Role of β3 Integrin in Acute Renal Allograft Rejection in Humans

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Background and objectives: β3 Integrin may play a role in the process of acute rejection by increasing leukocyte adhesion to the endothelium, cytotoxic T lymphocyte activation, and platelet aggregation.

Results: Although almost all monocytes express β3 integrin, its expression was also found on all leukocyte subsets, including T, B, and NK cells. The percentage of patients who experienced acute rejection was noted to be significantly higher in those with PI3/Pl3 (TT) genotype versus patients with the Pl3/Pl3 or Pl3/Pl1 (CT or CC) genotypes (33% for TT versus 20% for CT or CC). In a multivariate analysis, the Pl3/Pl3 (TT) genotype remained significantly associated with acute rejection. Patients with Pl3/Pl1 (TT) genotype also exhibited a higher number of acute rejection episodes per patient.

Conclusions: The PI3/Pl3 (TT) genotype is associated with an increased incidence of acute renal allograft rejection in humans, supporting a role for β3 integrin in the pathophysiology of acute rejection.

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eukocyte extravasation is a multistep process that begins with leukocytes rolling along the vascular endothelium. Conversion of rolling to firm adherence largely depends on the activation of leukocyte integrins. Integrins are a family of α/β heterodimeric membrane proteins involved in cell–extracellular matrix and cell–cell interactions (1). To date, 18 α subunits and eight β subunits, forming 24 different integrin αβ pairs, have been reported in mammals. The β3 subunit is shared in the two integrin molecules αVβ3 and αIIbβ3 (2). αVβ3 and αIIbβ3 integrins recognize ligands with arginine-glycine-aspartic acid (RGD) sequences such as vitronectin, fibronectin, fibrinogen, and von Willebrand factor (2).

A growing body of evidence demonstrates the potential role of β3 integrin in the process of allograft rejection. An increase in the expression of αVβ3 is shown in human samples of rejecting heart allografts (3,4). In a recent study (5) that used β3 knockout mice as recipients for vascularized cardiac allografts, we showed that lack of β3 integrin significantly inhibited acute transplant rejection and chronic rejection when combined with blockade of other integrins.

The mechanism by which β3 integrin is involved in transplant rejection is not fully understood but may relate to the tight adhesion of mononuclear cells to the endothelium and subsequent transendothelial migration, which is mediated by αVβ3 integrin (6). Furthermore, β3 integrin binding enhances cytotoxic T cell activity (7). Cytotoxic T lymphocytes were reported to degranulate efficiently upon exposure to MHC-bound peptide after a β3 integrin/fibronectin–mediated adhesion to endothelium. The importance of β3 in transplant rejection could also be exerted through the role of αIIbβ3 in regulating platelet function, because the accumulation of platelets and fibrin in rejecting renal transplants has been demonstrated directly by labeling autologous platelets labeled with indium-111 and indirectly in histologic studies (8,9). The αIIbβ3 integrin (also called platelet glycoprotein IIb/IIIa) is the most abundant integrin on the platelet surface and plays an important role in platelet activation and aggregation (1). Because platelets are known to release inflammatory cytokines on activation, it is likely that this contributes to inflammation of the transplant organ and rejection.

A single nucleotide substitution T → C in the protein that
encodes part of the ITGB3 gene (exon 2, 1565 T/C) of the β3 integrin subunit results in a leucine (PlA1 isoform) to proline (PlA2 isoform) substitution at amino acid 33 of the mature protein (10). This genetic variant has been found to affect functionally the platelet response to various agonists in vitro (11). The PlA polymorphism has been associated with a higher incidence of acute coronary syndromes as well as with a higher rate of restenosis after angioplasty (12). Salido et al. (13) demonstrated that the incidence of acute renal transplant rejection was influenced by the PlA genotypic variants of the recipients. In an attempt to assess the impact of the ITGB3 gene in a larger, racially diverse, multicenter cohort with more current immunosuppressive regimens, we examined the impact of PlA gene polymorphism on the incidence and frequency of acute renal allograft rejection.

