Detection of Cardiac Allograft Rejection and Response to Immunosuppressive Therapy With Peripheral Blood Gene Expression

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Background—Assessment of gene expression in peripheral blood may provide a noninvasive screening test for allograft rejection. We hypothesized that changes in peripheral blood expression profiles would correlate with biopsy-proven rejection and would resolve after treatment of rejection episodes.

Methods and Results—We performed a case-control study nested within a cohort of 189 cardiac transplant patients who had blood samples obtained during endomyocardial biopsy (EMB). Using Affymetrix HU133A microarrays, we analyzed whole-blood expression profiles from 3 groups: (1) control samples with negative EMB (n=7); (2) samples obtained during rejection (at least International Society for Heart and Lung Transplantation grade 3A; n=7); and (3) samples obtained after rejection, after treatment and normalization of the EMB (n=7). We identified 91 transcripts differentially expressed in rejection compared with control (false discovery rate < 0.10). In postrejection samples, 98% of transcripts returned toward control levels, displaying an intermediate expression profile for patients with treated rejection (P<0.0001). Cluster analysis of the 40 transcripts with ≥25% change in expression levels during rejection demonstrated good discrimination between control and rejection samples and verified the intermediate expression profile of postrejection samples. Quantitative real-time polymerase chain reaction confirmed significant differential expression for the predictive markers CFLAR and SOD2 (UniGene ID No. 355724 and No. 384944).

Conclusions—These data demonstrate that peripheral blood expression profiles correlate with biopsy-proven allograft rejection. Intermediate expression profiles of treated rejection suggest persistent immune activation despite normalization of the EMB. If validated in larger studies, expression profiling may prove to be a more sensitive screening test for allograft rejection than EMB. (Circulation. 2004;110:3815-3821.)

Key Words: immune system ■ transplantation ■ rejection ■ genes ■ diagnosis

Detection of allograft rejection is a major clinical concern in the care of heart transplant recipients. The optimal approach is to detect rejection before the onset of cardiac dysfunction and to treat it aggressively with augmented immunosuppression. It is equally important to reduce immunosuppression in patients who do not have rejection to minimize drug toxicity. The current standard to screen for rejection is the detection of inflammatory infiltrates on serial endomyocardial biopsy (EMB).1-2; however, EMB is an invasive procedure limited by patient discomfort, risk of complications, and cost.3 These barriers prevent frequent monitoring for rejection and limit optimal titration of immunosuppressive therapy.

Rejection is a complex immune response that involves T-cell recognition of alloantigens in the cardiac allograft, costimulatory signals, elaboration of effector molecules by activated T cells, and an inflammatory response within the graft.4-10 Activation and recruitment of circulating leukocytes to the allograft is an essential part of this process, which makes peripheral blood monitoring of the immune response an attractive method for the noninvasive detection of rejection. The purpose of the present study was to test the hypothesis that gene-expression profiles obtained from peripheral blood correlate with histological cardiac allograft rejection on serial EMBs. Our findings raise the possibility that peripheral blood gene-expression profiles...
could serve as a noninvasive method to screen for cardiac allograft rejection.

Methods

Patient Population
We prospectively collected 409 blood samples from 189 consecutive cardiac transplant patients referred for routine surveillance EMB at the Hospital of the University of Pennsylvania between March and July 2002. All subjects gave written informed consent, and the University of Pennsylvania Institutional Review Board approved the study protocol.

Sample Collection
Blood samples were obtained from a central venous sheath immediately before EMB and were collected in RNA preservation solution (PAXgene Blood RNA Tubes, Qiagen Inc) for immediate RNA stabilization and storage at −80°C. EMB specimens were assessed by a cardiac pathologist at the University of Pennsylvania, and rejection grade was determined with the International Society for Heart and Lung Transplantation (ISHLT) grading system. This system categorizes biopsies into several grades (0, 1A, 1B, 2, 3A, 3B, and 4) based on the extent of lymphocyte infiltration, myocyte necrosis, and presence or absence of hemorrhage. Augmented immunosuppression is indicated for ISHLT grade 3A or higher rejection.

Study Design
We performed a nested case-control study of peripheral blood gene expression within our cohort of biopsy patients. Case patients ("rejection") were chosen on the basis of the presence of rejection expression within our cohort of biopsy patients. Case patients during and after resolution of clinically significant rejection. This group of postrejection specimens ("postrejection") allowed us to analyze changes in gene-expression profile over time in the same rejection patients after treatment with augmented immunosuppression with corticosteroids, antimetabolites, calcineurin inhibition, and/or sirolimus.

