SUPPLEMENTARY APPENDIX

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

Eva-Maria Dauber,[1] Wolfgang R. Mayr,[1] Hein Hustinx,[2] Marlies Schönbacher,[1] Holger Budde,[3] Tobias J. Legler,[3] Margit König,[4] Oskar A. Haas,[4] Gerhard Fritsch[4] and Günther F. Körmöczi[1]

[1]Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Austria; [2]Blood Transfusion Service, Swiss Red Cross (SRK), Bern, Switzerland; [3]Department of Transfusion Medicine, University of Göttingen, Germany and [4]Children's Cancer Research Institute, St. Anna Hospital, Vienna, Austria

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Correspondence: *GÜNTHER F. KÖRMÖCZI*

guenther.koermoeczi@meduniwien.ac.at

Online Supplement

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

Eva-Maria Dauber[1], Wolfgang R. Mayr[1], Hein Hustinx[2], Marlies Schönbacher[1], Holger Budde[3], Tobias J. Legler[3], Margit König[4], Oskar A. Haas[4], Gerhard Fritsch[4], and Günther F. Körmöczi[1]

[1] Department of Blood Group Serology and Transfusion Medicine, Medical University of Vienna, Vienna, Austria

[2] Blood Transfusion Service, Swiss Red Cross (SRK), Bern, Switzerland

[3] Department of Transfusion Medicine, University of Göttingen, Göttingen, Germany

[4] Children's Cancer Research Institute, St. Anna Hospital, Vienna, Austria

Materials and Methods

Studied patients

Two female Caucasoid individuals (proposita A and proposita B, aged 69 and 35 years, respectively) from Switzerland without a history of transfusion or hematopoietic stem cell transplantation came to attention with unexplained mixed-field agglutination in routine serologic 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 SRK Institutional Review Board. Written informed consent was obtained for extended testing and inclusion into this investigation.

Serologic blood group typing and red cell flow cytometry

Serologic blood group typing, anti-erythrocyte antibody screening and direct antiglobulin testing was carried out by gel centrifugation technique using Bio-Rad (Cressier, Switzerland) reagents and equipment, as described previously.[1] In addition, further monoclonal anti-c reagents were used: 951 (Diagast, Loos, France), c1.16C.15A (BAG, Lich, Germany), MS35 (Immucor, Rödermark) and MS42 (Ortho Clinical Diagnostics, Neckargemünd, Germany).

Expression of c and C antigens of RBCs from both propositae and of control RBC samples was determined by flow cytometry (FACSCalibur with CellQuest acquisition software, BD Biosciences, San Jose, CA) after indirect immunofluorescence staining with polyclonal anti-c and anti-C reagents (Molter, Neckargemünd, Germany). Polyclonal reagents had been prepared for use by repeat adsorption onto antigen-positive group O RBCs (*ccddee* and *Ccddee* with anti-c and anti-C, respectively), washing and subsequent acid elution (DiaCidel, Bio-Rad).[2] As secondary reagent, Alexa Fluor 488-labeled Fab fragment goat anti-human IgG (Jackson Immuno Research, Ely, UK) was used. Adequate fluorescence marker placements allowed for the quantification of c-positive RBC fractions.

Sorting of nucleated cell subsets from peripheral blood

Cell subsets of ethylenediamine tetraacetic acid (EDTA)-anticoagulated blood samples were quantified by four-color FACSCalibur flow cytometry after isotonic RBC lysis with ammonium chloride buffer and dual platform cell staining.[3] For cell sorting, three-laser FACSAria (BD Biosciences) equipment with DIVA software (BD Biosciences) was used. Two vials with 10^6 leukocytes each were prepared per blood sample, and sorted

using the following fluorescent dye and murine monoclonal antibody combinations: Syto41 (Molecular Probes, Eugene, OR), Anti-CD8 FITC (clone DK25, Dako,Glostrup, Denmark), Anti-CD56 PE (clone NCAM16.2, BD Biosciences, San Jose, CA), Anti-CD3 PE Texas Red (clone UCHT1, PharMingen, San Diego, CA), Anti-CD45 peridin chlorophyll protein (PerCP; clone 2D1, BD), Anti-CD4 PE-cyanin7 (clone SK3, BD), Anti-CD71 allophycocyanine (APC; clone L01.1, BD) and Anti-CD14 APC-cyanin7 (clone MoP9, BD) for CD4+, CD8+, natural killer cells and normoblasts; and Syto41, Anti-CD15 FITC (clone C3D1, Dako), Anti-CD33 PE (clone P67.6, BD), Anti-CD45RA PE Texas Red (clone 2H4, Coulter, Miami, FL), Anti-CD45 PerCP, Anti-CD34 PE-cyanin7 (clone 8B12, BD) and Anti-CD19 APC (clone SJ25C1, BD) for CD34+ cells, monocytes, granulocytes and B cells. The purity of sorted cell subsets generally exceeded 98%.