Concise Methods

Study Participants

We carried out a cross-sectional study involving 424 renal transplant recipients. A total of 135 of these patients received a transplant at Brigham and Women’s Hospital (BWH), and the remaining 289 patients received a transplant at the University of Alabama at Birmingham (UAB). All of these patients were treated with a calcineurin inhibitor. They had a median follow up of 30 mo. Except for a few patients who were followed for the minimum of 6 mo, almost all patients were followed for a minimum of 1 yr. The range of follow-up was 6 mo to 5 years. Institutional review board approval was obtained at both institutions. After informed consent was granted, blood samples were collected from hospitalized inpatients or patients who presented to the outpatient clinic. The following information was obtained from the medical chart: Date of transplantation, age, gender, race of recipient, type of donor (deceased donor or living), cold ischemia time, time to

Figure 1. Surface expression of β3 integrin (CD61) on leukocyte subsets. (A) Representative FACS plots from a single blood donor of size-gated, live, unstimulated lymphocytes and monocytes stained with the indicated antibodies. Number is percentage of leukocyte subset that is positive for CD61. (B) Mean percentage of leukocyte subset indicated expressing more CD61 than isotype control ± SEM for five (CD4 and CD14) or 11 donors (CD8, CD19, and CD56). Note that nearly 100% of monocytes express β3 integrin. (C). Median expression level of CD61 fluorescence in dual-positive cell subset (top right quadrant in A) ± SEM for five (CD4 and CD14) or 11 donors (CD8, CD19, and CD56).
initial graft function, number of HLA mismatches, number of acute rejection episodes, immunosuppressive therapy (cyclosporine versus tacrolimus), and serum creatinine. Acute rejection was defined either histologically (29.8% of cases) or clinically (an acute rise in serum creatinine that was subsequently ameliorated by antirejection therapy). Acute rejection episodes were initially treated with intravenous steriods, and steroid-resistant rejection was treated with OKT3 or ATG. Immunosuppression consisted of a calcineurin inhibitor (>93% of the patients), steriods, and mycophenolate mofetil. Humanized anti-IL-2 receptor antibody (Daclizumab; Hoffman LaRoche, Nutley, NJ) and Thymoglobulin was used as an induction therapy in 20% of cases.

### Determination of ITGB3 Genotypes

DNA extraction was performed as described previously (14). Previously published primers (sense 5’-CTTAGCTATGGAAGTGG-TAGG-3’ and antisense 5’-ACTGACTTGAGCTCGGGAG-3’) were used specifically to amplify a fragment of intron 1 and exon 2 of the ITGB3 gene, followed by restriction enzyme digestion with MspI (New England Biolabs, Ipswich, MA). PCR was performed in diluted 1 x master mix with 2.0 mM MgCl2, 0.175 mM dNTP, 0.3 μM of each primer, and 1 U of Platinum TaqDNA polymerase (Invitrogen, Carlsbad, CA) added. A thermal cycler (Eppendorf Mastercycler) was used to institute an initial denaturation of 95°C for 3 min followed by 35 cycles of 95°C for 1 min, 62°C for 1 min, and 72°C for 1 min and a final extension of 72°C for 10 min. Our PCR reactions yield products of 256 bp. Five microilters of the amplification product was digested. The PlA1 allele (Leu33) remained as a 256-bp band, whereas the PlA2 allele (Pro33) yielded two bands of 154 and 102 bp after MspI digestion.

### FACS Analysis

FACS was performed on a FACScan instrument (BD Biosciences, San Diego, CA) using freshly isolated peripheral blood mononuclear cells (PBMC) from healthy donors. PBMC were stained with FITC-labeled anti-CD61 murine mAb obtained from Accurate Chemical (Westbury, NY) and leukocyte subtyping mAb and isotype control antibodies from BD. Lymphocytes and monocytes were gated by size, and only live cells were analyzed on the basis of a lack of propidium iodide staining.

### Statistical Analyses

In our analysis, patient genotypes were used as independent categorical variables in analyzing recent serum creatinine (continuous variable), presence or absence of rejection (binomial variable), and number of acute rejection episodes (ordinal variable). Nominal logistic fit was used to assess the association of PLA genotypes with the incidence of acute rejection. Those that were significantly associated with risk for acute rejection were then used as covariates in a multivariate regression model that included the PlA allele (Leu33) as a 256-bp band, whereas the PlA2 allele (Pro33) yielded two bands of 154 and 102 bp after MspI digestion.

### Results

**β3 Integrin Is Constitutively Expressed on Leukocytes**

Despite growing evidence for an important role of β3 integrin in allograft rejection, surprisingly little is known about the surface expression of β3 integrin on specific leukocyte subsets. To understand more fully which leukocytes normally express β3 integrin (CD61) on their cell surface, we performed FACS analysis on healthy volunteers using freshly isolated PBMC stained with specific directly labeled mAb and isotype controls (Figure 1A). The expression of β3 integrin (CD61) is shown by percentage of expressing cells and median fluorescence intensity (Figure 1, B and C, respectively). Although all leukocyte subsets express β3 integrin, monocytes express the most. Figure 1C shows that whereas between 5 and 20% of all lymphocyte subclasses (CD4+, CD8+, CD19+, and CD56+) had low-level expression (median fluorescence intensity of approximately 30), nearly 100% of CD14+ monocytes had almost 10-fold higher levels of expression.

