Angiotensin II Type 1–Receptor Activating Antibodies in Renal-Allograft Rejection

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ABSTRACT

BACKGROUND
Antibodies against HLA antigens cause refractory allograft rejection with vasculopathy in some, but not all, patients.

METHODS
We studied 33 kidney-transplant recipients who had refractory vascular rejection. Thirteen had donor-specific anti-HLA antibodies, whereas 20 did not. Malignant hypertension was present in 16 of the patients without anti-HLA antibodies, 4 of whom had seizures. The remaining 17 patients had no malignant hypertension. We hypothesized that activating antibodies targeting the angiotensin II type 1 (AT1) receptor might be involved.

RESULTS
Activating IgG antibodies targeting the AT1 receptor were detected in serum from all 16 patients with malignant hypertension and without anti-HLA antibodies, but in no other patients. These receptor-activating antibodies are subclass IgG1 and IgG3 antibodies that bind to two different epitopes on the second extracellular loop of the AT1 receptor. Tissue factor expression was increased in renal-biopsy specimens from patients with these antibodies. In vitro stimulation of vascular cells with an AT1-receptor-activating antibody induced phosphorylation of ERK 1/2 kinase and increased the DNA binding activity of the transcription factors activator protein 1 (AP-1) and nuclear factor-κB. The AT1 antagonist losartan blocked agonistic AT1-receptor antibody–mediated effects, and passive antibody transfer induced vasculopathy and hypertension in a rat kidney-transplantation model.

CONCLUSIONS
A non-HLA, AT1-receptor–mediated pathway may contribute to refractory vascular rejection, and affected patients might benefit from removal of AT1-receptor antibodies or from pharmacologic blockade of AT1 receptors.
Many aspects of T-cell–mediated responses in allograft rejection have been elucidated, yet humoral mechanisms are relatively unexplored. Vascular rejection that is refractory to intensified immunosuppression is the most important predictor of early and late graft loss.\(^1\) The association of antidonor humoral reactivity against HLA antigens and vascular rejection has been established.\(^2\) Other targets of the allograft-directed host response remain elusive. However, alloantibodies against the polymorphic non-HLA system were found in serum obtained before transplantation from patients in whom refractory rejection developed after they received kidney transplants from HLA-identical siblings.\(^3\) Allograft endothelium may be a primary target of the cytotoxic actions of non-HLA antibodies, triggering endothelial-cell activation or apoptosis.\(^4,5\) Identification of non-HLA antigens that are relevant to rejection might provide insight into underlying mechanisms, define risk-related phenotypes, and facilitate development of specific therapies.

We studied kidney-transplant recipients who had severe allograft dysfunction but did not have anti-HLA antibodies; in these patients, rejection was invariably accompanied by accelerated hypertension, and even convulsions, in a manner reminiscent of preeclampsia. In fact, the first patient we examined in this group (the index patient) had had preeclampsia 16 years earlier. Immunologic similarities between graft rejection and fetal survival in pregnancies complicated by preeclampsia are well recognized.\(^6\) We previously reported that agonistic antibodies that target the angiotensin II type 1 (AT\(_1\)) receptor may develop in women with pre-eclampsia after the 20th week of gestation,\(^7\) and we observed agonistic AT\(_1\)-receptor activity in nine kidney-transplant recipients during an episode of rejection.\(^8\) We then reasoned that similar mechanisms might be present in refractory allograft rejection and initiated a comprehensive investigation of this issue.

**Methods**

**Study Patients and Biopsies**

We studied all patients with steroid-refractory acute renal-allograft rejection seen among those who received kidney transplants at the Charité University Hospital Campus Mitte in Berlin from January 1, 2000, through July 31, 2004. Serum samples were obtained prospectively from all patients during rejection episodes and were screened for donor-specific anti-HLA antibodies and also analyzed for agonistic antibodies targeting the AT\(_1\) receptor. Serum samples from patients with steroid-refractory acute vascular rejection from three collaborating centers (the Charité University Hospital Campus Virchow Clinic, and the University Hospital Erlangen–Nürnberg, both in Germany, and the Ospedale Civile in Bergamo, Italy) were also examined. Written informed consent to use serum samples for research purposes was obtained from each patient while he or she was awaiting transplantation. The institutional review board of Charité University Hospital Berlin approved the protocols.

