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Tolerance to incompatible ABO blood group antigens is not observed following homograft implantation

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Abstract

Failure to develop antibodies to nonself A and B blood group antigens is well described after infant ABO-incompatible heart transplantation and suggests that exposure to incompatible ABO antigens early in life may lead to tolerance rather than immunogenicity. If this finding is also true following ABO-incompatible cryopreserved homograft implantation, then such patients who require transplantation may be able to accept certain ABO-incompatible organs. In this study, we measured anti-A and -B antibody titers (isohemagglutinins) and allosensitization to human leukocyte antigens (HLA) in 21 patients after homograft placement (12 of whom were <1 year of age at initial homograft exposure) in childhood. We also examined homograft explant specimens for endothelial preservation and expression of HLA and A and B blood group antigens. We observed no differences in isohemagglutinins between patients who received ABO-incompatible versus ABO-compatible homografts. Allosensitization to HLA was present in 88% of patients (9 of 9 ABO-incompatible recipients and 5 of 7 ABO-compatible recipients). In 7 homograft explant specimens (median implant duration 10.1 years), the vasa vasorum endothelium was intact with ABO blood group antigen expression on 3 of 5 non-O homografts. These data suggest that tolerance to incompatible A and B blood group antigens does not occur following placement of ABO-incompatible homografts in childhood.

Keywords

Blood transfusion; Homograft; Immunology; Pediatric; Transplantation; Heart

1. Introduction

Cryopreserved, cadaveric pulmonary artery and aortic tissue (homograft) is commonly used in the surgical approach to a variety of congenital heart defects. A major drawback to the use of homografts is the strong potential for development of anti-human leukocyte antigen (HLA) antibodies (allosensitization). West and colleagues have demonstrated immunologic tolerance after infant ABO-incompatible (ABOi) heart transplantation [1], suggesting that exposure to incompatible ABO antigens early in life may lead to tolerance rather than immunogenicity. Whether homografts also induce tolerance to nonself blood group antigens is unknown. If this occurs, then patients awaiting transplantation outside of infancy may also be able to accept certain ABOi organs. We sought to explore the tolerogenic potential of homografts with regard to A and B blood group antigens by comparing the anti-A and -B blood group antibody (isohemagglutinin) titers among children who had received ABOi versus ABO-compatible (ABOc) homografts. We hypothesized that infants who received ABOi homografts would fail to develop isohemagglutinins to incompatible blood group antigens in a manner analogous to infants who receive ABOi heart transplants.

2. Subjects and methods

2.1. Study population and specimens

We recruited 21 patients at the Heart Center at Children's Hospital of Pittsburgh of UPMC who had previously undergone surgery using homograft material. Patients who were younger than 18 months old post homograft placement were not eligible. This criterion was used to exclude any possibility of transient antibodies from perioperative blood products confounding the results and to ensure that patients were of a reasonable age and time after homograft placement to allow for appropriate development of isohemagglutinins. After enrollment, each patient's medical record was reviewed to collect clinical data including blood group, underlying diagnoses, date(s) and details of prior surgery using homograft, type of homograft (aorta or pulmonary artery), homograft supplier and serial number, and blood group of the homograft donor. When the homograft blood group was not able to be determined from the medical record, we contacted the homograft supplier to obtain this information. Blood was collected upon enrollment and kept at room temperature for up to 24 hours before centrifugation. Serum was stored at -20°C until analysis for isohemagglutinins and anti-HLA antibodies.

