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Clinical Impact of Anti-Endothelial Cell Antibodies in Kidney and Pancreas Transplantation

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Abstract

Background Anti-endothelial cell antibodies (AECA) have been associated with graft dysfunction and rejection, but lack of scalable, cheap testing has hindered routine screening. Indirect immunofluorescence on human umbilical vein endothelial cells (HUVEC) aims to fill this niche, and a high incidence of severe multiple rejections has been reported in a large study of deceased-donor kidney transplant recipients.

Methods We retrospectively studied 119 kidney transplants and 21 pancreas transplants consecutively performed in 128 recipients at our Centre.

Results 13% showed positive pretransplant AECA. Of them, 47% showed negativization of AECA at various posttransplant times. At a median follow-up of 14 months (range : 6-24), patients with positive pretransplant AECA showed a higher risk of biopsy-proven acute cell-mediated pancreas rejection (OR = 24; P = 0.02); post-transplant persistence of AECA correlated with an even higher risk of pancreas rejection (OR = 78 ; P = 0.01). No correlation between pretransplant AECA and risk of kidney rejection or graft loss was found. De novo AECA developed in 4% of all patients, but did not correlate with any transplant-related event.

Conclusion Our study demonstrates a lack of importance of pretransplant and *de novo* AECA screening in kidney transplantation. Larger studies on pancreas transplantation will be necessary to confirm the relevance in such setting.

Keywords Kidney transplantation; Pancreas transplantation; Autoantibodies; Graft rejection; Anti-endothelial cell antibodies; AECA; Indirect immunofluorescence; Titerplane™

Abbreviations AECA: Anti-Endothelial Cell Antibodies; ANA: Anti-Nuclear Antibodies; ATG: Antithymocyte Globulin; DSA: Donor-Specific Anti-HLA Antibodies; EPC: Endothelial Progenitor Cells HUVEC: Human Umbilical Vein Endothelial Cell; IIF: Indirect Immunofluorescence

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Introduction

The role of anti-graft antibodies in graft rejection has been dominated for decades by donor-specific anti-HLA antibodies (DSA) [1,2]. Accumulating evidence supports the role of autoantibodies, and especially of anti-endothelial cell antibodies (AECA) [3], leading to antibody-mediated and cell-mediated rejection, and finally graft dysfunction [4-6]. This has to date proven especially true in kidney [4,7-9] and heart [10-14] transplantation.

Until now, AECA detection has mostly relied on flow cytometry crossmatch between donor-specific Tie-2+ circulating endothelial progenitor cells (EPC) and recipient serum (XmOne™, Absorber) [15-17]. Unfortunately, the XmOne™ assay requires living donor cells (not always available after deceased donor transplantation), high technical skills, and is very expensive.

Anyway, apart from MHC class I polypeptide-related sequence A (MIC-A) [18], AECA are mostly directed towards non-polymorphic antigens (e.g. angiotensin receptor type 1 [8,9,19], vimentin [7,12,20], endothelin-1 type A receptor [14]), so that they can be detected using non-donor-specific endothelial cells [21]. Even though some ELISA kits have become commercially available for titrating antibodies to single endothelial cell antigens (e.g. angiotensin receptor type 1 [22]), a broad-spectrum and cheap assay would be highly desirable. The newly marketed, EC- and IVD-labelled Titerplane™ kit (Euroimmun GmbH, Lubeck, Germany) employs a mix of 3 human umbilical vein endothelial cell (HUVEC) lines to detect AECA by indirect immunofluorescence (IIF). The Titerplane™ assay is much cheaper and faster than the XmOne™ assay. Both tests require discrimination of real AECA from confounding anti-HLA antibodies: while in the XmOne™ assay the donor EPC carry previously typed HLA antigens, the HUVEC lines adsorbed on the Titerplane™ slides vary from lot to lot and need to be HLA typed in order to make results interpretable. A major difference is that HUVEC, on the contrary of EPC, do not constitutively express HLA class II molecules (data on file), although HLA class II expression can occur *in vivo* in endothelial cells during inflammation. Recently, Sun et al. [23] reported a large study on 226 deceased-donor kidney recipients in China, showing that, at a median follow-up of 3 years, *de novo* AECA correlated with multiple, severe acute kidney rejections. Anyway, some authors criticized the lack of HLA typing on the HUVEC lots used, potentially leading to misclassification of HUVEC-specific anti-HLA antibodies as AECAs [5].