Allograft-biopsy specimens were processed by standard techniques and graded according to the Banff 97 classification, with updated scoring of the degree of humoral rejection.\(^9,10\) Biopsy specimens were stained with polyclonal antibodies for C4d (Biomedica)\(^11,12\) and tissue factor (provided by T. Luther, Dresden).

Initial immunosuppressive therapy consisted of a calcineurin inhibitor, mycophenolate mofetil, methylprednisolone, and antibody against interleukin-2 receptor for induction. Patients who were positive for donor-specific anti-HLA antibodies or C4d or who had vascular rejection manifested as endarteritis or necrotizing vasculitis with fibrinoid necrosis were treated with plasmapheresis together with intravenous immune globulin. Patients initially treated with cyclosporine were switched to high-dose tacrolimus when refractory rejection was detected.

**Bioassays and Analysis of Epitopes**

Patients’ serum samples were examined for the presence of AT\(_1\)-receptor–activating IgG in a functional bioassay that involves spontaneously beating cultured neonatal-rat cardiomyocytes that express several G-protein–coupled receptors, including the AT\(_1\) receptor. Changes in the activation state of the cells is measured directly by counting the beating rate by means of computerized image analysis. Isolation, cultivation, and detection procedures have been described in detail previously.\(^13\) The dose–response relationship between activating IgG or angiotensin II concentration and the spontaneous beating rate is linear.

To study the specificity of the AT\(_1\) receptor–mediated response, IgG-stimulated cells were treated with an AT\(_1\)-receptor blocker or angiotensin II type 2 (AT\(_2\))–receptor blocker. Short, overlapping
assays

Surface-Plasmon-Resonance Analysis

Binding of AT<sub>1</sub>-receptor antibody was verified and quantified by surface-plasmon-resonance analysis (BIAcore). The IgG fraction from affected patients or control human IgG was allowed to pass over biotinylated peptides loaded on a streptavidin-immobilized chip in a biosensor (BIAcore) at different flow rates. After the association phase and the dissociation phase, the relative increase in response of AT<sub>1</sub>-receptor peptide and control peptide (β<sub>2</sub>-adrenergic receptor) flow channels was determined. The kinetic variables were calculated with BIA evaluation software (Biosensor, version 3.1).

Assays

Human coronary-artery endothelial cells and vascular smooth-muscle cells were stimulated with AT<sub>1</sub>-receptor antibodies in the presence or absence of AT<sub>1</sub>-receptor blocker or AT<sub>2</sub>-receptor blocker. Protein extraction, sodium dodecyl sulfate–polyacrylamide-gel electrophoresis, and membrane treatment were performed as described by Dechend et al. Primary antibodies against polyclonal extracellular signal–regulated kinase (ERK) 1/2 and phosphorylated ERK 1/2 were used. Triplicate experiments with six patients’ IgG preparations were performed. Electromobility-shift assays in vascular smooth-muscle cells stimulated with angiotensin II, AT<sub>1</sub>-receptor antibodies, or control IgG were performed as described previously.

Animal Model of Kidney Transplantation

The low-responder Fischer 344-to-Lewis life supporting rat kidney-transplantation model was used to test the physiological effects of various antibodies. AT<sub>1</sub>-receptor antibodies (a pool derived from two donors), control IgG, or vehicle was continuously infused into recipient rats by means of intraperitoneal, seven-day-release, osmotic minipumps (Alzet, Charles River Laboratories). After implantation of the minipumps, one kidney was transplanted into each rat (cold-ischemia time was two hours), and kidneys were harvested after seven days (n=4 per treatment group). For continuous blood-pressure monitoring, telemetric transmitters were placed into the abdominal aortas of six additional rodent recipients, before transplantation. The histologic features of the rat allografts were analyzed after harvesting with standard techniques. Direct immunofluorescence with fluorescein isothiocyanate–labeled antihuman IgG (Dako) was used to determine intragraft binding of AT<sub>1</sub>-receptor antibodies; cryosections were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (Vector).

