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

Regula J. Mueller,[1] Georg Stussi,[1,2] Gisella Puga Yung,[1,3] Milica Nikolic,[4] Davide Soldini,[5] Jörg Halter,[6] Sandrine Meyer-Monard,[6] Alois Gratwohl,[6] Jakob R. Passweg,[7] Bernhard Odermatt,[5] Urs Schanz,[2] Barbara C. Biedermann,[8] and Jörg D. Seebach[1,3]

[1]Laboratory for Transplantation Immunology, Department of Internal Medicine and; [2]Clinic of Hematology, University Hospital Zurich; [3]Division of Immunology and Allergology, University Hospital and Medical Faculty Geneva; [4]Institute of Pharmaceutical Sciences, Swiss Federal Institute of Technology, Zurich; [5]Institute for Clinical Pathology, Department of Pathology, University Hospital Zurich; [6]Division of Hematology, University Hospital Basel; [7]Division of Hematology, University Hospital Geneva, and [8]Department of Research, Molecular Nephrology, University Hospital Basel, Switzerland

Funding: this study was supported by the Swiss National Foundation (NFP46), by the Research Commission and Young Investigator Promotion of the University of Zurich, by the Krebsliga of the Kanton of Zurich, by UBS AG on behalf of a client, as well as by the contributions of the Hans Wilsdorf Foundation, and the late Christian Loepfe in support of transplantation research, Switzerland.

Acknowledgments: we thank the late Andreas Zisch and Solange Moll for critical reading of the manuscript; Katja Huggel for microscopic reevaluation; Claudia Ledermann and the team of Molecular Diagnostics, University Hospital Basel, for their superb help with the STR analysis; Silvia Behnke and her team for expert advice on immunohistochemical issues; Astrid Morger for excellent technical assistance and Jens Kelm for his help with microscopic analyses.

Manuscript received on July 8, 2010. Revised version arrived on September 13, 2010. Manuscript accepted on September 27, 2010.

Correspondence:
Jörg D. Seebach, Division of Immunology and Allergology, Department of Internal Medicine, University Hospital Geneva, 4 Rue G. Perret Gentil, CH-1211 Geneva 14, Switzerland.
Phone: +41.22.3729372.
Fax: +41.22.3729481.
E-mail: joerg.seebach@hcuge.ch

The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
The possibility that allogeneic hematopoietic stem cell transplantation performed across the ABO blood group-barrier is associated with an increase of graft-*versus*-host disease, in particular endothelial damage, has not been elucidated so far. For this reason, we investigated the level of endothelial cell chimerism after allogeneic hematopoietic stem cell transplantation in order to delineate the role of hematopoietic stem cells in endothelial replacement.

Design and Methods
The frequency of donor-derived endothelial cells was analyzed in 52 hematopoietic stem cell transplant recipients, in 22 normal skin biopsies, in 12 skin samples affected by graft-*versus*-host disease, various tissues from five autopsies and four secondary solid tumors by ABH immunohistochemistry, XY fluorescence *in situ* hybridization and short tandem repeat analysis of laser captured endothelial cells.

Results
Skin biopsies from two patients transplanted with minor ABO-incompatible grafts (i.e. O in A) showed 3.3% and 0.9% H antigen-positive donor-derived endothelial cells by ABH immunohistochemistry. Tumor biopsies from two recipients showed 1.2% and 2.5% donor-derived endothelial cells by combined immunohistochemistry/ fluorescence *in situ* hybridization. All other skin samples, heart, liver, bone-marrow, and tumor tissues failed to reveal donor-type endothelial cells up to several years after ABO-incompatible hematopoietic stem cell transplantation.

Conclusions
Endothelial cell replacement by bone marrow-derived donor cells after allogeneic hematopoietic stem cell transplantation is a rare event. It does not seem to represent a major mechanism of physiological *in vivo* blood vessel formation, tumor neo-angiogenesis, vascular repair after graft-*versus*-host disease episodes or acceptance of ABO-incompatible grafts.

Key words: endothelial cell, chimerism, hematopoietic stem cell transplantation, GVHD.

Citation: Mueller RJ, Stussi G, Puga Yung G, Nikolic M, Soldini D, Halter J, Meyer-Monard S, Gratwohl A, Passweg JR, Odermatt B, Schanz U, Biedermann BC, and Seebach JD. Persistence of recipient-type endothelium after allogeneic hematopoietic stem cell transplantation. Haematologica 2011;96(1):119-127. doi:10.3324/haematol.2010.030288

Introduction

Transdifferentiation of hematopoietic stem cells into non-hematopoietic tissue has received great attention in transplantation medicine as a potential mechanism of tissue repair.[1] In 1965, Medawar[2] hypothesized that long-term acceptance of solid organ transplants may be the result of replacement of their vascular endothelial cells by recipient-type cells leading to an endothelial cell chimerism. In particular, bone marrow-derived endothelial progenitor cells may replace damaged graft endothelium after rejection episodes. This hypothesis has been corroborated both by animal models and data from human transplants.[3-9] However, there are also equivocal results possibly due to the different methods used to detect chimerism, pre-existing male chimerism in female donors after giving birth to male children or receiving male blood transfusions, and finally physiological differences in the extent of chimerism in various organs and cell types.[10,11] The concept of plasticity has also been questioned by the finding that stem cells can fuse with other cells and mimic the appearance of transdifferentiation; however this has not been shown formally for endothelial cells.[12,13]

The occurrence of endothelial cell chimerism following hematopoietic stem cell transplantation (HSCT) is likewise controversial. In theory, bone marrow-derived donor-type endothelial cells may replace damaged host endothelium in tissues affected by graft-*versus*-host disease (GVHD) as well as normal endothelium during physiological cell replacement. In support of this notion donor chimerism has been described in the endothelium of skin, gut, heart and bone marrow in patients after HSCT.[14-16] Furthermore, it was recently suggested that donor-derived endothelial cells participate in endothelial repair during the effector phase of acute GVHD,[17] and donor-derived endothelial cells were detected in 25% of sex-mismatched skin biopsies following HSCT, especially in patients with acute GVHD, and at later time points.[18] Among others, ABH histo-blood group antigens expressed on endothelial cells represent a potential target for GVHD. Allogeneic HSCT is performed across the ABO-blood group barrier in about 30-50% of patients. Whether ABO-incompatibility affects the rate of GVHD, overall survival, transplant-related mortality and relapse is a matter of a longstanding debate. Recent studies showed that minor and major ABO incompatibility can be associated with a higher incidence of GVHD and may also have a negative impact on outcome.[19-21] Endothelial replacement and chimerism by donor bone marrow-derived cells might play a role in ABO-incompatibility; theoretically it could be a result of rejection and eventually favor the development of ABO tolerance.

