

Increased Transcript Levels of TNF- α , TGF- β , and Granzyme B in Endomyocardial Biopsies Correlate With Allograft Rejection

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Abstract

Objectives: Endomyocardial biopsies are the criterion standard in diagnosing acute cardiac transplant rejection. This study sought to analyze mRNA expression profiles of various immune-response-related genes in endomyocardial biopsies of heart transplant patients and to correlate the results with histologic findings.

Materials and Methods: Forty-three biopsies obtained from 6 heart transplant recipients experiencing acute rejection were analyzed for granzyme B, CTLA4, IL-6, TGF β , and TNF α expression using real-time polymerase chain reaction. The results were compared with the histologic findings. Biopsies obtained before, during, and after acute rejection episodes were grouped according to the International Society of Heart and Lung Transplantation standard biopsy grading from 1990. Group 1 consisted of biopsies with International Society of Heart and Lung Transplantation grade 0 (n=12), group 2 of International Society of Heart and Lung Transplantation grade 1A (n=14), and group 3 of International Society of Heart and Lung Transplantation grades 1B, 2, 3A, and 4 (n=17).

Results: A strong correlation was seen between histologic groups and gene expression of granzyme B, which showed the highest overall transcript levels. CTLA4 was elevated in group 2, but no further increase in the rejecting group 3 was seen. For IL-6, TGF β and TNF α gene expression was strongly elevated in group 3 compared with groups 1 and 2. On analysis of biopsies with International Society of Heart and Lung Transplantation, grade 0 and 1A, relative to the time point of rejection, we found a substantial increase in mRNA expression of all analyzed immune response-related genes before a rejection episode. The strongest up-regulation was seen for granzyme B, TNF α , and TGF β .

Conclusions: Our data suggest that analyses of gene expression provides valuable information in diagnosing heart transplant rejection. Furthermore, analyses of granzyme B, TGF β , and TNF α might not only confirm an ongoing rejection episode, but also may have a positive predictive value.

Key words: Renal transplant, Renal failure, Uric acid, Elderly, Recipients

Introduction

After heart transplant, proinflammatory cytokines produced by graft infiltrating cells play an important role in allograft rejection.¹ These cytokines released after ischemic injury and reperfusion of the graft result in proliferation and activation of T cells.^{2, 3} Furthermore, allograft rejection caused by mismatched HLA antigens occurs as a result of humoral and cell-mediated responses to these HLA antigens expressed on donor tissue.⁴ The recognition of alloantigen expressed by donor tissue owing to recipient T cells activates cytotoxic graft infiltrating

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lymphocytes.⁴⁻⁶ Although immunosuppressive therapy is mandatory for avoiding activation and proliferation of alloreactive T-cells, acute rejection cannot completely be prevented. Particularly, during the first 6 months, acute rejection occurs more frequently. Besides T cells, the effector mechanism mediating allograft rejection includes macrophages, natural killer cells and B cells. Immune response-related genes produced by these cell types such as cytokines, chemokines, and membrane receptors play a central role in the alloimmune response.^{7, 8} In particular, granzyme B released from activated cytotoxic T-cells and natural killer cells has been closely associated with acute cellular rejection in solid organ transplants.⁵ The transforming growth factor- β (TGF β) is a key fibronectin cytokine. It has been shown to increase collagen, fibronectin, and other matrix proteins, so it plays a role in the immunoregulation and repair processes before and after rejection.^{9, 10}

Therefore, increased expression of these immune response-related genes may be associated with rejection episodes in clinical heart transplant. For monitoring and diagnosing rejection episodes in heart transplanted patients, histologic analysis of endomyocardial biopsies is regarded as the most-informative technique.^{11, 12} We analyzed gene expression of TNF- α , IL6, CTLA4 (CD152), granzyme B, and TGF- β in biopsies of heart transplanted patients and correlated these expression levels with histologic grade and time point of acute rejection.

