Angiotsensin II Receptors From Peritransplantation Through First-Year Post-Transplantation and the Risk of Transplant Coronary Artery Disease

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OBJECTIVES
We evaluated whether the angiotensin II (Ang II) receptors from perioperation through one-year post-transplantation predict the transplant coronary artery disease (TCAD) progression.

BACKGROUND
The role of Ang II receptors (type 1: AT1R; type 2: AT2R) in TCAD is uncertain. We investigated 28 heart donors and the corresponding recipients. The levels of AT1R and AT2R messenger ribonucleic acid (mRNA) were examined in lymphocytes from the donor spleen and in the donor heart at one-week and one-year posttransplantation to determine their association with the progression of TCAD, measured as changes in maximal intimal thickness (CMIT) and plaque volume (CPV) by intravascular ultrasound (IVUS) examinations.

RESULTS
The AT1R mRNA in lymphocytes from the donor spleen (CMIT: r = 0.73, p < 0.0001; CPV: r = 0.69, p < 0.0001) and in the donor hearts at one-week (CMIT: r = 0.52, p = 0.005; CPV: r = 0.56, p = 0.002) and at one-year (CMIT: r = 0.63, p < 0.0001; CPV: r = 0.43, p = 0.004) post-transplantation along with AT2R mRNA in the donor hearts at one-year post-transplantation (CMIT: r = 0.3, p < 0.0001; CPV: r = 0.53, p = 0.009) were univariate predictors, whereas AT1R mRNA in lymphocytes and in the donor hearts at one-year post-transplantation proved to be multivariate predictors of the progression of TCAD.

CONCLUSIONS
These data suggest a role for Ang II receptors in the pathogenesis of TCAD and support a novel concept that TCAD may have its origin in the donor per se and may be modulated by the recipient's inherent biological factors. (J Am Coll Cardiol 2004;43:1565–73) © 2004 by the American College of Cardiology Foundation

Transplant coronary artery disease (TCAD) is a disease of the vasculature of the donor heart in a recipient (1) and involves both intramyocardial and epicardial branches of the transplant coronary arteries (2). Whereas coronary angiography demonstrates TCAD in 10% to 20% of heart transplant recipients (3), intravascular ultrasound (IVUS) identifies abnormal intimal thickness in 50% of heart transplant recipients at one-year post-transplant (4). Also, TCAD is a major cause of morbidity and mortality in those surviving past the first year following cardiac transplantation (5). Although immune mechanism has been implicated in its pathogenesis (6), the exact molecular mechanisms underlying the progression of TCAD are uncertain.

Recent investigations have elucidated the role of the renin-angiotensin system (RAS) in the pathobiology of native coronary artery disease (7). The biological effects of angiotensin II (Ang II) are mediated through two major subtypes of receptors—Ang II type 1 receptor (AT1R) and Ang II type 2 receptor (AT2R). The role of Ang II receptors in the pathogenesis of TCAD is not known. Because TCAD involves the donor heart artery, we attempted to investigate Ang II receptor expression in the donor before cardiac procurement and subsequently in the transplanted heart. We hypothesized that Ang II receptor expression is increased in the donor before donor heart procurement, which may contribute to the development of TCAD in a recipient. We assessed AT1R and AT2R in lymphocytes derived from the donor spleen obtained before transplantation and in donor heart at one-week and one-year post-transplantation. We performed paired-IVUS examinations to estimate the progression of TCAD and serial endomyocardial biopsies to assess heart rejection. The purpose of these experiments was to determine whether: 1) the possible alterations in Ang II receptors in the donor before transplantation persists in the donor heart after transplantation, and 2) whether such alterations relate to subsequent development of transplant vasculopathy.

