newborn. D (RH1) and c (RH4) are clinically the most important Rh antigens, as the frequently encountered anti-D and anti-c alloantibodies have pronounced hemolytic potential. All Rh antigens reside on RhD and RhCcEe polypeptides encoded by the RHD and RHCE genes, respectively, mapped to the short arm of chromosome 1 (p34-36).1,2

Unambiguous Rh typing is mandatory to account for the clinical relevance of these antigens. However, Rh-mismatched transfusion or hematopoietic stem cell transplantation (iatrogenic chimerism) may lead to concurrent presence of Rh antigen-positive and -negative red blood cells (RBCs) in the circulation. Importantly, mixed-field agglutination in serological Rh typing was noted also in non-iatrogenic
settings, usually regarding the D antigen (and often haplotypically linked C or E antigens). Apart from inborn forms of chimerism,4 acquired Rh antigen loss was preferentially observed in patients with clonal myeloid diseases;4,11 in some cases with cytogenetic chromosome 1 alterations.12–14 Also hematologically healthy subjects were observed to have this phenomenon.4,14,15 As the dominant mechanism of acquired Rh phenotype splitting, mosaicism based on myeloid lineage-restricted loss of heterozygosity (LOH) of variable stretches of chromosome 1 was identified with loss of one RH haplotype.4 In one case, somatic RHD mutation was described,19 whereas in other cases, RHD and RHCE gene deletion was reported.4,20,21

In this study, the phenotypic and molecular characteristics of spontaneous c antigen anomaly in 2 unrelated individuals were investigated. For the first time, data are provided that demonstrate two different forms of somatic chromosome 1 mosaicism at different stages of ontogenetic development, as evidenced by involvement of different cells and tissues. The clinical significance of this phenomenon with regard to transfusion medicine and as a potential marker for hemat-oncologic disease is discussed.

Methods

Patients

Two female Caucasian individuals (proposita A and proposita B, aged 69 and 35 years, respectively) from Switzerland without any history of transfusion or hematopoietic stem cell transplantation, came to attention with unexplained mixed-field agglutination in routine serological blood group typing. The latter was performed in the course of pretransfusion testing for knee surgery (proposita A) and as part of routine pregnancy monitoring (proposita B). This study was approved by the Swiss Red Cross Institutional Review Board. Written informed consent was obtained for extended testing and inclusion in this investigation.

Serological blood group typing and red cell flow cytometry

Serological blood group typing, anti-erythrocyte antibody screening and direct antiglobulin testing was carried out using gel centrifugation technique (Bio-Rad, Cressier, Switzerland), as described.25 In addition, monoclonal anti-c reagents from Diagast (Loos, France), BAG (Lich, Germany), Immucor (Rödermark) and Ortho Clinical Diagnostics (Neckargemünd, Germany) were used.

Expression of c and C antigens of RBCs from both propositae was determined by flow cytometry (FACSCalibur with CellQuest software, BD Biosciences, San Jose, CA, USA) after indirect immunofluorescence staining with polyclonal anti-c and anti-C reagents (Molter, Neckargemünd, Germany).

Sorting of nucleated cell subsets from peripheral blood

Cell subsets of ethylenediamine tetraacetic acid (EDTA)-anticoagulated blood samples were quantified and sorted as previously described.4,25

Erythropoietic burst forming unit cultures

Cultures for erythropoietic burst-forming units (BFU-E), scoring and individual clonal picking for subsequent DNA isolation was performed as previously described.4

DNA isolation

Genomic DNA from EDTA-anticoagulated blood was extracted with the GenoPrep Cartridge B 350 on a GenoM-6 instrument (GenoVision, Vienna, Austria). DNA from buccal swabs, hair samples, finger nails, and single BFU-E colonies with the Qiamp DNA Investigator or Mini Kit (Qiagen, Valencia, CA, USA). DNA from sorted peripheral blood cells was extracted with Chelex.24

Molecular blood group RH genotyping

For RHD and RHCE genotyping, testing for variant RHD alleles and RHD zygosity of blood samples, polymerase chain reaction (PCR) kits (RBC Ready Gene CDE, Zygofast or RHd, Innotrain, Kronberg, Germany) were used.20 The RHCE* allele was detected from DNA isolated from single BFU-E colonies with sequence specific monoplex real-time PCR using primers, probes and real-time PCR reagents as previously described,26 with a modified cycle protocol for increased sensitivity.

Microsatellite analysis

DNA prepared from whole blood and hair roots was tested in a multiplex-PCR of 15 highly polymorphic autosomal microsatellite loci to check for the existence of a possible chimerism (AmpFISTR IDentifiler PCR Amplification Kit, Applied Biosystems, Foster City, CA, USA).

