Persistence of recipient-type endothelium after allogeneic hematopoietic stem cell transplantation

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Supplementary Design and Methods

Tissue sampling

A total of 52 patients were included in this analysis; skin punch biopsies were obtained from 22 of these patients in a prospective manner during routine bone marrow punctures before and after HSCT. In addition, diagnostic skin biopsies from 21 patients suspected of having GVHD, nine tissue samples (skin, heart, bone marrow and liver) from five autopsies, and four tumor biopsies were analyzed retrospectively. In summary, a total of 46 skin samples were analyzed (22 from patients during routine bone marrow punctures without clinically suspected skin GVHD; 21 patients suspected of having skin GVHD including a patient from whom two biopsies were taken; and two autopsy samples). The average size of the skin biopsies was 0.0025 m^3. Our analysis included 29 ABO-incompatible and 23 ABO-compatible HSCT, 28 gender-mismatched and 24 gender-matched HSCT. Seventeen patients received a graft with minor ABO-incompatibility [O in A (n=9), O in B (n=4), A in AB (n=1), and B in AB (n=3)], ten patients received a graft with major ABO incompatibility [A in O (n=7); B in O (n=3)] and two patients had a bidirectional ABO-incompatible HSCT [A in B (n=1), B in A (n=1)].

The study included 13 patients (25%) with acute myelogeneous leukemia, 17 (33%) with chronic myelogeneous leukemia, six (12%) with acute lymphoblastic leukemia, six (12%) with multiple myeloma, three (6%) with chronic lymphocytic leukemia, two (4%) with myelodysplastic syndrome, three (6%) with myeloproliferative syndrome, one (2%) with non-Hodgkin's lymphoma and one (2%) with aplastic anemia. The median age at HSCT was 41 years (range, 14-63 years) and 34 of 48 (71%) recipients were male. Biopsies were taken after a median of 194 days (range, 7-3,476 days).

Ten patients (19%) had a history of acute GVHD grade III-IV, 32 (62%) had acute GVHD grade I-II and 10 (19%) had no signs of acute GVHD. At the time of biopsy 12 patients (23%) had extensive chronic GVHD, 11 (21%) had limited chronic GVHD and 20 (39%) did not have any signs of chronic GVHD (Table 1). When all 46 histological samples examined for endothelial cell chimerism were analyzed for the presence of skin GVHD only 12 revealed signs of acute (n=3) or chronic (n=9) GVHD (*data not shown*).

Immunohistochemistry

Serial 3 μm-sections were incubated with monoclonal anti-A, B and H antibodies (Dako, Carpintera CA, USA) and monoclonal antibodies against CD45, CD31 (PECAM-1), CD34 and von Willebrand factor (VWF). Antibody binding was visualized with a secondary biotin-conjugated rabbit anti-mouse antibody followed by immuno-peroxidase staining. Counterstaining was done for 2-4 minutes in standard acidic hemalaun solution. The morphology of the tissues was analyzed with additional hematoxylin/eosin (H/E) staining. Endothelial cell chimerism was quantified by counting cells in the endothelial lining with a clearly recognizable nucleus and well-defined endothelial morphology that stained positively for ABH antigen. In addition, the cell count was normalized for variable section sizes by calculating the ratio between the number of endothelial cells and the number of cross-sectioned blood vessels on every section. Results were verified by a second observer unaware of the donor/recipient blood groups.

Combined fluorescence in situ hybridization for X and Y chromosomes and immunohistochemical staining for von Willebrand factor

Sections (4 μm) were deparaffinized in xylol, dehydrated in ethanol and incubated in 0.2 M HCl at room temperature for 20 min. Thereafter, they were immersed in Pretreatment Solution (Vysis®, Downer's Grove IL, USA) for 10 min at 80°C and digested with Protease I (Vysis®, Downer's Grove IL, USA) for 15-20 min at 37°C. After dehydration in ethanol, CEP Y Spectrum Red probe and CEP X Spectrum Green (Vysis®, Downer's Grove IL, USA), which cover classical satellite III sequences on the X and Y chromosomes, were co-incubated simultaneously at 80°C for 6 min and allowed to hybridize overnight at 42°C. The samples were then washed in 2xSSC/0.3 NP40 at 72°C for 5 min and 2xSSC for 2 min at room temperature. For subsequent immunohistochemical staining, the slides were incubated overnight at 4°C with monoclonal mouse anti-VWF antibodies (Dako, Glostrup, Denmark) followed by a secondary fluorescent goat anti-mouse antibody (Alexa Fluor 647, Molecular Probes®). Finally, 10 μL of 4'6-diamidino-2-phenylindole (DAPI II, Vysis®, Downer's Grove IL, USA) were applied to the samples for chromatin counterstaining. Isotype controls and omission of the primary antibody were used as negative controls. FISH signals and fluorescent immunostaining were evaluated with a Leica DM Fluorescence Microscope (with Z-stack analysis) and the corresponding LAS Leica Fluorescence Software AF 6000 DFC. For the quantification only morphologically clearly distinguishable VWF-positive endothelial cells which displayed a signal of

two chromosomes in their nucleus were counted, i.e. X and X for females and X and Y for males. Cells with only one visible signal were counted separately. In some samples chromogen *in situ* hybridization (CISH) was performed to confirm the results obtained with FISH. SPOT-Light® chromosome X/Y probe cocktail and SPOT-Light® CISH™ detection kit (Zymed® Laboratories Inc., San Francisco CA, USA) were used according to a previously described protocol.[1]