METHODS
Study population. We investigated 28 heart donors and the corresponding recipients. The lymphocytes were isolated from the donor spleen. Recipients of heart transplantation had serial surveillance endomyocardial biopsies.
through the first-year post-transplantation. Endomyocardial biopsies obtained at one week and at one year after transplantation were used to determine the expression of AT1R and AT2R messenger ribonucleic acid (mRNA). Baseline (within four weeks’ post-transplantation) and one-year follow-up IVUS examinations were performed to measure the progression of TCAD. The protocol was approved by the ethics review committee of our institution.

**Study end points.** The prespecified end points were: 1) expression of AT1R and AT2R mRNA in lymphocytes and in donor hearts at one week and one year following transplantation, 2) average biopsy score of the transplanted heart, and 3) changes in maximal intimal thickness (CMIT) and in plaque volume in transplant coronary arteries over one year.

**Experimental protocol. ISOLATION OF LYMPHOCYTES FROM DONOR SPLEEN.** Donor spleen was injected with Hank’s balanced salt solution (HBSS) and the discharge containing cells collected and centrifuged. Cells at interface were transferred to conical tubes, diluted with HBSS, and centrifuged. The cell pellet was resuspended in fresh HBSS and centrifuged. Cells were then mixed with Lympho-Kwik T/B, incubated at 37°C for 20 min, diluted with fresh HBSS, and centrifuged. Finally, the lymphocyte pellet was washed by HBSS and resuspended in 0.5% bovine serum albumin (8).

**ENDOMYOCARDIAL BIOPSY.** Right ventricular endomyocardial biopsies were obtained using the standard transjugular approach. Endomyocardial tissues were divided into parallel parts for histological analysis and ribonucleic acid (RNA) isolation. Specimens for histological analysis were fixed in formalin, routinely processed, and embedded in paraffin. Sections were stained with hematoxylin-eosin to determine the grade of cellular rejection in accordance with the criteria established by the Working Formulation of the International Society for Heart and Lung Transplantation (9). Recipients of heart transplantation had approximately 13 endomyocardial biopsies through the first year of post-transplantation. Each biopsy was allocated a score (1–6) depending on the degree of cellular rejection. Average biopsy score was determined as the sum of individual scores divided by the number of biopsies. Patients were classified into low (biopsy score <1.0), intermediate (biopsy score 1.0–1.5), and high (biopsy score >1.5) biopsy score categories.

**ISOLATION OF TOTAL RNA.** Total RNA from lymphocytes: Ambion RNAqueous TM-4PCR kit (Ambion, Austin, Texas) was used to isolate total RNA from lymphocytes derived from donor spleen. The lymphocytes were treated with lyses solution and 64% isomyl alcohol and then applied to filter cartridge placed into a collecting tube. After centrifugation, the flow-through was discarded. Preheated elution solution was applied to the filter and then centrifuged for 30 s to recover the eluate containing total RNA.

Total RNA from endomyocardial biopsies: Biopsy specimens were retrieved from frozen blocks and were rapidly processed to isolate total RNA using the Totally RNA kit (Ambion) following the manufacturer’s instructions. Briefly, tissue was homogenized in 200 μl of denaturation solution. An equal volume of phenol:chloroform was added, vortexed, and then stored on ice for 5 min. After centrifugation, the aqueous phase was transferred to a new tube, and 1/10 volume of sodium acetate solution was added. Next, acid: chloroform was added, vortexed, and stored on ice for 5 min before centrifugation. The upper aqueous phase was transferred to a new tube, precipitated with isopropanol, and stored at −20°C for 30 min before centrifugation. The pellet was washed with 70% ethanol and resuspended in diethyl pyrocarbonate-treated distilled water.

Assessment of RNA yield: The concentration and purity of RNA was determined by its absorbency in a spectrophotometer. The yield of total RNA was 400 to 800 ng per specimen. Therefore, only during initial experiments, a portion of recovered total RNA was electrophoresed to determine its purity.

**REVERSE TRANSCRIPTION.** The RNA samples were reverse-transcribed using TaqMan reverse transcription kit (Applied Biosystems, Foster City, California). The mix was aliquoted in individual tubes, and template RNA was added. Samples were incubated for 90 min at 25°C, 45 min at 48°C, and 5 min at 95°C. A tube with no reverse transcriptase was included to control for deoxynucleic acid contamination.

**REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR).** Both AT1R and AT2R primers and probes for RT-PCR were designed using the PRIMER express program. The BLASTN search was conducted (GenBank, EMBL) to confirm the gene specificity and absence of DNA polymorphism. The oligonucleotide sequences of TaqMan probe and primers were as follows: AT1R—TaqMan probe FAM-1422 ATCCACCAA-GAACCTGCAACACCATGTTT-TAMRA, forward primer 1350 AGCCAAATCCCCACTCATCCT, reverse primer 1470 TCGAACATGTCACTCAACTCCTA;
AT2R—TaqMan probe FAM 948 CTGGCCCTTCAT-CATTGTGGTCCCTCC-TAMRA; forward primer 919 AAGTCCAGATGCACGTG; reverse primer 1055 CAGTCAATGACTGCTATAACTTCG.

To measure gene expression, a reaction mix was prepared on ice with TaqMan buffer, MgCl2, dATP, dCTP, dGTP, dUTP; AmpErase UNG, and AmpliTaq Gold DNA polymerase, 185 ribosome forward and reverse primers and probe (50 nM), AT1R or AT2R forward and reverse primers and probes (100 nM). The RT-PCR reaction was performed in a final volume of 50 μl in duplicate using ABI Prism 7700 (Applied Biosystems). Each RT-PCR run included a no-template control, the calibration, and patient’s complementary deoxyribonucleic acid. The thermal cycling conditions comprised an initial denaturation at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 62°C for 1 min. Data were analyzed by the Sequence Detector Version 1.5 (Applied Biosystems). Using the $2^{-ΔΔCT}$ method of relative quantification, we reported the fold change in gene expression (10).

IMMUNOFLUORESCENCE STUDIES. Paraffin section preparation: Immunofluorescence labeling for AT1R and von Willebrand factor (vWF) were performed on 5-μm-thick formalin-fixed and paraffin-embedded sections. Paraffin sections were dewaxed with xylene and hydrated with decreasing concentrations of ethanol. The sections were washed in 0.01 mol/l phosphate buffered saline (0.01 mol/l PBS, pH 7.4) and incubated in 2% bovine serum albumin for 30 min at 37°C to eliminate nonspecific staining.

Immunofluorescence stain for AT1R: The above sections were incubated with antibody to AT1R (chicken polyclonal antibodies to rat AT1R, Jackson Immuno Research Laboratories, West Grove, Pennsylvania) in 1:100 dilutions for 1 h at room temperature and additional 24 h at 4°C. The slides were then washed and incubated with FITC-conjugated anti-chicken secondary antibody (Jackson Immuno Research Laboratories).

Immunofluorescence stain for vWF: To identify the vascular endothelium within the endomyocardial biopsy section, immunofluorescence stain for vWF was performed. The sections were incubated with mouse monoclonal antibody to vWF (Dako, Carpinteria, California) in 1:100 dilutions for 1 h at room temperature and an additional 24 h at 4°C. The slides were then washed and incubated with rhodamine-conjugated anti-mice IgG secondary antibody (Jackson Immuno Research Laboratories).

Dual immunofluorescence staining for AT1R and vWF: The dual labeling was performed by sequential treatment of endomyocardial biopsy sections by primary antibodies to AT1R and vWF and their respective secondary antibodies as described above.

Image analysis: Sections were examined by LEICA DMR immunofluorescence microscope (Heidelberg, Germany) and images were captured using the Micromacs digital camera (Princeton Instruments, Trenton, New Jersey) and Image-Pro Plus software (Mediacybernautics, Silver Spring, Maryland).