### Renal Transplant Recipient Demographics and Genotype Frequencies

Of 424 renal allograft recipients studied, 55% were male and 68% were white. Compared with the BWH, the UAB cohort consists of a much higher number of black patients (Table 1). As shown in Table 2, 41% of the allografts were from living donors. Thirteen percent of recipients experienced DGF that required post-transplantation hemodialysis. The incidence of acute rejection was 29%, and 6.2% of the patients had more than one rejection episode. As shown in Table 3, 333 (75%) patients were homozygous for PlA1/PlA1 (TT), whereas 101 (23%) patients were heterozygous (CT). Only 11 (2%) patients were homozygous for PlA1/PlA2 (CC). The aforementioned gene frequencies were similar to those previously reported and were in agreement with those predicted by Hardy-Weinberg equilibrium (11,15). Seventy-five percent of white and 74% of black patients were homozygous for the PlA1/PlA1 (TT) genotype.

### PIA Gene Polymorphism Is Associated with the Risk for Acute Rejection

The association between the PlA1 genotype and the incidence of renal allograft rejection was studied in univariate and multivariate analyses to address the impact of confounding risk factors. Given that almost 93% of patients were treated with a calcineurin inhibitor, we included only these patients in our analysis. A total of 19% of patients were treated with tacrolimus. These risk factors included HLA-A, B, and DR mismatches; living versus cadaveric donors; DGF; PRA; black race; immunosuppressive therapy (cyclosporine versus tacrolimus); and induction therapy and were chosen because they were previously shown to be associated with higher risk for acute allograft rejection. Those that were significantly associated with risk for rejection were then used as covariates in a multivariate regression model that included the PlA1 genotype. Examining the data

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BWH</th>
<th>UAB</th>
</tr>
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<tbody>
<tr>
<td>Male (%)</td>
<td>50</td>
<td>57</td>
</tr>
<tr>
<td>Age (yr; mean ± SD)</td>
<td>47 ± 11</td>
<td>44 ± 12</td>
</tr>
<tr>
<td>White (%)</td>
<td>83</td>
<td>61</td>
</tr>
<tr>
<td>Black (%)</td>
<td>16</td>
<td>38</td>
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</tbody>
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*BWH, Brigham and Women’s Hospital; UAB, University of Alabama.
from the BWH patients, HLA-A (P = 0.02) and HLA-B mismatch (P = 0.002), black race (P = 0.06), and PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) genotype (P = 0.04) were significantly associated with higher risk for acute allograft rejection, whereas PRA, induction therapy, DGF, and donor source were not. In the multivariate analysis, the PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) genotype (odds ratio [OR] 3.4; P = 0.04) and HLA-B mismatches (OR 9.3; P = 0.01) remained significantly associated with the incidence of acute rejection. An analysis of the UAB patients showed an association between black race and higher incidence of acute rejection (P = 0.03) but no association with HLA-A mismatch (P = 0.5) or HLA-B mismatch (P = 0.1). Although there was a trend toward higher risk for acute rejection in the PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) group, it did not reach statistical significance (OR 1.7; P = 0.16). Furthermore, no difference was found when the analysis was performed using tacrolimus or cyclosporine as an independent variable.

A univariate analysis on the combined cohort showed that black race (P = 0.09), PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) genotype (P = 0.01), HLA-A mismatch (P = 0.56), and HLA-B mismatch (P = 0.002) were again associated with higher incidence of acute rejection. In a multivariate analysis that incorporated these risk factors, HLA-B mismatch (OR 2.4; P = 0.01) and PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) genotype (OR 2.1; P = 0.01) were associated with higher risk for acute rejection, whereas no influence of HLA-A mismatch (P = 0.80) and black race (P = 0.19) was noted on the incidence of acute rejection (Table 4). The association between the PI\textsuperscript{A1} genotype and the incidence of renal allograft rejection of the combined BWH and UAB cohorts is shown in Figure 2. The percentage of patients who experienced acute rejection was noted to be significantly higher in those with PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) genotype versus patients with the PI\textsuperscript{A1}/PI\textsuperscript{A2} or PI\textsuperscript{A2}/PI\textsuperscript{A2} (CT or CC) genotypes (33% for TT versus 20% for CT or CC; OR 1.95; 95% CI 1.134 to 3.364; P = 0.01). We also studied the impact of PI\textsuperscript{A1}/PI\textsuperscript{A2} or PI\textsuperscript{A2}/PI\textsuperscript{A2} (CT or CC) genotypes on the incidence of acute rejections in patients with biopsy-proven rejection in our whole cohort. Our analysis of these patients showed PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) genotype (OR 1.86; P = 0.03) was associated with higher risk for acute rejection.