From each patient who underwent surgery to replace a homograft conduit ($n = 7$), a piece of explanted homograft was taken from the operating room table and placed immediately into formalin. The tissue remained in formalin for up to 24 hours before paraffin embedding. One heavily calcified specimen (specimen 4) was decalcified using 25% formic acid before embedding. Slides were then prepared from the paraffin blocks and stained with hematoxylin and eosin for standard light microscopy. Immunoper-oxidase staining with primary anti-A and -B blood group antibodies (Ortho-Clinical Diagnostics, Raritan, NJ) and HLA class I (ab70328) and class II (ab55152) antibodies (Abcam, Cambridge, MA) was performed using the Ventana Benchmark XT automatic slide stainer (Ventana Medical Systems, Tucson, AZ) with either high pH (HLA) or no antigen retrieval. Similarly, preservation of the endothelium was separately confirmed by staining for CD31 using murine monoclonal (clone JC70A) antibody (Dako, Carpinteria, CA) with high pH antigen retrieval. Slides were incubated with primary antibody for 32 minutes at 37°C and then with the iView DAB detection system (Ventana) and counterstained with hematoxylin. Antibodies were diluted in Tris-bovine serum albumin-buffered solutions to the following dilutions: anti-A 1:400, anti-B 1:400, class I HLA 1:7,500, class II HLA 1:500, and CD31 1:200. For each antibody, positive results required diffuse granular membranous brown staining. Complete absence of endothelial staining was the requirement for negative cases.

Positive and negative controls were run in each batch and deemed adequate. A single pathologist (CG) who was blinded to all clinical information reviewed all specimens.

2.2. Isohemagglutinins

Screening for anti-A and anti-B antibodies was performed by standard reverse typing methods [2]. When present, immunoglobulin (Ig)-M and IgG anti-A and anti-B titers were determined using a standard saline-based, doubling-dilution technique [2]. Agglutination reactions were also quantified on a numerical scale of 0 to 12 according to the Marsh criteria [3].

2.3. Anti-HLA alloantibodies

Serum samples were batch analyzed for the presence of IgG antibodies to class I and II HLA using the Luminex technique [4]. Briefly, all samples were first tested against color-coded microbeads coated with a mixture of HLA class I and class II antigens (LABScreen mixed, One Lambda, Canoga Park, CA) and assayed using a flow analyzer (LABScan 100 flow analyzer, One Lambda). Reactive or equivocal samples were then tested with microbeads coated with single HLA antigens (LABScreen single antigen, One Lambda) to determine specificity and relative median fluorescence intensity.

2.4. Statistical analysis

Patients who received at least 1 ABOi homograft were categorized as ABOi recipients, and patients who received only ABOc homografts were categorized as ABOc recipients. Data are presented as median and range or count and frequency, as appropriate. Comparisons of Marsh scores were performed by the rank sum test, and categorical assessment of presence versus absence/inappropriately low isohemagglutinins titer(s) was performed using Fisher's exact test. Categorical assessment used normal isohemagglutinin titer ranges that accounted for age and recipient blood group [5]. Data analysis was performed using Stata 10.1 (StataCorp LP, College Station, TX) and all comparisons used a two-sided α of 0.05. All work was conducted after approval by the University of Pittsburgh Institutional Review Board and was carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

3. Results

3.1. Homografts and ABO compatibility

Thirty-three homograft exposures occurred in 21 patients (16 males and 5 females). Underlying diagnoses were hypoplastic left heart syndrome ($n = 7$), tetralogy of Fallot $\pm$ pulmonary atresia ($n = 6$), aortic stenosis status post Ross procedure ($n = 4$), common arterial trunk ($n = 3$), and d-transposition of the great vessels with doubly committed ventricular septal defect ($n = 1$). Twenty-six homografts were supplied by LifeNet Health (Virginia Beach, VA) and 6 were supplied by CryoLife (Kennesaw, GA). The supplier of 1 homograft and the blood group of 6 homograft donors were not able to be determined. Homografts were processed between 1990 and 2008 and implanted between 1993 and 2008.

Nine patients received at least 1 ABOi homograft (ABOi group) and 7 patients received only ABOc homografts (ABOc group). Five patients were unable to be categorized because of an inability to determine the blood group of at least 1 of their homografts. Twelve patients (6 ABOc and 6 ABOi) were under 1 year of age at initial homograft exposure. Table 1 presents the characteristics of the study groups and those of the patients who could not be categorized.

