Pretransplant Detection of Anti-Endothelial Cell Antibodies Could Predict Renal Allograft Outcome

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Abstract

Objectives: **Endothelial cells that line the vasculature are targets for immune-mediated assault through anti-endothelial cell antibodies. The aim of this work was to detect anti-endothelial cell antibodies and describe the association with kidney allograft rejection and graft survival.**

Materials and Methods: **The study included 60 patients who had undergone live-donor kidney transplant. Inclusion criteria included first kidney transplant, panel reactive antibody titer less than 5%, cause of end-stage renal disease not including vasculitis or systemic lupus erythematosus, and age > 18 years. Patients were classified into 2 groups: 40 patients with anti-endothelial cell antibodies (referred to as the *positive group*) and 20 patients without anti-endothelial cell antibodies (referred to as the *negative group*).**

Results: **Serum creatinine level was higher in the positive group at 1 month and 1 year (*P* = .04). The occurrence of acute rejection was not significantly different in the positive group (18 patients [45.0%]) compared with the negative group (5 patients [25.0%], *P* = .5). However, the number of acute rejection episodes was higher in the positive group (22 episodes) compared with the negative group (6 episodes, *P* = .04). In patients who experienced acute rejection, chronic nephropathy was more frequent in the positive group (6 of 18 patients, 33.3%) compared with the negative group (1 of 5 patients, 20.0%) (*P* = .03). One-year and 5-year graft survival was 91% and 79% in the positive group, and 100% and 91% in the negative group, respectively. The difference at 5 years was significant (*P* = .04).**

Conclusions: **The presence of anti-endothelial cell antibodies was associated with a higher number of acute rejection episodes and lower long-term graft survival in kidney transplants. It could be an informative test to identify patients at high risk for immunological graft loss.**

Key words: *Kidney transplant, Outcome, Renal allograft, Acute rejection, Chronic rejection.*

Introduction

Endothelial cells lining the vasculature are targets for immune-mediated assault through anti-endothelial cell antibodies. These have been detected in such an impressive diversity of conditions associated with widespread vasculitis that they certainly represent a heterogeneous family of autoantibodies (1). The presence of anti-endothelial cell antibodies in many diseases does not necessarily imply causation, since their production may follow rather than precede endothelial cell damage (2). However, some evidence suggests that they contribute to the pathophysiology of these diseases, although this has not been demonstrated definitively (3, 4).

Several reports have shown an association between rejection and the detection of anti-endothelial cell antibodies in renal transplant recipients (5-8). In 1995, al-Hussein and colleagues showed that specific antigens on endothelial monocytes were the targets of circulating antibodies in the sera of patients with graft failure, and they assumed that the antibodies had direct specificity to class 2 antigens (5). In addition, the presence of antibodies to major histocompatibility complex was strongly associated with rejection (5). In other

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reports, poor graft function has been associated with the presence of antibodies in the pretransplant and posttransplant sera to non-major histocompatibility complex targets, such as endothelial and/or monocyte antigens (6), kidney cell genes (7), and cultured cells or hybridomas of endothelial cell lines such as EA.hy or A549 cell lines (8). Therefore, vascular endothelial antigens of the allograft kidney represent the primary target for injury in most forms of rejection (9). Immunohistopathological studies of hyperacute, accelerated, acute, and chronic rejection showed endothelial damage that appeared to be mediated by humoral and/or cellular immune processes (10).

Interest in the possible role of endothelial-specific antibodies has grown steadily over the years. Such antibodies escape detection in conventional crossmatch tests (11). The aim of this work was to detect anti-endothelial cell antibodies and describe the correlation with kidney allograft rejection and graft survival.

Materials and Methods

Sixty patients with end-stage renal disease who had undergone a live-donor kidney transplant at the Urology & Nephrology Center, Mansoura University, Egypt between December 1998 and September 2001 formed the basis for this study. Inclusion criteria included first kidney transplant, panel reactive antibody titer less than 5%, cause of end-stage renal disease not including vasculitis or systemic lupus erythematosus, and age > 18 years. Details of the demographic characteristics are outlined in Table 1. Written consent for participation in the study was obtained from all patients. The study was undertaken in accordance with the Declaration of Helsinki (1964) and all subsequent amendments, and was approved by the local ethics committees.

