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Pre-transplant serum concentrations of anti-endothelial cell antibody in panel reactive antibody negative renal recipients and its impact on acute rejection

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Abstract

Background: Endothelial cell antigens are important targets in acute rejection (AR). Our goal was to measure the serum concentrations of pre-transplant anti-endothelial cell antibody (AECA) in panel reactive antibody (PRA) negative recipients and its impact on AR within 6 months following renal transplantation.
Methods: We retrospectively examined pre-transplant sera from 392 patients using cellular enzyme linked immunosorbent assay (ELISA) with substrate from a permanent endothelial cell line *EAhy926*. Equal volumes of serum from 40 healthy volunteers were mixed and used as the negative control.
Results: The positive rate of AECA was 15.8%. There were no significant differences with respect to age, gender, original disease, dialysis history, immune suppressive regimen, cytomegalovirus (CMV) antigen positive rate, complement dependent cytotoxicity (CDC) level and soluble CD30 (sCD30) levels between the AECA positive group and AECA negative group. AR rate in the AECA positive group was higher than that in the AECA negative group (35.5% vs. 22.4%, $p=0.023$). The AECA positive patients had significantly higher rates of acute grade II T-cell mediated rejection (TMR) and acute antibody mediated rejection (AMR) compared with AECA negative patients. The concentrations of sCD30, and AECA were independent risk factors for AR within 6 months; the odds ratios were 7.005 and 2.469, respectively.
Conclusions: Positive AECA was an independent risk factor for AR and appeared to correlate with relatively severe rejection subtypes.
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Keywords: acute rejection (AR); antibody; endothelial cell; kidney; transplantation.

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Introduction

The vascular endothelium of transplanted organs is the first line barrier between the allograft and the host. It represents an important target for allograft-directed immune responses (1). By expressing both class I and class II human leukocyte antigens (HLA), activated graft endothelium may become a target for anti-HLA antibodies leading to acute or chronic allograft rejection (2–4). Although it is clear that HLA antigens expressed on activated graft endothelial cells can become targets of the immune system, the role of non-HLA antigens such as endothelial cell antigens in renal transplantation have been proposed (5).

Anti-endothelial cell antibody (AECA) has been found in a variety of autoimmune diseases, including Wegener's granulomatosis, rheumatoid vasculitis, and scleroderma, with possible pathogenic contributions (6–8). It represents a heterogeneous group of antibodies directed against a variety of antigenic determinants on endothelial cells.

Following transplantation, the presence of antibodies to non-HLA antigens has been reported to be a target for transplant rejection (9, 10). Opelz reported that an analysis of the clinical outcomes of 4084 HLA-identical sibling transplantations, 10 years graft survival was 72.4% for patients without anti-HLA antibodies compared with 63.3% for patients with 1%–50% panel reactive antibodies (PRA), and 55.5% for patients with PRA >50%. This may demonstrate a role for non-HLA directed immune response (11). Lucchiari et al. studied nine cases of irreversible vascular rejection occurring in the absence of detectable anti-HLA antibodies. In eight of nine eluates from kidneys with acute vascular rejection, they found the presence of antibodies reacting with human umbilical endothelial cells, but not with HLA antigens (12). In a series of 80 consecutive patients undergoing cardiac transplantation, Fredrich et al. reported significant correlation between the occurrence of humoral rejection and the presence of AECA, as detected in a cell-based enzyme linked immunosorbent assay (ELISA) using human umbilical vein endothelial cells (HUVECs) (13).

In this study, we retrospectively analyzed pre-transplant serum AECA concentrations using an endothelial cell-based ELISA in 392 cases of PRA negative patients. Our goal was to determine the role of AECA concentrations and its impact on the episodes of acute rejection (AR) within 6 months following renal allograft transplantation.

Materials and methods

Patients' population

The study protocol conformed to the provisions of the Declaration of Helsinki and informed consents were obtained from all the patients and volunteers. We collected blood samples prior to transplantation from 392 renal allograft recipients (272 males and 120 females) between December 1998 and August 2003. Patients undergoing a second transplantation or with positive PRA were excluded. The average age was 39.9 years (11–70 years). PRA was measured with the lambda antigen tray (LAT) quantitative method by ELISA (One Lambda, Canoga Park, CA, USA). A value of >10% was defined as positive. The control group included 40 healthy volunteers (25 males and 15 females). Their sera was AECA negative as tested by indirect immunofluorescence using HUVEC as substrate (Euroimmun, Lübeck, Germany). Equal volumes of serum from these 40 healthy volunteers were mixed and used as negative control. All sera was stored at –80°C until tested.