3.2. Isohemagglutinins

As indicated in Table 2, all patients demonstrated the presence of appropriate isohemagglutinin(s) with respect to their native ABO blood group. We determined no significant differences in median Marsh scores between the ABOi and ABOc groups: anti-A IgM, 50 (27–75) versus 50.5 (43–58), $p = 0.99$; anti-B IgM, 38 (15–63) versus 28 (13–47); $p = 0.22$; anti-A IgG, 68 (21–75) versus 48 (38–58), $p = 0.44$; anti-B IgG, 35 (3–58) versus 26.5 (8–50), $p = 0.38$. When the analysis was restricted to homografts placed before 1 year of age, there was no difference between ABOi ($n = 6$) and ABOc ($n = 6$) group median anti-A Marsh scores (IgM, 57.5 (41–75) vs 50.5 (43–58), $p = 0.64$; IgG, 68.5 (41–75) vs 48 (38–58), $p = 0.17$). The ABOi group had higher anti-B Marsh scores; however, the differences were not statistically significant (IgM, 50 (19–63) vs 26 (13–47), $p = 0.08$; IgG, 42 (13–58) vs 23 (8–50), $p = 0.18$).

Analysis of the data by titers revealed 2 males in the ABOi group with lower than expected isohemagglutinin titers; however, only 1 of these patients had low titers against the homograft blood group (patient blood group A, homograft group B at 2.3 years of age, anti-B titer 2, expected titer 8–512). The other patient had a lower than expected titer against a blood group from which he had not received an incompatible homograft (patient blood group O, homograft group B at 12.8 years of age, anti-A titer 4, expected A titer 16–256, anti-B titer 4, expected B titer 4–512).

3.3. Anti-HLA antibodies

Fourteen patients in the ABOi or ABOc groups (88%) were allosensitized to class I HLA and 12 (75%) to class II HLA by Luminex single antigen beads. By group, all 9 ABOi recipients versus 5 ABOc recipients were allosensitized to HLA (100% vs 71%; $p = 0.18$). When the analysis was restricted to homografts placed before 1 year of age, there was no significant difference in HLA allosensitization between the ABOi and ABOc groups (100% vs 67%; $p = 0.46$).

3.4. Homograft explant histology

Summary data on the 7 homograft explants are presented in Table 3. On histologic examination, the intimal and medial layers of the homograft conduit lumen appeared sclerotic with frequent foci of calcification (Fig. 1). There was no identifiable endothelium on the conduit lumen in 4 specimens and only sparse patches of luminal endothelium (CD31 positive) on 3 homografts (Fig. 2A and B). Staining for class I HLA was positive on these rare endothelial patches, as was staining for A (specimen 3) and B (specimen 4) antigens on the 2 non-O blood group specimens (Fig. 2C–E). On all homograft explants the vasa vasorum indicated prominent CD31 and class I HLA staining (Fig. 3A and B). Blood group antigen staining of the vasa vasorum was present on both blood group A homografts and on 1 of 3 blood group B homografts (Fig. 3c and D).

4. Discussion

Homografts result in allosensitization to nonself HLA antigens in neonates, children, and adults [6–8]; however, the immunologic significance of exposure to an ABOi homograft is uncertain. Whereas some groups have reported no difference in homograft longevity on the basis of ABO compatibility [9–11], others have demonstrated decreased freedom from homograft failure for ABOi homografts compared with ABOc homografts when used in the surgical treatment of congenital heart disease [12–14]. This uncertainty, combined with the knowledge that tolerance to incompatible ABO antigen(s) occurs following ABOi heart transplantation in infants [1,15], led us to undertake this study. Specifically, we wanted to

address the hypothesis that exposure early in life to incompatible A and B blood group antigens on implanted homografts might lead to tolerance to these antigens.

Our data suggest that isohemagglutinin profiles of infants and children who receive ABOi homografts are consistent with what would be expected based on patient blood group alone. In particular, there were no statistically significant differences in Marsh scores for anti-A and anti-B antibodies among the patients who received ABOi versus ABOc homografts. We also determined no significant difference in the number of patients with inappropriately low titers between the ABOi and ABOc groups. Although 2 patients in the ABOi group had inappropriately low isohemagglutinin titers, one of these patients' low titers was against a nonhomograft blood group. Furthermore, interpatient variability in isohemagglutinins has been demonstrated to predominate over age-related variability [16]. Overall, our findings do not support a hypothesis that implantation of ABOi homografts in infants and young children leads to systematic failure to produce relevant isohemagglutinins during long-term follow-up.