DNA samples from whole blood, buccal swabs (only proposita B), single hair roots, nucleated blood cell subsets, or BFU-E colonies were analyzed with up to 16 different primer pairs targeting polymorphic dinucleotide microsatellite markers located on chromosome 1.

Fluorescence in situ hybridization analyses

Dual-color fluorescence in situ hybridization (FISH) analyses on fixed peripheral blood cells of both propositae were performed as previously described.21 P1-based artificial chromosome clones that encompass the RHD/RHCE and AF1q gene loci, respectively, were used. At least 200 cells per proband were scored and the signal patterns recorded separately for segmented and round nuclei.

Results

Spontaneous Rh blood group anomaly in 2 unrelated individuals

Routine serological blood group determination revealed unexpected mixed-field agglutination with respect to c antigen typing in 2 unrelated females without known hematologic disorder (proposita A and B). This was evident with all employed anti-c typing reagents (six monoclonal and one polyclonal). The proportion of c-positive red cells by flow cytometry was 53% and 50% in proposita A and B, respectively (Table 1). Apart from this, both individuals showed a normal C+D+E+E+ Rh phenotype. All other tested blood groups (ABO, MNS, P1Pk, Lutheran, Kell, Duffy, Kidd) were of normal phenotype (Table 1). No unexpected red cell antibodies were found in the plasma of these individuals, and the direct antiglobulin test with their erythrocytes was negative.

Routine RHD/RHCE genotyping combined with RHD zygosity determination of blood-derived DNA from both propositae yielded RHD heterozygosity (Dd) and predicted common CcEe phenotypes.

The c antigen quantities of their c-positive RBC subsets were similar to CcDdee phenotype control RBCs (Figure
1). Antithetical C antigen expression of both propositae was higher than in CcDdee controls, approaching the higher quantities seen in CCDDee controls (Figure 2).

Exclusion of congenital or acquired chimerism as cause of Rh phenotype anomaly

Twin chimerism or dispermy, as well as artificial chimerism (due to blood transfusion or organ transplantation) could be the reason for mixed blood group phenotypes. However, both propositae denied having a twin or a history of blood transfusions or organ grafts. Moreover, the analysis of 15 microsatellite loci with DNA of whole blood (loci located on chromosomes 2-5, 7, 8, 11-13, 16, 18, 19, and 21) ruled out chimerism: exclusively homozygous or well-balanced heterozygous allelic peaks were found, with a maximum of two alleles present at each locus (data not shown).

Loss of heterozygosity on chromosome 1 at an early stage of ontogenetic development in proposita A

As the RHD/RHCE loci are located on the short arm of chromosome 1, the possibility of mosaicism was tested by use of heterozygous chromosome 1 microsatellite markers (for full details, see the Online Supplementary Appendix). In proposita A, the analysis of D1S468 (21 Mb telomeric of RH*D), D1S234 (0.5 Mb telomeric of RH*D), and D1S233 (5.7 Mb centromeric of RH*D) using DNA from whole blood, and sorted leukocyte subpopulations (CD4+ T cells, CD8+ T cells and granulocytes) showed in all samples a clear-cut imbalance of the peak heights. This indicated the presence of 2 cell populations at each locus (data not shown).

Loss of heterozygosity on chromosome 1 confined to myeloid cells in proposita B

In proposita B, the analysis of microsatellites in the region between D1S507 (10.3 Mb telomeric of RH*D) and D1S2890 (32.1 Mb centromeric of RH*D) using DNA from whole blood showed in all samples a peak height imbalance diagnostic of LOH, thus demonstrating the existence of 2 cell populations in which 1 lost 1 allele. D1S252 located centromeric of D1S2890 exhibited no LOH. Hairs showed no LOH in all loci tested.

The alleles of D1S2890 were further investigated using DNA from buccal swab, single hair roots, sorted leukocyte subpopulations (CD4+ T cells, CD8+ T cells, and granulocytes), and BFU-E colonies. A myeloid lineage-restricted pattern of LOH was found, with LOH detected in sorted granulocytes and in 4 out of 22 BFU-E colonies. In contrast, hairs (n=3), buccal cells, and lymphocyte subsets showed no LOH (Figure 3). Further details of these analyses are provided in the Online Supplementary Appendix.