We report here a retrospective study on kidney and, for the first time, pancreas transplantations performed consecutively at a single center, using a single lot of HLA-typed HUVEC.

Results

The HUVEC lines used in the single Titerplane™ lot were HLA-typed as follows, showing a mix of at least 2 different donor sources: A*01,*02,*03,*31; B*07,*08,*15,*51; C*01,*02,*03,*07; DRB1*03,*11,*13,*15; DQB1*02, *03, *06. DPB1, DQA1, and DPA1 typing was not performed.

17 (13%) of patients tested AECA$^+$ pretransplant. The main baseline characteristics of pretransplant AECA$^+$ vs. AECA$^-$ patients are reported in Table 1.

	pre-transplant AECA$^+$ (n= 17)	pre-transplant AECA$^-$ (n= 111)	*P*
Age (yrs)	43.5 ± 11.4	46.4 ± 12.4	0.366
Sex (M/F)	9 / 8	78 / 33	0.171
Immune-mediated comorbidities	12 (70.5 %)	49 (44.1%)	0.07
anti-HLA antibodies against class I and/ or II (any PRA)	8 (47.1 %)	22 (19.5 %)	0.027
Donor age (yrs)	46.4 ± 13.5	50.3 ± 17.7	0.386
Previous transplants	3 (17.6 %)	11 (9.7 %)	0.398
Cold ischemia time (hours)	6.8 ± 5.6	9.3 ± 7.6	0.196
Preemptive	4 (23.5 %)	25 (22.3%)	1
Maintenance dialysis	13 (76.5 %)	83 (80.2%)	1
peritoneal dialysis	0	14 (16.9%)	0.204
hemodialysis	13 (100%)	71 (85.5%)	0.36
Time on dialysis (months)	36.4 ± 33.7	27.9 ± 23.2	0.253
Anti-thyroid antibodies	4 (23.5 %)	11 (9,7%)	0.115
Anti-nuclear antibodies	1 (5.9%)	22 (20.5%)	0.306
Living donor	8 (47%)	44 (38.9%)	0.603
CMV IgG+	15 (88.2 %)	86 (76.8%)	0.523
Maintenance immunosuppression			
Tac + MMF/MYF + Ster	16 (94.1 %)	106 (95.5 %)	0.583
CsA + MMF/MYF + Ster	0	2 (1.8 %)	1
other	1 (5.9 %) (tac+ster)	3 (2.7 %) (2 eve+MYF+ster, 1 tac+ster)	0.456
Induction therapy			
basiliximab	8 (47.1 %)	89 (80.4 %)	0.006
ATG	9 (52.9 %)	22 (19.6 %)	

Table 1. Baseline characteristics of the pre-transplant AECA$^+$ vs. AECA$^-$ patients. Comparisons between means were performed using two-tailed Student's *t* test.

No difference was found in age, donor age, years on dialysis, type of dialysis, previous transplantation, prevalence of ANA or anti-thyroid antibodies, or CMV seropositivity between the 2 groups. A higher frequency of pretransplant immune-mediated comorbidities leading to end-stage renal failure (including focal segmental glomerulosclerosis, IgA nephropathy, lupus nephritis, membranous glomerulonephritis) was found in the AECA$^+$ group (70.5 % vs. 44.1 %, P = 0.07). Similarly, pretransplant HLA antibodies were more common in the AECA$^+$ group (47.1% vs. 19.5%, P = 0.027), causing a bias towards "stronger" ATG induction therapy in this group (52.9 % vs. 19.6%, P = 0.02).

