

Anti-Angiotensin Type 1 Receptor Antibodies Associated With Antibody Mediated Rejection in Donor HLA Antibody Negative Patients

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Background. Angiotensin type 1 receptor (AT₁R) mediates most physiologic and pathophysiologic actions of its endogenous ligand, angiotensin II, with overactivity leading to vascular remodeling and hypertension. Antibodies to AT₁R are implicated in several vascular pathologies. The aim of our study was to determine the impact of antibody to AT₁R on clinical outcomes including antibody mediated rejection (AMR), with or without C4d deposition, in patients whose sera contained no donor human leukocyte antigen (HLA)-specific antibody (HLA-DSA).

Methods. Pretransplant sera from 97 recipients and sera obtained at the time of acute rejection (AR) were tested by Luminex-based single-antigen bead assays to determine HLA-DSA and antibodies to major histocompatibility class I chain-related gene A (MICA). The presence of antibody to AT₁R was determined by a cell-based ELISA method using a cutoff of 17 units to distinguish high from low binding.

Results. Sera from 63 recipients were determined to have no HLA-DSA and no donor-specific MICA antibodies pretransplant and at the time of AR, and 16 of these recipients were diagnosed with AR including 7 with AMR and 9 with cellular AR (cell-mediated rejection). High-binding AT₁R antibodies were identified for six of seven in the AMR+ group and zero of nine in the cell-mediated rejection+ group ($P=0.0009$).

Conclusions. A strong association was observed between the presence of high binding to AT₁R and AMR in recipients whose sera contained no antibody to donor HLA or MICA. Assessing the AT₁R antibody status along with the HLA-DSA provides additional information to determine the immunologic risk for recipients.

Keywords: Antiangiotensin II type 1 receptor antibodies, Antibody-mediated rejection, Donor HLA-specific antibodies, Kidney transplantation.

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Antibody responses to non-human leukocyte antigens (HLA) have been reported in solid organ transplantation and may occur as alloantibodies or autoantibodies. The antigens detected include major histocompatibility class I chain-related gene A (MICA) and major histocompatibility class I chain-related gene B (1–3), glutathione S-transferase T1 (4, 5), angiotensin II type 1 receptor (AT₁R) (6–8), endothelial

cell antigens (9, 10), vimentin (11, 12), and non-HLA IgM antibodies (13). Our study focuses on the antibody response to the AT₁R (6–8) in kidney transplant recipients.

The human gene for angiotensin type 1 receptor (AT₁R) is located on chromosome 3 and encodes a G protein-coupled receptor with seven transmembrane domains. Antibodies to this receptor recognize a structure formed by the second extracellular loop. AT₁R is responsible for most of the physiologic cardiovascular effects mediated by angiotensin II including regulation of arterial blood pressure and water-salt balance (14). Autoantibodies to AT₁R have been initially associated with hypertension in preeclampsia and malignant

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hypertension (15, 16). Overactivity of angiotensin II/AT₁R complex has also been reported to be associated with cardiac, renal, and vascular remodeling leading to mortality and morbidity from cardiovascular conditions (17). Binding of agonistic antibodies to AT₁R causes activation of the phosphatidylinositol-calcium second messenger system, phosphorylation of extracellular signal-regulated kinase 1/2, Activator Protein-1 activation, and increases DNA-binding activity of nuclear factor- κ B (7, 18). The single copy of human AT₁R contains four exons. Alternative splicing of exons 1, 2, and 3 onto exon 4 yields four main transcripts with markedly different rates of translation, indicating that mRNA processing may play an important role in determining the level of AT₁R expression. The most extensively studied A1166C polymorphism is associated with increased responsiveness to angiotensin II and various cardiovascular and renal pathologies (19). A hypothetical consequence is that A1166C polymorphism of the donor, which is associated with increased AT₁R expression in vascular cells, could potentiate AT₁R antibody actions because of increased antibody target interactions. Antibodies detected in transplant patients recognize two neighboring epitopes located at the extracellular loop of the receptor. There is no described polymorphism yet, which would affect expression of these epitopes. Patients can develop antibodies to AT₁R polymorphisms through the usual routes of allosensitization including pregnancy, blood transfusion, and transplantation (8).