RH genotype splitting confirmed by molecular analysis of single erythroid progenitor cells

DNA samples from separate BFU-E colonies were subjected to real-time PCR genotyping for RHCE*c. Six BFU-E samples each of both individuals with mixed Rhc phenotype were analyzed and displayed a similar pattern: 3 out of 6 tested BFU-E DNA samples showed heterozygous results for the RHCE*c allele; in contrast, the other half indicated LOH at this locus (Table 2). Importantly, only BFU-E colonies with RHCE*c heterozygosity were found to be also heterozygous for RHD (Dd), whereas LOH was uniformly associated with homozygous or potentially hemizygous RHD-positive typing (DD or D-) (Table 2).

Table 1. Blood group phenotypes of the 2 propositae with spontaneous c antigen (RH4) mixed-field typing.

| Proposita | Rh  | ABO | MNS | P1Pk | Lutheran | Kell | Duffy | Kidd |
|-----------|-----|-----|-----|------|----------|------|-------|------|
| A         | c± (53% c+) | D+C+ | e-+ | Ow- | A     | M-N+S+s+ | P1- | Lu(a-b+) | k-k+, Kp(a-b+) | Fy(a+b+) | Jk(a+b+) |
| B         | c±  (50% c+) | D+C+ | e-+ | Ow- | A     | M+N+S+s+ | P1- | Lu(a-b+) | k-k+, Kp(a-b+) | Fy(a+b+) | Jk(a+b+) |

±: mixed-field agglutination.

Figure 1. Flow cytometric analysis of red cell c antigen (RH4) expression of propositae A and B. Immunofluorescence histograms of erythrocytes indirectly stained with polyclonal anti-c are shown. Note c-negative and c-positive cell subpopulations (black histograms). For comparison, a CcDdee control (open histograms) is included.
These results underlined the haplotypic nature of the observed blood group anomaly and indicated the co-existence of 2 RBC lines: 1 of normal c-positive phenotype encoded by 2 parental RH haplotypes (CDe/cde) and a second with c-negative phenotype encoded by the LOH-modified RHCE*c-negative parental haplotype only (homozygous CDe/CDe or hemizygous CDe/---).

Rh blood group anomaly caused by somatic recombination-associated duplication

To determine whether the Rhc-negative cell clone resulted from a hemizygous deletion (CDe/---) or a more complex somatic recombination-associated duplication of the CDe haplotype, dual-color FISH analyses on fixed peripheral blood cells obtained from both studied individuals were performed. In both proposita A and B, FISH analysis showed the diploid presence of the RH loci in all segmented and round nuclei (Figure 4). Despite there being no proof by chromosomal sequencing, these results indicated somatic recombination-associated loss of the RHCE*c-positive/RHD-negative and duplication of the RHCE*c-negative/RHD-positive haplotype as cause for the observed RBC phenotype splitting. Hence, the LOH-affected RHCE*c-negative cell lines of both propositae most probably harbored homozygous CDe/CDe haplotypes.

Discussion

Two individuals with an unexplained mixed-field agglutination in routine serological Rhc typing have been observed. Common causes of mixed Rhc phenotype, such as RBC transfusion or hematopoietic stem cell transplantation, were ruled out in the 2 propositae. Extended molecular testing was performed to define the underlying mechanism of this condition. Microsatellite analysis across different chromosomes excluded spontaneous chimerism known to bring about mixed blood group phenotypes.27

Using chromosome 1 microsatellite markers, somatic mosaicism with partial haploid loss of 1p involving the RH locus was found to be responsible for the observed Rhc phenotype anomaly in both individuals studied, encompassing at least 26.7 and 42.4 Mb, respectively.

The high red cell expression of the antithetical C (RH2) antigen, nearly approaching levels of RHCE*C homozygous controls, indicates that the deletion of a part of 1p (eliminating the RHCE*C allele) has been repaired by a duplication of homologous stretches of the other chromosome harboring the RHCE*C allele. This view is further supported by the FISH results, showing the uniform presence of two RH loci in all examined cell nuclei. Accordingly, also LOH-affected cells did not show an RH

Table 2. Molecular RHCE*c analysis and Rhd typing of single erythroid progenitor cells of both individuals with Rh phenotype splitting.

| Proposita | BFU-E colony | RHCE*c | Rhd |
|-----------|--------------|--------|-----|
| A         |              |        |     |
| A1        | LOH          |        |     |
| A2        | heterozygous |        | Dd  |
| A3        | heterozygous |        | Dd  |
| A4        | LOH          |        | Dd  |
| A5        | heterozygous |        | Dd  |
| A6        | LOH          |        | Dd  |
| B         |              |        |     |
| B1        | heterozygous |        | Dd  |
| B2        | LOH          |        | Dd  |
| B3        | LOH          |        | Dd  |
| B4        | heterozygous |        | Dd  |
| B5        | heterozygous |        | Dd  |
| B6        | LOH          |        | Dd  |

LOH: loss of heterozygosity; BFU-E: erythropoietic burst-forming units.

