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TGF- β POLYMORPHISMS AND CARDIAC ALLOGRAFT REJECTION¹

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Abstract

Background—Cytokine gene polymorphisms regulate cytokine expression. We analyzed TGF- β allelic variation in codon 25 in susceptibility to acute rejection episodes in cardiac transplant recipients.

Methods—Between June 1997 and December 2001, 123 *de novo* heart transplants were performed at UAB with analysis based on 109 patients. Clinical and laboratory data were recorded at intervals up to 1 year post transplant. Recipient genotypes for TGF- β (codon 25) were determined using PCR sequence specific primers. Correlations between TGF- β genotypes and acute rejection were made using Kaplan-Meier plots and parametric hazard models.

Results—Of those enrolled, 72% had ≥ 1 rejection and 46% had multiple rejections in the first year post transplant. Among those ≥ 55 years of age at transplant, patients with the GG genotype had significantly fewer rejections as compared to those with the CC or GC genotype (1.25 vs 2.5, $p < 0.01$). There was no difference in the risk of rejection between the genotype groups among patients < 50 years of age at transplant ($p=0.43$). Similar results were observed when we used time to cumulative 2R or greater rejection as the outcome.

Conclusion—The GG TGF- β genotype may protect against acute rejection in older recipients during the first year post transplantation.

Keywords

Polymorphism; TGF; cardiac rejection

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Cardiac allograft rejection is a longstanding and unsolved complication of heart transplantation. While advances in pharmacology have decreased the mortality associated with rejection, generalized immunosuppression is itself a significant morbidity factor post-transplant. In addition, the only consistent and time proven method for detecting acute rejection remains the endomyocardial biopsy (1,²). This procedure is invasive and has a well documented set of associated morbidities. Thus, an important research goal is the identification of demographic, genetic or environmental factors predicting risk for rejection thereby allowing physicians to tailor immunosuppressant regimens and limit performance of biopsies to those at highest risk of rejection. (1).

During the first months post cardiac transplantation, almost 40% of adults will have one or more rejection episodes with most experiencing at least one episode of rejection in the first six months (2). It is clear that some donor and recipient demographic and clinical characteristics place the recipient at higher risk of initial and cumulative rejections at various time points after transplant (2). Despite these clinical risk factors, our ability to predict rejection is still limited, meaning that other mechanisms underlying the pathophysiology of acute rejection must be explored and understood in order to improve predictive measures of rejection risk.

TGF- β is a key cytokine important in acute inflammation, and the regulation of atherogenesis (4,⁵). Accordingly, TGF- β may play a central role in both the initiation and propagation of acute and chronic rejection in the cardiac allograft (4). Circulating TGF- β levels are determined by genetics (4) via a polymorphism in codon 25 (G915C) of the gene. Interestingly, those homozygous for the *G* allele produce twice the concentration of the TGF- β than *C* allele carriers (5). In light of this polymorphism's regulatory role and TGF- β 's putative relationship to the acute and chronic inflammatory responses thought to initiate rejection, we hypothesize that this polymorphism may affect the relative risk for rejection in recipients homozygous for the *G* allele.

The objective of our study was therefore to determine the relationship between this polymorphism and the risk for acute rejection in patients undergoing *de novo* cardiac transplants. Additionally, as the majority of heart transplantations are performed on individuals between the ages of 55 and 64 years our secondary goal was to determine whether there was a genotype and recipient age interaction that may impact the risk of rejection (6). Such knowledge could reduce the mortality and morbidity associated with rejection, both through correct tailoring of immunosuppressant therapy to recipient risk and by restricting the use of invasive procedures to those at highest risk.

MATERIALS AND METHODS

Patients

Between June 1997 and December 2001, 123 *de novo* heart transplants were performed at UAB. 108 of these individuals were prospectively enrolled in our study and provided complete information on the TGF- β genotype and other important covariates of interest. This study was approved by the Institutional Review Board at UAB.