Statistical Analysis

Continuous data are presented as medians (with ranges). We performed comparisons between groups with use of Fisher’s exact test for categorical values and the Mann–Whitney U test for continuous variables.

Results

Thirty-three patients with steroid-refractory rejection were identified (23 at Charité University Hospital Campus Mitte and 10 at collaborating centers). Thirteen had detectable donor-specific anti-HLA antibodies. Sixteen patients who did not have donor-specific anti-HLA antibodies had malignant hypertension, followed by convulsions in four. Four patients had neither donor-specific anti-HLA antibodies nor malignant hypertension. Figure 1 shows representative biopsy specimens from patients with malignant hypertension and refractory rejection who had no donor-specific anti-HLA antibodies; these showed either endarteritis (Fig. 1A) or fibrinoid necrosis (Fig. 1B). Patients whose biopsy specimens had evidence of fibrinoid necrosis underwent magnetic resonance angiography studies that showed multiple perfusion defects consistent with cortical infarctions (Fig. 1C). Subsequently, patients with anti-HLA antibodies had substantially better graft survival than did those who had malignant hypertension but did not have anti-HLA antibodies (Fig. 1D).

We studied the AT<sub>1</sub>-receptor agonistic response and IgG subclasses with a bioassay that records the chronotropic response (the increase in the number of beats per minute) of spontaneously beating neonatal-rat cardiomyocytes when exposed to immunoglobulin from patients. Analysis of serum obtained from the 13 anti-HLA–positive patients and from the 16 anti-HLA–negative patients with malignant hypertension before transplantation and at
the time of rejection indicated differences between the groups. Figure 2A demonstrates a mean increase of 26 to 30 beats per minute in cells exposed to IgG obtained before transplantation or at the time of rejection from patients without anti-HLA antibodies. Serum from 13 patients with humoral rejection mediated by donor-specific anti-HLA antibodies did not induce such increases. Immunoglobulin from the four remaining patients, who had steroid-refractory rejection without malignant hypertension or HLA-antibodies, did not induce a response (data not shown).

All 16 patients whose serum led to an increased chronotropic response were screened for hereditary and autoimmune causes of thrombophilia as a possible trigger of the observed thrombotic angiopathy; all tests were negative, as were additional serologic tests for autoimmune disorders. Acute infection with cytomegalovirus was ruled out by pp65 antigen and reverse-transcriptase polymerase-chain-reaction analysis (in three participating German centers) or on clinical grounds (in Bergamo, Italy).

Specific angiotensin II–receptor antagonists were used to test whether the agonistic response to patients’ IgG was specific for the AT$_1$ receptor. Losartan, but not the AT$_2$-receptor antagonist PD 123319, abolished the functional bioassay response (Fig. 2B). Angiotensin II and IgG from patients achieved results in the bioassay. Thus, IgG fractions of our patients’ serum contained AT$_1$-receptor antibodies.

The demographic data from the 16 patients who were positive for AT$_1$-receptor antibodies...
were compared with those for the 13 patients with donor-specific anti-HLA-antibodies. As shown in Table 1, patients with anti-HLA antibodies were more likely to have staining for C4d in their renal-biopsy specimens and had less rapid allograft loss. Otherwise, there were no significant differences in clinical or demographic characteristics between the two groups.

To estimate the incidence and prevalence of steroid-refractory allograft rejections in patients with AT1-receptor antibodies, we studied all episodes of rejection that occurred after the 278 consecutive kidney transplantations performed at the Charité University Hospital Campus Mitte in Berlin between January 1, 2000, and July 31, 2004. During this period, 119 biopsy-proved episodes of rejection were
that are rejected because of the presence of anti-
fractory vascular rejection. Vascular lesions in grafts
antibodies played a role in the pathogenesis of re-
formed, on the basis of our hypothesis that these
rate constant at 1.05±0.29×10⁻³ per second, revealing a high bind-
ing affinity of AT₁-receptor antibodies to the AT₁
receptor. Further studies were performed, on the basis of our hypothesis that these antibodies played a role in the pathogenesis of re-
fractional vascular rejection. Vascular lesions in grafts
are rejected because of the presence of anti-
HLA antibodies are generally attributed to comple-
ment activation. However, patients with AT₁-recep-
tor antibodies did not have complement-fixing cy-
totoxic anti-HLA antibodies in their serum, and
their renal-biopsy specimens generally did not show
staining for C4d. AT₁-receptor antibodies were ex-
amined to see whether they could initiate signaling
mediated by ERK 1/2 in vitro by means of endothe-
ial cells and vascular smooth-muscle cells (Fig. 3A
and 3B). AT₁-receptor antibody induced phosphory-
ation of ERK 1/2 (Fig. 3A and 3B), which peaked
at 10 minutes without a change in total ERK 1/2 in
the cells.

