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Molecular Testing for Early Detection and Monitoring of Graft Rejection by Heart Transplant Recipients

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Abstract

Heart transplantation is a life-prolonging procedure for many patients with stage D heart failure and other forms of advanced heart disease. However, even with the latest advances in immunosuppression, graft rejection is a major cause of death among heart transplant patients. It would be desirable to be able to detect rejection early enough and specifically enough to prevent allograft dysfunction without unnecessary over-immunosuppression. Hitherto, the main technique employed to monitor rejection status has been endomyocardial biopsy, which is invasive, prone to tissue sampling error, and placed in question by interobserver variability, but is unmatched by any non-gene-based noninvasive technique. Currently, a multi-parametric approach is employed that comprises clinical examination for signs or symptoms of heart failure, endomyocardial biopsies, drug level monitoring, allograft function tests (mainly echocardiographic studies), and screening for allograft vasculopathy. Gene expression profiling can now be used in the USA to screen heart transplant patients for risk of current rejection, thereby sparing the majority from endomyocardial biopsy, and the possibility of its application in Europe is currently being studied, as is its performance in comparison to endomyocardial biopsy in regard to relevant clinical outcomes, quality of life, and resource utilization. In future it may also be useful for classification of patients as regards risk of future rejection, for monitoring weaning from steroids, and for detection of allograft vasculopathy and antibody mediated rejection. **(Trends in Transplant. 2009;1:35-40)**

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Key words

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Introduction

Heart transplantation is a life-prolonging procedure for many patients with stage D heart failure and other forms of advanced heart disease[1]. However, even with the latest advances in immunosuppression, graft rejection is a major cause of death among heart transplant patients[2]. According to the registry of the International Society for Heart and Lung Transplantation (ISHLT), rejection causes 12% of deaths occurring between one and 12 months after heart transplantation[3], and 20-50% of heart transplant patients suffer at least one rejection episode during the first year following transplantation[4]. It would be desirable to be able to detect rejection early enough and specifically enough to prevent allograft dysfunction without unnecessary over-immunosuppression. Gene expression profiling seems to be a promising tool for this purpose[5].

Screening for and monitoring cardiac rejection: clinical methods

Hitherto, the main technique employed in monitoring the rejection status of a transplanted heart has been endomyocardial biopsy (EMB), which allows rejection to be screened for and monitored on the basis of the extent and distribution of lymphocytic infiltrates and associated myocardial damage[6]. The goal of periodic EMB is to detect acute rejection before allograft dysfunction occurs. The latest version of the ISHLT EMB grading scheme[7] establishes four categories: 0R (absence of rejection), 1R (mild rejection, defined as the presence of an interstitial and/or perivascular infiltrate, with or without a focus of myocyte damage, 2R (moderate rejection, the presence of two or more infiltrate foci with associated myocyte damage, and 3R (severe rejection, the presence of a diffuse infiltrate with multifocal myocyte damage and or edema, and/or vasculitis and/or hemorrhage). The letter "R" denotes "Revised Classification" to avoid confusion with the previous scheme, the 1990 working formulation[8].

Endomyocardial biopsy has significant limitations. It is invasive, it is expensive[9], its sensitivity is limited by sampling efficacy, it suffers from considerable interobserver variability, and it is difficult to interpret nodular endomyocardial infiltrates (so-called "Quilty lesions")[10]. Also, although the incidence of complications is very low when EMB is performed by experienced staff, severe complications can arise, including pneumothorax, bleeding, pericardial tamponade secondary to perforation of the right ventricle, arrhythmias, fistulae between a coronary artery and the right ventricle, tricuspid regurgitation, damage to the carotid or femoral artery, and arterial-venous fistulae[11]. There is no consensus among heart transplant centers or countries regarding the frequency with which EMB should be performed, or for how long they should continue to be performed, but the current trend is to reduce their number; most U.S. centers limit periodic EMB to the first five years posttransplantation[12], and most Spanish centers to the first year[13]. The role of EMB for rejection screening continues to be debated[14,15].

Although many noninvasive techniques have been investigated as regards their capacity for early detection of rejection, including echocardiography, radionuclide imaging, magnetic resonance imaging, intramyocardial electrogram, immune system monitoring and biochemical parameters[16-18], none has so far proved able to match the performance of EMB. Currently, a multi-parametric approach is employed that comprises clinical examination for signs or symptoms of heart failure, EMB, drug level monitoring, allograft function tests (mainly echocardiographic studies), and screening for allograft vasculopathy. At the same time it is mandatory to be on the look-out for side effects or complications of immunosuppressive therapy, particularly nephrotoxicity, infection, and cancer. Table 1 lists the most important procedures, including some that are currently still being validated for clinical use.