Patient demographics including age, gender, original disease, history of dialysis, cytomegalovirus (CMV) antigen positive rate, the levels of complement dependent cytotoxicity (CDC), HLA mismatch and pre-transplant serum soluble CD30 (sCD30), and immune suppressive regimen and AR episodes within 6 months post-transplantation were recorded. HLA typing was measured in A, B and DR loci using polymerase chain reaction. The sCD30 concentration was measured and classified according to previous work performed in our laboratory (14). High sCD30 concentrations were classified when measured values were >170 U/mL while values less than this were considered to be low. Episodes of AR episodes were diagnosed by the clinical manifestations or allograft biopsy and classified into acute T-cell mediated rejection (TMR) and acute antibody mediated rejection (AMR) according to Banff 05 criteria (15). AMR criteria were defined as positive peritubular capillary (PTC) C4d staining by immunohistochemistry concomitant with polymorphonuclear leukocyte infiltration in PTC and vasculitis or glomerulitis. Clinical rejection was diagnosed as acute renal allograft dysfunction with no evidence of obstruction, blood supply compromise or drug toxicity, without biopsy and recovery following intensive immunosuppressive therapy.

Cell culture and cellular ELISA

A cellular ELISA kit, prepared in our laboratory, was used for detection of AECA. A permanent HUVEC line *EAhy926* (16), provided by Professor Edgell (University of North Carolina, USA), was used as the substrate in the ELISA. The culture medium consisted of a high glucose Dulbecco minimum essential medium (DMEM) medium (GIBCO BRL, Gainthersburg, MD, USA) with 10% fetal bovine serum (FBS), 10 mmol/L glutamine. The cells were passaged at subconfluence and dissociated with 0.25% typsin. After reaching confluence following the 5th passage, cells were collected and seeded into the wells (5×10^3 cells per well) of 96-well cell culture plate (Costar, NY, USA). All the plates were prepared simultaneously using the same passage of *EAhy926* cells proliferated from one original cell.

The cellular ELISA kits were prepared as follows: after seeding, the 96-well cell culture plates were incubated at 37°C in 5% CO_2 for 48 h until confluence, washed three times with PBS-0.05% Tween, fixed at 4°C for 10 min with PBS-0.1% glutaraldehyde and blocked at 37°C for 2 h with PBS-2% bovine serum albumin (BSA). After allowing to dry, the plates were stored at –20°C. Cellular ELISA was performed as follows: after washing twice with PBS-0.05% Tween, patient sera (in duplicate) were added to wells at a dilution of 1:50, incubated at 37°C for 2 h. For each plate, there were four wells for negative control serum and four wells for the blank control (no serum added). Following two washes with PBS-0.05% Tween, horseradish peroxidase-conjugated mouse antihuman immunoglobulin (Dako, Glostrup, Denmark) was added to each well and incubated at 37°C for an hour. After washing three times and adding tetramethyl benzidine, the plates were measured at 490 nm with an ELISA reader (Bio-Rad, Hercules, CA, USA). The absorbance value (A) was calculated as the average value of the wells for the duplicate determinations for each sample. The AECA level was calculated as P/N value. $P/N=(A_{\text{patient}}-A_{\text{blank control}})/(A_{\text{negative control}}-A_{\text{blank control}})$.

Statistical analysis

Numerical results were reported as the mean±standard deviation (SD). Differences between groups were compared using the χ^2-test or one way analysis of variance. Multivariate analysis with stepwise logistic regression was applied to determine the risk of AR. These analyses were performed with SPSS 13.0 software (SPSS Inc, Chicago, IL, USA). A $p<0.05$ was considered to be statistically significant.

Results

AECA levels and clinical characteristics

The P/N value of pre-transplant sera was 1.32±0.34, while the P/N value of sera from healthy controls (negative control) was 1.02±0.29. A P/N value >1.60 (1.96 SD over the average value of negative control) was considered to be AECA positive. There were 62 AECA positive patients out of all 392 patients (positive rate 15.8%).

Clinical data for the AECA positive group and AECA negative group are shown in Table 1. There were no significant differences with respect to age, gender, original disease, dialysis history, immune suppressive regimen, CMV antigen positive rate, CDC and sCD30 concentrations between the AECA positive group and the AECA negative group. The HLA-mismatch level was higher in the AECA positive group compared with the AECA negative group (5.4±0.7 vs. 4.6±1.0, $p=0.043$).