Materials and Methods

Patients

Together, 45 patients transplanted at our institution between 2001 and 2004 were screened regularly using protocol biopsies. From this cohort, 6 patients (all men; aged, 45 to 65 y) had relevant rejection episodes and were eligible for further analyses. From these patients, endomyocardial biopsies before, during, and after a rejection episode were available for analysis (n=43). The average HLA-mismatch of our patient cohort was 4.5 for HLA-A, B, and DR. Patients received standard triple immunosuppressive therapy (cyclosporine or tacrolimus, azathioprine, or mycophenolate mofetil and prednisolone). Rejection episodes $\geq 3A^{11}$ were treated primarily by intravenous methylprednisolone; and

for continued rejection, with antithymocyte globulin. The study was performed according to the guidelines of the local ethics committee and conformed to the ethical guidelines of the 1975 Helsinki declaration. Written, informed consent was obtained from all patients.

Histopathology

A total of 43 snap frozen endomyocardial allograft biopsies taken from 6 rejecting patients were snap frozen for histopathologic analysis. Biopsies were taken in median 23.5 days after transplant (range, 6 to 473 d). Throughout the study, grading was performed according to the International Society of Heart and Lung Transplantation (ISHLT) standard biopsy grading for acute rejection from 1990¹¹: 0 (no rejection), 1A (focal perivascular), 1B (diffuse but sparse infiltrate), 2 (aggressive focal infiltrate with focal myocyte damage), 3A (multifocal aggressive infiltrates with myocyte damage), 3B (diffuse inflammatory processes with necrosis), and 4 (diffuse aggressive polymorphous infiltrate with edema, hemorrhage, vasculitis, and necrosis).

Real-time quantitative polymerase chain reaction

For mRNA analysis, recovered biopsies were immediately embedded in RNAlater (QIAGEN, Hilden, Germany) and stored at -80°C. Total RNA was isolated using the QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany). cDNA synthesis was performed according to standard protocols. Quantitative real-time polymerase chain reaction (qRT-PCR) amplification was performed in triplicates on the StepOne sequence detector (Applied Biosystems, Foster City, CA, USA), using a cycling profile of 20 seconds at 95°C, followed by a total of 40 two temperature cycles with 1 second at 95°C, and 20 seconds at 60°C. Primer sets and probes were commercially synthesized by Eurofins MWG GmbH (Ebersberg, Germany) according to published sequences (Table 1). To generate PCR standards, the respective PCR product was cloned into a TOPO cloning vector (Invitrogen Corporation, Carlsbad, CA, USA) and sequenced. Standard curves with known concentrations of template copy numbers were used to determine the expression of the amplified target. The samples were normalized against the expression of the housekeeping gene 18srRNA. The results are given as relative expression units.

Table 1. The oligonucleotide sequences.

Gene	Orientation	Sequence	Product Size	Ref.
Granzyme B	for	5'-GCG AAT CTG ACT TAC GCC ATT A-3'	94	19
	rev	5'-CCA GAG TCC CCC TTA AAG GAA-3'		
	probe	5'-FAM-CCC ACG CAC AAC TCA ATG GTA CTG TCG TA-TAMRA-3'		
TGF- β	for	5'-CGA GAA GCG GTA CCT GAA C-3'	127	20
	rev	5'-TGA GGT ATC GCC AGG AAT TGT -3'		
	probe	5'-FAM-CAG CAC GTG GAG CTG TAC CAG AAA TAC AGC-TAMRA-3'		
TNF- α	for	5'-ATC TTC TCG AAC CCC GAG TGA-3'	84	21
	rev	5'-CGG TTC AGC CAC TGG AGC T-3'		
	probe	5'-FAM-CCC ATG TTG TAG CAA ACC CTC AAG CTG A-TAMRA-3'		
CTLA4	for	5'-CCC AGA TTT ATG TAA TTG ATC CAG AA-3'	78	22
	rev	5'-CCG AAC TAA CTG CTG CAA GGA-3'		
	probe	5'-FAM-CGT GCC CAG ATT CTG ACT TCC TCC TCT-TAMRA-3'		
IL-6	for	5'-TGA CAA ACA AAT TCG GTA CAT C-3'	96	23
	rev	5'-AGT GCC TCT TTG CTG CTT TCA C-3'		
	probe	5'-FAM-CAG CCC TGA GAA AGG AGA CAT GTA ACA A-TAMRA-3'		
18s rRNA	for	5'-CGC CGC TAG AGG TGA AAT TC-3'	101	24
	rev	5'-CGA ACC TCC GAC TTT CGT TCT-3'		
	probe	5'-CCG GCG CAA GAC GGA CCA GA-3'		

Statistical Analyses

The results are given as the mean per group \pm SD, which was derived from the mean per graft. Statistical analyses were performed using a 1-way ANOVA. Differences between groups are considered significant when $P < .05$.