Dragun et al. (7) have reported the presence of antibodies against AT₁R in 16 renal allograft recipients who had severe vascular rejection and malignant hypertension but no donor HLA-specific antibodies (HLA-DSAs). The studies were prompted by the observation of one patient who received a zero HLA-A, -B, -DR-mismatched allograft and developed accelerated vascular rejection refractory to steroids and antilymphocyte antibody preparation (7). This index patient had a history of preeclampsia. Other patients in the study had severe vascular pathologic condition including hypertensive crisis accompanied by seizures in some of them and lack of response to steroids or antilymphocyte preparations. A larger study of sera from 154 renal transplant recipients showed the incidence of early antibody-mediated rejection (AMR) because of non-HLA-DSA to be 2.3% or lower (8). Six patients who had both HLA-DSA and AT₁R antibodies had more accelerated graft loss than patients with HLA-DSA alone. Scornik et al. (20) reported that posttransplant antibodies to AT₁R did not correlate to C4d-positive rejection.

The presence of C4d in vessels of the grafts serves as a good marker for AMR diagnosis (21) although it is not entirely sensitive, and there is accumulating evidence for C4d-negative AMR (22, 23). Loupy et al. (23) reported that 48% of 45 patients with HLA-DSA at transplant had an incomplete form of subclinical AMR with glomerulitis and peritubular capillaritis but no C4d deposition on 3-month protocol biopsies, whereas 31% had subclinical AMR with C4d deposition on biopsies at the same time point. Both groups showed decreased graft function at 1 year posttransplant. AMR associated with HLA-DSA is typically manifested margination of neutrophils and monocyte or macrophages in glomerular and peritubular capillaries (PTCs) with associated endothelial injury. Non-HLA antibodies may also initiate the cascade leading to vascular endothelial activation, damage, and apo-

ptosis (6). Although antibodies to AT₁R have been shown to be the IgG1 and IgG3 subclasses, which fix complement, Dragun et al. (7) reported C4d was detected in only 5 of 16 patients with such antibodies and renal allograft rejection. These results suggest the pathogenesis of rejection in the presence of anti-AT₁R antibodies may be distinct from that observed when HLA-DSA is present.

The aim of our study was to determine the impact of antibody to AT₁R on clinical outcomes including AMR, with or without C4d deposition, in patients whose pretransplant sera contained no donor-specific antibody (DSA) to HLA or MICA.

RESULTS

Sera from patients with a clinical history of AMR, cell-mediated rejection (CMR), or both not attributable to HLA-DSA, and pretransplant sera from patients with no HLA-specific antibodies transplanted between November 2006 and October 2009 were included in this study. Pretransplant sera from 97 kidney recipients were tested by the cell-based ELISA method for the levels of antibodies to AT₁R (24) as detailed in the *Materials and Methods*. In addition, DSA to HLA (including HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQB, -DQA, and -DPB) and MICA were also determined. High levels of anti-AT₁R antibody (>17 units) were observed for 33% (32/97) of these pretransplant sera (Table 1, cause of end-stage renal disease). No significant difference in the cause of end-stage renal disease was observed between patients with high versus low levels of anti-AT₁R. The percentage of patients with any HLA-specific antibody detected by solid-phase assays was 72% vs. 58% for the high and low anti-AT₁R groups, respectively ($P=0.35$; Table 1, pretransplant HLA-specific antibody status).

No DSA to HLA or MICA was observed both pretransplant and at the time of rejection for sera from 63 of these patients. Sixteen of these recipients were diagnosed with AMR or CMR (Table 2). Seven of the recipients were diag-

TABLE 1. Levels of AT₁R and cause of end-stage renal disease and pretransplant HLA-specific antibody status

	High AT ₁ R levels (>17 units)	Low AT ₁ R levels (<17 units)
Cause of ESRD		
Polycystic kidney disease	3	5
Diabetes mellitus	8	15
DM+HTN	2	3
Glomerulonephritis	12	18
HTN	1	9
Other/unknown	6	15
Total	32 (33%)	65 (67%)
Pretransplant HLA-specific antibody status ^a		
HLA-specific antibody positive ^b	23 (72%)	38 (58%)
HLA-specific antibody negative	9	27

^a $P=0.35$, Fisher's exact test.