Short tandem repeat analysis

Single endothelial cells were captured out of 6 μm cryostat sections previously stained either for CD45 (negative selection) or for VWF antigen (positive selection) following careful microscopic evaluation with a laser-capture microdissector (LCM Arcturus VERITAS™, Molecular Devices, USA). Nuclei from 20-40 endothelial cells per section were needed for complete short tandem repeat (STR) amplification; however the sensitivity of this method has been proven to go down to the single-cell level. STR analysis was successfully performed on single blood cells and on laser-captured spleen endothelial cells.[2] The thermoplastic polymer film of the LCM caps (Arcturus CapSure® HS LCM caps, Molecular Devices, USA) containing the isolated cells was added directly to the Amp FJSTR® Profiler® STR multiplex PCR amplification kit (Applied Biosystems, Rotkreuz, Switzerland), amplifying nine different STR loci and the amelogenin locus, discriminating X and Y chromosomes. DNA amplification was performed with a multiplex STR polymerase chain reaction (PCR) and PCR fragments were separated by capillary electrophoresis on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Fragment size and peaks were analyzed using GeneScan analysis software (Applied Biosystems).

References

1. Mueller RJ, Stussi G, Odermatt B, Halter J, Schanz U, Seebach JD. Major ABO-incompatible hematopoietic stem cell transplantation: study of post-transplant pure red cell aplasia and endothelial cell chimerism. Xenotransplantation. 2006; 13(2):126-32.
2. Espina V, Milia J, Wu G, Cowherd S, Liotta LA. Laser capture microdissection. Methods Mol Biol. 2006;319:213-29.

Online Supplementary Table S1. Summary of numbers of samples and different technical determinations in the study population.

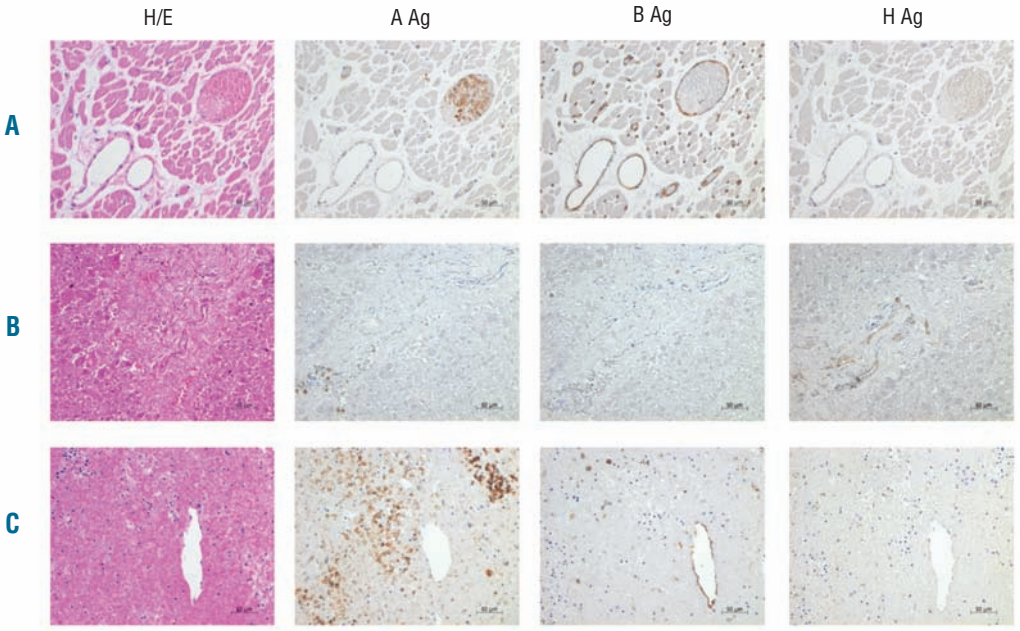
A

	N	ABOi only	ABOc only	ABOi and gender MM	ABOc and gender MM
Skin biopsies*	44	10	12	13	9
Autopsy samples†	9	6	0	3	0
Tumor samples	4	0	0	1	3
Total samples	57	16	12	17	12

B

	N	ABOi only	ABOc only	ABOi and gender MM	ABOc and gender MM
ABH-IHC only	35	14	12	3	6
Karyotype only	3	0	0	0	3
ABH-IHC and karyotype	14	0	0	11	3
ABH-IHC and STR	2	2	0	0	0
ABH-IHC, karyotype and STR	3	0	0	3	0
Total	57	16	12	17	12

*(**A**) Number and kind of material collected classified by ABO blood group incompatibility (ABOi) and compatibility (ABOc); and gender mismatch (MM). (**B**) Number of samples (skin, autopsy and tumor) analyzed by ABH immunohistochemistry (ABH-IHC), karyotype analysis by FISH and/or CISH (karyotype); and short tandem repeats (STR). *Patient #31: two skin biopsies taken at 94 and 903 days after transplantation. †Patient #44 and 45: skin autopsy samples.*



Online Supplementary Figure S1. ABH antigen (Ag) staining in autopsy-derived tissue. Representative pictures from ABH Ag-stained heart, solid bone marrow and liver sections. (A) Heart and (B) bone marrow from a 61-year-old patient (#45), who died of grade IV acute GVHD 145 days after bidirectional ABO-incompatible HSCT (A in B). (C) Liver from a 60-year-old patient (#48), who died of gastrointestinal cytomegalovirus-infection 102 days after major ABO-incompatible HSCT (A in O). Hematopoietic cells are positively stained for donor-specific A antigen, whereas recipient-specific B antigen (A and B) and H antigen (C) persisted in all endothelial cells. H/E, morphology staining with hematoxylin/eosin.