Since previous studies often suffered from technical difficulties hampering reliable distinction between donor and recipient endothelial cells, we meticulously investigated the level of endothelial cell chimerism after allogeneic HSCT by using three different methods. After ABO-incompatible HSCT, ABH antigen expression on the vascular endothelium was analyzed by ABH immunohistochemistry.[22,23] In addition, tissues from gender-mismatched HSCT recipients, accounting for approximately 50% of patients undergoing allogeneic HSCT,[19] were analyzed by fluorescence *in situ* hybridization (FISH) for X and Y chromosomes. Finally, polymerase chain reaction-based short tandem repeat (STR) analyses were performed on selected samples using single laser-capture micro-dissected endothelial cells to identify their donor or recipient origin. Physiological endothelial cell turnover was analyzed in normal skin, autopsy tissue, tissues affected by GVHD and secondary tumor biopsies.

Design and Methods

Tissue sampling and immunohistochemistry

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 from five autopsies, and four tumor biopsies were analyzed retrospectively. The study protocol was approved by the local ethical committee of the University Hospital Zurich (EK-951).

Immunohistochemical staining of ABH antigens and endothelial cell markers was performed according to a previously published protocol[22] with monoclonal antibodies against A, B and H antigens (Dako, Carpintera CA, USA) and against CD45, CD31 (PECAM-1), CD34 and von Willebrand factor (VWF). Further details on the samples, patients' characteristics, ABH antigen staining and analysis of chimerism are provided in the *Online Supplementary Material*.

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

Tissue samples after gender-mismatched HSCT were analyzed by combined FISH and immunohistochemical staining for the endothelial cell-specific marker VWF.[24] Sections (4 μm) were stained for X and Y chromosomes. The technical details of the FISH and VWF-staining and methods of analysis are provided in the *Online Supplementary Material*.

Short tandem repeat analysis

Single endothelial cells were captured out of cryostat sections previously stained for either CD45 (negative selection) or VWF antigen (positive selection). Nuclei from 20-40 endothelial cells per section were needed for complete STR amplification. The technical details of STR analysis are provided in the *Online Supplementary Material*. To determine donor- and recipient-specific patterns of STR loci, peripheral blood mononuclear cells of the donor and the recipient were analyzed prior to transplantation.

The respective numbers of skin, autopsy and tumor biopsies that were tested with one or any combination of the three methods used to determine endothelial chimerism are summarized in *Online Supplementary Table S1*.

Results

Persistence of recipient-type short tandem repeat patterns in skin endothelial cells after hematopoietic stem cell transplantation

At the time of taking the skin biopsy, hematopoietic chimerism was determined in peripheral blood samples in 34 out of the investigated 52 patients. Hematopoietic donor-chimerism was 100% in 30 patients including the two with low levels of donor endothelial chimerism (#25, #28) shown below. Three patients had low levels [#16

Table 1. Patients' characteristics and evaluation of endothelial cell chimerism.

Patient	Age	Disease	Day*	GVHD†	ABO R/D	ABO R‡	ABO D‡	Gender R/D	Gender R§	Gender D§	STR¶
Prospective skin biopsy study											
1	32	CML	102	0/no	A/O	46/21	0/14	m/m	NA	NA	R
2	46	AML	151	I/no	A/O	257/71	0/45	f/m	46	0	ND
3	43	CML	245	II/no	A/O	84/29	0/27	f/m	ND	ND	ND
4	39	CML	615	IV/no	A/A	75/39	NA	m/f	ND	ND	ND
5	40	CML	103	0/no	A/A	79/28	NA	m/m	NA	NA	ND
6	40	CML	102	I/no	A/A	40/15	NA	m/m	NA	NA	ND
7	41	AML	381	I/ext.	A/A	91/33	NA	m/m	NA	NA	ND
8	41	AML	192	0/ext.	A/A	116/27	NA	m/m	NA	NA	ND
9	48	ALL	420	I/ext.	A/A	74/23	NA	m/f	ND	ND	ND
10	51	ALL	181	0/no	A/A	109/35	NA	m/m	NA	NA	ND
11	56	AML	264	I/no	A/A	21/8	NA	f/m	ND	ND	ND
12	34	CML	94	II	B/O	203/95	0/50	m/m	NA	NA	ND
13	45	AML	547	0/no	B/O	74/38	0/31	f/m	25	0	ND
14	52	AML	193	I/lim.	B/O	49/13	0/15	m/m	NA	NA	ND
15	41	AML	849	IV/no	O/A	28/14	0/12	m/m	NA	NA	R
16	27	MPS	100	0/no	O/A	34/16	0/18	f/m	6	0	R
17	26	CML	718	II/ext.	O/B	114/56	0/51	f/m	29	0	R
18	57	MPS	132	I/lim.	O/B	59/49	0/45	m/f	7(8)**	0	R
19	20	ALL	189	I/ext.	O/O	22/17	NA	m/m	NA	NA	ND
20	28	AML	100	I/lim.	O/O	64/24	NA	f/m	ND	ND	ND
21	52	AML	101	II/ext.	O/O	92/61	NA	f/m	ND	ND	ND
22	58	MM	51	II	O/O	2,906/781	NA	m/f	21**	0**	ND
Retrospective skin biopsy study											
23	41	CML	1915	III/no	A/B	106/29	0/25	m/f	5§	0**	ND
24	15	AML	1928	II/lim.	A/O	293/45	0/33	m/f	4(9)	0	ND
25	**28**	**ALL**	**1552**	**I/lim.**	**A/O**	**148/72**	**5/68**	**m/f**	**11****	**0****	**ND**
26	57	CLL	83	II	A/O	273/87	0/41	f/m	19**	0**	ND
27	31	CML	7	III	A/O	23/3	0/3	m/f	10(12)	0	ND
28	**48**	**MM**	**1001**	**II/lim.**	**A/O**	**432/164**	**4/82**	**m/m**	**NA**	**NA**	**ND**
29	14	CML	613	II/ext.	A/A	123/46	NA	f/m	8**	0**	ND
30	35	AML	918	II/ext.	A/A	110/42	NA	f/f	NA	NA	ND
31	63	MM	94 903	III III/lim.	A/A A/A	327/103 1,111/463	NA NA	f/f f/f	NA NA	NA NA	ND ND
32	35	ALL	1412	II/lim.	B/O	418/142	0/35	m/f	29(12)**	0**	ND
33	42	CML	195	I/no	B/B	221/58	NA	m/f	3**	0**	ND
34	53	MPS	489	0/lim.	O/A	44/26	0/22	m/m	NA	NA	ND
35	30	ALL	999	II/ext.	O/A	266/91	0/65	f/f	NA	NA	ND
36	45	CML	34	I	O/O	35/18	NA	m/m	NA	NA	ND
37	24	CML	1121	I/no	O/O	92/69	NA	f/m	ND	ND	ND
38	44	CLL	866	0/lim.	O/O	18/92	NA	m/m	NA	NA	ND
39	54	CLL	89	II	O/O	117/19	NA	m/m	NA	NA	ND
40	28	CML	3476 3476	II/ext. II/ext.	AB/A AB/A	579A/158 442B/126	0/66 0/66	m/m m/m	NA NA	NA NA	ND ND
41	38	MDS	35 35	III III	AB/B AB/B	69A/33 35B/19	0/12 0/12	m/m m/m	NA NA	NA NA	ND ND
42	59	MDS	188 188	I/lim. I/lim.	AB/B AB/B	290A/84 336B/101	0/50 0/50	m/f m/f	19(24) NA	0 NA	ND ND
43	32	MM	1548 1548	I/no I/no	AB/B AB/B	444A/106 433B/109	0/73 0/73	m/m m/m	NA NA	NA NA	ND ND
Autopsy study											
44	57	AML	96	IV	A/O	577/267[S]	0/228[S]	m/m	NA	NA	ND
45	61	MM	145 145 145	IV/no IV/no IV/no	B/A B/A B/A	1,950/665[S] 18,734/134[H] 974/78[BM]	0/461[S] 0/14[H] 0/13[BM]	m/m m/m m/m	NA NA NA	NA NA NA	ND ND ND