In addition, we selected follow-up blood specimens from the rejection patients after treatment with augmented immunosuppression and resolution of rejection to grade 2 or lower on repeat EMB. This group of postrerejection specimens ("postrejection") allowed us to analyze changes in gene-expression profile over time in the same patients during and after resolution of clinically significant rejection.

Microarray Sample Preparation and Hybridization
Control (n=7), rejection (n=7), and postrejection (n=7) samples were selected as described above and purified with a commercial nucleic acid isolation column (PAXgene Blood RNA Column, Qiagen Inc). Total RNA samples were analyzed by Agilent bioanalyzer and OD260/OD280 ratio for RNA quality and quantification. Individual complementary DNAs (cDNAs) were prepared from each RNA isolate with reverse transcriptase (Superscript II primed by a poly (T) oligomer/T7 promoter). Each cDNA was subsequently used as a template to make biotin-labeled cRNA with an in vitro transcription reaction, which resulted in a single cRNA for each blood sample. Each cRNA was hybridized with an individual Affymetrix Hu133A oligonucleotide array, which was subsequently processed and scanned according to the manufacturer’s instructions. All arrays (n=21) were hybridized on the same day by a single technician to avoid variability in hybridization conditions. Each array quantifies the expression of 22,215 transcripts (including full-length mRNA sequences and expressed sequence tags) derived from build 133 of the UniGene database (available at www.affymetrix.com). Data were saved as raw image files and converted into probe-set data (.cel files) with Microarray Suite (MAS 5.0).

Microarray Analysis
There are several methods to convert Affymetrix probe-set data into normalized measures of gene expression, including software provided by the manufacturer (MASS), model-based methods (dChip), and robust multiarray analysis (RMA). We chose RMA on the basis of its superiority in the analysis of small data sets. Software for RMA is available (www.bioconductor.org) for use in the R 1.70 package for statistical computing (www.r-project.org).

Differentially Expressed Genes in Rejection Compared With Control Samples
To determine candidate markers of rejection, we applied 3 criteria to the normalized data. First, data were filtered to include genes present above background on at least 1 array. Second, significance analysis of microarrays (SAM; available at http://www-stat.stanford.edu/~tibs/SAM) was used to correct for multiple comparisons and to select candidate markers of rejection using genes that were differentially expressed with an estimated overall false-discovery rate <0.10. Third, we required at least a 25% change in expression between rejection and control samples for a transcript to be of interest. The identities of differentially expressed genes were determined with annotation databases (available at www.netaffx.com) or via BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) of the corresponding expressed sequence tags.

Response to Treatment
To determine whether our candidate markers of rejection responded to immunosuppressive therapy, we analyzed expression data for these transcripts in postrerejection samples. If our candidate genes were markers of rejection, we hypothesized that genes that were overexpressed (underexpressed) in rejection versus control should also be overexpressed (underexpressed) in rejection versus postrejection. Alternatively, if our candidate genes were identified owing to confounding factors (eg, differences in age between the rejection and control subjects), then we would not expect the pattern of differential expression to be recapitulated in the paired rejection versus postrejection comparison.

We repeated this process 10,000 times, and used the repeated samples to determine our probability value, ie, the probability of a chance occurrence of the observed or better concordance.

Cluster Analysis
The capacity of our candidate markers to distinguish control, rejection, and postrerejection samples was assessed by hierarchical clustering. Clusters were constructed with average linkage clustering and Pearson correlation coefficients as a measure of similarity with Cluster software and displayed with Treeview software (available at http://rana.lbl.gov).

Validation
Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to validate changes in selected genes. Because there was insufficient RNA after the microarray studies to validate these data from all of the original samples, validation was performed with mRNA harvested from additional samples from the original biopsy cohort by the same selection criteria. RNA isolates were treated with DNase to remove any contaminating genomic DNA and were subsequently converted to cDNA with an in vitro transcription reaction. cDNAs were used as templates for Taqman qRT-PCR with
ABI Assays-on-Demand on an ABI Prism 7900 sequence detection system. The specific assays used were Hs00153439_m1 (CFLAR), Hs00167309_m1 (SOD2), and Hs99999905_m1 (GAPDH). All samples were run in triplicate, and GAPDH was used as an internal control to normalize transcript abundance. Triplicates were averaged to calculate an expression value for each sample. Data were compared among control, rejection, and postrejection samples by the Wilcoxon rank-sum test, with P<0.05 indicating statistical significance.