Pretransplant data for the patients and the donor were collected and are shown in Table 1. The patients were prepared for renal transplant by performing HLA typing for class 1 and 2 antigens and crossmatch tests. Tissue typing for class 1 and 2 antigens was performed using the lymphocytotoxicity test. Lymphocytes were isolated from peripheral whole blood by density gradient centrifugation on. Class 1 antigen typing was performed using the complement-dependent lymphocytotoxicity test. Class 2 antigen typing was performed using B cells isolated by immunomagnetic beads.

In preparation for detecting anti-endothelial cell antibodies, the plates were prepared using the EA.hy 926 cell line. The cell line was cultured and maintained in Dulbecco's modified Eagle's medium with 1% glutamine, 1% penicillin streptomycin, fetal calf serum, HAT media, and trypsin (Sigma, St. Louis, MO, USA). The culture was maintained for 1 week at 37°C in a 5% CO_2 incubator. Trypsinization of the cells was performed using GIBCO trypsin-EDTA (Invitrogen, Carlsbad, CA, USA). The cell count was adjusted (80 000 – 90 000 cells/mL), and 200 μL of cells was placed in the 60 central wells of a 96-well flat bottom plate. The plates were incubated for at least 48 hours and used within 1 week.

To detect anti-endothelial cell antibodies, the culture medium was eliminated, 100 μL of 0.1% glutaraldehyde (Sigma) was dispensed into each well, followed by incubation for 10 minutes at 4°C. The culture medium was discarded, and the plates were washed 4 times with 1% phosphate-buffered saline with bovine serum. The nonspecific binding sites were saturated for 3 hours or overnight at 37°C with 200 μL of 3% phosphate-buffered saline with bovine serum albumin. The test serum was diluted to 1/800 using 0.5% phosphate-buffered saline with bovine serum albumin, and 100 μL of the diluted serum was added in triplicate. The plates were incubated for 1 hour at 37°C and washed 4 times; Then, a solution of 100 μL of diluted Ig AGM Kappa lambda peroxidase (1/5000) (Sigma) was added. The plates were incubated for 1 hour at 37°C and washed 4 times. Finally, 100 μL of substrate was added. The plates were kept in the dark for 5 minutes. The reaction was blocked with 50 μL H_2SO_4 for 3 minutes. Reading was performed at 492 nm against blank.

According to the presence or absence of anti-endothelial cell antibodies, patients were classified into 2 groups. The first group consisted of 40 patients with anti-endothelial cell antibodies (referred to as the *positive group*), and the second group was composed of 20 patients without anti-endothelial cell antibodies (referred to as the *negative group*). As shown in Table 1, 22 patients in the positive group and 12 patients in the negative group received induction therapy. Initial immunosuppression was provided using 500 mg methylprednisolone on the day of transplant, and oral prednisolone for 2 days at a dosage of 3.5 mg/kg per day and for 5 days at a dosage of 1.5 mg/kg per day.

Table 1. Demographic and clinical characteristics of both groups.