continued on the next page

continued from previous page

46	39	NHL	350 350	IV/no IV/no	O/A O/A	14,256/94[H] 41/18[BM]	0/13[H] 0/20[BM]	m/f m/f	26 H ** ND	0 H ** ND	ND ND
47	44	CML	115	IV/no	O/A	ND	ND	m/f	20(20) H	0 H	ND
48	60	AML	102 102	II II	O/A O/A	12,456/81[H] 246/27[L]	0/3[H] 0/25[L]	m/m m/m	NA NA	NA NA	ND ND
Tumor tissues											
49	47	MM	181	II/ext.	O/B	74/37	0/28	m/f	ND	ND	ND
50	**14**	**CML**	**12 years**	**II/no**	**A/A**	**ND**	**ND**	**f/m**	**34**	**2**	**ND**
51	**12**	**AA**	**13 years**	**0/no**	**A/A**	**ND**	**ND**	**f/m**	**31**	**4**	**ND**
52	41	CML	21 years	0/no	O/O	ND	ND	f/m	13	0	ND

**Days after HSCT. †Grade of GVHD (acute 0-IV; lim: chronic limited; ext: chronic extensive). ‡Number of ABH antigen-expressing endothelial cells shown as a ratio to the number of counted blood vessels on the same tissue section. §Counted numbers of VWF-positive endothelial cells showing either XX (f: female), XY (m: male) karyotype or only the Y chromosome (indicated in brackets). **Results confirmed by CISH. S: skin, H:heart, BM: bone marrow, L: liver. ¶Type of STR pattern found. Patients showing endothelial cell chimerism are highlighted in bold. ALL: acute lymphatic leukemia, AML: acute myelogenous leukemia, CML: chronic myelogenous leukemia, D: donor, MDS: myelodysplastic syndrome, MM: multiple myeloma, MPS: myeloproliferative syndrome, NA: not applicable, ND: not determined due to technical limitations, NHL: non-Hodgkin's-lymphoma, R: recipient.*

(3%), #26 (8%), and #44 (5%)] and one patient [#33 (77%)] had high levels of remaining recipient hematopoietic cells in the peripheral blood at the time of biopsy.

STR analysis was performed on DNA extracted from vascular endothelial cells in skin biopsies from five patients. In each case, seven or more highly polymorphic STR loci, including the amelogenin locus, were successfully analyzed. All of the captured endothelial cells contained DNA with a STR pattern matching the previously analyzed STR pattern of the recipient and no donor repeats could be detected (Table 1; Figure 1). Unfortunately, the analysis of endothelial cells in the skin biopsies of patients #25 and #28, who showed low levels of donor-type endothelial cell chimerism by ABH antigen staining, was unsuccessful because of poor DNA quality in these formalin-fixed and paraffin-embedded biopsy samples.

Persistence of recipient-type ABH antigen expression in skin endothelial cells after hematopoietic stem cell transplantation

Skin biopsies from 25 patients were evaluated for endothelial cell chimerism by immunohistochemistry for ABH antigens in the context of ABO-mismatched HSCT. Normal skin was analyzed for ten patients and GVHD-affected skin samples for 15 patients.

As expected, ABH antigens were found on both endothelial cells and erythrocytes of the dermal layers. In general, the expression pattern of ABH antigens was comparable in all types of blood vessels, with the exception of H antigen staining in biopsies from type O recipients, where capillaries showed stronger expression than arterioles and venules. On the other hand, H antigen staining was weaker than A and B antigen staining. The granular layer of the epidermis, sweat glands and hair follicles were positively stained in about 80% of the samples.

Representative examples of ABH antigen staining in skin biopsies and the average quantification of each recipient group according to the different blood groups (i.e. recipients of type A, B, O and AB) are shown in Figure 2. Staining for the pan-leukocyte marker CD45, the endothelial cell markers CD31, CD34 and VWF and additional hematoxylin/eosin (H/E) staining were performed on serial sections in order to evaluate the presence of leukocytes and the number of blood vessels in the tissue sample in parallel. A median of 45 vessels (range, 3-781) and 108 endothelial cells (range, 18-2,906) on every skin biopsy section were analyzed for expression of ABH antigens, resulting in a total of 11,282 counted endothelial cells. In 23 out of 25 patients, the skin biopsies exclusively showed recipient-derived vascular endothelial cells (Figure 2E-H). The remaining two skin biopsies, derived from recipients of minor ABO-incompatible grafts (patients #25 and #28), exhibited low levels of donor-type endothelial cells. In proportion to the total amount of evaluated endothelial cells on the sections, these two patients showed 3.3% and 0.9% of H antigen-positive cells in the endothelial lining, respectively (Table 1). Both patients suffered from limited chronic GVHD at the time of the biopsy and had a history of mild acute GVHD (grade I and II, respectively), but no histological signs of skin inflammation or skin GVHD on the biopsy.