Results

Patient Characteristics
The frequency of rejection was low in the present study population (Figure 1). Of 409 EMB samples, 81% showed minimal or no evidence of allograft rejection (ISHLT grades 0, 1A, or 1B), and 6% showed clinically significant rejection (grade 3A or higher) that required increases in the immunosuppression regimen. The characteristics of patients chosen for study are outlined in Table 1. All control samples had grade 0 rejection on biopsy, and all rejection samples were obtained from patients with rejection graded 3A or higher. The postrejection samples were obtained a median of 55 days after rejection was first detected.

Microarray Analysis

Candidate Markers of Rejection
Of the 22 215 transcripts on each array, 10 826 (49%) were expressed at levels higher than background in at least 1 of the 21 samples. Of these, 91 gene products were differentially expressed in rejection compared with control (Figure 2, red) with a false-discovery rate <0.10 after SAM analysis. Seven genes were overexpressed and 84 genes were underexpressed in rejection. These genes were regarded as candidate markers for high-grade rejection. Overall, there was good reproducibility in gene expression in these candidates. The average coefficient of variation within each group (control or rejection) was 4%; however, reproducibility was different for each gene, ranging from a minimum coefficient of variation of 1% to a maximum of 11%.

We assessed changes in our candidate markers after treatment of rejection by measuring expression levels in follow-up samples from the same patients. As shown in Figure 2 (blue), expression of nearly all of the candidate markers moved closer to a fold change of 1 after immunosuppressive therapy, which indicates a return toward levels in control. This finding

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*Rejection and postrejection represent the same patients during and after treatment of grade 3A or higher rejection with augmented immunosuppression.
†Double therapy indicates mycophenolate, azathioprine, or sirolimus plus cyclosporine or tacrolimus; triple therapy indicates double therapy plus corticosteroids.
is consistent with the response to therapy noted on EMB; however, expression in the postrejection samples did not fully normalize to a fold change of 1, which suggests that treated rejection has an intermediate expression profile between control and rejection. By randomly resampling gene-expression data, we estimated the probability of finding a set of 91 genes that by chance showed differential expression in rejection with concordant changes after rejection. Only 1 in 10,000 randomly selected sets of 91 genes showed this pattern ($P=0.0001$); therefore, it is extremely unlikely that the observed intermediate expression profile occurred owing to chance. These findings suggest that we have identified an expression profile that correlates with active rejection in these patients.

**Cluster Analysis**

We used hierarchical clustering as an additional method to characterize the ability of our candidate markers to distinguish control, rejection, and postrejection samples. Hierarchical clustering is a computational method that groups experimental samples according to similarity in patterns of gene expression across a large number of genes.$^{18}$ We selected 40 transcripts that showed at least a 25% change in expression between control and rejection and performed cluster analysis on this panel of genes. As shown in Figure 3, samples clustered into 2 main branches, with complete partitioning of control and rejection samples into separate branches. Postrejection samples were present in both the control and rejection branches of the dendrogram, consistent with an intermediate expression profile for treated rejection.

**Gene Function**

The identities of our 40 candidate markers of rejection included 30 unique transcripts (Table 2). The majority of these are involved in the following cellular pathways: (1) transcription or translation, (2) cell-cycle regulation, (3) tumorigenesis/tumor suppression, (4) immune response, (5) apoptosis, and (6) intracellular signaling. Also included in Table 2 are a number of expressed sequence tags of unknown function. Several transcripts are represented by multiple probe sets on the HU133A array. These replicate probe sets showed consistent changes during rejection that resolved at postrejection biopsy time points (Figure 3). The marker with the largest number of internal replicates was the gene CASP8 and FADD-like apoptosis regulator (CFLAR), an inhibitor of apoptosis that is downregulated in rejection.

**Quantitative PCR**

We verified transcriptional changes using qRT-PCR for 2 genes: CFLAR and superoxide dismutase 2 (SOD2). Consistent with the microarray analysis, both genes were significantly downregulated during rejection, with a mean fold change of $0.76 \pm 0.06$ ($P=0.01$) for CFLAR and a mean fold change of $0.74 \pm 0.09$ ($P=0.02$) for SOD2, as shown in Figure 4. Thus, peripheral blood gene-expression changes observed by microarray profiling were confirmed in comparisons of rejection and control samples. In postrejection samples, CFLAR expression trended back toward control levels, with a fold change closer to 1.0, but SOD2 did not. The partial return toward baseline for CFLAR and the lack of return for SOD2 likely reflect persistent partial activation of circulating leukocytes in these samples, which were taken at variable times after histological resolution of rejection.

**Discussion**

This study demonstrates the principle that peripheral blood gene expression correlates with cardiac allograft rejection detected on EMB. We identified 40 transcripts that are altered in acute cellular rejection and returned toward normal in response to augmented immunosuppression. Moreover, we observed in 2 separate analyses that treated rejection has an intermediate expression profile, which suggests persistent immune activation despite resolution of rejection on biopsy. These findings raise the possibility that expression profiling may prove to be a more sensitive screening test for rejection than EMB.