Posttransplant outcome of AECA$^+$ vs. AECA$^-$ patients is summarized in Table 2. The 2 groups had similar cold ischemia times, and no difference was found in outcome for renal transplantations. On the contrary, a statistically significant difference was found in the number of cell-mediated pancreas rejections (50% vs. 4%; OR = 24; P = 0.02), even higher when AECA persisted posttransplant (100% vs. 4%; OR = 78; P = 0.01).

	AECA$^-$ (n= 111)	AECA$^+$ (n=17)	P
de novo anti-HLA class I and/or II	34 (30.6 %)	8 (47.1 %)	0.27
Biopsy-proven acute renal rejections (Banff classification)	11 (10.2 %) out of 108 KT	1 (6.3 %) out of 16 KT	1
focal C4d+	3 (27.3 %)	0	1
diffuse C4d+	1 (9.1 %)	0	1
suspected	4 (36.4 %)	0	1
CMR I	3 (27.3 %)	1 (100%)	0.416
CMR II	3 (27.3 %)	0	1
CMR III	1 (9.1 %)	0	1
AMR I	2 (18.2 %)	0	1
AMR II	1 (9.1 %)	0	1
AMR III	0	0	1
Biopsy-proven acute pancreas rejection (Drachenberg classification)	1 (4 %) out of 25 PTx	3 (50 %) out of 6 PTx	0.02
undetermined	0	0	n.d.
CMR I	0	1 (33,3 %)	
CMR II	1 (100 %)	2 (66,7 %)	
CMR III	0	0	
AMR I	0	0	
AMR II	0	0	
AMR III	0	0	
Early (<2 weeks) acute renal rejections	1 (9.1 %)	0	1
Early (<2 weeks) acute pancreas rejections	0	0	n.d.
Multiple acute renal rejections	4 (36.4 %)	0	1
Multiple acute pancreas rejections	0	1 (33.3 %)	1
Renal graft loss	1 (0.9 %)	0	1
Pancreas graft loss	1 (4 %)	0	1
Death	0	0	n.d.
CMV reactivations	26 (23.4 %)	3 (17.6 %)	0.761
EBV reactivations	3 (2.7 %)	1 (5.9 %)	0.439
Serum creatinine drop (mg/dl)z	-0.03 ± 0.24	-0.13 ± 0.37	0.14

Table 2. Outcome of transplantation according to pretransplant AECA status. CMR = cell-mediated rejection; AMR = antibody-mediated rejection; n.d. = not determinable. Comparisons between means were performed using two-tailed Student's t test.

After transplantation, 8 of 17 AECA$^+$ patients became negative at different time points; on the contrary 4 of 111 patients (all deceased donor kidney recipients) developed *de novo* AECA. Overall, 4 groups could be identified, whose details are summarized in Table 3.

No AECA$^+$ patient had concurring positive HUVEC-specific anti-HLA antibodies.

	AECA pre$^-$ post $^-$ (n= 107)	AECA pre$^-$ post $^+$ (n=4)	AECA pre$^+$ post$^+$ (n=9)	AECA pre$^+$ post $^-$ (n=8)
de novo anti-HLA class I and/or II post-tx	33 (30.8 %)	1 (25 %)	5 (55.6 %)	3 (37.5 %)
biopsy-proven acute renal rejections (Banff classification)	11 (10.6 %) out of 104 KT	0 out of 4 KT	0 out of 8 KT	1 (12.5 %) out of 8 KT
focal C4d+	3 (27.3 %)	0	0	0
diffuse C4d+	1 (9.1 %)	0	0	0
Suspected	4 (36.4 %)	0	0	0
CMR I	3 (27.3 %)	0	0	1 (100 %)
CMR II	3 (27.3 %)	0	0	0
CMR III	1 (9.1 %)	0	0	0
AMR I	2 (18.2 %)	0	0	0
AMR II	1 (9.1 %)	0	0	0
AMR III	0	0	0	0
biopsy-proven acute pancreas rejections (Drachenberg classification)	1 (4.2 %) out of 24 PT	0 out of 1 PT	2 (100%) out of 2 PT	1 (25%) out of 4 PT
undetermined	0	0	0	0
CMR I	0	0	1 (50%)	0
CMR II	1 (100 %)	0	1 (50%)	1 (100 %)
CMR III	0	0	0	0
AMR I	0	0	0	0
AMR II	0	0	0	0
AMR III	0	0	0	0
Early (<2 weeks) acute renal rejections	1 (9.1 %)	0	0	0
Early (<2 weeks) acute pancreas rejections	0	0	0	0
Multiple acute kidney rejections	4 (36.4 %)	0	0	0
Multiple acute pancreas rejections	0	0	1 (100%)	0
Renal graft loss	1 (0.9 %)	0	0	0
Pancreas graft loss	1 (4.2 %)	0	0	0
Death	0	0	0	0
CMV reactivations	26 (24.3 %)	0	0	3 (37.5 %)
EBV reactivations	3 (2.8 %)	0	0	1 (12.5 %)
Serum creatinine drop	-0.03 ± 0.24	0.01 ± 0.06	-0.02 ± 0.15	-0.22 ± 0.48