Moreover, three out of ten patients after ABO-identical HSCT (O in O 1; A in A 2) showed, in addition to their own blood group type, aberrant B antigen (O in O and A in A) or H antigen (A in A) expression in skin biopsies.

Persistence of recipient-type X and Y chromosome karyotype in skin endothelial cells after hematopoietic stem cell transplantation

In situ hybridization for X and Y chromosomes confirmed the findings of ABH immunohistochemistry in patients with gender-mismatched HSCT (Table 1; Figure 3A). Skin biopsies were available from 22 patients after gender-mismatched HSCT. Chimerism was analyzed in 15 patients by combined immunohistochemistry for VWF/FISH and was confirmed in eight patients by chromogen *in situ* hybridization (CISH) analysis. Skin biopsies derived from seven patients could not be analyzed due to severely impaired tissue morphology. A total of 242 endothelial cells were counted with a signal of two chromosomes in their nucleus in relation to 204 endothelial cells which only displayed one X or the Y chromosome due to a cross-sectioned nucleus. None of the endothelial cells analyzed in this study had more than two sex chromosomes in the nucleus, making cell fusion as potential repair mechanism unlikely.

Recipient-type endothelial cells persisted in all skin

biopsies. In contrast, donor leukocytes were frequently found in the perivascular areas, most likely representing infiltrating donor leukocytes. The HSCT of patient #25 was gender-mismatched, and this patient showed 3.3% donor-type endothelial cell chimerism by ABH antigen staining (Table 1). However, evidence for donor-derived endothelial cells was not detected with immunohistochemistry/FISH or with CISH.

Donor-type endothelial cell chimerism at a low level in tumor neoangiogenesis after hematopoietic stem cell transplantation

Finally, tumor samples from four patients were analyzed for endothelial cell chimerism. One patient had an extramedullary relapse of multiple myeloma after ABO-incompatible HSCT (patient #49, B in O). In this case, ABH immunohistochemistry of the tumor biopsy 181 days after HSCT exclusively showed recipient-type H antigen expression on endothelial cells and none of the cells expressed donor-type B antigen. Furthermore, three patients developed secondary carcinomas 12 to 21 years after ABO-identical, gender-mismatched HSCT and were analyzed for endothelial cell chimerism by combined immunohistochemistry/FISH. Low numbers of donor-derived endothelial cells were detected in tumor vessels in two patients, 1.2% of the total amount of VWF-positive endothelial cells in a mucoepidermoid carcinoma of the parotid gland (patient #50; Figure 3B) and 2.5% in a papillary thyroid carcinoma (patient #51). In contrast, one patient with invasive ductal carcinoma of the breast had no signs of donor-derived endothelial cells (patient #52). In conclusion, the large majority of endothelial cells in the investigated tumors arose from the endogenous progenitor cell pool and not from the transplanted hematopoietic stem cells.

No evidence for donor-type endothelial cell chimerism in autopsy-derived tissues after hematopoietic stem cell transplantation

Post-mortem tissue was obtained from five patients. Three patients died from grade IV acute GVHD (patients #44, #45 and #46), one patient had general pancytopenia and subsequently died of bronchiolitis obliterans and subarachnoid hemorrhage (patient #47), and one patient died of gastrointestinal cytomegalovirus infection (patient #48). Using ABH immunohistochemistry on samples from the skin (4 patients), heart (3 patients), bone marrow (2 patients) and liver (1 patient), and additional *in situ* hybridization for X and Y chromosomes in heart tissue of two patients, endothelial cells from blood vessels in skin, heart, liver and solid bone marrow showed no evidence of donor-type endothelial cell chimerism (Table 1, *Online Supplementary Figure S1*).

Discussion

This study provides evidence that replacement of endothelial cells by bone marrow-derived donor stem cells does not represent a major repair mechanism for blood vessels after allogeneic HSCT. Overall, donor-derived cells did not engraft systematically into the recipient's endothelium after HSCT, despite the presence of donor-derived hematopoiesis. The results of endothelial cell chimerism in animal models are controversial, although recently published reports are in line with our findings,[25-28] and reports regarding humans remain scarce. So far, endothelial cell chimerism has not been analyzed after ABO-incompatible HSCT, but it has been described after gender-mismatched HSCT. Two earlier studies, which did not find donor-type endothelial cells in the stromal constituents of bone marrow up to 3 years after HSCT,[29,30] were contrasted by several reports showing endothelial cell chimerism in skin, gut, heart and bone marrow after gender-mismatched HSCT.[14-18,31,32] All these studies used XY *in situ* hybridization for the detection of donor-derived cells. Reported numbers of donor-type bone marrow-derived endothelial cells after HSCT varied from 2% in non-GVHD-affected skin and gastrointestinal tract up to 40% in the lung. In any case, the detection of endothelial cell chimerism depends crucially on the meth-