Endomyocardial Biopsy

Routine biopsies were obtained weekly for the first six weeks. Subsequently, biopsies were obtained every 30 days until month 5; then every 3 months until 2 years; and then yearly. Biopsies were graded originally based on the 1990 ISHLT criteria and reclassified using the 2005 criterion for the present analyses. Only clinically relevant rejections that resulted in treatment changes or therapy were used for analysis.

Demographics and Data Collection

Demographic information was prospectively collected on donors and recipients through chart review and from data extracted from the Cardiac Transplant Research Database (CTRD). This included information regarding donor and recipient age, race, and gender. Clinical variables recorded included a detailed history of immunosuppressant usage, including type, dose, level, and duration of use; rejection history including total number of treated rejections as well as the severity and timing of each rejection; number of HLA mismatches; use of OKT-3 induction; and use of a ventricular assist device. Blood samples were collected from both the donor and recipient for genotyping after informed consent.

Genotyping

DNA was extracted from whole blood using standard methods. Single-nucleotide polymorphisms in the TGF- β codon 25 were PCR-amplified using sequence-specific primers (PCR-SSP) capable of differentiating the G915C polymorphism as described by Densem et al (7).

Statistical Plan

The summary demographic and clinical variables of transplant recipients with the TGF- β *GG* versus either the *GC* or *CC* genotypes were compared using the chi-square or Fisher's exact test for dichotomous variables and Student's t-test for continuous variables.

Cumulative rejection plots and parametric analyses were used to assess differences between genotype groups with respect to the risk of rejection during the first year post-transplant. Multivariable analyses in the hazard function domain with backwards selection were used to control for known risk factors for rejection. The full model of interest included indicator variables for TGF- β recipient genotype group (*GG* vs. *GC/CC*), median age of recipient at time of transplant (<55 or ≥ 55 years), female donor, female recipient, African-American donor, African-American recipient, positive CMV serology, and OKT-3 induction and use of a left ventricular assist device. To examine whether any of the other risk factors modified the effect of the genotype on the risk of rejection, we also included any two-way interactions with the genotype groups provided that the cross-classification between genotype groups and the factor of interest had a minimum of five in each cell. This led to the inclusion of two-way interaction terms between the genotype groups and age, female recipient, OKT-3 induction, and use of a ventricular assist device in the full model. With the exception that we did not allow the removal of main effects terms for any factor until the interaction term involving that factor had been removed, at each step, the term with the highest p-value (provided it was greater than 0.10) was removed from the model and the model was refit with all remaining terms. This process was continued until no remaining terms could be removed. All statistical analyses were performed using SAS version 9.1.

RESULTS

Of the 108 patients enrolled in this study, 77 (71%) had at least one treated rejection, 49 (45%) had ≥ 1 treated rejection with a total of 166 rejections observed during the first year post-transplant. Of these rejections, 52 (31%) were classified as 1R and 99 (60%) as 2R or greater. There were 2 additional treated rejections with a grade of zero and 13 for which the classification was unknown. All 15 of these treated rejections were associated with hemodynamic compromise determined clinically and confirmed by echocardiography and/or hemodynamics. Eight patients died during the first year. All but one died after having at least one rejection.

Demographic and Genotype Correlations

Table 1 summarizes the demographic and clinical variables. The use of immunosuppressants was fairly uniform in this population, with the majority of patients receiving Cyclosporine, Mycophenolate, and statins at some point during the first year post-transplant. All patients received prednisone, which were tapered according to protocol to a final dose of 10mg orally every other day by year one. 18 patients (17%) received left ventricular assist devices (LVADS) as a bridge to transplant. Table 2 summarizes these variables by TGF- β recipient genotype status. There were no significant differences between genotype groups with respect to any of the variables of interest.

Rejection and Genotype Correlations

Figure 1 shows a plot of the mean cumulative treated rejections during the first year for all 108 patients in the study. By the end of the third month, the patients in this sample had suffered one treated rejection episode on average. The average number of treated rejections increased to slightly more than 1.5 treated rejections by the end of the first year.