Results

Intra-graft gene expression in relation to histologic rejection grades

To correlate gene expression profiles with histologic findings, endomyocardial biopsies were grouped according to histologic criteria as suggested by Akdere and colleagues.¹³ Grading of the endomyocardial biopsies was performed using the 1990 version of the ISHLT grading system.¹¹ Biopsies with grade 0 (no rejection) were combined in group 1 (n=12), whereas an ISHLT grade 1A rejection with focal perivascular infiltrate (but no necrosis) was assigned to group 2 (n=14) and histologic grades 1B, 2, 3A, and 4 with different stages of infiltrates and the need of a therapeutic intervention was assigned to group 3 (n=17). A strong increase in mRNA expression of all analyzed immune response related genes could be observed in group 3 compared to groups 1 or 2 (Figure 1). However, only for granzyme B, was a constant increase from group 1 to group 3 detected. Although, CTLA4 transcripts were augmented in group 2, no further increase in the rejecting group 3 was seen. For IL-6, TGF β , and TNF α , no relevant difference between groups 1 and 2 was observed. However, in biopsies from the rejecting group 3, gene expression for TGF β and TNF α was significantly elevated, and showed a

similar trend for IL-6. The overall lowest expression level was detected for IL-6, whereas granzyme B revealed the highest increase and maximum expression level.

mRNA expression in endomyocardial biopsies with grade 0 or 1A before and after rejection episodes.

On analysis, we observed a certain variability in gene expression in biopsies with ISHLT grade 0 and 1A. We therefore addressed the question whether the time point the endomyocardial biopsy was recovered relative to the onset of the rejection episode influenced the level of gene expression. For analyses, all biopsies with ISHLT grade 0 and 1A were divided into 2 groups. The first group (n=8) included endomyocardial biopsies taken directly before a rejection episode (median, 27 days; range, 5 to 49 d before a rejection episode). The second group consisted of endomyocardial biopsies taken directly after a rejection episode (n=10; median, 32.5 d; range, 7 to 58 d after rejection episode). To enhance the informative value, negative endomyocardial biopsies obtained during times without any evidence of rejection were added to group 2 (n=9). Interestingly, there was a substantial increase in mRNA expression of all analyzed immune response related genes (Figure 2). The strongest up-regulation was seen for granzyme B, TNF α , and TGF β , whereas less-meaningful differences were seen for IL-6 and CTLA4.

Discussion

Acute cellular rejection of cardiac allograft is a multifactorial process with proinflammatory