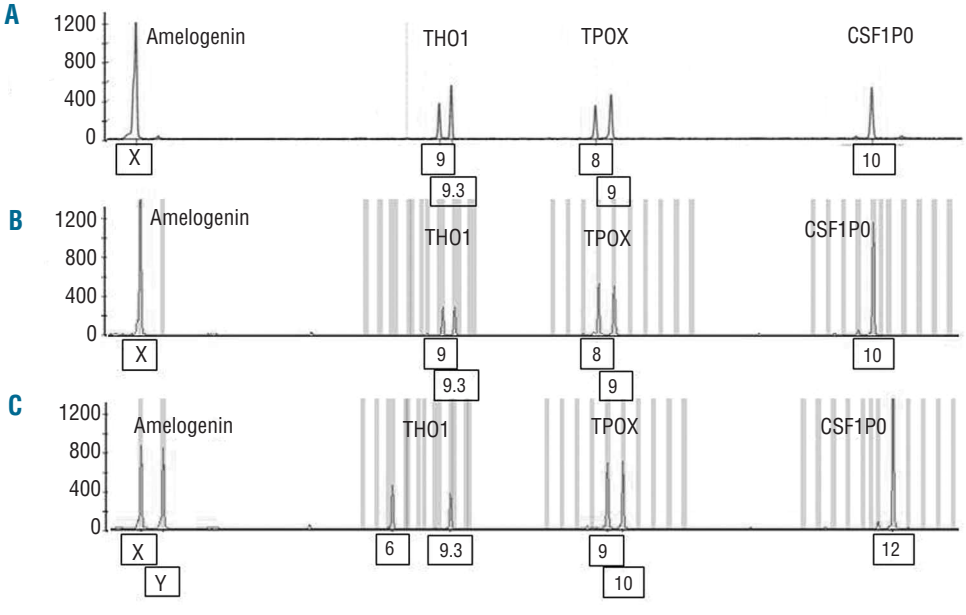

Figure 1. (A) Representative picture of the STR analysis of DNA isolated from 40 endothelial cells from a skin biopsy from a 27-year-old female (patient #16), who was treated for osteomyelofibrosis with hematopoietic stem cells from her brother. Skin biopsy 100 days post-HSCT. Four STR loci (THO1, TPOX, CSF1PO and amelogenin) are shown. (B) STR pattern of the recipient determined pre-HSCT. (C) STR pattern of the donor determined pre-HSCT.

ods applied for chimerism analysis.

X and Y chromosome analysis by *in situ* hybridization is generally performed in combination with immunohistochemical staining of a single endothelial cell marker such as CD31, CD34 or VWF, with potential pitfalls. In our experience, several immunohistochemical endothelial and hematopoietic cell markers, a careful morphological analysis and XY *in situ* hybridization are necessary to conclusively determine endothelial cell chimerism. For instance, CD34 is expressed not only on endothelial cells but also on hematopoietic precursor cells and, without leukocyte-specific CD45 staining,[33] perivascular cells dislocated into the luminal part of the vessels and extravasating or adhering leukocytes (in particular monocytes) could be misinterpreted as endothelial cells. In addition, cell morphology is often severely impaired due to the pretreatment (heat, enzyme digestion) of the tissue required for XY *in situ* hybridization.

ABH immunohistochemistry may also have several technical and biological limitations. The levels of expression of ABH antigens may vary in different tissues and vessels within the same patient, but there is also a considerable inter-patient variability.[24] In our study, samples were obtained from both normal skin and from skin suspected of having GVHD; the observed inter-patient variability may, therefore, be due to a loss of vessels and to denudation of the vessel wall depending on the grade of GVHD.[34]

In the present study we combined three different methods, i.e. immunohistochemical staining, XY *in situ* hybridization and STR analysis. The STR analysis consistently detected DNA with recipient-specific pattern in endothelial cells and DNA with a donor-specific pattern in blood cells; third-party DNA was never detected. Moreover, no cells with more than diploid sex chromosomes were detected in any tissue specimen analyzed by XY *in situ* hybridization, making cell fusion unlikely.[12,13]

Low-level endothelial cell chimerism was observed in two patients who received grafts with minor ABO-incompatibility (O in A), although this finding could not be confirmed by XY *in situ* hybridization. Alternative explanations for this apparent chimerism are invading leukocytes