^b Determined by solid-phase assays.

AT₁R, angiotensin type 1 receptor; ESRD, end-stage renal disease; DM, diabetes mellitus; HLA, human leukocyte antigen; HTN, hypertension.

TABLE 2. AMR associated with high levels of AT₁R-specific antibody in patients with no donor HLA-specific antibodies

Acute rejection	High anti-AT ₁ R (>17 units) antibodies	Low anti-AT ₁ R (<17 units) antibodies
AMR	6	1
CMR	0	9

Total number of patients with no donor-specific antibody to HLA or MICA is 63.

$P=0.0009$, Fisher's exact test.

AMR, antibody-mediated rejection; CMR, cell-mediated rejection; AT₁R, angiotensin type 1 receptor; HLA, human leukocyte antigen; MICA, major histocompatibility class I chain-related gene A.

nosed with AMR by biopsy or presumed AMR based on clinical parameters; two of these patients also had biopsy findings consistent with CMR. Presumed AMR is defined as a rapid deterioration of renal function within 24 to 48 hr of transplant after having evidence of improving renal function. This is usually associated with increases of serum creatinine, significant reductions in urine output and development of significant increased resistivity, and decreased blood flow to the allograft as determined by Doppler flow ultrasound.

A C4d-positive biopsy was observed for one patient including biopsy findings of C4d staining in PTCs with pronounced glomerulitis, focal margination of leukocytes in cortical PTCs, and very focal arteritis. Two patients were diagnosed with presumed AMR based on clinical findings. Four patients had biopsies negative for PTC C4d staining but histologic findings consistent with AMR including glomerulitis, transplant glomerulopathy, focal interstitial hemorrhage, very focal capillary thrombosis, and margination of leukocytes (neutrophils and CD68-positive mononuclear cells) in the PTC. Two of these patients also had biopsy findings consistent with CMR. Nine patients had biopsy findings consistent with CMR, without histologic or immunohistologic findings of AMR. High AT₁R antibody levels were observed for six of seven patients diagnosed with AMR or presumed AMR, and no patients diagnosed with CMR alone ($P=0.0009$). One patient with clinically defined presumed AMR had low levels of AT₁R binding and no DSA to HLA or to MICA.

The posttransplant development of high levels of antibody to AT₁R was observed for one additional patient with AMR diagnosed at 110 days posttransplant (Table 3). This patient was not included in the aforementioned study. No HLA-DSA was detected in sera obtained 6 months pretrans-

plant or at the time of transplant. Antibodies to AT₁R were low (<2.5 and 3.7 units) for the same time points. The patient received a deceased donor allograft with zero mismatches for HLA-A, -B, -DR, and one mismatch for HLA-C and -DQ. The patient became noncompliant, stopped taking all immunosuppressive drugs, and was diagnosed with AMR at 110 days posttransplant. At that time, strongly binding HLA-DSA were detected to both HLA-C4 and DQ5 (>200,000 standard fluorescence intensity or >10,000 median fluorescence intensity). The AT₁R binding had also increased to a very high level of 30.1 units.

DISCUSSION

Here, we report that high levels of anti-AT₁R antibody were found associated with AMR in 10% (6/63) of patients with no HLA or MICA DSA at the time of transplant and diagnosis of AMR. This percentage is higher than the 2% to 4% previously reported (7, 8, 20), perhaps because the selection of patients was not random and skewed in part with patients who had experienced AMR, CMR, or both not attributable to HLA-DSA. Thus, the frequency of AMR attributable to anti-AT₁R non-HLA antibodies cannot be determined from this study.

High levels of anti-AT₁R were found in 33% (32/97) of pretransplant sera tested; however, there did not seem to be any significant difference in the anti-AT₁R levels with different causes of end-stage renal disease (Table 1, cause of end-stage renal disease). Most high levels of AT₁R were not associated with hypertensive nephrosclerosis but to a variety of diseases. These findings are consistent with those reported previously (25). Angiotensin II-mediated overactivation of the AT₁R has been traditionally associated with hypertension and malignant hypertension (17, 26). However, recent transplant studies in AT₁R tissue-specific deficient mice implicate that renal but not systemic AT₁R expression is instrumental for development of hypertensive responses (27). Four of the patients in this study developed malignant hypertension posttransplant. Three were in the low antibody group and one in the high antibody group. Possibly center-related differences in early posttransplant antihypertensive management may greatly contribute to variability in hypertensive responses observed among different studies (7, 8, 20).