	Positive group (n=40)	Negative group (n=20)	*P* Value
Recipient age, y	29.8 ± 5.6	27.9 ± 5.1	.3
Recipient sex			
Men	33 (82.5%)	15 (75.0%)	.5
Women	7 (17.5%)	5 (25.0%)	
Donor age, y	34.9 ± 9.9	35.6 ± 9.4	.5
Donor sex			
Men	17 (42.5%)	8 (40.0%)	.5
Women	23 (57.5%)	12 (60.0%)	
Causes of ESRD			.3
Membranous nephropathy	2 (5.0%)	0	
Mesangiocapillary glomerulonephritis	1 (2.5%)	1 (5.0%)	
Chronic interstitial nephritis	5 (12.5%)	4 (20.0%)	
Reflux nephropathy	1 (2.5%)	2 (10.0%)	
Polycystic kidney disease	3 (7.5%)	2 (10.0%)	
Hypertensive nephrosclerosis	4 (10.0%)	3 (15.0%)	
Congenitally small kidneys	1 (2.5%)	0	
Unknown	23	8	
Donor source			
Related	16 (40.0%)	19 (95.0%)	.2
Unrelated	24 (60.0%)	1 (5.0%)	
Blood group			
Same	33 (82.5%)	13 (65.0%)	.4
Different but compatible	7 (17.5%)	7 (35.0%)	
Pretransplant blood transfusion	25 (62.5%)	8 (40.0%)	.1
< 5 transfusions	20 (50.0%)	6 (30.0%)	.2
≥ 5 transfusions	5 (12.5%)	2 (10.0%)	
Donor-specific transfusion	25 (62.5%)	8 (40.0%)	.5
HLA 0-4 mismatches			.07
0	7 (16.7%)	2(10%)0	
2	0	XX14 (70%)	
3	26 (66.6%)	4(20%)0	
4	7 (16.7%)	0	
Ischemia time, minutes	50.9 ± 17.X	48.2 ± 18.X	.4
Induction therapy	22 (55.0%)	12 (60.0%)	.6
Basiliximab	12 (54.6%)	8 (66.7%)	
Dacliximab	10 (45.4%)	4 (33.3%)	
ATN	2 (5.0%)	1 (5.0%)	.8

Data are presented as mean ± standard deviation or number (percentage).
The positive group represents patients with anti-endothelial cell antibodies; the negative group represents patients without anti-endothelial cell antibodies.
Abbreviations: ATN, acute tubular necrosis; ESRD, end-stage renal disease.

Maintenance immunosuppression consisted of oral prednisolone, tapered until it reached 0.15 mg/kg per day in the ninth month and continued thereafter. Cyclosporine was given 2 days before transplant at a dosage of 6 mg/kg per day in 2 divided doses; the dosage was adjusted using monoclonal antibodies (TDx, Abbott Diagnostics, Abbott Park, IL, USA) to keep the whole blood trough level between 200 and 300 ng/mL during the first 2 months and between 100 and 150 ng/mL thereafter. Mycophenolate mofetil 500 mg twice daily was started on the second day after transplant, increased to 1000 mg twice daily 1 week after transplant, and then titrated according to the leukocyte count and occurrence of adverse effects. Acute rejection episodes were treated with 500 mg methylprednisolone pulses daily for 5 days and with antithymocyte globulin in cases of steroid-resistant rejection.

Follow-up data were obtained for patients regarding rejection episodes and graft survival. Rejection was described by its occurrence, number, time after transplant, severity of biopsy-proven acute rejection episodes, and histopathological diagnosis of chronic allograft nephropathy. Graft survival was calculated at 1 year and 5 years using actuarial Kaplan-Meier survival curves.

Continuous data were reported with mean and standard deviation (SD); ordinal data, with number and percentage. Statistical analyses were carried out using the statistical software, SPSS (Statistical Product and Services Solutions, version 10.0, SPSS Inc, Chicago, IL, USA). For univariate analysis, *t* test and chi-square tests were used. Graft survival was calculated using Kaplan-Meier survival curves, and differences were compared using the log-rank test. The value of $P < .05$ was considered statistically significant.

Results

The study included 48 men and 12 women with mean age of 28.8 ± 11.1 years.

Demographic data for patients and their donors were comparable in both groups, as shown in Table 1. Serum creatinine levels were higher in the positive group at 1 month and at 1 year ($P = .04$) but were not significantly different at other times (3 months, 6 months, 5 years). Mean serum creatinine at 1 year was 1.6 ± 1.0 mg/dL in the positive group and 1.3 ± 0.3 mg/d in the negative group ($P = .04$).