We also examined the possibility that the
AT₁-receptor antibodies could activate the tran-

Table 1. Characteristics of Patients with AT₁-Receptor Antibodies and Patients with Donor-Specific Anti-HLA Antibodies but without AT₁-Receptor Antibodies.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>AT₁-Receptor Antibodies (N=16)</th>
<th>Anti-HLA Antibodies (N=13)</th>
<th>P Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplant from living donor (no.)</td>
<td>2</td>
<td>4</td>
<td>0.36</td>
</tr>
<tr>
<td>Male sex (no.)</td>
<td>10</td>
<td>5</td>
<td>0.12</td>
</tr>
<tr>
<td>First kidney allograft (no.)</td>
<td>8</td>
<td>7</td>
<td>1.00</td>
</tr>
<tr>
<td>Cold-ischemia time (hr)</td>
<td>1.6–20.0</td>
<td>1.4–16.2</td>
<td>0.86</td>
</tr>
<tr>
<td>No. of HLA mismatches</td>
<td></td>
<td></td>
<td>0.80</td>
</tr>
<tr>
<td>Median</td>
<td>11.2</td>
<td>11.3</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1.6–20.0</td>
<td>1.4–16.2</td>
<td></td>
</tr>
<tr>
<td>Peak panel-reactive antibodies (%)</td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>Median</td>
<td>34</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>0–100</td>
<td>0–96</td>
<td></td>
</tr>
</tbody>
</table>
| Panel-reactive antibodies at trans-
plantation (%)                        |                                 |                           | 0.30     |
| Median                               | 1                               | 0                         |          |
| Range                                | 0–99                            | 0–84                      |          |
| Age at transplantation (yr)          |                                 |                           | 0.38     |
| Median                               | 37.6                            | 44.1                      |          |
| Range                                | 15.1–67.2                       | 20.1–65.2                 |          |
| Time from transplantation to rejec-
tion (days)                           |                                 |                           | 0.10     |
| Median                               | 4                               | 9                         |          |
| Range                                | 2–1217                          | 3–680                     |          |

* P values were determined with use of Fisher’s exact test for categorical variables and the Mann–Whitney U test for continuous variables.
scription factor activator protein 1 (AP-1) downstream from ERK 1/2 (Fig. 3C). Both AT₁-receptor antibodies and angiotensin II itself induced AP-1 activity in vascular smooth-muscle cells, whereas control IgG caused no such induction. DNA-binding activity of nuclear factor-κB (NF-κB), a transcription factor pivotal in initiating immune responses and inflammatory disease,16 when studied in nuclear extracts from vascular smooth-muscle cells, was activated by AT₁-receptor antibodies, but not by IgG from control patients (Fig. 3D).

Tissue factor, which initiates the extrinsic coagulation pathway, is a target gene for AP-1 and NF-κB. Biopsy specimens obtained from patients during episodes of rejection mediated by AT₁-receptor antibodies revealed intense staining of tubular cells, inflammatory infiltrate, and peritubular capillaries (Fig. 4A). In contrast, only weakly positive staining was seen in biopsy specimens obtained after patients had been treated with losartan and plasmapheresis (Fig. 4B). Treatment consisting of plasmapheresis, intravenous immune globulin, and 100 mg of losartan daily in seven patients with AT₁-receptor antibodies resulted in significantly
angiotensin ii type 1–receptor antibodies and rejection

improved allograft survival, as compared with that in patients receiving standard antirejection treatment (Fig. 4C). Serum from four patients with the longest rejection-free follow-up (who are still receiving losartan) became negative for AT$_1$-receptor antibodies (Fig. 4D).