Biopsies were performed in six of seven cases diagnosed as AMR; two cases were diagnosed and treated based on clinical indications. Only one of the biopsies was C4d positive. A lack of association between anti-AT₁R antibodies and acute clinical C4d-positive allograft rejection in the absence of malignant hypertension has been reported (20). Nickleit and

TABLE 3. Early AMR in recipient of HLA-A, -B, and -DR zero mismatched deceased donor graft: association with HLA-DSA and AT₁R antibody

Time point	Anti-class I DSA	Anti-class II DSA	Anti-AT ₁ R (units)
6 mo pretransplant	Negative	Negative	<2.5
Transplant	Negative	Negative	3.7
~100 d posttransplant	C4 >200,000 SFI/10,000 MFI	DQ5 >200,000 SFI/10,000 MFI	30.1

AMR, antibody-mediated rejection, because of noncompliance; AT₁R, angiotensin type 1 receptor; HLA-DSA, donor human leukocyte antigen-specific antibody; DSA, donor-specific antibody; SFI, standard fluorescence intensity; MFI, median fluorescence intensity.

Mihatsch (28) reported that 40% to 50% of rejection with severe vascular changes were C4d negative, suggesting the involvement of noncomplement fixing antibodies. Dragun (25) reported severe vascular pathology plus, tubulitis, and interstitial infiltrates characteristic of acute CMR in patients with antibody AT₁R. In our study, the four C4d-negative biopsies had histologic findings consistent with AMR including glomerulitis, transplant glomerulopathy, focal interstitial hemorrhage, very focal capillary thrombosis, and margination of neutrophils and monocyte/macrophages in the PTC. Two of these biopsies also had findings consistent with CMR. These results suggest that antibodies to AT₁R may be both complement and noncomplement fixing. The diagnosis of AMR in the absence of C4d deposition is a newly emerging area of study. Sis et al. (22) have reported that endothelial gene expression can be increased in association with HLA-DSA in the absence of C4d deposition. Loupy et al. (23) reported that humoral lesions without C4d deposition may represent a milder but progressive form of AMR. Single markers such as C4d deposition or DSA alone may be poor predictors of AMR.

Both autoreactive and alloreactive antibodies may develop against AT₁R (28). The autoantibodies associated with transplantation are generally of the IgG class requiring T-cell help (29). T-cell self-tolerance may be broken by an infectious inflammatory trigger, which may also generate different cryptic T-cell epitopes. Autoantibodies can be the result of molecular mimicry and crossreactivity with microbial antigens such as seen for antibodies directed against G-protein receptors in myasthenia gravis and Chagas' disease (30, 31). This molecular mimicry could trigger activation of autoreactive and memory T cells. The IgG3 isotype of AT₁R antibodies recognizes an epitope also occurring on parvovirus B19 capsid protein (32).

De novo antibody to AT₁R was seen in sera from a noncompliant patient who also developed antibody to HLA-DSA (Table 3). The interpretation of de novo formation of antibody to AT₁R in this noncompliant patient may be the result of increased expression of AT₁R in the kidney damaged by alloresponses. Endothelial cell activation may act as a danger signal and may be a prerequisite for the induction of severe non-HLA antibody-related phenotypes. Alternatively, the kidney donor but not the recipient may express the A1166C functional polymorphism resulting in an allogeneic response. The majority of patients with high levels of anti-AT₁R antibodies did not develop AMR, suggesting that no disparate donor polymorphism was detected or autoantibodies did not crossreact with the donor AT₁R. Determination of the functional A1166C polymorphism that regulates tissue expression of AT₁R is not routinely performed in HLA laboratories and has been the focus of cardiovascular medicine. Thus, it was not possible to determine the frequency of this polymorphism in our patient and donor population studied. More comprehensive studies on this matter are warranted.