Previous investigators have used molecular markers to develop better screening tests for cardiac allograft rejection. For example, expression of immune stimulatory and activation markers (CD40, CD27, TIRC7), cytokines (interferon-γ, interleukin [IL]-2, IL-4, IL-6, IL-8), and cytotoxic T-cell effector molecules (perforin, granzyme B, FasL) are elevated in biopsy samples of rejecting myocardium.$^{4-10}$ These markers could be used to enhance the sensitivity of biopsy-detected rejection, but they do not eliminate the need for invasive procedures. Other groups have correlated levels of circulating markers, such as cytokine or cytokine mRNA levels, with cardiac allograft rejection in an effort to develop noninvasive screening tests.$^{19,20}$ In particular, Morgun et al$^{21}$ performed quantitative PCR analysis on peripheral mononuclear cell candidate transcripts and found correlations between EMB results and candidate mRNA expression levels.
These studies support the hypothesis that peripheral blood gene expression may reflect organ-level rejection, but they are limited by the short list of candidate markers tested with PCR-based technologies.

In contrast to previously employed methods, microarray technology offers the possibility of simultaneously screening thousands of potential candidate genes in an unbiased fashion. These advantages allow for the identification of gene-expression profiles that may be much more sensitive and specific than any one candidate marker, as has been shown in previous studies of hematologic malignancies and renal transplantation.22–25 The relatively small fold changes in gene expression observed in the present study (<2.5-fold) support the hypothesis that an aggregate marker composed of multi-
ple genes, which integrates small changes in a large number of component markers, will prove to be the most robust diagnostic approach for detecting allograft rejection noninvasively.

In addition to viewing our candidate genes as diagnostic markers of rejection, a portion of them may mediate components of rejection. As shown in Table 2, the known or proposed function of our candidates involves cellular processes that are plausible components of an immune response, such as regulation of DNA transcription or translation, cell-cycle and apoptosis regulators, and markers of immune system activation. It is possible that changes in expression of genes involved in the regulation of programmed cell death, such as **CFLAR**, promote clonal expansion of specific lymphocyte populations as part of the rejection process; however, our ability to make specific biological inferences is limited by the mixed cell population examined and the observational nature of the present study. Determining which, if any, of our candidate genes contribute to rejection will require experimental approaches.

Expression profiling is a powerful technique, but it creates substantial challenges that result from the analysis of many genes in a small number of samples. We addressed these concerns at multiple levels. First, we used conservative normalization and gene-selection strategies that are superior in the analysis of relatively small data sets. Second, we incorporated serial measurements in the same patients, which reduces the impact of interpatient variability. Third, we validated selected findings using quantitative PCR. Fourth, we performed our analyses on immediately preserved whole-blood isolates, which minimizes the impact of sample pre-processing procedures, such as cell sorting or buffy coat isolation, on the gene-expression profile and is more convenient to implement in a clinical setting. The major limitation

**Figure 3.** Cluster analysis. We analyzed 40 candidate markers with hierarchical clustering (see Table 2 for full names and functional annotation of 40 candidates). Results are displayed with an Eisen plot, which consists of a dendrogram to demonstrate relationships among samples and color-coded heat map to display level of expression of individual genes. For each gene, red indicates higher-than-median expression, and green indicates lower-than-median expression. As shown in the dendrogram, our candidate markers partition rejection (R) and control (C) samples into 2 main branches. Postrejection samples (p) are present in both main branches, which indicates intermediate expression profiles for this group. Genes chosen for subsequent qRT-PCR validation are indicated with blue squares.

**Figure 4.** We quantified transcript abundance of 2 candidate markers, **CFLAR** and **SOD2**, using qRT-PCR. Data are displayed as fold changes in expression in rejection (n=10) and postrejection (n=8), each compared with control (n=9). In agreement with microarray findings, both **CFLAR** and **SOD2** expression were decreased in rejection. **CFLAR** expression returned toward control levels in postrejection samples, and **SOD2** expression remained low, consistent with persistent partial activation of circulating leukocytes after treatment of rejection. *P*<0.05 compared with control by Wilcoxon rank-sum test.
of this proof-of-principle study is the small sample size, which limits our ability to assess the influence of confounding factors, such as age, on changes in peripheral gene expression.

In conclusion, we have demonstrated the principle that peripheral blood gene expression correlates with cardiac allograft rejection. Further studies are necessary to test our panel of markers prospectively with the goal of developing a clinically useful, noninvasive test for cardiac allograft rejection.

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References


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