To demonstrate that the agonistic antibodies could induce vascular rejection, AT$_1$-receptor antibodies were infused into rats that had received kidney transplants. One week after transplantation, kidneys from all experimental animals showed evidence of endarteritis and intravascular infiltrates (Fig. 5A). Transplants from control animals infused with control IgG showed only endothelial activation (Fig. 5B). Infusion of AT$_1$-receptor antibodies had no effect on native kidneys in uninephrectomized animals (data not shown). The act of transplanting non-native kidneys into recipients appeared to make the organs prone to the effects of AT$_1$-receptor antibodies. AT$_1$-receptor antibodies containing human AT$_1$-receptor–activating antibody–positive IgG but not control IgG could be detected within the muscular layer of injured arteries in the rats (Fig. 5C and 5D). The mean arterial pressure, measured by radiotelemetry, was higher in the group of rats with AT$_1$-receptor antibodies two weeks after transplantation than in controls (Fig. 5E).

**Discussion**

Patients with refractory vascular allograft rejection and accelerated hypertension but without anti-HLA
antibodies were found to have pathogenic antibodies directed at two epitopes of the second extracellular loop of the AT₁ receptor in their serum. AT₁-receptor antibodies induced phosphorylation of ERK 1/2, AP-1 activation, and NF-κB activation in vascular cells. An AT₁-receptor antagonist blocked the effects mediated by AT₁-receptor antibodies. Passive infusion of AT₁-receptor antibodies in a rat renal-transplantation model induced vascular changes and increased blood pressure. These data suggest that AT₁-receptor antibodies may be directly involved in the pathogenesis of vascular rejection.

Earlier we had observed AT₁-receptor activity during steroid-refractory, C4d-negative renal-allograft rejection in nine patients with hypertension, but not in eight patients with steroid-sensitive cellular rejection. In the present study, we increased the number of patients studied and clearly defined the pretransplantation activity of IgG1- and IgG3-subclass antibodies that bound to AT₁-receptor epitopes and induced signaling cascades. The clinical presentation of transplant recipients with AT₁-receptor antibodies appears similar to that of transplant recipients with anti-HLA antibodies, but with significantly worse allograft survival.

Similar agonistic antibodies against the AT₁ receptor have been reported in patients with preeclampsia.⁷ Recently, other investigators confirmed the existence of AT₁-receptor antibodies in patients with preeclampsia and also demonstrated that the antibodies induced mobilization of intracellular calcium and activation of a transcription factor, nuclear factor of activated T cells, in AT₁-receptor–transfected Chinese-hamster-ovary cells.¹⁷ The index patient in the present report had had preeclampsia many years earlier. Nevertheless, the antibodies in this patient and the other transplant recipients bound to an epitope that did not entirely coincide with the antibodies described earlier in the patients with preeclampsia.⁷

Antibodies to G-protein receptors have been identified in some patients with idiopathic dilated cardiomyopathy or malignant hypertension.²⁸ In Graves’ disease, autoantibodies activate the thyrotropin G-protein receptor, increasing target-organ activity.²⁹ Anti–endothelial-cell antibodies are non–HLA antibodies that may be related to the pathogenesis of allograft rejection.²¹,²² However, research on anti–endothelial-cell antibodies is hampered by the heterogeneity of endothelial cells in various vascular beds, a fact that makes identifying a standard detection method difficult.²³ Moreover, initially detected target antigens of anti–endothelial-cell antibodies were of unclear relevance.²³

We suggest that AT₁-receptor antibodies have similarities to anti–endothelial-cell antibodies since endothelial cells have one AT₁ receptor,²⁴ and AT₁-receptor antibodies induce phosphorylation of ERK 1/2 in endothelial cells. We further suggest that binding of AT₁-receptor antibodies to the AT₁ receptor is a critical step for activating the downstream signaling cascade, mimicking the action of angiotensin II and inducing damage to the allograft. Emerging information has established that angiotensin II acts as an inflammatory cytokine participating in various vascular disorders.²⁵

AT₁-receptor antibodies may induce inflammatory responses and contribute to allograft rejection by means of activation of NF-κB target genes. Blockade of NF-κB activity with decoy oligodeoxynucleotides reduced tubulointerstitial infiltration in rat renal allografts.²⁶ Expression of tissue factor as regulated by NF-κB and AP-1 may increase procoagulatory activity of the injured vessels.