We also analyzed the impact of these covariates on the incidence of acute rejection within the subgroups of black and white patients. There was no influence on the incidence of acute rejection of any of the covariates when only the black patients were considered; however, when the same analysis was performed for the white subgroup, the PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) genotype (P = 0.018) and HLA-B mismatch (P = 0.001) were still noted to be associated with higher risk for acute rejection. In a multivariate analysis of white patients from the combined BWH and UAB cohorts, HLA-B mismatch (OR 3.7; P = 0.004) and PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) genotype were associated with a higher risk for acute rejection (OR 2.4; P = 0.02).

### Table 2. Transplant data

<table>
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<th>Parameter</th>
<th>%</th>
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<tr>
<td>Calcineurin inhibitors</td>
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<tr>
<td>Delayed graft function</td>
<td>13.1</td>
</tr>
<tr>
<td>No. of rejection episodes</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>70.8</td>
</tr>
<tr>
<td>1</td>
<td>23.0</td>
</tr>
<tr>
<td>&gt;1</td>
<td>6.2</td>
</tr>
<tr>
<td>Previous transplant</td>
<td>7.5</td>
</tr>
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<td>Living donors</td>
<td>41.0</td>
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</tbody>
</table>

### Table 3. Genotype frequencies of PLA

<table>
<thead>
<tr>
<th>PLA Genotype</th>
<th>Frequency (%)</th>
<th>Expected (n)</th>
<th>Observed (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT)</td>
<td>75</td>
<td>330</td>
<td>333</td>
</tr>
<tr>
<td>PI\textsuperscript{A1}/PI\textsuperscript{A2} (CT)</td>
<td>23</td>
<td>106</td>
<td>101</td>
</tr>
<tr>
<td>PI\textsuperscript{A2}/PI\textsuperscript{A2} (CC)</td>
<td>2</td>
<td>9</td>
<td>11</td>
</tr>
</tbody>
</table>

### Table 4. Multivariate risk estimates for association with acute rejection

<table>
<thead>
<tr>
<th>Variable</th>
<th>Odds Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-A mismatch</td>
<td>0.90</td>
<td>0.80</td>
</tr>
<tr>
<td>HLA-B mismatch</td>
<td>2.40</td>
<td>0.01</td>
</tr>
<tr>
<td>TT (reference group CT/CC)</td>
<td>2.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Black</td>
<td>1.37</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Figure 2. Percentage of patients who experienced acute rejection by PI\textsuperscript{A1} genotype. Note an almost two-fold increase in the incidence of acute rejection (%) in the PI\textsuperscript{A1}/PI\textsuperscript{A1} (TT) group versus the PI\textsuperscript{A1}/PI\textsuperscript{A2} (CT) or PI\textsuperscript{A2}/PI\textsuperscript{A2} (CC) group.
Lack of Association of Genotypes with Long-Term Renal Allograft Function

We tested the impact of the PLA gene polymorphism on renal function. In a univariate analysis, no association was found between the most recent serum creatinine level and the genotypic variants of PLA. Creatinine clearance as estimated by the Cockcroft-Gault formula was 77.31 ± 4.4 ml/min for the TT and 75.6 ± 3.2 for CT and CC genotypes, respectively (P = 0.3). Although no association was found between the genotypic variant and long-term renal allograft survival, there was an association between acute rejection and graft survival. Patients with graft loss had a significantly higher rate of acute rejection (P < 0.001).

Discussion

Our findings support β3 integrin as a clinically relevant molecule in renal transplant rejection. We were able to demonstrate low-level expression of CD61 on small percentages of lymphocytes of various lineages and much higher expression in nearly all monocytes from normal blood donors, indicating constitutive expression in a variety of leukocytes. We also demonstrate that the risk for experiencing acute rejection was significantly higher in renal allograft recipients who had the PLA1/PLA1 (TT) genotype of β3 integrin (OR 2.2; 95% CI 1.3 to 3.8; P = 0.003). In addition, patients with this genotype exhibited a higher number of acute rejections than the combined group of PLA1/PLA2 (CT) heterozygotes and PLA1/PLA2 (CC) homozygotes (0.43 versus 0.19; P = 0.002). Finally, by multivariate analysis, the PLA1/PLA1 (TT) genotype remained significantly associated with acute rejection risk when known risk factors and race were considered (OR 2.2; 95% CI 1.3 to 4.0; P = 0.008). It is interesting that the association was principally seen in white recipients despite a similar frequency and power in black patients. This is an unexpected finding that may be explained by a genetic background effect. This observation may explain the higher OR for developing acute rejection in patients with the TT genotype for the BWH cohort, which consisted of higher numbers of white patients than the UAB cohort, which had larger numbers of black patients. These human data are also supported by our recent mouse study showing that compared with wild type, heart allograft survival is significantly prolonged in β3 integrin−/− recipients and was associated with reduced T cell infiltration into grafts (5). These data highlight the functionality of this gene polymorphism.