Table 3. Outcome of transplantation according to pre- and post-transplant AECA status.

Discussion

Our study attempts to replicate on a Caucasian cohort the findings previously reported by Sun et al. [23] on Chinese patients, expanding investigation to pancreas transplantation and living donor kidney transplantation. Based on the fact that most AECA are actually autoantibodies, we investigated whether a correlation existed between AECA and other autoantibodies (anti-thyroid, ANA), biopsy-proven autoimmune cause of end-stage nephropathy or comorbidities: this hypothesis proved false, despite inclusion of suspected but not biopsy-proven autoimmune nephropathies would have led to statistical significance. Accordingly, the association between anti-HLA antibodies and AECA that we found reinforces the continuum between auto- and alloimmunity [4,14,24,25].

An original finding is the higher frequency of patients experiencing negativization of AECA than in the study by Sun et al. [23] (8 out of 17 vs. 5 out of 52). Negativization of AECA in serum is unlikely to be due to sequestration by donor endothelium, since, as stated in introduction, most antigens targeted by AECA are autoantigens. Unfortunately, at the top of our knowledge, there is no way to selectively stain for AECA in a kidney biopsy (C4d staining being highly aspecific and poorly sensitive), and anyway no patient in our series had a kidney biopsy.

In our study 24% of patients (mostly the anti-HLA antibody-positive) received anti-thymocyte globulins (ATG) as induction immunosuppression (vs. 0% in the Chinese study), and ATG use was accordingly more common in the pretransplant AECA$^+$ group (P = 0.006), but overall type of induction immunosuppression didn't affect posttransplant kinetics of AECA (data not shown). Anyway we can't exclude that the stronger induction with ATG could represent a potential bias at preventing clinical consequences due to AECA.

Cytomegalovirus infection/reactivation is associated with expansion of effector T lymphocytes causing endothelial cell damage [26,27]: despite this, we could not find any difference in IgG CMV seropositivity in AECA$^+$ vs. AECA$^-$ patients before transplantation, and CMV or EBV infections/reactivations didn't impact on development of *de novo* AECA.

As previously reported by Sun et al. [23], our study confirmed the lack of predictive power by preformed AECA on outcome of renal grafts (early, single or multiple rejection episodes, graft loss or renal function). These findings apply independently from post-transplant kinetics of AECA but contrary to what was proposed by Sun et al. [23], we couldn't find any correlation between kidney rejection episodes and de novo AECA. This finding should be interpreted cautiously due to small size of de novo AECA in our sample (4 patients vs. 22 in the study by Sun et al. [23]).

Interestingly, for the first time we report an association between preformed AECA and cell-mediated pancreas rejection (P < 0.01); unfortunately the de novo AECA group included only a single pancreas transplant recipient, and we couldn't extend this correlation.