Figure 2 shows separate plots of the mean cumulative treated rejections during the first year by recipient TGF- β genotype. This plot suggests that patients with the GG genotype had fewer treated rejections as compared to those with the CC or GC genotype (mean of 1.25 vs. 2.0 treated rejections during the first year, $p = 0.05$).

To further address this perceived trend, we fit a multivariable parametric model in the hazard domain using backwards selection as described above. The final multivariable model included donor and recipient race, as well as a significant interaction between the TGF- β recipient genotype group and the age at the time of transplant on the risk of treated rejection (Table 3, $p < 0.01$). African-American recipients had a marginally significant increased risk of rejection ($p = 0.06$), while patients with transplants from African-American donors had a significant decreased risk of rejection ($p = 0.04$). Figures 3A and 3B show the mean cumulative treated rejections between the TGF-Beta recipient CC/GC genotype (versus the GG genotype), separately by age at the time of transplant. Among those patients ≥ 55 years of age at the time of transplant, patients with the GG genotype had significantly fewer treated rejections as compared to those with the CC or GC genotype (mean of 1.0 vs. 2.5 treated rejections during the first year, $p < 0.01$). In contrast, there was no difference in the risk of treated rejection between the genotype groups among patients < 55 years of age at the time of transplant ($p = 0.40$). This suggests that in the age group most commonly utilizing cardiac transplantation as a treatment modality for end stage heart disease the presence of the CC or GC genotype is a potential risk factor for rejection.

Secondary Analysis

In order to assess whether the significant effects were observed among those rejections of greatest concern, we refit the final multivariable model using time to grade 2R or 3R rejection as the outcome (as opposed to time to any treated rejection). Of the 108 patients enrolled in this study, 59 (55%) had ≥ 1 2R/3R rejection during the first year.

The interaction between the TGF- β recipient genotype and transplant age was confirmed using this subset ($p = 0.01$). Figure 4A shows that, among those patients ≥ 55 years of age at the time of transplant, patients with the GG genotype had a significantly lower risk of serious rejection as compared to those with the CC or GC genotypes (mean of 0.75 vs. 1.5 rejections during the first year, $p = 0.02$). Similar results were observed when we refit the final multivariable model using cumulative 2R/3R rejections or rejection with hemodynamic compromise as the outcome (Figure 4B).

DISCUSSION

The salient result of this study is a novel protective effect of the TGF- β GG in mitigating acute rejection in cardiac allograft recipients ≥ 55 years of age. This finding is significant and particularly interesting in the context of what is known about this single nucleotide polymorphism's effect on circulating levels of TGF- β and the well-characterized role of TGF- β in allograft rejection mechanisms. The GG polymorphism documented in this study results in almost twice the level of circulating TGF- β seen in individuals carrying a CC or CG allele. (5). Given the strong association between the GG polymorphism and decreased incidence of acute rejection, a consideration of the possible TGF- β mechanism underlying this effect is warranted.

In light of its impact on immune modulation, smooth muscle cell physiology, macrophage behavior, and extracellular matrix accumulation, TGF- β appears to have a profound effect on chronic rejection in cardiac (5,⁸) as well as other solid organ transplantation. For example, TGF- β plays a significant role in the fibrotic changes noted in chronic renal rejection and recipients with the high-producing (GG) genotype have a greater incidence of late renal dysfunction (9,¹⁰). Similarly, liver transplant patients with high TGF- β producing genotypes have decreased survival rates and more chronic rejection (8). Furthermore, an increase in the expression of TGF- β is associated with an increased development of obliterative bronchiolitis in lung transplant recipients (11). Therefore, it appears that high levels of TGF- β and hence, the high producing TGF- β genotypes, may play a role in the development of chronic rejection, via graft fibrosis.

Interestingly, our results and prior studies outline an additional role for TGF- β genotypes in acute rejection. It is known that TGF- β inhibits pro-inflammatory mediators in vitro and in vivo (13) and inhibits IL-2 dependent T cell proliferation, IL-1 dependent murine thymocyte proliferation, B-cell IgG, and decreases HLA-DR (14). Thus, in these ways elevated levels of TGF- β may mitigate acute rejection, providing a reasonable explanation for the dramatic effect of the high TGF- β (GG) genotype on clinical outcomes noted in this study. Importantly, it should be noted that this protective effect is clearly more evident in the first several months post transplant when the likelihood of rejection is the greatest.