Gene-based methods in heart transplantation

The traditional genetic approaches of the pre-genomic era were designed to identify single loci or genes responsible for Mendelian disorders such as familial hypercholesterolemia. In these conditions, alteration of a single DNA codon results in pathological changes in protein abundance or function, and persons with the clinical signs of the pathology in question invariably exhibit the genetic alteration. However, pathologies of this kind are usually rare. The genetic component of more common disorders

Table 1. Immune and functional monitoring of heart transplant recipients

Monitoring tool	Type	Value
Endomyocardial biopsy	Histology and immunohistochemistry or immunofluorescence	Gold standard for the diagnosis of rejection. Disadvantage of being invasive and susceptible to sampling errors and variability in interpretation.
Drug monitoring and pharmacogenomics	Drug level or AUC	Trough levels are usually monitored for practical reasons, although peak levels usually correlate better with AUC; gene polymorphisms of CYP3A5 and MDR1 correlate with calcineurin inhibitor levels.
Functional monitoring	Diastolic parameters	Moderate correlation with significant rejection.
	Tissue Doppler	Δ tissue Doppler systolic velocities vare sensitive although less specific for the diagnosis of significant rejection.
	B-type natriuretic peptide	Correlates with significant rejection; no specific threshold has good discrimination capacity.
Genomic markers of rejection	AlloMap® Mollecular Expression Test (GEP)	Sensitive marker for cellular rejection although lower specificity; not validated for antibody mediated rejection.
T-cell function assays	ImmuKnow®	Marker of T-cell activation, currently under validation in heart transplantation.
	ELISpot	Marker of cytokine-producing T-cells; currently under validation.
Antibody monitoring	Donor-specific antibodies	The presence of DSA has been associated with an increased risk of rejection and allograft vasculopathy.

AUC: area under curve; GEP: gene expression profile: DSA: donor-specific antibodies.
Modified from Hunt, et al.[12]

generally involves variant alleles of multiple genes that interact to modulate the individual's response to non-genetic risk factors[19]. The problem for the clinical geneticist is to identify pathological patterns of variation. Human populations exhibit about three million single nucleotide polymorphisms (SNP), i.e. about 0.1% of the three billion base pairs in the human genome are polymorphic[5].

In the field of transplantation, several SNP have been associated with heart transplant outcome, which correlates with the possession of variant alleles by donor or recipient. These SNP are clustered in genes involved in alloimmune or pharmacogenetic interactions, the renin-angiotensin-aldosterone system, proclivity to renal dysfunction, and transforming growth factor-beta (TGFβ) signaling[5,20-24]. However, studies seeking to establish the relevance of a candidate polymorphism are often hampered by their observational character and small sample size, while meta-analyses are faced with selection bias (the non-publication of studies with negative results) and study weaknesses such as lack of clarity and uniformity regarding outcome measures, or poor evaluation of the ethnic characteristics of populations[5]. As a result, although SNP have thrown light on heart transplant outcomes, the possibility of using them to predict propensity for rejection remains uncertain.

Gene expression profiling

An approach that currently promises to be of much more immediate utility than SNP analysis is based on correlations between clinical states and the expression of certain genes. Although DNA defines a person's biological potential, it is the active transcription of DNA to RNA, followed by translation to protein, that realizes this potential in accordance with his or her history, environmental context, and clinical situation. If a gene expression profile can be identified that is sufficiently characteristic of a given physiological state, this profile can then be used to test whether the individual patient exhibits the state in question. If the gene expression profile becomes manifest before clinical, biochemical, or histologic signs, this allows earlier detection of disease states, and if it appears in tissue that can be obtained noninvasively, it may allow noninvasive diagnosis of conditions that were previously best diagnosed invasively. In certain cases, a gene expression profile may indicate not a current or imminent disorder, but a physiological state that correlates with the future development of disease.

Genes that are upregulated during acute cellular rejection after heart transplantation are involved in a wide range of functions, including T-cell activation and migration, natural killer cell activation, stem cell mobilization, hematopoiesis, platelet function, alloimmune recognition, and steroid responsiveness. Gene expression profiles have been obtained both from heart tissue[25] and from peripheral blood mononuclear cells (PBMC)[26,27]. For screening and monitoring purposes, the latter source has the great advantage over EMB of being noninvasive, which not only eliminates the risk of EMB-related complications but also allows more frequent testing.

Schoels, et al.[26] took 58 blood samples from 44 heart transplant patients at the time of EMB, and used real-time quantitative PCR to study the expression of 39 genes, including genes for cytokines and chemokines, in PBMC. The PBMC from patients with ISHLT EMB grades ≥ 2 (according to the 1990 classification[8]) differed significantly from those of patients graded < 2 as regards expression of the genes for perforin, CD95 ligand, granzyme B, RANTES, CXCR3, COX2, ENA 78, and TGFβ-1, and a group of five was identified (perforin, CD95L, RANTES, COX2 and SEC7/TIC) that, with appropriate thresholds, discriminated between the two EMB grade groups with a sensitivity of 84% and a specificity of 82%.