In the positive group, acute rejection episodes were seen in 18 of 40 patients (45.0%), with 15 patients (83.3%) experiencing 1 rejection episode and the remaining 3 patients (16.7%) experiencing more than 1 episode; 2 episodes were steroid-resistant. In the negative group, acute rejection occurred in 6 of 20 patients (30.0%), with 5 patients (83.3%) experiencing 1 rejection episode; no acute rejection episodes were steroid-resistant. The overall occurrence of acute cell-mediated rejection was not significantly different in the 2 groups ($P = .5$). However, the number of acute rejection episodes was higher in the positive group (22 episodes in 18 patients) compared with the negative group (6 episodes in 5 patients) ($P = .04$). Although there was a trend to more severe episodes of acute rejection in the positive group as graded by the Banff classification, this did not reach statistical significance ($P = .07$), as shown in Table 2. Mean time to the first acute rejection episode after transplant was 57.3 ± 33.9 days in the positive group versus 39.8 ± 31.7 days in the negative group, with no statistically significant difference ($P = .2$).

In the patients who experienced acute rejection, chronic nephropathy was more frequent in the positive group (6 of 18 patients, 33.3%) compared with the negative group (1 of 5 patients, 20.0%) ($P = .03$). One-year and 5-year graft survival was 91% and 79% in the positive group, and 100% and 91% in the negative group. These differences were not significant at 1 year ($P = .07$), but were significant at 5 years ($P = .04$). Graft loss due to immunological causes (acute and chronic rejection) was more frequent in the positive group (4 patients) compared with the negative group (1 patient) ($P = .04$) (Table 2). Clinical grading of the recipients at the last follow-up is illustrated in Table 2. Patients who had functioning grafts were classified as to graft function estimated by serum creatinine; 5 patients lost their grafts due to immunological causes (chronic rejection or frequent acute rejection). In 1 patient in the positive group, death occurred despite a functioning graft. There was no difference in clinical graft function between the 2 groups ($P = .8$).

Table 2. Posttransplant follow-up in both groups.

	Positive group (n=40)	Negative group (n=20)	*P* Value
Acute rejection	18 (45.0%)	5 (25.0%)	.5
No. of acute rejection episodes	22	6	.04
1	15 (83.3%)	4 (80.0%)	
2	2 (11.1%)	1 (20.0%)	
≥ 3	1 (5.6%)	0	
Severity of acute rejection			
Cell-mediated	21 (95.4%)	5 (83.3%)	.5
Antibody-mediated	1 (4.6%)	1 (16.7%)	
Resistant to steroid treatment	2 (9.1%)	0	.09
Histopathological grading of graft biopsy			
Borderline changes	10 (45.5%)	4 (66.7%)	.07
Grade 1 (mild)	7 (31.8%)	1 (16.65%)	
Grade 2 (moderate)	4 (18.2%)	0	
Grade 3 (humoral)	1 (4.5%)	1 (16.65%)	
Chronic allograft nephropathy	6 (33.3%)	1 (20.0%)	.03
Clinical grading (at last follow-up)			.8
Creatinine ≤ 1.5 mg/dL	22 (55.0%)	14 (70.0%)	
Creatinine > 1.5 to < 3 mg/dL	12 (30.0%)	5 (25.0%)	
Creatinine ≥ 3 mg/dL	1 (2.5%)	0	
Immunological graft failure (CR, AR)	4 (10.0%)	1 (5.0%)	
Death with functioning graft	1 (2.5%)	0	

Data are presented as number (percentage).
The positive group represents patients with anti-endothelial cell antibodies; the negative group represents patients without anti-endothelial cell antibodies.
Abbreviations: AR, acute rejection; CR, chronic rejection.