Erythropoietic burst forming unit cultures

Cultures for erythropoietic burst-forming units (BFU-E) were performed as described elsewhere.[4] After cultivation for two to three weeks, all colonies were scored according to standard criteria.[5] Single BFU-E colonies were picked using sterile Pasteur pipettes and resuspended in 100 µL of PBS in 0.5 mL microcentrifuge tubes for subsequent DNA isolation.

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). DNA from sorted peripheral blood cells was extracted with Chelex.[6]

Molecular blood group *RH* genotyping

For routine *RHD* and *RHCE* genotyping, testing for variant *RHD* alleles and *RHD* zygosity of blood samples, commercially available typing kits (RBC Ready Gene CDE, Zygofast or RHd, Inno-train, Kronberg, Germany) were used employing polymerase chain reaction (PCR) technique with sequence-specific priming (SSP).[7]

The *RHCE*c* 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,[8] with a modification of the cycle protocol for increased sensitivity. Because very low DNA concentrations were observed, the internal control was replaced by an external real-time PCR using primers and probe specific for the single copy gene beta globin on the same PCR plate.[9] Reaction conditions were 95°C for 10 minutes, 50 two-step cycles with 60°C for 60 seconds and 95°C for 15 seconds. The temperature profile for the ß-Globin PCR and the *RHCE*c* PCR was identical. An ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA) was used. *RHD/RHd* zygosity of single BFU-E colonies was tested PCR-SSP using the RBC Ready Gene Zygofast kit with an ABI 9700 PCR cycler (Applied Biosystems). The reaction conditions were 94°C for 2 minutes, followed by 5 cycles with 94°C for 20 seconds and 70°C for 60 seconds, 10 cycles with 94°C for 20 seconds, 65°C for 60 seconds and 72°C for 45 seconds, 25 cycles with 94°C for 20 seconds, 61°C for 50 seconds and 72°C for 45 seconds and a final step with 72°C for 5 minutes. Amplicons were visualized in agarose gel stained with ethidium bromide.

Microsatellite analysis

DNA prepared from whole blood and hair roots was tested in a multiplex-PCR of 15 highly polymorphic autosomal microsatellite loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, VWA, TPOX, D18S51, D5S818, FGA) in order to check for the existence of a possible chimerism (AmpFlSTR IDentifiler PCR Amplification Kit, Applied Biosystems, Foster City, CA).
DNA samples from whole blood, buccal swabs (only proposita B), single hairs roots, nucleated blood cell subsets, or BFU-E colonies were analyzed with Human Mapping Primers v2.5 according to the manufacturer's instructions (Applied Biosystems) targeting the following polymorphic dinucleotide microsatellite markers located on chromosome 1: D1S468, D1S507, D1S2697, D1S2644, D1S199, D1S2864, D1S234, D1S233, D1S255, D1S2890, D1S2841 (only proposita A), D1S206, D1S252 (1p) and D1S498, D1S2635 and D1S2836 (1q). The instrument Geneamp PCR System 9700 and ABI Prism 310 or 3130 Genetic Analyzer (Applied Biosystems) were used for amplification and further analysis.

Fluorescent in-situ hybridization analyses

Dual-color fluorescent in-situ hybridization (FISH) analyses on methanol/acetic acid fixed peripheral blood cells of both propositae were performed as described.[10] P1-based artificial chromosome (PAC) clones RP11-335G20 (accession No. AL928711; kindly provided by Mariano Rocchi, Insitituto di Genetica, Bari, Italy) and RP11-316M1 (Research Genetics, Huntsville, AL) that encompass the *RHD*/*RHCE* and *AF1q* gene loci, respectively, were used. Signals were recorded after overnight incubation at 37°C. At least 200 cells per proband were scored and the signal patterns recorded separately

for segmented and round nuclei. Images were visualized using a Zeiss Axioplan microscope equipped with 100x/1.45 alpha Plan-Fluar lens and acquired using a Photometrics (Tucson, AZ) charge-coupled device camera with IPLAB software (VYSIS, Stuttgart, Germany).