IVUS. The technique of IVUS was earlier reported in detail (11). Briefly, using standard technique for intracoronary catheter delivery, the operator advances the imaging device into the coronary artery to the most distal position that could be safely reached and then retracts at a constant speed using an automated pull-back system while recording serial cross-sectional images on a super-VHS tape. Proximal, mid, and distal segments of the three major epicardial coronary arteries, defined according to Coronary Artery Surgery Study classification, were targeted for imaging (12). Matched sites were analyzed at baseline and at one year after transplantation to assess: 1) change in maximal intimal thickness (CIMIT), and 2) change in plaque volume (CPV). The threshold to define transplant vasculopathy was a CIMIT ≥0.3 mm per year (11).

Data analysis. Shape of the distribution curve of the individual variables was determined from Z-score histograms, which show variability among measurements. In normal distribution, 2.5% of the observations will be below the mean minus 2 SDs. Data were expressed as mean with SD when the variables showed a normal distribution. Data that did not follow the normal distribution were expressed as median and interquartile range (25th to 75th percentile). The Pearson correlation was used to express relationships. Univariate models were constructed to determine the association of gene expression and progression of TCAD and multivariate regression models to identify the relative predictive power of gene expression. The order of incorporation of co-variates in multiple regression models was lymphocyte AT1R, donor heart AT1R at one week and one year after transplantation, and donor heart AT2R at one year following transplantation. The Student t test was used to compare subgroups when the variables were normally distributed. The Mann-Whitney test was used to compare groups when the variables were skewed, such as levels of AT1R and AT2R. The Kruskal-Wallis test was used to compare three subgroups according to average biopsy score, where the numbers of patients were unequal and variables were skewed in distribution.

Categorical variables were compared by the Fisher exact test, and the strength of association between AT-R in lymphocytes or in donor hearts at one year after transplantation and the development of transplant vasculopathy was studied using receiver operating characteristic curves. The area under the curve was estimated using the Hanley and McNeil method (13). Differences were considered significant at p < 0.05. Because of small sample size in this exploratory study, no adjustment for the other confounders was made and no model-building strategy was adopted.

RESULTS

Patients. The mean age of the donors was 35.1 ± 12.9 years, and for recipients it was 55 ± 11.8 years. There were
19 males and 9 females among the recipients. Etiology of heart failure was ischemic cardiomyopathy in 15 (54%) and idiopathic dilated cardiomyopathy in 13 (46%) patients. Mean ischemic time was 170 ± 53 min. All patients were on triple immunosuppressive drugs including prednisone, cyclosporine, and mycophenolate mofetil. Eighteen patients were on angiotensin I-converting enzyme (ACE) inhibitors for the treatment of hypertension.

**Laboratory characteristics.** The overall average biopsy score at one year was 1.25 ± 0.50, CMIT was 0.46 ± 0.37 mm, and maximal change in plaque volume was 2.75 ± 1.99 mm². **Figure 1** shows IVUS images of a transplant coronary artery at baseline and at one-year follow-up. Expression values of AT1R mRNA and AT2R mRNA in lymphocytes derived from donor spleen (9.9, 3.1 to 23.8 fold; 0.32, 0.12 to 1.80 fold), in donor hearts at one week (1.04, 0.6 to 2.0 fold; 2.33, 1.23 to 3.84 fold), and at one year (1.5, 0.6 to 3.8 fold; 0.55, 0.78 to 1.01 fold) after transplantation were presented as the median and interquartile range (25th to 75th percentile). Levels of AT1R mRNA in lymphocytes from the donor were correlated with those in the donor heart at one week (CMIT: r = 0.52, R² = 0.27, p = 0.005; CPV: r = 0.56, R² = 0.32, p = 0.002) and at one year (CMIT: r = 0.63, R² = 0.40, p < 0.0001; CPV: r = 0.43, R² = 0.18, p = 0.004) after transplantation. Only the level of AT1R mRNA in the donor heart at one year following transplantation was associated with CMIT (r = 0.73, R² = 0.54, p < 0.0001) or CPV (CMIT: r = 0.53, R² = 0.28, p < 0.009). **Figure 2** displays regression plots between AT1R mRNA and CMIT or CPV. No relationship was observed between average biopsy score and CMIT (r = 0.05, p = 0.74) or CPV (r = 0.15, p = 0.30).