Risk factors for AR

Ninety-six of 392 recipients developed AR within 6 months following transplantation. The AR rate in the AECA positive group was higher than the AECA negative group (35.5% vs. 22.4%, $p=0.023$), as shown in Table 2. With respect to AR subtypes, the AECA positive group had significant higher rates of grade II TMR and AMR than patients in the AECA negative group (see Table 2). There was no significant difference in the rate of clinical rejection between the two groups.

The analysis of risk factors for AR (Table 3) indicated that the positive rates of AECA and CMV antigen, and sCD30 concentrations in the AR group were sig-

Table 1 Clinical characteristics in the AECA negative and the AECA positive recipients.

	AECA negative (330 cases)	AECA positive (62 cases)	p-Value
P/N value	1.22±0.26	1.84±0.25	
Age, years	39.7±11.4	40.8±11.4	0.474
Gender, male:female	228:102	44:18	0.448
Original disease			0.611
Chronic glomerulonephritis	298	57	
Polycystic renal disease	9	4	
Hypertensive nephropathy	3	0	
Diabetic nephropathy	2	0	
Lupus nephritis	5	0	
Gouty nephropathy	5	0	
Obstructive nephropathy	2	0	
Others	6	1	
Dialysis			0.133
Undialyzed	46	6	
HD≤12 months	248	46	
HD>12 months	18	8	
PD≤12 months	18	2	
Immune suppressive regimen			0.403
Pred+Aza+CsA	26	2	
Pred+MMF+CsA	233	42	
Pred+MMF+FK506	46	14	
FK506 replaces CsA	9	1	
MMF replaces Aza	13	3	
Others	3	0	
CMV antigen			0.096
Negative	105	19	
Positive	115	33	
CDC level	4.1±1.9	3.8±1.6	0.248
HLA mismatch	4.6±1.0	5.4±0.7	0.043
sCD30 level, U/mL	140.21±63.62	139.46±52.31	0.930

AECA, anti-endothelial cell antibody; HD, hemodialysis; PD, peritoneal dialysis; Pred, predinisolone; Aza, azathioprine; CsA, cyclosporin A; MMF, mycophenolate mofetil; FK506, tacrolimus; CMV, cytomegalovirus; CDC, complement dependent cytotoxicity; HLA, human leukocyte antigens; sCD30, soluble CD30.

Table 2 Rates and types of acute renal allograft rejection in relation to the pre-transplant AECA concentration.

	AECA negative (330 cases)	AECA positive (62 cases)	χ^2	p-Value
Acute rejection	74 (22.4%)	22 (35.5%)	4.814	0.023
Acute T-cell mediated rejection	39 (11.8%)	13 (21.0%)	4.520	0.032
Grade I	29 (8.8%)	7 (11.3%)	0.929	0.231
Grade II	10 (3.0%)	6 (9.7%)	6.948	0.019
Acute antibody mediated rejection	12 (3.6%)	6 (9.7%)	5.331	0.033
Clinical rejection	23 (7.0%)	3 (4.8%)	0.081	0.532

AECA, anti-endothelial cell antibody.

Table 3 Analysis of risk factors of acute rejection.

	No acute rejection (296 cases)	Acute rejection (96 cases)	p-Value (χ^2)
Age, years	40.5±11.7	37.9±10.1	0.046
Gender, male:female	206:90	66:30	0.485
AECA positive rate	13.5% (40/296)	22.9% (22/96)	0.023 (4.814)
CMV antigen positive rate	50.0% (96/192)	65.0% (52/80)	0.016 (5.122)
CDC level	4.0±1.8	4.3±2.0	0.201
HLA mismatch	4.8±1.0	4.5±0.8	0.343
sCD30 level, U/mL	129.29±55.75	173.39±68.14	<0.001

AECA, anti-endothelial cell antibody; CMV, cytomegalovirus; CDC, complement dependent cytotoxicity; HLA, human leukocyte antigens; sCD30, soluble CD30.

Table 4 Effects of other factors on acute rejection episodes as estimated by stepwise logistic regression analysis.

Variable	Odds ratio	p-Value	95% CI
AECA[a]	2.469	0.010	1.236–4.932
sCD30[b]	7.005	<0.001	3.819–12.849

[a]AECA was considered as positive or negative; [b]sCD30 enter into the equation with low or high level.

nificantly higher than those without AR. The average age of patients in the AR group was lower than in those without AR. The variables included in the multivariate analysis with stepwise logistic regression were age, gender, AECA, CMV antigen, the degree of HLA-mismatch, and CDC and sCD30. This confirmed that sCD30 concentrations, and AECA positivity were independent risk factors for AR within 6 months post-transplant, with an odds ratio of 7.005 and 2.469, respectively (Table 4).