Polymerase chain reaction methods can only handle up to about 50 genes at a time. Much more efficient for the purposes of identifying gene expression profiles are DNA microarrays, which can carry representatives of all the genes in the genome. In a study in which they compared PBMC from seven patients of EMB grade ≥ 3A (1990 classification[8]) and seven of grade ≤ 1A with regard to 22,215 DNA transcripts, Horwitz, et al.[27] identified 91 transcripts (not all from different genes) that had significantly altered levels in the patients with rejection, and were able to discriminate between the groups with and without rejection by applying a clustering algorithm to data for 40 of these 91. Furthermore, when patients with rejection had been treated and their rejection largely resolved, they exhibited profiles intermediate between their previous (rejection) profiles and those of the patients without rejection, thus confirming the relevance of the corresponding genes to rejection. The involvement of the apoptosis-related gene *CFLAR* and the oxidative stress-related gene *SOD2* was confirmed by real-time quantitative PCR.

In the Cardiac Allograft Gene Expression Observational study (CARGO)[28], to date the largest and most systematic investigation of gene expression profiling for diagnosis of heart graft rejection, an RNA microarray of 50 mer oligonucleotides representing 7,370 genes identified 97 genes as candidate biomarkers when used to analyze gene expression in 285 PBMC samples from 98 patients (247 corresponding to ISHLT grade 0 EMB specimens, and 38 to specimens with ISHLT grade ≥ 3A). Together with a further 155 genes known to be related to these 97 or otherwise involved in transplant rejection, their utility as markers of rejection was further examined by statistical work-up of data from quantitative PCR analyses of 36 PBMC samples corresponding to grade ≥ 3A rejection and 109 corresponding to grade 0 EMB (most of these samples had not been included in the set screened for gene expression by microarray analysis). Eventually, a discriminator equation involving a group of 11 genes was developed that, on the basis of its application to a set of 281 samples from patients ≥ 1 year posttransplantation that was representative of the clinically observed EMB grade distribution, and using a diagnostic score threshold favoring negative predictive value, was estimated to have a negative predictive value of 99.6%, a positive predictive value of 6.8%, for grade ≥ 3A rejection. Using this equation, PCR of PBMC can allow patients with sub-threshold scores to be spared the risk involved in routine surveillance EMB. The CARGO II study is currently being carried out to validate these results in an independent, mainly European, population.

An unexpected and interesting feature of the CARGO data is that grade 1B EMB samples were associated with PBMC test scores similar to those of grade ≥ 3A samples and significantly higher than those of grade 2 samples (as well as grades 1A and 0)[28,29]. Although the import of this finding is still unclear, it suggests (if not the result of a combination of tissue sampling error and misclassification of EMB specimens by pathologists) that rejection, as reflected by PBMC gene expression profiles, may be a two-phase process. It was also noted that test scores in general tended to rise during the first year post-heart transplantation, and with them the optimal threshold for detection of rejection[28].

Starling, et al.[30], using the test version recently given FDA market clearance as the AlloMap® Molecular Expression Test (XDx, USA), found that this rise appeared to be largely due to post-transplantation weaning from steroids, and concluded that the AlloMap® test might be useful for monitoring this process as well as for ruling out rejection. Allograft vasculopathy has also been reported to be associated with higher AlloMap® scores[31].

The CARGO/AlloMap® studies mentioned above correlated test scores with current EMB status. The same research group also found that test scores could predict EMB-proven rejection weeks or months prior to the event[32], and that testing shortly after heart transplantation allowed the classification of patients as having low, medium, or high risk of future rejection[33]. In the other direction, early posttransplant ischemic injury has been reported to be associated with higher AlloMap® scores recorded an average 34 months post- heart transplantation[34].

Although the performance of the AlloMap® test has been tested in a large number of patients using EMB results as a reference method, EMB has significant shortcomings, notably tissue sampling error and between-pathologist differences in interpretation. The IMAGE study (for Invasive Monitoring Attenuation through Gene Expression) is currently being carried out to compare the AlloMap® with EMB in regard to relevant clinical outcomes (graft dysfunction, rejection with hemodynamic compromise, death, EMB-related complications), quality of life, and resource utilization[35].

In those of the above studies that concerned rejection, it was cellular rejection that was considered. An important recent development has been the analysis of CARGO study data to identify a gene expression profile for antibody mediated rejection[36]. This work is still in progress, but the results obtained so far are promising and indicate cross-linking between the processes of cellular and antibody mediated rejection.

In conclusion, the potential benefits of gene expression profiling of organ graft recipients are many[37]. In the first place, it may eventually be possible to diagnose rejection noninvasively and before tissue injury occurs; currently, it certainly seems possible to restrict the use of invasive techniques to a high-risk subgroup of patients identified by gene expression profiling at low cost. It has been estimated that the use of the AlloMap® test could save U.S. hospitals over $15.7 million annually[5,9]. Gene expression profiling may also allow prognosis of the outcome of rejection and of responsiveness to therapy, prediction of future allograft function, and the individualization or optimization of immunosuppressive therapy and changes thereof. Finally, it may help in the development of mechanism-based therapy. Further studies are needed to address these important issues fully.

Conflicts of interest: Dr Crespo-Leiro acknowledges participation in the on-going study CARGO II.

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