Overall, the study by Sun et al [23] reported a far higher incidence of pretransplant AECA$^+$ recipients than in our cohort (52/226 = 23% vs. 17/128 = 13%), and a higher frequency of *de novo* AECA (22/174 = 12.6% vs. 4/111 = 3%). We speculate that their observation was mostly related to higher percentage of deceased donors in the Chinese cohort (100% vs. 76/128 = 41% in our cohort), leading to significantly longer cold ischemia times (17 vs. 8 hrs. in our cohort), higher necrosis and finally sensitization to endothelial antigens An alternative explanation relies on HUVEC-specific anti-HLA antibodies as potential confounders: contrary to Sun et al [23], we retrospectively tested sera using a single lot of HUVEC line that we extensively HLA-typed in order to rule out false positives. Although in our case series this effort proved useless (no AECA$^+$ patient had HUVEC-specific anti-HLA antibodies), such control should always be applied to correctly interpret test results, and retesting on a different Titerplane™ lot is advised in case of anti-HUVEC HLA-specific antibodies.

Lack of DPA1, DPB1, and DQA1 typing of HUVEC lot didn't affect results since no patients in our series had antibodies against these loci.

Overall, the number of patients enrolled in this study was relatively small and the average follow-up was shorter (14 months compared to 36 months of Sun et al [23]). Nevertheless some results, especially the ones concerning pancreas transplantation, deserve further investigation.

Materials and methods

Patients

We initially considered eligible 168 kidney and/or pancreas transplants consecutively performed at the Pisa Kidney-Pancreas Transplant Centre between October 2009 and June 2012. As reported in Figure 1, 40 patients were excluded because of pretransplant desensitization (because of ABO blood group incompatibility or positive crossmatch), lack of paired pre- and posttransplant sera, follow-up shorter than 6 months, or use of plasmapheresis for treatment of underlying nephropathy. The main characteristics of the remaining 128 patients enrolled in the study were as follows: median age 46 years; M/F ratio 87/41; 41% (n= 52) had living donors; 27.3% (n = 35) had received a previous transplant; 45.8% (n= 59) of kidney transplantations were preemptive, while the remaining patients had been on maintenance dialysis for a median of 29 months. Of the 128 patients, 45 (35.2 %) received a single (n = 30) or double (n = 15) kidney transplantation, 52 (40.6 %) a living donor kidney transplantation, 21 (16.4 %) a simultaneous pancreas and kidney transplantation, 5 (3.9 %) a pancreas after kidney transplant, 4 (3.1 %) a pancreas transplant alone, 1 (0.8 %) a simultaneous deceased donor pancreas and living donor kidney transplant. Serum sampling was performed immediately before the transplant, and then around month 1, 3, 6, 12, and 24.

Induction immunosuppression consisted of antithymocyte globulin (ATG) 0.8-1 mg/kg body weight for up to 10 doses or basiliximab 20 mg on day 0 and +4. Maintenance immunosuppression consisted of tacrolimus (Tac) (targeting of 7-10 ng/ml), mycophenolate mofetil or sodium mycophenolate (MMF/MYF) (up to 2 g/day or 1.4 mg/day, respectively) and prednisone (Ster) (tapered to 5 mg/day); cyclosporine or everolimus replaced tacrolimus in some patients. Clinical data were collected until last visit or graft loss/death. Renal and pancreatic biopsies were performed upon clinical suspicion of rejection. Acute rejection episodes were treated according to histology with 3 i.v. boluses of 500 mg/day methylprednisolone each or ATG 0.5-1 mg/kg up to 14 days. Patients treated with plasmapheresis of i.v. immunoglobulins were excluded from the study in order to avoid confounders on AECA determination.