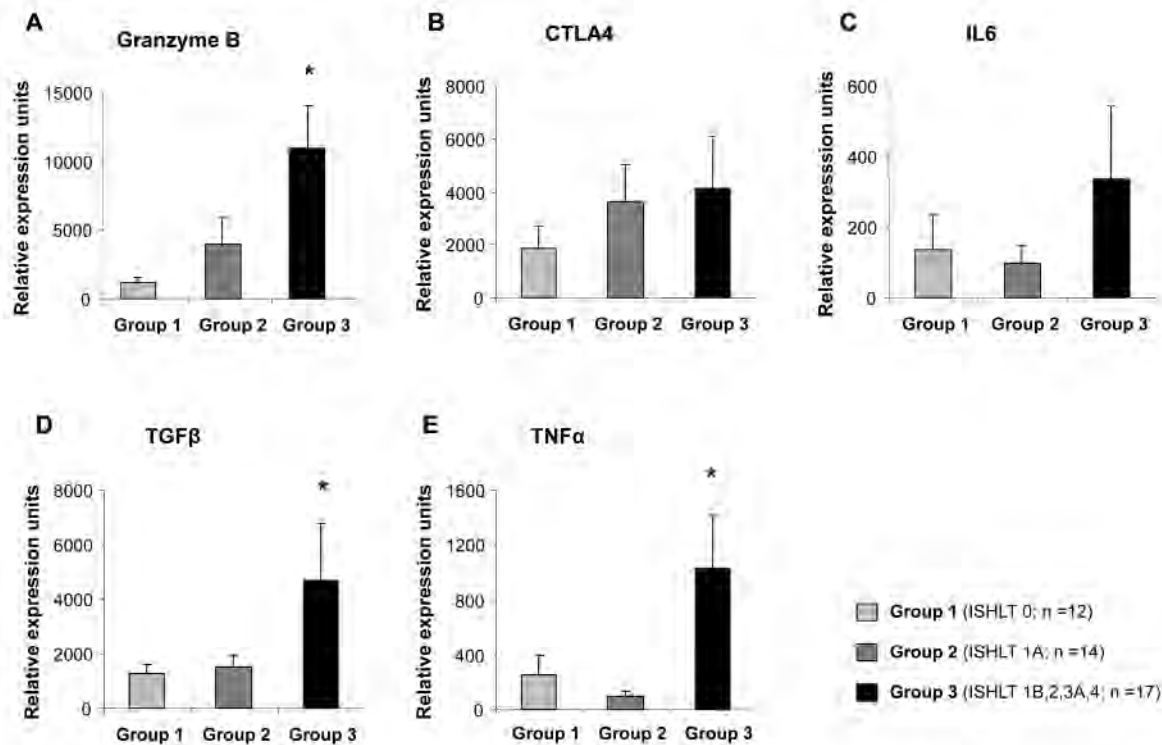


Figure 1. Quantitative mRNA expression levels within endomyocardial biopsies. Analysis was performed for granzyme B (A), CTLA4 (B), IL-6 (C), TGF- β (D), and TNF- α (E) mRNA. Depending on their histologic ISHLT-core, biopsies were divided into 3 groups: group 1 (ISHLT grade 0; n=12), group 2 (ISHLT grade 1A; n=14), and group 3 (ISHLT grades 1B, 2, 3A, and 4; n=17). Data are shown as the mean for each group \pm SEM (* $P < .05$).