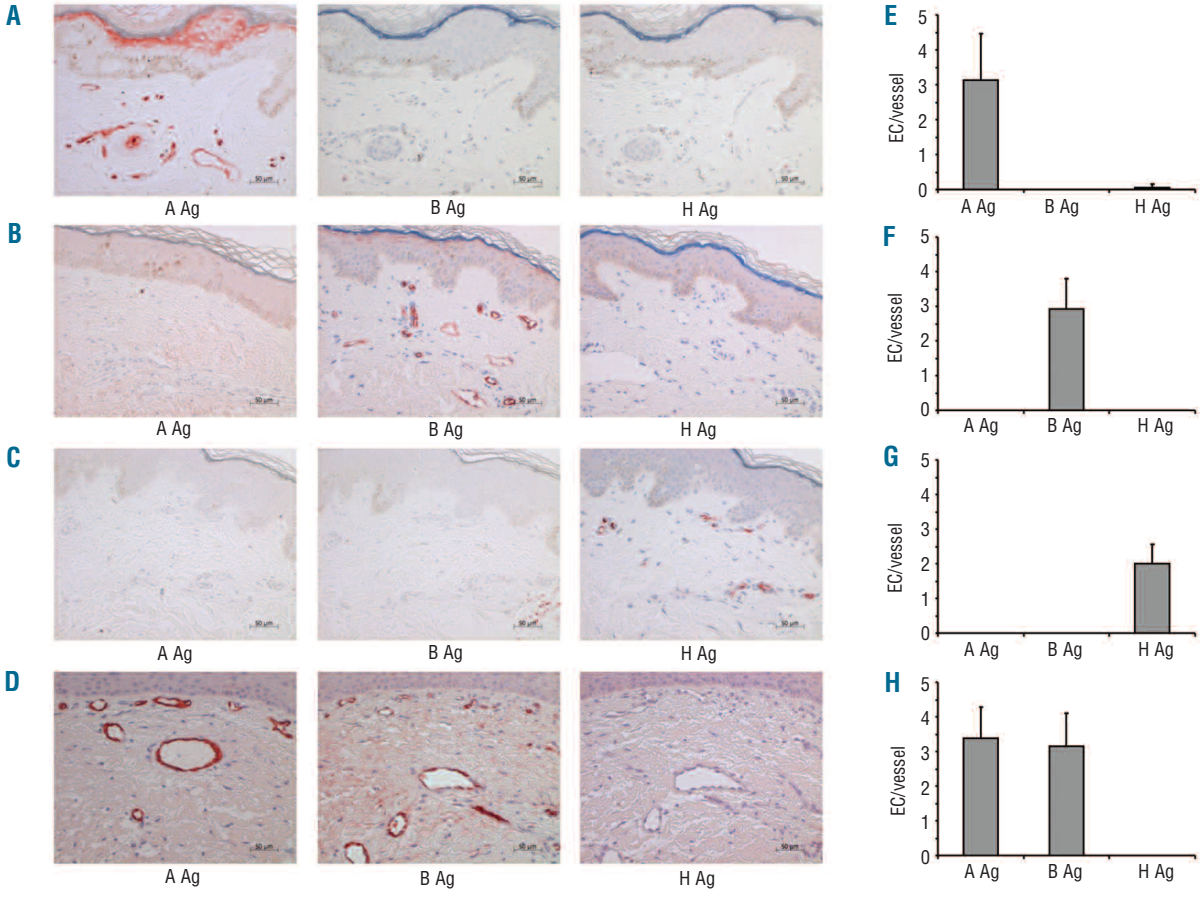

Figure 2. ABH antigen expression in skin biopsies. (A-D) Representative pictures from ABH antigen (Ag)-stained skin biopsy sections. (A) 46-year old patient (#2) 151 days after minor ABO-incompatible HSCT (O in A). (B) 34-year old patient (#12) 94 days after minor ABO-incompatible HSCT (O in B). (C) 26-year old patient (#17) 718 days after major ABO-incompatible HSCT (B in O). (D) 28-year old patient (#40) 3,476 days after minor ABO-incompatible HSCT (A in AB). (E-H) Quantification of ABH Ag-positive endothelial cells in biopsies derived from 25 patients after ABO-incompatible HSCT. The number of ABH Ag-expressing endothelial cells is shown as the ratio to the number of counted blood vessels in the same tissue section (y-axis). (E) B or O donor in A recipient, n=10. (F) A or O donor in B recipient, n=5. (G) A or B donor in O recipient, n=6. (H) A or B donor in AB recipient, n=4. Ag: antigen; EC: Endothelial cells.

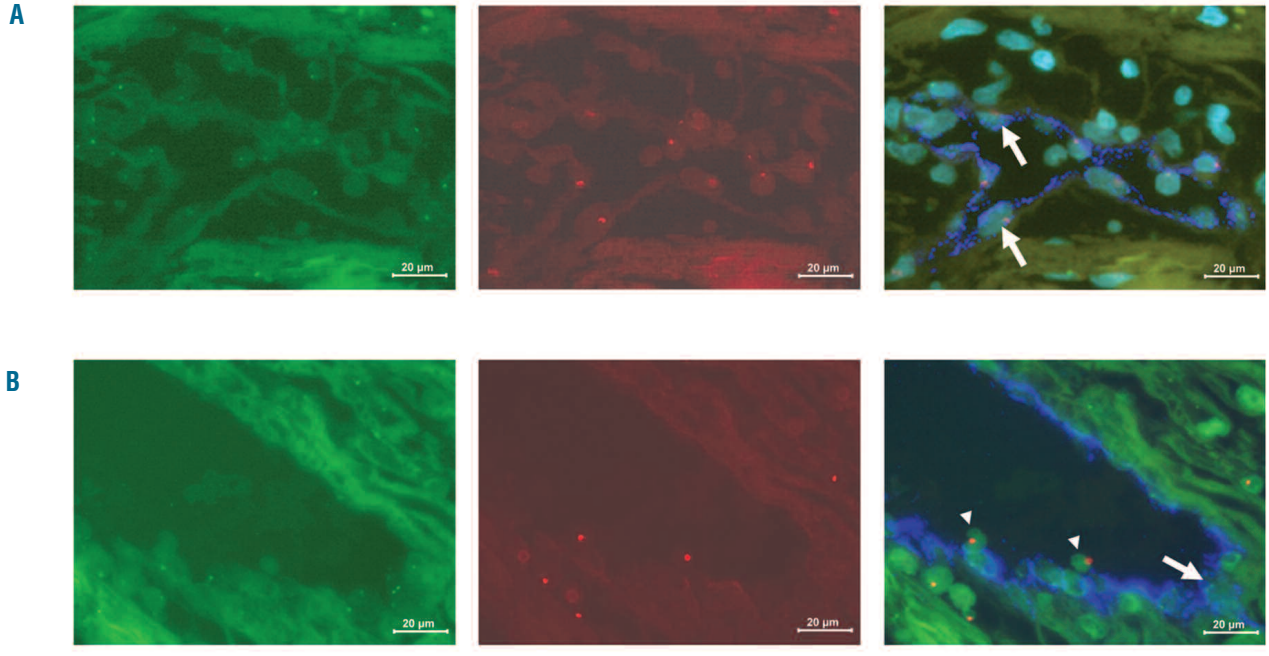

Figure 3. FISH in endothelial cells after gender-mismatched HSCT. FISH shows the X chromosome marked with CEP X Spectrum Green (green, left column) and the Y chromosome with CEP Y Spectrum Red (red, middle column). Endothelial cells are stained for VWF (blue). Overlays of the three different channels are shown in the right column. (A) Skin biopsy from a 59-year old male patient (#42), who was treated for myelodysplastic syndrome with hematopoietic stem cells from a female donor. Biopsy 188 days post-HSCT. Endothelial cells show recipient-specific XY karyotype (arrows). (B) Mucoepidermoid carcinoma of the parotid gland from a 26-year old female patient (#50) who was treated for chronic myeloid leukemia with hematopoietic stem cells from a male donor 12 years earlier. Hematopoietic cells show donor-specific XY karyotype (arrowheads), whereas endothelial cells show recipient-specific XX karyotype (arrow).

or altered ABH antigen expression caused by changes in A and B glycosyltransferases due to leukemia.[35] The amount of CD45-positive leukocytes counted in the two skin biopsies was not higher than in other samples without endothelial cell chimerism. However, H antigen expression was also seen in skin biopsies of blood group type A obtained from two patients before HSCT. In addition, aberrant ABH antigen expression was seen in three patients after ABO-identical HSCT. Patient #9 (A in A) showed A and H antigens, patient #30 (A in A) showed A and B antigens and patient #38 (O in O) showed H and B antigens. This could be explained by genetic mutations of the ABO-gene and/or aberrant ABH antigen expression, a phenomenon described previously in rejection sites after liver transplantation or in gastric and colonic tumors of blood group O individuals.[36,37]

Murata *et al.*[17] elegantly demonstrated cells of donor-specific male XY karyotype concurrently determined as CD31-positive and CD45-negative endothelial cells in GVHD-affected dermis of 13 gender-mismatched transplanted patients. Strikingly, the maximal percentage of chimeric endothelial cells was 9.5% during the effector phase of acute GVHD, but, after this phase, the proportion of chimeric cells fell to 2%. Furthermore, Willemeze *et al.*[18] recently reported even higher percentages of donor-derived endothelial cell chimerism (up to 25% in skin biopsies) related to both repair of damaged endothelium and maintenance of vascular homeostasis; in contrast, skin epithelial cell chimerism (85%) did not seem to correlate with tissue damage.