Discussion

Evidence is accumulating that non-HLA antigens are involved in allograft survival, even if extensive matching of the donor and recipient tissue are performed before kidney transplant (11). Serological and immunohistochemical studies have indicated that alloantibodies eluted from rejected kidneys recognize organ-specific antigens from human kidney and components of microvascular endothelial and tubular epithelial cells (12-14). The present study demonstrated that recipients with positive anti-endothelial cell antibodies had higher levels of serum creatinine at 1 month and 1 year after transplant, and this affects graft failure and survival at 5 years. These

results are in accordance with the findings of Mizutani and colleagues (14) who found that 92% of patients with graft failure had anti-endothelial cell antibodies, as opposed to 70% of patients with functioning grafts ($P < .001$). The results of other studies differed from our results, as Le Bas-Bernardet and colleagues (4) noted nonsignificantly higher levels of serum creatinine at 10 years at different periods, and Yard and colleagues (15) showed that anti-endothelial cell antibodies did not correlate with serum creatinine levels 1 year after transplant. One reason for this difference might be the longer observation period in our study.

In the present study, patients with multiple episodes of rejection showed significantly higher levels of anti-endothelial cell antibodies than those with 1 episode of rejection. These results were in accordance with the results of Shin and colleagues (16) who found that serum anti-endothelial cell antibody IgG titers increased significantly in recipients with acute rejection (6.9 ± 3.1 versus 13.5 ± 9.9 U/mL, $P < .01$), but decreased to 5.6 ± 3.0 U/mL ($P < .01$) after formal rejection therapy. At the same time, our results regarding acute rejection showed some disagreement with Paul and colleagues (10) who found a highly significant correlation ($P < .001$) between irreversible rejection and donor-specific endothelial antibodies, and with Nakagawa and colleagues (9) who reported significant differences in the histological findings of biopsies between groups with and without anti-endothelial cell antibodies. Although our results showed a trend toward more severe histopathological findings in recipients with anti-endothelial cell antibodies, this did not reach statistical significance. While Kooijmans-Coutinho and colleagues (17) found that anti-endothelial cell antibodies were associated with vascular rejection in all patients studied, we did not find this association. This may be related to the lower occurrence of antibody-mediated rejection in live-donor kidney transplants. Moreover, an association between anti-endothelial cell antibodies and hyperacute rejection was reported after kidney transplant from identical HLA donors (18). These results strongly suggest that antibodies that are kidney-specific, particularly microvascular endothelial cell-specific, play a pivotal role in acute allograft rejection.

Contrary to La Bas-Bernardet and colleagues (4) who found no significant difference in kidney allograft survival at different periods after transplant ($P = .9$) related to anti-endothelial cell antibodies, we demonstrated that the presence of anti-endothelial cell antibodies in renal transplant recipients is associated with renal transplant failure. Graft survival after 5 years was significantly better in the negative group versus the positive group. These results are in agreement with the results of Yard and colleagues (15) who found that allospecific anti-endothelial cell antibodies were associated with the occurrence of vascular rejection and graft loss after renal transplant, and Kooijmans-Coutinho and colleagues (17) who found an association between the presence of anti-endothelial cell antibodies and graft loss in 75% of their patients.

One limitation of this study was that the levels of panel reactive antibody, donor-specific antibodies, crossmatches, and anti-endothelial cell antibodies were not measured after; in particular, antibodies were not measured at the time of rejection episodes. It would have been of particular interest in patients with biopsy-proven acute rejection to examine the possible correlation between the presence or increase of anti-endothelial cell antibodies and graft rejection or loss. Also, we were unable to exclude the presence of weak anti-HLA antibodies or antibodies that arose after transplant as a cause of the observed differences in outcomes between groups.

In conclusion, we wish to stress the clinical significance of anti-endothelial cell antibodies in renal allograft recipients, because of the significant association between the presence of anti-endothelial cell antibodies and more frequent graft rejection and lower long-term graft survival in patients after living-donor kidney transplant. However, our data should be interpreted cautiously owing to the small number of patients analyzed and the relatively short follow-up. Nevertheless, detection of anti-endothelial cell antibodies before transplant could be performed prospectively to identify patients with higher risk of acute rejection and immunological graft loss after renal transplant.

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