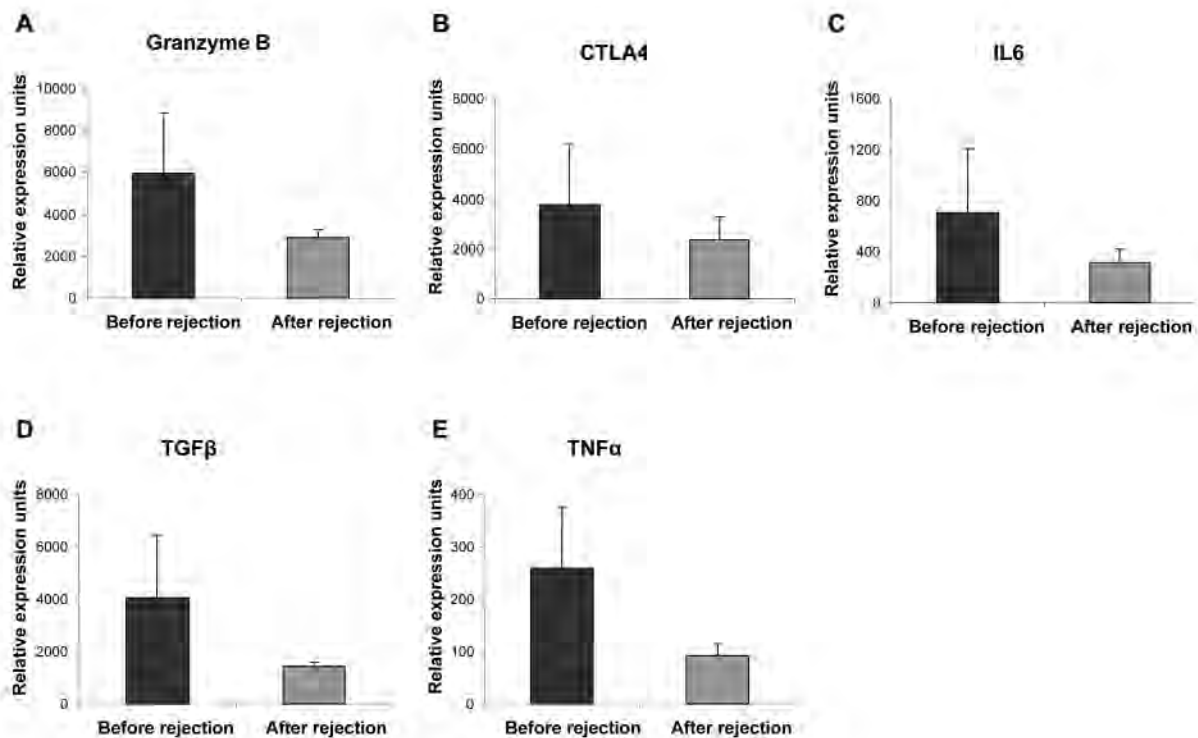


Figure 1. Quantitative mRNA expression levels within endomyocardial biopsies depending on the time point the endomyocardial biopsies were taken relatively to the rejection episode. Analysis was performed for granzyme B (A), CTLA4 (B), IL-6 (C), TGF- β (D), and TNF- α (E). Endomyocardial biopsies were taken directly before (n=8) or after (n=10) a rejection episode. Additionally, negative endomyocardial biopsies obtained during times without any evidence of rejection were added to the postrejection group (n=9). Data are shown as the mean for each time point \pm SEM (before/after rejection) (* $P < .05$).

cytokines activating cytotoxic T-cells, macrophages, natural killer cells, B cells, adhesion molecules, and apoptotic pathways.⁷ Besides clinical symptoms, the diagnosis of acute cellular rejection is based on the histologic evaluation of endomyocardial biopsies according to the ISHLT guidelines.¹¹ Endomyocardial biopsies are a reliable method to recognize acute rejection in a solid organ transplant and still the criterion standard, despite being an invasive procedure. The accuracy of diagnosing acute rejection is of high importance, owing to its clinical and therapeutic consequences. To increase the gain of information from endomyocardial biopsies, additional analysis of transcript levels of various immune-response related genes was investigated over the last 2 decades.^{7, 14, 15}

Granzyme B has been shown as one the most-promising markers for detecting allograft rejection. In particular, it was demonstrated that granzyme B is a reliable indicator of renal allograft rejection.^{5, 16} However, little data about granzyme B exist in heart transplants.¹⁵ Our study confirmed the importance of granzyme B in acute allograft rejection after a heart transplant. Its level of expression correlated with the biopsy groups according to the ISHLT classification. Whereas hardly any granzyme B transcripts were found in endomyocardial biopsies without signs of rejection (group 1), the highest levels were seen in endomyocardial biopsies from group 3.

CTLA4 was the only other gene that showed an increase in expression from group 1 to group 2. CTLA4, expressed on activated T-cells and conveying inhibitory functions, has also been found at increased levels in acutely rejecting lung transplants.¹⁷ In our study, however, the increment between samples with ISHLT grade 1A (group 2) and those with more-severe signs of rejection (group 3) was small, indicating that CTLA4 expression might not add additional information to the histologic grading. On the other hand, IL-6, TGF β , and TNF α showed no relevant differences between samples obtained from ISHLT grade 0 (group 1) and grade 1A (group 2), but a strong rise in samples from group 3. Because IL-6 showed only a low relative level of expression overall, it seems less useful as a diagnostic marker.

An interesting finding was obtained on comparing samples taken before and after rejection episodes. When endomyocardial biopsies without histologic signs of rejection obtained before and after rejection episodes were compared, we could clearly

demonstrate that in particular granzyme B, TGF β , and TNF α were already up-regulated before the onset of acute rejection. Therefore, these markers might not only confirm an ongoing rejection episode, but also may have a positive predictive value.

One aspect that has not been the subject of this study was whether gene expression profiles from peripheral blood also might identify patients at risk of having a rejection episode. Such a noninvasive diagnostic is being developed for kidney transplant recipients using urinalysis.¹⁶ One study in heart transplant recipients found that gene expression profiles in peripheral blood mononuclear cells might be a valuable tool for noninvasive diagnosis of higher grades (\geq ISHLT grade 2) of transplant rejection.¹⁸ However, a recent study comparing gene expression from biopsies or bronchoalveolar lavage with peripheral blood samples from lung transplant recipients showed that peripheral blood was not indicative of a rejection episode.¹⁷ Although these results will have to be validated in further studies, they indicate that biopsies will remain the criterion standard in the near future, but that expression profiles of suitable immune response-related genes might add considerable informative value.

In summary, our findings affirm gene expression profiling as an additive tool in the diagnosis of heart transplant rejection. Furthermore, analysis of granzyme B, TGF β , and TNF α might not only confirm an ongoing rejection episode, but also have a positive predictive value.

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