These findings occurred despite the persistence of blood group antigen expression on some homografts as late as 16 years after implantation. When the analysis was restricted to those who received homografts before 1 year of age, we also observed no diminishment in isohemagglutinins or prevalence of HLA allosensitization of patients who received ABOi homografts.

Our findings regarding explant histology expand what has previously been reported on this topic. Consistent with other reports [17,18], we determined these explanted cryopreserved homograft conduits to be fibrotic with minimal cellularity in the intimal and medial layers. However, we also observed rare patches of endothelium with blood group antigen expression on the conduit luminal surface in 3 of 7 homografts, as well as diffuse expression of ABO blood group antigens on the well-preserved endothelium of the vasa vasorum. To our knowledge these findings have not previously been reported.

It remains unclear why tolerance was not observed after receipt of an ABOi homograft, yet occurs after ABOi heart transplantation in infants [1,15]. The extent of allosensitization to HLA antigens that we observed is consistent with the experience of others following homograft exposure in children [6-8]. Thus, it cannot be argued that these homografts were fully devoid of immunogenicity. Differences in the quantity and/or quality of A and B antigen expression between cryopreserved homografts and cardiac allografts could contribute to different immune responses to the incompatible ABO antigens expressed on each, as could differences in vascularization of a whole organ transplant compared with a homograft. Varying expression and/or persistence of homograft donor antigens has been reported because of differences in homograft handling and processing (*e.g.*, duration of warm ischemia, antibiotic disinfection) [19]. Although our previous work demonstrated intense and uniform ABO blood group antigen expression on the vasa vasorum of cryopreserved homografts at the time of implantation [20], in the current study only 3 of 5 non-O homograft explants expressed A or B blood group antigens despite the presence of uniformly intact endothelium of the vasa vasorum. By contrast, diffuse expression of donor ABO blood group antigens has been demonstrated on endomyocardial biopsy specimens as late as 4 years after ABOi heart transplantation [1]. The importance of persistence of nonself blood group antigens for the development of tolerance is also supported by animal models [21,22] and by human data indicating that transient exposure to foreign blood group antigens does not result in tolerance to incompatible ABO antigens [1].

The main limitation of this study is the small sample size. This limits our ability to definitively conclude there are no differences in isohemagglutinins between ABOi and

ABOc homograft recipients. Nonetheless, if tolerance to incompatible blood group antigens after ABOi homograft placement occurred at a frequency similar to what has been observed following infant ABOi heart transplantation, then we could reasonably expect to have observed some evidence for it in this series. An earlier study published only in abstract form has also suggested similar findings [23]. Other difficulties encountered in performing this research that were not anticipated at the outset include obtaining “pure” groups of patients who received only ABOi or ABOc homografts in infancy as well as retrospectively obtaining the homograft donor blood group information. Often this information was not available in the medical record or even from the supplier. Data on time intervals from donor death to homograft harvest or start of cryopreservation were also not available. A prospective study in which all necessary data are maintained from the time of implantation and in which serial, postimplantation assessments of isohemagglutinins and anti-HLA antibody responses are performed in a large cohort is best suited to definitively address this topic.

In summary, we have observed no diminishment of isohemagglutinins in patients who received ABOi homografts relative to ABOc recipients, including those who received ABOi homografts under 1 year of age. Thus, tolerance to incompatible A and B blood group antigens does not appear to result in this setting. Further work is needed to understand why tolerance may not occur after ABOi homograft placement yet occurs routinely following ABOi heart transplantation in infancy.

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Fig. 1. Representative sections of homograft explant specimens on hematoxylin and eosin staining. (A) Sclerotic intima (I) and media (M) with no identifiable endothelial cell lining are observed. The adventitia (Ad) contains small vessels (arrow) of viable vasa vasorum. (B and C) Frequent foci of calcification (Ca) and rare foci ossification (Os) are noted (10x magnification).

Fig. 2. Representative sections of sparse endothelium on homograft explant conduit luminal surfaces as seen by (A) hematoxylin and eosin and (B) CD31 staining. These endothelial patches constituted an estimated 5% of the conduit luminal surface. (C) Staining for class I HLA was positive on these endothelial patches, as was staining for (D) blood group A and (E) blood group B antigen on 2 non-O blood group specimens (20x magnification).