In the present study, endothelial cell chimerism was detected at lower levels (0.9% and 3.3%) in two out of 43 patients with skin biopsies (#25 and #28), and in none out of five patients examined post-mortem after HSCT. Both patients with endothelial cell chimerism suffered from limited chronic GVHD at the time of the biopsy and had a history of mild acute GVHD (grade I and grade II, respectively), but no histological signs of skin inflammation or skin GVHD on the biopsy. In general, when all 46 histological samples examined for endothelial cell chimerism by ABO were also analyzed for the presence of skin GVHD, only 12 revealed signs of acute (n=3) or chronic (n=9) GVHD. On the other hand, 45 patients of the total study population of 52 had a history of clinically determined GVHD (positive score for skin, gut and liver manifestations) as noted in Table 1. Due to these low numbers an association of donor endothelial chimerism with GVHD could not be analyzed statistically. Taken together, our results indicate that GVHD does not result in widespread endothelial cell destruction, at least not in the skin, as observed in hyperacute rejection in solid-organ transplantation, yet the exact mechanism of immunological escape after HSCT remains unexplained.[34]

Analysis of tumor tissue arising after HSCT revealed detectable amounts, i.e. 1.2% and 2.5%, of donor-derived endothelial cells incorporating into the growing vascular bed of the recipients. In line with this result, Peters *et al.* also reported that secondary tumors after HSCT can induce mobilization of bone marrow-derived stem cells to areas of neovascularization.[38] They showed that 4.9% of the analyzed human tumor endothelial cells were of bone marrow origin.[38] Similarly, mouse models showed

that during angiogenesis bone marrow-derived endothelial progenitors are recruited to the blood vessel wall but they do not form part of the endothelium.[39,40] In contrast, Lyden *et al.* showed that mouse bone marrow-derived precursor cells are necessary for tumor angiogenesis.[41] Recent observations in mouse tumor and ischemia models clarified this issue by showing transplanted donor-derived progenitor cells temporarily incorporated in the endothelium only in the early phase of neoangiogenesis, but these cells disappeared subsequently.[42]

The mechanism of involvement of bone marrow-derived stem cells in postnatal angiogenesis and vessel repair is still under debate. It has been shown in a mouse model that neighboring endothelial cells are responsible for vascular remodeling rather than bone marrow-derived cell incorporation.[43] Thus, bone marrow-derived stromal cells may play a pivotal role by producing arteriogenic cytokines leading to paracrine stimulation and proliferation of local endothelial cells,[43] suggesting that paracrine signaling might be an important mediator of cell therapy and indicating that bone marrow-derived stem cells can, in the adult, act as "cytokine factories" promoting vascular remodeling.

In conclusion, the absence of endothelial cell chimerism in the majority of patients examined after HSCT has several implications. First, it supports the concept of a relative resistance of endothelial cells to GVHD. Second, it excludes endothelial cell chimerism as a mechanism to explain tolerance after ABO-incompatible HSCT.[44] Third, it indicates that, although the existence and potential of bone marrow-derived endothelial progenitor cells have both been shown beyond any doubt, transplanted hematopoietic stem cells play a minor role in the physiological turnover of endothelial cells, vascular repair, and tumor neoangiogenesis after allogeneic HSCT.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

References

1. Rovo A, Gratwohl A. Plasticity after allogeneic hematopoietic stem cell transplantation. Biol Chem. 2008;389(7):825-36.
2. Medawar PB. Transplantation of tissues and organs: introduction. Br Med Bull. 1965;21(2):97-9.
3. Lagaaij EL, Cramer-Knijnenburg GF, van Kemenade FJ, van Es LA, Bruijn JA, van Krieken JH. Endothelial cell chimerism after renal transplantation and vascular rejection. Lancet. 2001;357(9249):33-7.
4. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, et al. Chimerism of the transplanted heart. N Engl J Med. 2002;346(1):5-15.
5. Tanaka Y, Haga H, Egawa H, Okuno T, Miyagawa-Hayashino A, Tsuruyama T, et al. Intragraft expression of recipient-type ABO blood group antigens: long-term follow-up and histological features after liver transplantation. Liver Transpl. 2005;11(5):547-54.
6. Hove WR, van Hoek B, Bajema IM, Ringers J, van Krieken JH, Lagaaij EL. Extensive chimerism in liver transplants: vascular endothelium, bile duct epithelium, and hepatocytes. Liver Transpl. 2003;9(6):552-6.
7. Fogt F, Beyser KH, Poremba C, Zimmerman RL, Khettry U, Ruschoff J. Recipient-derived hepatocytes in liver transplants: a rare event in sex-mismatched transplants. Hepatology. 2002;36(1):173-6.
8. Xu W, Baelde HJ, Lagaaij EL, De Heer E, Paul LC, Bruijn JA. Endothelial cell chimerism after renal transplantation in a rat model. Transplantation. 2002;74(9):1316-20.
9. Hillebrands JL, Klatter FA, van Dijk WD, Rozing J. Bone marrow does not contribute substantially to endothelial-cell replacement in transplant arteriosclerosis. Nat Med. 2002;8(3):194-5.
10. Bianchi DW, Zickwolf GK, Weil GJ, Sylvester S, DeMaria MA. Male fetal progenitor cells persist in maternal blood for as long as 27 years postpartum. Proc Natl Acad Sci USA. 1996;93(2):705-8.
11. Koopmans M, Kremer HI, Baelde HJ, Fernandes RJ, De Heer E, Bruijn JA, et al. Chimerism in kidneys, livers and hearts of normal women: implications for transplantation studies. Am J Transplant. 2005;5(6):1495-502.
12. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al Dhalimy M, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature. 2003;422(6934):897-901.
13. Bailey AS, Willenbring H, Jiang S, Anderson DA, Schroeder DA, Wong MH, et al. Myeloid lineage progenitors give rise to vascular endothelium. Proc Natl Acad Sci USA. 2006;103(35):13156-61.
14. Jiang S, Walker L, Afentoulis M, Anderson DA, Jauron-Mills L, Corless CL, et al. Transplanted human bone marrow contributes to vascular endothelium. Proc Natl Acad Sci USA. 2004;101(48):16891-6.
15. Kvasnicka HM, Wickenhauser C, Thiele J, Varus E, Hamm K, Beelen DW, et al. Mixed chimerism of bone marrow vessels (endothelial cells, myofibroblasts) following allogeneic transplantation for chronic myelogenous leukemia. Leuk Lymphoma. 2003;44(2):321-8.
16. Thiele J, Varus E, Wickenhauser C, Kvasnicka HM, Lorenzen J, Gramley F, et al. Mixed chimerism of cardiomyocytes and vessels after allogeneic bone marrow and stem-cell transplantation in comparison with cardiac allografts. Transplantation. 2004;77(12):1902-5.
17. Murata H, Janin A, Leboeuf C, Soulier J, Gluckman E, Meignin V, et al. Donor-derived cells and human graft-versus-host disease of the skin. Blood. 2007;109(6):2663-5.
18. Willemze AJ, Bakker AC, von dem Borne PA, Bajema IM, Vossen JM. The effect of graft-versus-host disease on skin endothelial and epithelial cell chimerism in stem-cell transplant recipients. Transplantation. 2009;87(7):1096-101.
19. Seebach JD, Stussi G, Passweg JR, Loberiza FR Jr, Gajewski JL, Keating A, et al. ABO blood group barrier in allogeneic bone marrow transplantation revisited. Biol Blood Marrow Transplant. 2005;11(12):1006-13.
20. Kimura F, Sato K, Kobayashi S, Ikeda T, Sao H, Okamoto S, et al. Impact of ABO-blood group incompatibility on the outcome of recipients of bone marrow transplants from unrelated donors in the Japan Marrow Donor Program. Haematologica. 2008;93(11):1686-93.
21. Ludajic K, Balavarca Y, Bickeboller H, Rosenmayr A, Fischer GF, Fae I, et al. Minor ABO-mismatches are risk factors for acute graft-versus-host disease in hematopoietic stem cell transplant patients. Biol Blood Marrow Transplant. 2009;15(11):1400-6.
22. 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.
23. Le Pendu J, Caillard T, Mollicone R, Couillin P, Oriol R. Expression of ABH and X (Lex) antigens in various cells. Biochimie. 1988;70(11):1613-8.
24. Page C, Rose M, Yacoub M, Pigott R. Antigenic heterogeneity of vascular endothelium. Am J Pathol. 1992;141(3):673-83.
25. Bailey AS, Jiang S, Afentoulis M, Baumann CI, Schroeder DA, Olson SB, et al. Transplanted adult hematopoietic stems cells differentiate into functional endothelial cells. Blood. 2004;103(1):13-9.
26. Ziegelhoeffer T, Fernandez B, Kostin S, Heil M, Voswinckel R, Helisch A, et al. Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ Res. 2004;94(2):230-8.