The mechanism behind these findings could be the importance of β3 integrin in increased monocyte adhesion to the endothelium, cytotoxic T lymphocyte activation, and platelet activity. Our finding of significant and nearly universal expression on fresh monocytes indicates a need for future investigation of the effect of this polymorphism on monocyte function.

In contrast to our results, Salido et al. (13) previously showed an association between the PLA1/PLA allele and a higher incidence of acute rejection. Their cohort of 119 patients was smaller, with a much higher incidence of acute rejection (43%) than our study, which may have reflected use of a less potent immunosuppressive protocol. Our study was undertaken in a racially diverse population that included a significant proportion (29%) of black patients and had more intense immunosuppression. The association noted in the Salido study was thought to result from patients with the PLA2 genotype as having greater platelet/coagulative system activity and a higher incidence of thrombotic events. It is interesting that the difference noted between these two studies has similarly been described in other reports that examined the importance of the PLA1 polymorphism in patients with ischemic heart disease with different demographics and treatment protocols (12,15–17).

The importance of the PLA1 gene polymorphism could vary according to the specific nature of the disease or organ system, as well as patient demographics. In this regard, Mikkelsson et al. (12) reported that whereas PLA1/PLA gene polymorphism is more prone to early atherosclerosis and more rapid progression of stable coronary artery disease, the PLA2 allele carries higher risk for thrombotic complications. The differential interaction of αVβ3 with endothelium versus smooth muscle cells has been thought to contribute to these differences. It is possible that the same pathogenetic mechanisms are applicable in the transplant population. Thus, the influence of PLA1 gene polymorphism in the pathogenesis of acute rejection may depend on the pathophysiology of acute rejection, along with heterogeneity in patient population and treatment protocols. Further studies will be needed to elucidate the functional linking of αVβ3 integrin and PLA polymorphisms with the process of transplant rejection.

Our study has strengths and limitations. This is a relatively large study of transplant recipients with a considerable amount of clinical information allowing multivariate analysis of the association. Also, the study was conducted using phenotype definitions blinded to genotype and vice versa. We should emphasize that this is a retrospective, cross-sectional study in which the end point of acute rejection was defined histologically in only one third of the cases. The remainders were defined clinically, which can occasionally yield false-positive results; however, these would be expected to be randomly distributed in the groups. Although we found no association with long-term renal function, we were also somewhat limited in our ability to assess this during and immediately after the acute rejection episodes, and any effect of genotype would be confounded by such clinical factors as the rapidity, intensity, and efficacy of immunosuppression. As with any genetic association, it is also possible that this association is not due to this polymorphism but instead may be related to closely genetically linked variants that are the true functional variant. This possibility is decreased by multiple in vitro studies that demonstrated that this variant is functional and by our own recent data indicating a role for this gene in murine transplant rejection. Finally, although the number of statistical comparisons was limited, we did not correct the data for multiple comparisons and it is possible that the association noted here is due to chance and therefore replication in larger studies will be necessary.

This study not only highlights the need to examine the significance of αVβ3 integrin but also indicates the importance of platelets in the process of acute rejection. The accumulation of platelets and fibrin in rejecting renal transplants and their importance in acute rejection has long been known; however, compared with lymphocytes, they have received little attention (8). Xu et al. (18) showed that platelet-derived CD154 plays an important role in acute rejection and that targeting CD40/
CD154 results in significant prolongation of cardiac allograft survival in mice. Humanized anti-CD154 trials resulted in significant thromboembolic events and the cessation of further drug studies for the present time (19).

Conclusions
It is still too early to recommend pretransplantation genotyping or modulation of immunosuppressive therapies on the basis of PI^A polymorphisms. Nonetheless, we conclude that PI^A polymorphisms may influence the incidence of acute renal allograft rejection. Elucidation of how the aVb3 and aIIb3 molecules function in rejection may identify a novel target for new immunosuppressants and prognostic markers.

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Disclosures
None.

References