Fig. 3. HLA and A and B blood group expression on homograft explant vasa vasorum. (A) CD31 immunostaining highlights viable vasa vasorum. (B) Class I HLA, (C) blood group A, and (D) blood group B antigen staining (20x magnification).

Table 1

Group demographics

	ABOi (<i>n</i> = 9)	ABOc (<i>n</i> = 7)	Uncategorized (<i>n</i> = 5)
Male	6 (67%)	6 (86%)	4 (80%)
White	9 (100%)	6 (86%)	5 (100%)
Blood group (A:B:O)	4:0:5	5:1:1	4:1:0
Number of homografts	1.8 ± 0.7	1.3 ± 0.5	1.6 ± 0.5
Age at first homograft implant	69 days (0 days–6.5 years)	5 days (0 days–1.8 years)	335 days (9–11.7 years)
Age at titer	14.4 years (2.4–17.2 years)	11.8 years (1.7–18.6 years)	9.9 years (8.1–24.8 years)
Time from first implant to titer	11.7 years (2.4–14.7 years)	11.8 years (1.5–16.8 years)	9.3 years (7.4–14.9 years)

Data are presented as count (%), ratio, mean ± standard deviation, and median (range).

ABOi = ABO incompatible; ABOc = ABO compatible.

Table 2

Isohemagglutinin titers after homograft placement

Group	Age at implant ^a	ABO blood group				Anti-A titer		Anti-B titer	
		Patient	HG 1	HG 2	HG 3	IgM	IgG	IgM	IgG
ABOi	0 days	O	B	A	O	256	128	128	64
ABOi	3 days	O	B	—	—	32	16	4	2
ABOi	5 days	A	A	B	—	—	—	32	16
ABOi	8 days	O	B	AI	—	32	64	8	8
ABOi	69 days	A	O	B	—	—	—	16	8
ABOi	229 days	O	B	—	—	128	128	32	16
ABOi	369 days	O	O	B	—	4	4	4	4
ABOi	2.3 years	A	B	O	—	—	—	2	1
ABOi	6.5 years	A	B	—	—	—	—	32	8
ABOc	0 days	A	O	—	—	—	—	8	2
ABOc	1 days	B	B	—	—	64	64	—	—
ABOc	3 days	A	O	—	—	—	—	16	32
ABOc	5 days	A	O	O	—	—	—	8	8
ABOc	9 days	O	O	O	—	32	16	8	8
ABOc	77 days	A	A	—	—	—	—	2	2
ABOc	1.8 years	A	A	—	—	—	—	16	8

ABOc = ABO compatible; ABOi = ABO incompatible; HG = homograft.

^a For ABOi patients, age at implant refers to age of receipt of first ABOi homograft.

Table 3

Homograft explant characteristics and histology

HG No.	HG type	Patient age at implant	Duration of implant	Blood group		Conduit lumen			Vasa vasorum						
				Homograft	Patient	A	B	CD31	HLA cI	HLA cII	A	B	CD31	HLA cI	HLA cII
1	Ao	3 days	3.0 years	B	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Diff+	Diff+	Neg
2	PA	3 days	11.8 years	O	A	Neg	Neg	Focal+	Focal+	Neg	Neg	Neg	Diff+	Diff+	Neg
3	PA	8 days	2.4 years	A1	O	Focal+	Neg	Focal+	Focal+	Neg	Diff+	Neg	Diff+	Diff+	Neg
4	Ao	191 days	9.3 years	Unknown	B	Neg	Focal+	Focal+	Focal+	Neg	Neg	Neg	Diff+	Diff+	Neg
5	PA	229 days	11.7 years	B	O	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Diff+	Diff+	Neg
6	PA	1.8 years	16.8 years	A	A	Neg	Neg	Neg	Neg	Neg	Focal+	Neg	Diff+	Diff+	Neg
7	PA	6.4 years	10.1 years	Unknown	A	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Diff+	Diff+	Focal+

Ao = aorta; diff = diffusely; HG = homograft; HLA cI = HLA class I; HLA cII = HLA class II; neg = negative; PA = pulmonary artery.