Figure 2. Red cell C antigen (RH2) expression levels of proposita A and B compared to normal controls. Mean fluorescence intensities of erythrocytes indirectly stained with polyclonal anti-C after subtraction of negative control obtained with ccddee cells are shown. CcDdee (n=3) and CcDdee (n=3) control values are depicted as average with standard deviation.
deletion on their altered 1p that would be recognized by only one RH-FISH signal, as demonstrated previously. Instead, they appear to have retained RH loci on both 1p, not distinguishable from normal cells in this assay. The mechanism of the mixed-field agglutination in serological Rhc typing is, therefore, probably a somatic recombination with partial chromosome loss followed by a duplication.

Further studies designed to identify the cell lines and tissues that were affected by LOH revealed a differential configuration in the 2 propositae (see insert of Figure 3). In proposita B, LOH was observed in a lineage-specific distribution, occurring in a fraction of myeloid cell subsets but not in lymphoid compartments or non-hematopoietic tissues. Accordingly, only some, but not all, of the studied BFU-E colonies showed LOH. These results are compatible with the predominant genetic background of spontaneous Rh phenotype splitting as investigated in an earlier study. In the vast majority of individuals with mixed RhD and RhC or RhE phenotype, myeloid-lineage restricted mosaicism caused by LOH of variable chromosome 1 stretches encompassing the RHD/RHCE loci had been identified. In the present study, for the first time, this genetic background was documented with respect to spontaneous Rhc phenotype anomaly.

In contrast, proposita A showed a different spectrum of tissue involvement by LOH. Besides some of the myeloid stem cells and BFU-E colonies, also lymphocytes and hair roots were affected by this somatic change. These results indicate that LOH developed in a pluripotent stem cell line at an early stage of ontogenetic development, still capable of differentiating into hematopoietic as well as hair root cells. Such a constellation has not so far been described.

Copy-neutral LOH on 1p can compromise expression of many different genes, including those encoding Rh blood group antigens. The analysis of discrepant blood grouping...
results with mixed-field agglutination patterns is essential for safe transfusion therapy of such patients. Unequivocal blood group typing is a prerequisite for transfusion support and prenatal investigations evaluating fetomaternal incompatibility. At many institutions, not only ABO and RhD typing is performed, but also further highly immunogenic antigens including c, K and others are increasingly taken into account for transfusion matching. Such extended matching strategy was markedly shown to reduce the alloimmunization rate of transfusion recipients, an effect especially desirable for multi-transfused patient cohorts or women of childbearing age. For both propositae, neither anti-c nor anti-C alloimmunization is to be expected, as both antigens are present. Hence, no particular transfusion strategy seems to be required regarding these two antigens.

Of note, mixed blood group phenotypes often escape serological detection but may be unveiled by molecular screening. The latter is of particular relevance for blood donor testing: it could have avoided a number of documented anti-D immunizations by red cell concentrates from serologically D-negative blood donors with an undetected D-positive cell subset.

Apart from these implications for transfusion medicine, the blood group anomaly may only be the first evidence of an underlying genetic alteration of possibly extended clinical relevance. While it is increasingly recognized that somatic mosaicism including LOH may not be uncommon in apparently healthy subjects, a LOH-based blood group discrepancy may well represent a surrogate marker of myeloid diseases. Apart from acute myeloid leukemia and myelodysplastic syndrome, allelic loss on 1p was also detected in many other malignancies, such as colorectal cancer, neuroblastoma, lung cancer and hepatocellular carcinoma. Hence, this chromosomal region is probably home to tumor suppressor genes. It may be concluded that, depending on individual tissue distribution of LOH on 1p, the potential loss of tumor suppressor gene function could increase the risk for malignant transformation in affected organs. Alternatively, copy-neutral LOH may also result in duplication of oncogenic mutations with a subsequently increased likelihood of cancer. Recent data indicate that detection of LOH may not only have diagnostic but also prognostic potential for myeloid neoplasms. Taken together, when encountering a patient with spontaneous blood group phenotype splitting, clinical and laboratory screening investigations for hematologic disease should be considered.

**Figure 4.** Dual-color fluorescence in situ hybridization signal patterns of selected cell nuclei obtained with PAC clones that encompass the RH/D/RHCE (FITC, green) and, as a control, AF1q gene sequences (Cy3, red). Representative results of fixed peripheral blood cells of proposita B are shown: all segmented (top) and round (bottom) nuclei contained two signals each and, thus, two RH gene loci. An identical pattern was seen in proposita A (not shown). Original magnification x1000.
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