The protective effect of the GG genotype did not extend in to the cohort of cardiac allograft recipients <55 years of age. The reason for this distinction is not clear, although it is known that both circulating and intra-organ TGF- β levels are known to increase with age. Therefore, we can conclude that the protective effect of the high TGF- β (15,¹⁶) genotype occurred in the context of already elevated (age-related) levels of TGF- β . Whether the cumulative effects of age and GG genotype activate a "threshold effect" for TGF- β , leading to mitigation of acute rejection seems a reasonable theory. However, any conclusion regarding the *lack* of a protective effect of the GG genotype in population <55 years of age should be made with great caution, as other age-related factors may be involved.

A separate analysis of *donor* TGF- β genotypes showed no correlation with rejection (data not shown). Thus the reduction in rejection is likely related to genotype related activity of circulating/infiltrating cell types (inflammatory or other) from the recipient and not donor specific cells. It should be emphasized that future investigations evaluating both the genotype specific activity of graft infiltrating cells and intragraft TGF- β levels are necessary to confirm this important translational finding.

In applying these findings to clinical practice, this age genotype interaction, once confirmed in a multi-center study, could serve as an additional and complementary risk factor for predicting acute rejection for some cardiac transplant patients. Genotyping is a non-invasive test and when combined with other readily obtainable clinical factors could, for the "low risk"

patient, reduce the number of routine invasive biopsies performed. In addition, genotyping combined with other clinical factors could allow for a simplification of immunosuppression for the low risk patient with the hope of avoiding potential side effects seen with these drugs, particularly infection. As an illustration, a younger recipient and/or those with the *CC* or *GC* genotype may continue to need more potent immunosuppressive therapy in the first year post transplant secondary to their elevated risk of overall acute rejection. In contrast, older recipients with the *GG* genotype, identified as “low risk” for rejection, may be prescribed less potent immunosuppressants or reduced frequency of endomyocardial biopsies. Immunosuppressant blueprints like these could potentially mitigate long term immunosuppressant toxicity like renal insufficiency, infection, and post transplant lymphoproliferative disorder and improve overall quality of life for our transplant community.

LIMITATIONS

This study also has several acknowledged limitations. While having all transplants performed at one center gives us a universal population, it does not allow us to account for variations in pre and post transplant care as well as techniques that may occur among different transplant centers. Additionally, despite long term follow up for all 108 patients, our study population was relatively small and future studies with additional patients are warranted to confirm these results and thus these analyses should be considered exploratory. However, the small sample size did not preclude meaningful comparisons among these subjects and our multivariable analysis demonstrated that the effect of TGF- β genotype remains significant after adjusting for other known variables associated with acute rejection. Importantly, circulating and or tissue levels of TGF- β and other relevant polymorphisms (17) were also not evaluated in the present study and could add relevant confirmatory information.

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ABBREVIATIONS

UAB	University of Alabama at Birmingham
CTRD	Cardiac Transplant Research Database
ISHLT	International Society for Heart and Lung Transplantation

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Figure 1.

Mean cumulative rejections during the first month for all patients in the study (N=108). Most rejections occurred in the first three months, while the average number of rejections increased to approximately 1.5 rejections by the end of the first year post-transplant.

Figure 2.

Mean cumulative rejections during the first year by recipient TGF-Beta genotype status. There was a marginally significant difference in the risk of rejection for the two genotype groups ($p=0.05$). During the first year post-transplant, those with the *CC* or *GC* genotypes suffered an average of 2 rejection episodes whereas those with the *GG* genotype suffered an average of 1.25 rejection episodes.

Figure 3.

Figure 3A. Mean cumulative rejections during the first year by recipient TGF-Beta genotype status for those patients less than 55 years of age at the time of transplant. There was no difference in the risk of rejection between the genotype groups for this subset of patients ($p=0.40$).