Discussion

Mounting evidence suggests that antibodies targeting vascular endothelial cell are involved in allograft rejection (9, 10, 12, 13). However, the vascular endothelial cell antigen system is a minor histocompatibility system, genetically linked to HLA antigens (1). Some researchers have claimed that the number of AECA positive sera tested by cellular ELISA decreased dramatically when the sera were first incubated with platelets to absorb anti-HLA antibodies (17). In the present study, we only inspected pre-transplant, PRA negative recipients to avoid cross reaction with anti-HLA antibodies. We found the positive rate of AECA in this population to be 15.8%. The AR rate in the AECA positive group was 35.5% higher compared with the AECA negative group. The positive AECA level was confirmed to be an independent risk factor for AR, with a 95% confidence interval (CI) of 1.236–4.932. It appeared to correlate with relatively severe rejection subtypes, such as grade II TMR and AMR. Researchers at another Chinese center also reported three renal allograft recipients who developed C4d-positive AR with detectable circulating AECA (18). These patients had severe dialysis-dependent graft dysfunction and two patients lost their grafts following rescue therapy; one had an AECA titer increase from 1:10 to 1:80, while the other remained AECA positive during treatment. However, in the patient that recovered, the AECA titer decreased to become undetectable from an initial titer of 1:40. They also analyzed circulating AECA concentrations in 653 renal recipients and found that circulating AECA was positive in 13 of 47 cases with acute vascular rejection. These were mostly resistant to steroid treatment and were associated with a significantly lower 1-year graft survival rate. More patients with AECA positive acute vascular rejection experienced one or more episodes of AR during the 1 year of follow-up (19).

AECA concentrations may rise secondary to pre-existing endothelial injury or viral infection. Toyoda et al. claimed that CMV infection was associated with increased humoral immune response to endothelial cell antigens (20), and it could induce polyclonal AECA which recognize endothelial cell antigens and antigens on other cells (21). In our study, the CMV antigen positive rate in AECA positive patients (63%) was higher compared with AECA negative patients (52%), but this was not statistically different ($p=0.096$). This may be due to the exclusion of PRA positive patients and sample size.

It is now clear that vascular endothelial cells are heterogeneous, include macro- and microvasculature, exhibit tissue-specific differences and different pathogenic significance (22). There are differences in antigen composition and reactions to stimuli between the endothelium from large and small vessels, and between endothelial cells derived from various microvascular endothelial beds (23). The *EAhy926* cell line was derived by fusing HUVECs with a permanent human epithelial cell line *A549* (16). In a retrospective assay, a cell-based ELISA using *A549* cell line did not show any relationship between the presence of antibodies against *A549* and renal allograft rejection or transplant failure (24). Endothelial cell antigens may explain the positive relationship between positive AECA concentrations and AR episodes in this study. However, in a study using cellular ELISA with target cells of HUVEC, human glomerular endothelial cells (HGEC) or microvascular endothelial cells (MvEs) from 22 renal transplant recipients, only the AECA concentration measured with the HGEC based ELISA showed significant correlation with AR episodes within 3 months following transplantation (25). These results appear contradictory with ours and may be caused by the different numbers of patients preparation of the ELISA. Although cellular ELISA is convenient for testing multiple samples, results are affected by antigen loss between cell generations and uneven distributions in cell proliferation. In this study, we used the same fifth passage of *EAhy926* cells proliferated from one original cell to make all of our ELISA plates at the same time, in order to avoid differences between generations. Before testing, we use negative control serum and five patients sera samples to evaluate the intra-plate deviation (each serum tested randomly in eight wells in one plate). The average deviation was 4.89%. During testing, we also used the negative control and blank control to evaluate deviation between different plates. The average deviation was 8.87%. With these results, we concluded that this cellular ELISA using *EAhy926* was a reliable method to measure AECA concentrations. However, further experimental validation is required at other centers.

In conclusion, antibodies against endothelial cells may exist in pre-transplant PRA negative sera. A positive AECA level was found to be one of the risk factors for AR within 6 months following transplantation and appeared to correlate with relatively severe rejection subtypes.

Conflict of interest statement

The authors declare that there are no conflicts of interest to report.

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