We previously reported NF-κB and AP-1 subunit expression in biopsy specimens from several kidney-transplant recipients.⁸ We now report that biopsy specimens of patients with rejection associated with AT₁-receptor antibodies had evidence of increased tissue factor expression and secondary thrombotic occlusions and that tissue factor was
reduced after losartan treatment. It has previously been recognized that the coagulation system has a role in the pathogenesis of rejection.\textsuperscript{27} An anti-cardiolipin antibody–mediated increase in tissue factor expression contributes to thrombosis in patients with the antiphospholipid syndrome.\textsuperscript{28} In rat cardiac allografts, tissue factor plays a critical role in clotting abnormalities and transplant arteriosclerosis.\textsuperscript{29} Lesions in allografts in rats infused with \( AT_1 \)-receptor antibodies resemble those observed in kidney-transplant recipients. It is unknown whether \( AT_1 \)-receptor antibodies...
function only through proinflammatory and procoagulatory activity or also by means of specific immune responses. Experimental studies have implicated the renin–angiotensin system in the regulation of the specific immune response and immune-mediated renal injury. Since AT$_1$ receptors are present on human mononuclear cells, an effect of AT$_1$-receptor antibodies on T lymphocytes, monocytes, and dendritic cells appears to be likely. Furthermore, rejection in association with circulating anti-HLA antibodies is commonly characterized by a high incidence of severe vascular lesions with fibrinoid necrosis, whereas endarteritis and mononuclear cell tubulitis predominate in T-cell–mediated rejection.

We first observed endarteritis and later fibrinoid necrosis, tubulitis, and interstitial-cell infiltration in patients’ biopsy specimens. These characteristics may define a distinct type of kidney-transplant rejection mediated by AT$_1$-receptor antibodies. The incidence of rejection among patients positive for AT$_1$-receptor antibodies in this study is similar to that of rejection due to donor-specific anti-HLA antibodies. However, it remains unclear why AT$_1$-receptor antibodies develop in certain patients and why these antibodies target the allograft.

Molecular mimicry may be important; cross-reactivity with microbial antigens has an important role in other pathologic processes associated with antibodies directed against G-protein receptors—for example, myasthenia gravis or the dilated cardiomyopathy seen in Chagas’ disease. We have not tested the possibility that the AT$_1$ receptors in patients in whom AT$_1$-receptor antibodies develop might harbor specific polymorphisms. We speculate that post-transplantation reperfusion injury may alter the intragraft expression of AT$_1$ receptor, change its density, or cause conformational changes. A permissive environmental phenomenon might enhance local intragraft immunoreactivity owing to an activated innate immune response.

Transplantation nephrologists have been reluctant to use angiotensin II blockers because of possible decreases in organ perfusion and filtration. However, this concern may be outweighed by the potential advantages of these drugs. The seven patients discussed here who were treated with losartan, plasmapheresis, and intravenous immunoglobulin had amelioration of the antibody-mediated rejection process and remain free of rejection while receiving losartan. However, their treatment was neither randomly assigned nor blinded, and the numbers of patients are too small to permit us to draw firm conclusions. We speculate that detection of AT$_1$-receptor antibodies in patients on a waiting list for a transplant might identify those at risk for refractory rejection.

Dr. Fritsche reports having served as a consultant to Novartis, Hexal, Shire, and GDL, being a stockholder in Novartis, receiving grant support from Novartis and Wyeth, and receiving lecture fees from Hoffmann–La Roche. Dr. Kuntscher reports having served on the advisory boards of Novartis and Takeda, receiving grant support from Boehringer Ingelheim, and receiving lecture fees from Takeda. Dr. Unger reports serving as a consultant to Novartis, Abbott, Takeda, and Merek and having received grant support from Takeda, Novartis, Bayer, and Boehringer Ingelheim. Dr. Neumayer reports having received consulting fees from Novartis and Shire and lecture fees from Novartis and Wyeth. Dr. Luft reports having served as a consultant and receiving grant support from Novartis, Boehringer Ingelheim, and CVRx.

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