Figure 3B. Mean cumulative rejections during the first year by recipient TGF-Beta genotype status for those patients 55 years of age or older at the time of transplant. There was a significant difference in the risk of rejection for the two genotype groups ($p<0.01$). During the first year post-transplant, those with the CC or GC genotypes suffered an average of 2.5 rejection episodes whereas those with the GG genotype suffered an average of 1.25 rejection episodes.

Figure 4.

Figure 4A. The mean cumulative number of 2R/3R rejections during the first year by recipient TGF-Beta genotype status for those patients 55 years of age or older at the time of transplant. There was a significant difference in the risk of rejection for the two genotype groups ($p=0.02$). During the first year post-transplant, those with the CC or GC genotypes suffered an average of 1.5 rejection episodes whereas those with the GG genotype suffered an average of 0.75 rejection episodes.

Figure 4B. Mean cumulative 2R/3R rejections OR rejections with hemodynamic compromise during the first year by recipient TGF-Beta genotype status for those patients 55 years of age or older at the time of transplant. There was a marginally significant difference in the risk of

rejection for the two genotype groups ($p=0.04$). During the first year post-transplant, those with the *CC* or *GC* genotypes suffered an average of 1.5 rejection episodes whereas those with the *GG* genotype suffered an average of 0.75 rejection episodes.

Table 1

Demographic and Clinical Variables

Characteristics	(n = 108)
Age at Transplant (Yrs)	52 ± 10.6
Recipient TGF-Beta GG Genotype	89 (82%)
Female Donor	24 (22%)
Female Recipient	23 (21%)
African-American Donor	15 (14%)
African-American Recipient	15 (14%)
Positive CMV Serology	83 (77%)
OKT-3 Induction	55 (51%)
Use of Ventricular Assist Device	18 (17%)
# of HLA Mismatches *	4.2 ± 1.17
Cyclosporine (Neoral) Use	103 (95%)
Mycophenolate (Cellcept) Use	102 (94%)
Statin Use	102 (94%)
Prednisone Use	107 (99%)

* # of HLA mismatches is missing for 11 patients

Table 2
Demographic and Clinical Variables by TGF-Beta Genotype

Characteristics	Recipient TGF-Beta Genotype		p-value
	CC/GC (n = 19)	GG (n = 89)	
Age $\geq$ 55 at Transplant	10 (53%)	43 (48%)	0.73
Female Donor	3 (16%)	21 (24%)	0.56
Female Recipient	5 (26%)	18 (20%)	0.55
African-American Donor	2 (11%)	13 (15%)	0.99
African-American Recipient	4 (21%)	11 (12%)	0.30
Positive CMV Serology	16 (84%)	67 (75%)	0.55
OKT-3 Induction	7 (37%)	48 (54%)	0.18
Use of Ventricular Assist Device	5 (26%)	13 (15%)	0.31
# of HLA Mismatches*	3.8 $\pm$ 1.33	4.3 $\pm$ 1.13	0.13

**

of HLA mismatches is missing for 2 CC/GC patients and 9 GG patients

Table 3

Final Multivariable Model to Predict Cumulative Rejections during First Year

Characteristics	Effect	p-value
African-American Recipient	Harmful	0.06
African-American Donor	Protective	0.04
TGF-Beta Recipient <i>GG</i> Genotype *		
< 55 Yrs. of Age at Transplant	N/A	0.40
≥ 55 Yrs. of Age at Transplant	Protective	<0.01

* p < 0.01 for test of interaction between TGF-Beta recipient genotype and age at time of transplant

Table 4

Final Multivariable Model to Predict Cumulative 2R/3R Rejections During First Year

Characteristics	Effect	p-value
African-American Recipient	Harmful	0.07
African-American Donor	Protective	0.19
TGF-Beta Recipient <i>GG</i> Genotype *		
< 55 Yrs. of Age at Transplant	N/A	0.15
≥ 55 Yrs. of Age at Transplant	Protective	0.02

* p = 0.01 for test of interaction between TGF-Beta recipient genotype and age at time of transplant