**Multivariate models.** We incorporated donor age, AT1R mRNA in donor lymphocytes, and donor heart AT1R mRNA at one week and at one year after transplantation, and donor heart AT2R mRNA at one year following transplantation in a multivariate regression model. The AT1R mRNA levels in donor lymphocytes (p = 0.04) and in donor heart at one year after transplantation (p = 0.01) were identified as independent predictors of CMIT, with a combined r value of 0.88 and combined R² of 0.80.

**Donor characteristic and Ang II receptor gene expression.** Donor age was correlated with the CMIT (r = 0.39, p = 0.03), CPV (r = 0.37, p = 0.051), and AT1R mRNA in lymphocytes (r = 0.51, p = 0.005). The cause of donor death was stroke in 11 patients and other traumatic causes in the remaining 17 subjects.

**Immunofluorescence studies.** **Figure 3** shows endomyocardial biopsy sections labeled for AT1R, vWF, and dual stain for both from a patient whose transplanted heart demonstrated increased expression of AT1R mRNA. The AT1R was expressed in vasculature and the surrounding interstitial tissue (Fig. 3a). As expected, vWF was localized to the arteriole (Fig. 3b). **Figure 3c** confirms colocalization of AT1R and vWF in the arteriole.

**Subgroup analysis.** **TRANSPLANT VASCULOPATHIC SUBGROUPS.** Fifteen (54%) heart transplant recipients developed transplant vasculopathy and were compared to the remaining 13 (44%) recipients who did not develop vasculopathy. Except for donor age, no differences existed in recipient age, gender, ischemic time, treatment with angiotensin-converting enzyme (ACE) inhibitors, positive cytomegalovirus (CMV) viremia, and rejection score.
in these groups, as shown in Table 1. Associations between AT\textsubscript{1}R mRNA or AT\textsubscript{2}R mRNA and IVUS indices of vasculopathy were adjusted to age. Expression of AT\textsubscript{1}R mRNA in lymphocytes derived from the donor spleen and in the donor hearts at one year after transplantation was significantly higher in the transplant vasculopathic group compared with the nonvasculopathic group (Table 1).

**SUBGROUPS ACCORDING TO AVERAGE BIOPSY SCORE.** Eight heart transplant recipients had low, 8 had intermediate, and 12 had high average biopsy score. No differences in CMIT (0.58 ± 0.50, 0.40 ± 0.39, 0.54 ± 0.37 mm, respectively; p = 0.25), CPV (2.9 ± 2.3, 1.7 ± 1.4, 3.2 ± 2.4 mm\(^3\), respectively; p = 0.24), development of transplant vasculopathy (5/8, 2/8, 8/12, respectively; p = 0.16), or levels of AT\textsubscript{1}R or AT\textsubscript{2}R mRNA in lymphocytes and transplanted hearts were observed among these subgroups (Table 2).

**SUBGROUPS OF HEART TRANSPLANT RECIPIENTS TREATED WITH, AND THOSE NOT TREATED WITH, ACE INHIBITORS.** Heart transplant recipients treated with (n = 18) and those not treated with (n = 10) ACE inhibitors were similar in age, ischemic time, and average biopsy score. No differences existed in the levels of AT\textsubscript{1}R mRNA, in lymphocytes (median and interquartile range: 8.5, 3.1 to 35.0 vs. 8.5, 3.2 to 22.0; p = 0.3), or in donor heart at one week (0.8, 0.5 to 1.6 vs. 1.2, 0.8 to 2.2; p = 0.1) and one year (1.0, 0.6 to 2.5