The antibody status to HLA-DSA and AT₁R may assist in treatment options or alter diagnosis of presumed rejection. Clinical intervention has been reported for patients during episodes of rejection mediated by AT₁R antibodies. Attenuated pathology was seen in biopsy specimens after patients were treated with losartan (AT₁R blocker), plasmapheresis, and intravenous immunoglobulin (7). This treatment proto-

col resulted in improved renal function and graft survival compared with patient who received standard treatment. However, concern remains about the use of drugs that inhibit the renin angiotensin system. Many positive pleiotropic effects of antirenin angiotensin system drugs including decreased interferon- γ mRNA levels and attenuated lymphocyte proliferation could encourage their use in selected patients (33). The diagnosis of presumed AMR is often made based on the clinical parameters of the patient. Knowledge of the antibody status to HLA-DSA and AT₁R may help to clarify the pathologic process involved and aid in targeted clinical intervention.

In conclusion, we observed a strong association between the presence of high binding to AT₁R and AMR in recipients whose sera contained no antibody to donor HLA or MICA. These results provide valuable information regarding the AMR process in the absence of HLA-DSA and with no PTC C4d deposition. Assessing the pretransplant AT₁R antibody status along with the HLA-DSA can help to determine the immunologic risk for recipients. This information can assist in the diagnosis of AMR and provide the opportunity for clinical intervention.

MATERIALS AND METHODS

The detection and specificity analysis of the anti-HLA antibodies was determined by solid-phase antibody testing methods, flow cytometry screening bead assay, and Luminex-platform single-antigen bead assays as described previously (34). Results are expressed as standard fluorescence intensity or median fluorescence intensity. Complement-dependent cytotoxicity antibody analysis was performed on sera shown to contain anti-HLA antibodies by solid-phase assays. The presence of antibody to HLA-A, -B, -C, -DRB1, -DRB3/4/5, -DQB, -DQA, and -DPB was determined. Antibody to MICA was determined by a Luminex screening assay. The presence of any MICA-DSA was determined by MICA single-antigen bead assay and the donor and recipient MICA typing as determined by DNA-SSO (One Lambda Inc., Canoga Park, CA). The patients and donor were typed for HLA-A, -B, -C, -DRB1, -DRB3/4/5, and -DQB. If anti-DQ antibodies were detected, the patient and donor were typed for DQA to determine whether any relevant DSAs were present. If anti-DP antibodies were detected, the patient and donor were typed for HLA-DP to determine whether any HLA-DP DSAs were present. No DSA to the HLA antigens listed earlier were detected at the time of transplant or at the time of AMR or CMR diagnosis. All T- and B-cell flow cytometry crossmatches at the time of transplant were negative for the 63 patients in this study.

We measured anti-AT₁R antibodies with a sandwich ELISA (CellTrend GmbH Luckenwalde, Germany). The microtiter 96-well polystyrene plates were coated with the extracts from Chinese hamster ovary cells overexpressing the human AT₁R. To maintain the conformational epitopes of the receptor, 1 mM calcium chloride was added to every buffer. Duplicate samples of a 1:100 serum dilution were incubated at 4°C for 2 hr. After washing steps, plates were incubated for 60 min with a 1:20,000 dilution of horseradish peroxidase-labeled goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) used for detection. To obtain a standard curve, plates were incubated with test sera from an anti-AT₁R autoantibody-positive index patient. The interassay variability was 8%; the intraassay variability was 5%. Persons who were unaware of the patients' characteristics performed the assays. All tests were conducted in duplicate. When compared with neonatal cardiomyocyte bioassay (7), the ELISA currently has 96% specificity and 88% sensitivity. Binding can be inhibited by the coincubation with peptides corresponding to the second extracellular loop and antibody-binding epitopes of the AT₁R, what confirms specific epitope recognition. Because recipient AT₁R polymorphisms regulate AT₁R expression in the target tissue, there is no polymorphism-related confounding effect on the test. Biopsy samples were read and graded according to the Banff '07 classification (35), and for all

biopsies, C4d staining was performed by indirect immunofluorescence in frozen tissue sections.

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