Full-Detail Results: Loss of heterozygosity as evidenced by microsatellite analyses

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 chromosome 1 microsatellite markers. In proposita A, 13 out of 16 chromosome 1 microsatellite loci were heterozygous (10/13 on 1p: D1S468, D1S507, D1S2644, D1S199, D1S2864, D1S234, D1S233, D1S2841, D1S206, D1S252 and 3/3 on 1q: D1S498, D1S2635 and D1S2836) while three were homozygous (3/13 on 1p: D1S2697, D1S255, D1S2890).

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 two cell populations in which one lost one 1p segment. Such a LOH was also seen in two of six single hair roots, while the other four exhibited heterozygosity with balanced peak heights. The collection of separate BFU-E colonies allowed for DNA isolation from single erythroid progenitor cells. The analysis of 19 BFU-E colonies showed that nine had complete LOH, while in the other ten colonies both alleles of each locus could be seen with similar expression.

The use of further four heterozygous loci situated between D1S468 and D1S234 (D1S507, D1S2644, D1S199, D1S2864, 10.3, 6.6, 5.7 and 2.8 Mb telomeric of RH*D, respectively) confirmed these results: LOH in whole blood as well as in the affected

hair roots (six hair roots, thereof two with LOH) and corroboration of the results in BFU-E (two BFU-E colonies with and two without LOH).

Other microsatellite loci more centromeric than D1S233 were also tested: D1S2841, D1S206, D1S252 (53.7, 75.9 and 91.5 Mb centromeric of *RH*D,* respectively), and three 1q loci (D1S498, D1S2635 and D1S2836). In all these loci, the alleles showed no imbalance indicating that there was no hint for LOH. The minimal expansion of LOH on 1p of the affected cell lines amounted to at least 26.7 Mb (see Figure 3 of the original paper).

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

In proposita B, eleven out of 15 chromosome 1 microsatellite loci showed heterozygosity (9 out of 12 on 1p D1S507, D1S2697, D1S199, D1S2864, D1S234, D1S233, D1S255, D1S2890, D1S252 and 2/3 on 1q D1S2635 and D1S2836), while four were homozygous (3/12 on 1p D1S468, D1S2644, D1S206 and 1/3 on 1q D1S498).

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*) [D1S2697, D1S199, D1S2864, D1S234, D1S233 and D1S255 8.9, 5.7, 2.8, 0.5 Mb telomeric, 5.7 and 11.9 Mb centromeric of *RH*D*, respectively] using DNA from whole blood showed in all samples a peak height imbalance diagnostic of LOH, thus demonstrating the existence of two cell populations in which one lost one 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 the following sources: 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. LOH was demonstrated testing DNA from sorted granulocytes. The analysis of 22 BFU-E colonies revealed that four had complete LOH, while both alleles with a similar expression were detected in the other 18 colonies. In contrast, hairs (n=3), buccal cells, and lymphocyte subsets showed no LOH (see Figure 3 of the original paper).

References

1. Körmöczi GF, Legler TJ, Daniels GL, et al. Molecular and serologic characterization of DWI, a novel "high-grade" partial D. Transfusion. 2004;44(4):575-580.

2. Wagner T, Körmöczi GF, Buchta C, et al. Anti-D immunization by DEL red blood cells. Transfusion. 2005;45(4):520-526.

3. Fritsch G, Witt V, Dubovsky J, et al. Flow cytometric monitoring of hematopoietic reconstitution in myeloablated patients following allogeneic transplantation. Cytotherapy. 1999;1(4):295 - 309.

4. Petzer AL, Gunsilius E, Zech N, et al. Evaluation of optimal survival of primitive progenitor cells (LTC-IC) from PBPC apheresis products after overnight storage. Bone Marrow Transplant. 2000;25(2):197-200.

5. Coulombel L, Eaves AC, Eaves CJ. Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. Blood. 1983;62(2):291-297.

6. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques. 1991;10(4):506-513.

7. Körmöczi GF, Förstemann E, Gabriel C, Mayr WR, Schönitzer D, Gassner C. Novel weak D types 31 and 32: adsorption-elution-supported D antigen analysis and comparison to prevalent weak D types. Transfusion. 2005;45(10):1574-1580.

8. Legler TJ, Lynen R, Maas JH, et al. Prediction of fetal Rh D and Rh CcEe phenotype from maternal plasma with real-time polymerase chain reaction. Transfus Apher Sci. 2002;27(3):217-223.

9. Lo YM, Tein MS, Lau TK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. Am J Hum Genet. 1998;62(4):768-775.

10. Körmöczi GF, Dauber EM, Haas OA, et al. Mosaicism due to myeloid lineage restricted loss of heterozygosity as cause of spontaneous Rh phenotype splitting. Blood. 2007;110(6):2148-2157.