Figure 2. Regression plots showing relationship between serial angiotensin II type 1 receptor (AT\textsubscript{1}R) messenger ribonucleic acid and intravascular ultrasound indices of transplant coronary artery disease. CMIT = changes in maximal intimal thickness.
vs. 2.5, 0.6 to 3.9; p = 0.6) after transplantation in the recipients who were treated and those not treated with ACE inhibitors. Levels of AT1R mRNA in lymphocytes (0.3, 0.1 to 1.4 vs. 0.5, 0.1 to 4.3; p = 0.4) and in the donor heart at one week (2.3, 0.9 to 3.8 vs. 3.1, 2.1 to 3.9; p = 0.3) and at one year (0.3, 0.05 to 0.9 vs. 0.8, 0.4 to 1.3; p = 0.3) after transplantation were similar in these groups.

**DISCUSSION**

The novel findings of this study are as follows. First, the AT1R mRNA level in lymphocytes from the donor spleen correlated with that of the donor heart at one week or at one year after transplantation, and these levels were directly associated with the subsequent progression of TCAD. Second, the upregulation of AT1R mRNA, initially in the donor before recipient antigen was presented and subsequently in the transplanted heart, increased the risk of transplant vasculopathy in recipients. Third, the AT1R was predominantly localized to the vasculature of the transplanted heart in recipients with transplant vasculopathy. Fourth, donor heart AT2R mRNA predicted the progression of TCAD only late after transplantation. These results potentially support a role for Ang II receptors in the pathogenesis of TCAD.

Our present investigations support a novel concept that vasculopathy of the donor heart in a transplant recipient may have its origin in the donor per se and may be subsequently modulated by the recipient’s inherent biological factors. This notion has been supported by our earlier investigation, which showed that peri-transplantation ischemic injury to the myocardium correlates with the subsequent transplant vasculopathy (14). We chose to investigate Ang II receptors in lymphocytes from the donor spleen because donor spleen comprises both resident and circulating pools of lymphocytes that have a relatively long half-life and thus potentially reflect the dramatic perturbations in systemic and regional (spleen) hormonal milieu in the donor prior to organ procurement. Both heart (15) and lymphocytes isolated from the spleen (16) are known to express Ang II receptors. Few experimental and clinical studies support a possible role of RAS including Ang II receptors in the pathogenesis of TCAD.

For instance, AT1R blockade in animal models and ACE inhibition in clinical studies slowed the progression of coronary intimal thickness after heart transplantation (17,18). Both donor and recipient ACE gene polymorphism correlate with increased risk of TCAD after heart transplantation (19,20). However, our observations in this study showed that the expression of AT1R in the transplanted heart was independent of treatment with ACE inhibitors. These findings may suggest an incomplete inhibition of the conversion of Ang I to Ang II, activation of ACE-independent pathways for the generation of Ang II, or
vascular system is poorly understood, it generally opposes intriguing issues. Although the role of AT2R in the cardio-development of TCAD in this study raises important and plantation(24). Through its direct and immune modulating tion, is an important marker of prognosis following trans-