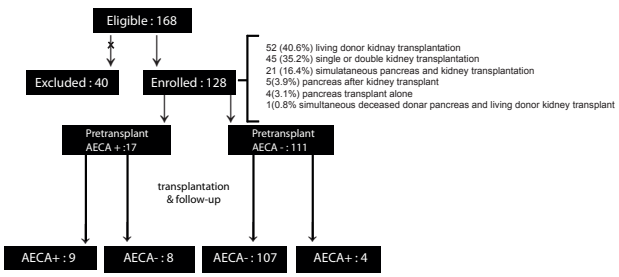

Figure 1. Details of study population

Laboratory Tests

Anti-nuclear antibodies (ANA), anti-thyroid antibodies, and CMV IgG were detected separately at the central hospital laboratory using routine methods. Anti-HLA antibodies were tested using Luminex™ LabScreen Mixed and, for positive ones, Single-Antigen assays (One Lambda, Canoga Park, CO), with a cut-off mean fluorescence intensity (MFI) of 500. The cutoff was chosen as low as possible (as recommended by manufacturers) in order to exclude false positives in AECA testing due to interfering donor-specific anti-HLA antibodies.

AECAs (and, as a side finding, ANAs) were detected by HUVEC Titerplane™ (Euroimmun Medizinische Labordiagnostika AG) technique according to manufacturer instructions. Briefly, serum samples were first diluted 1:100 in PBS-Tween, then 30 µl of diluted serum were applied to each reaction field of the reagent tray; reactions were started by fitting the BIOCHIP slides, containing fixed HUVECs, into the corresponding recesses of the reagent tray. The tray was then incubated for 30 minutes in the dark at room temperature. At the end of the incubation, the BIOCHIP slides were rinsed with PBS-Tween and immersed in a cuvette containing PBS-Tween for 5 minutes. Fluorescein-labeled anti-human immunoglobulin was applied (25 µl of conjugate) into each reaction field of a clean reaction tray. BIOCHIP slides were removed from PBS-Tween and put into the recesses of the reagent tray. They were incubated for 30 minutes and washed as previously. The slides were removed and the fluorescence was read under a microscope (Figure 2) . A positive AECA result was manifested by a granular fluorescence in the cytoplasm of the cell culture. A positive ANA result, a side finding of AECA testing, was manifested by a granular or homogeneous fluorescence within the nucleus. DAPI (4',6-diamidino-2-phenylindole) was used as nuclear counterstain.

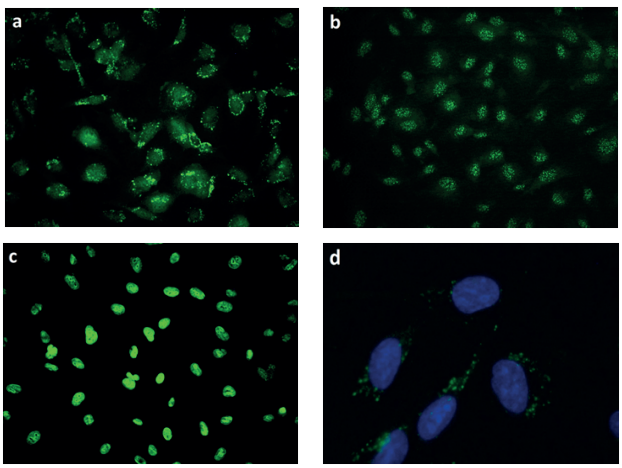

Figure 2. Microscope views of Titerplane™ tests showing (a) positive staining of "true" AECA (granular fluorescence in the cytoplasm of HUVEC); (b) positive granular or (c) homogeneous nuclear fluorescence staining marks ANA, a side finding of AECA testing; (d) positive staining of AECA (granular fluorescence in the cytoplasm) using DAPI (4',6-diamidino-2-phenylindole) as nuclear counterstain at larger magnification (blue nucleus).

3 µg of extracted DNA from the HUVEC lines used in the working Titerplane™ lot (lot F120112DF) was kindly provided by Euroimmun GmbH. Such DNA was HLA-typed by SSOP as previously reported [28].

Statistical Analysis

Comparisons between means were performed using two-tailed Student's *t* test; Fisher's exact test was instead used when comparing dichotomous variables. *P* values < 0.05 were considered statistically significant. Whenever a significant *P* was found, odds ratio were calculated. All calculations were run using MedCalc software v.13.3.3 (MedCalc Software bvba, Ostend, Belgium).

Conflict of Interest

We declare that we have no conflict of interest relevant to this publication.

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