27. Tsuzuki M. Bone marrow-derived cells are not involved in reendothelialized endothelium as endothelial cells after simple endothelial denudation in mice. Basic Res Cardiol. 2009;104(5):601-11.
28. Perry TE, Song M, Despres DJ, Kim SM, San H, Yu ZX, et al. Bone marrow-derived cells do not repair endothelium in a mouse model of chronic endothelial cell dysfunction. Cardiovasc Res. 2009;84(2):317-25.
29. Athanasou NA, Quinn J, Brenner MK, Prentice HG, Graham A, Taylor S, et al. Origin of marrow stromal cells and haemopoietic chimaerism following bone marrow transplantation determined by in situ hybridisation. Br J Cancer. 1990;61(3): 385-9.
30. Simmons PJ, Przepiorka D, Thomas ED, Torok-Storb B. Host origin of marrow stromal cells following allogeneic bone marrow transplantation. Nature. 1987;328(6129): 429-32.
31. Thiele J, Varus E, Wickenhauser C, Kvasnicka HM, Metz KA, Beelen DW. Regeneration of heart muscle tissue: quantification of chimeric cardiomyocytes and endothelial cells following transplantation. Histol Histopathol. 2004;19(1):201-9.
32. Suratt BT, Cool CD, Serls AE, Chen L, Varella-Garcia M, Shpall EJ, et al. Human pulmonary chimerism after hematopoietic stem cell transplantation. Am J Respir Crit Care Med. 2003;168(3):318-22.
33. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. Blood. 1996;87(1):1-13.
34. Biedermann BC, Sahner S, Gregor M, Tsakiris DA, Jeanneret C, Pober JS, et al. Endothelial injury mediated by cytotoxic T lymphocytes and loss of microvessels in chronic graft versus host disease. Lancet. 2002;359(9323):2078-83.
35. Brody JI, Beizer LH. Alteration of blood group antigens in leukemic lymphocytes. J Clin Invest. 1965;44:1582-9.
36. Bloom S, Fleming K, Chapman R, Neuberger J, Hubscher S. Inappropriate expression of blood group antigens in hepatic allografts. Hepatology. 1994;19(4): 876-81.
37. David L, Leitao D, Sobrinho-Simoes M, Bennett EP, White T, Mandel U, et al. Biosynthetic basis of incompatible histo-blood group A antigen expression: anti-A transferase antibodies reactive with gastric cancer tissue of type O individuals. Cancer Res. 1993;53(22):5494-500.
38. Peters BA, Diaz LA, Polyak K, Meszler L, Romans K, Guinan EC, et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med. 2005;11(3):261-2.
39. Purhonen S, Palm J, Rossi D, Kaskenpaa N, Rajantie I, Yla-Herttuala S, et al. Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. Proc Natl Acad Sci USA. 2008;105(18): 6620-5.
40. Wickersheim A, Kerber M, de Miguel LS, Plate KH, Machein MR. Endothelial progenitor cells do not contribute to tumor endothelium in primary and metastatic tumors. Int J Cancer. 2009;125(8):1771-7.
41. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med. 2001; 7(11):1194-201.
42. Nolan DJ, Ciarrocchi A, Mellick AS, Jaggi JS, Bambino K, Gupta S, et al. Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization. Genes Dev. 2007;21(12): 1546-58.
43. Kinnaird T, Stabile E, Burnett MS, Lee CW, Barr S, Fuchs S, et al. Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. Circ Res. 2004;94(5):678-85.
44. Stussi G, West L, Cooper DK, Seebach JD. ABO-incompatible allotransplantation as a basis for clinical xenotransplantation. Xenotransplantation. 2006;13(5):390-9.