Table 1. Subgroup Analysis: Transplant Vasculopathy

<table>
<thead>
<tr>
<th>Variables</th>
<th>Vasculopathy (n = 15)</th>
<th>Nonvasculopathy (n = 13)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor age (yrs)</td>
<td>39.8 ± 12.2</td>
<td>30.4 ± 11.9</td>
<td>0.03</td>
</tr>
<tr>
<td>Donor gender (male/female)</td>
<td>7/8</td>
<td>6/7</td>
<td>0.84</td>
</tr>
<tr>
<td>Cause of donor death (trauma/other)</td>
<td>6/9</td>
<td>10/3</td>
<td>0.13</td>
</tr>
<tr>
<td>Recipient age (yrs)</td>
<td>56.9 ± 9.9</td>
<td>56.0 ± 12.2</td>
<td>0.91</td>
</tr>
<tr>
<td>Recipient gender (male/female)</td>
<td>10/5</td>
<td>9/4</td>
<td>0.80</td>
</tr>
<tr>
<td>Ischemic time (min)</td>
<td>177 ± 58</td>
<td>164 ± 48</td>
<td>0.50</td>
</tr>
<tr>
<td>ACE inhibitor (n)</td>
<td>8</td>
<td>10</td>
<td>0.75</td>
</tr>
<tr>
<td>Average biopsy score</td>
<td>1.33 ± 0.57</td>
<td>1.2 ± 0.43</td>
<td>0.45</td>
</tr>
<tr>
<td>Positive CMV status</td>
<td>5</td>
<td>3</td>
<td>0.67</td>
</tr>
<tr>
<td>Lymphocyte AT1R mRNA</td>
<td>22.0 (9.3–29.4)</td>
<td>3.5 (2.2–6.9)</td>
<td>0.004</td>
</tr>
<tr>
<td>Donor heart AT1R at 1-wk post-Tx</td>
<td>1.2 (0.6–2.1)</td>
<td>0.8 (0.5–1.8)</td>
<td>0.35</td>
</tr>
<tr>
<td>Donor heart AT1R at 1-yr post-Tx</td>
<td>2.8 (1.4–3.9)</td>
<td>0.6 (0.4–1.3)</td>
<td>0.003</td>
</tr>
<tr>
<td>Lymphocyte AT2R</td>
<td>0.20 (0.02–1.80)</td>
<td>0.52 (0.30–2.10)</td>
<td>0.22</td>
</tr>
<tr>
<td>Donor heart AT2R at 1-wk post-Tx</td>
<td>2.10 (1.20–3.90)</td>
<td>3.10 (1.30–4.30)</td>
<td>0.71</td>
</tr>
<tr>
<td>Donor heart AT2R at 1-yr post-Tx</td>
<td>0.61 (0.21–1.40)</td>
<td>0.36 (0.05–0.98)</td>
<td>0.53</td>
</tr>
</tbody>
</table>

The mRNA expressions of AT1R and AT2R were presented as median (25th to 75th interquartile range).

The mRNA expressions of AT1R and AT2R were presented as median (25th to 75th interquartile range).

ACE = angiotensin-converting enzyme; AT1R and AT2R = angiotensin II type 1 and 2 receptor; CMV = cytomegalovirus; mRNA = messenger ribonucleic acid; post-Tx = post-transplantation; wk = week.

gradual reactivation of ACE activity over time with the use of ACE inhibitors (21). Consistent with these observations, AT1R blockers as add-on treatment to ACE inhibitor-containing regimen produce additional clinical benefit (22). These data support our finding that AT1R expression was significantly correlated with IVUS indices of TCAD regardless of ACE inhibitor treatment.

Transplant coronary artery disease is characterized by progressive increase in intimal thickness, especially within the first year following transplantation (23). The severity of TCAD, measured as intimal thickness by IVUS examination, is an important marker of prognosis following transplantation (24). Through its direct and immune modulating effects, Ang II might play an important role in the pathophysiology of TCAD. Ang II promotes cellular growth/apoptosis, fibrosis, inflammation, and extracellular matrix remodeling, all of which have implications in the pathogenesis of vascular disease (25). The vast majority of these effects are mediated through AT1R (25), the number of which may define the biological efficacy of Ang II (26).

An association between cardiac AT2R mRNA and the development of TCAD in this study raises important and intriguing issues. Although the role of AT2R in the cardiovascular system is poorly understood, it generally opposes the AT1R-mediated effects on the cardiovascular system. However, recent reports support a role of AT2R in vascular remodeling, apoptosis, and cardiac hypertrophy independent of its mere antagonistic interaction with AT1R (15,27). The increase in cardiac AT2R mRNA transcripts with increasing CMIT and plaque volume in transplant coronary arteries in the present investigation may imply a positive role of AT2R in the pathogenesis of TCAD or may be an overexpression to antagonize the biological effects of AT1R. The role of immune mechanisms in the progression of TCAD is not completely understood. Evidence suggests that the incidence of TCAD has, indeed, increased following introduction of cyclosporine (28). Moreover, reports investigating an association between allograft rejection and TCAD produced conflicting results (23,29,30). Our findings support earlier reports suggesting no association between graft rejection and the development of TCAD (23,29).

Some recent studies suggest that RAS may both stimulate and be stimulated by alloimmune responses (31). Immune cells synthesize Ang II and express Ang II receptors (32, 33). In a cardiac transplant model, Nataraj et al. (16) elucidated a molecular mechanism for the RAS-mediated regulation of cellular immune response and suggested that

Table 2. Angiotensin II Receptor Expression in Subgroups According to Average Biopsy Score

<table>
<thead>
<tr>
<th>Variables</th>
<th>Low (n = 8)</th>
<th>Intermediate (n = 8)</th>
<th>High (n = 12)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1R mRNA (fold)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor lymphocyte (spleen)</td>
<td>9.9 (4.1–23.4)</td>
<td>3.7 (2.1–17.5)</td>
<td>16.7 (3.3–28.6)</td>
<td>0.47</td>
</tr>
<tr>
<td>Donor heart at 1-week post-Tx</td>
<td>1.6 (0.9–1.8)</td>
<td>0.74 (0.3–1.5)</td>
<td>0.9 (0.5–2.3)</td>
<td>0.27</td>
</tr>
<tr>
<td>Donor heart at 1-yr post-Tx</td>
<td>0.7 (0.1–2.6)</td>
<td>1.2 (0.6–3.6)</td>
<td>1.2 (0.9–3.6)</td>
<td>0.60</td>
</tr>
<tr>
<td>AT2R mRNA (fold)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor lymphocyte (spleen)</td>
<td>0.4 (0.2–1.2)</td>
<td>1.4 (0.06–3.7)</td>
<td>0.32 (0.05–1.8)</td>
<td>0.97</td>
</tr>
<tr>
<td>Donor heart at 1-week post-Tx</td>
<td>2.3 (0.9–3.5)</td>
<td>2.7 (1.4–3.1)</td>
<td>2.9 (1.3–4.2)</td>
<td>0.78</td>
</tr>
<tr>
<td>Donor heart at 1-yr post-Tx</td>
<td>0.5 (0.1–0.6)</td>
<td>0.9 (0.2–1.5)</td>
<td>0.3 (0.1–0.9)</td>
<td>0.66</td>
</tr>
</tbody>
</table>

The mRNA expressions of AT1R and AT2R were presented as median (25th to 75th interquartile range).

Abbreviations as in Table 1.
Ang II may function as an autocrine factor for promoting T-cell proliferation. Consistent with an interaction between immune mechanism and RAS, treatment with immunosuppressive drugs, including cyclosporine A, alleviates Ang II-induced organ damage (34,35). Moreover, AT1R blockade reduces the risk of chronic rejection in animal transplant models (36).

This study has several limitations. The expression of Ang II receptors at the mRNA level would not provide information about protein receptor density, and the small amount of cardiac biopsy precluded assessment of these receptors at the protein level. Finally, our results are based on correlation and regression analyses, which do not establish a causal relationship between Ang II receptors and TCAD.

Conclusions. To our knowledge, this is the first human study demonstrating upregulation of AT1R in the donor and in the donor heart after transplantation in recipients who have developed transplant vasculopathy. We speculate that increased AT1R levels in the transplanted heart in recipients who prospectively developed TCAD may represent remnants of increased systemic and regional expression of these receptors in the donor prior to transplantation. Accordingly, the levels of AT1R mRNA in the donor lymphocytes were correlated with those of the transplanted heart and were identified as independent predictors for the progression of TCAD in recipients. The data would also suggest that AT1R might play a role in transplant vasculopathy late after transplantation. These findings support a role for Ang II receptors in the pathogenesis of transplant vasculopathy. We hope that our data will prompt studies aimed at evaluating donor and recipient characteristics as a continuum to elucidate the precise role of Ang II receptors or other biological molecules in the pathogenesis of TCAD.

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