Noninvasive Evaluation of Cardiac Allograft Rejection by Cellular and Functional Cardiac Magnetic Resonance

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OBJECTIVES We sought to use cardiac magnetic resonance (CMR) to establish sensitive and reliable indexes for noninvasive detection of acute cardiac allograft rejection.

BACKGROUND Appropriate surveillance for acute allograft rejection is vitally important for graft survival. The current gold standard for diagnosing and staging rejection after organ transplantation is endomyocardial biopsy, which is not only invasive but also prone to sampling errors. The motivation of this study is to establish a CMR-based alternative that is noninvasive and sensitive for early detection of allograft rejection before irreversible damage occurs.

METHODS We employed a noninvasive 2-pronged approach to detect acute cardiac allograft rejection using a rodent working heart and lung transplantation model. We used CMR to detect immune-cell infiltration at sites of rejection by monitoring the accumulation of dextran-coated ultra-small superparamagnetic-iron-oxide–labeled immune cells (in particular macrophages) in vivo. Simultaneously, we used CMR tagging and strain analysis to detect regional myocardial function loss resulting from acute rejection.

RESULTS Immune cells infiltration, mainly macrophages and monocytes, could be identified with CMR by in vivo labeling with ultra-small superparamagnetic-iron-oxide. Our data show that immune-cell infiltration in cardiac allograft rejection was highly heterogeneous. Thus, it is not surprising to find inconsistencies between rejection and endomyocardial biopsy results because of the limited number and small samples available. Tagged CMR and strain analysis showed that, as with immune-cell infiltration, ventricular functional loss was also heterogeneous. Although changes in global systolic function were generally not observed until the later stages of rejection, our data revealed that a functional index derived from local strain analysis correlated well with rejection grades, which may be a more sensitive parameter for detecting early rejection.

CONCLUSIONS CMR is noninvasive and provides a 3-dimensional, whole-heart perspective of the rejection status, potentially allowing more reliable detection of acute allograft rejection. (J Am Coll Cardiol Img 2009;2:731–41) © 2009 by the American College of Cardiology Foundation

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With the advances in immunosuppressive therapy and organ preservation, survival rate and quality of life after heart transplantation have improved considerably. Although decreased from the early 1980s, acute cellular rejection still occurs in 40% to 70% of patients during the first 6 months following surgery (1) and remains a challenge for battling mortality (2). The occurrence of acute rejection episodes often is the most predictive factor for the later development of chronic rejection. Therefore, the development of new, accurate, and noninvasive methods for monitoring of acute allograft rejection after heart transplantation is of vital importance.

Acute rejection is traditionally diagnosed by endomyocardial biopsy, which carries finite risks of morbidity and mortality due to its invasive nature, and is also prone to sampling error because of the limited sizes and locations of tissue available, particularly in pediatric patients. More importantly, discrepancies have been found between biopsy-based diagnosis and actual rejection (3–6). Humoral rejection, which accounts for most of the biopsy-negative episodes of rejection (7), is associated with accelerated graft coronary artery disease, increased graft loss, and increased mortality. The discordance among biopsy, actual rejection, and patient outcome demands a reliable and noninvasive alternative to biopsy for detecting acute rejection.

Uncoupling the threshold for antirejection therapy from histological biopsy grading and linking it more closely to graft dysfunction has been suggested (6,8). However, commonly used imaging modalities, such as 2-dimensional echocardiography and nuclear imaging, are relatively insensitive to subtle changes in cardiac function. Functional changes resulting from acute rejection usually are barely detectable by these methods until later rejection stages, when it may be too late for therapeutic intervention.

The goal of this study is to develop noninvasive imaging methodology to better detect early-stage rejection before the development of irreversible organ damage. Our laboratory has implemented a two-pronged approach with cardiac magnetic resonance (CMR) to noninvasively monitor both immune-cell infiltration and cardiac function of the rejecting grafts simultaneously. Immune cells, particularly macrophages, are tracked with CMR by labeling them with dextran-coated ultra-small superparamagnetic-iron-oxide (USPIO) nanoparticles in vivo (9–13). Cardiac tagging and strain analysis are used to evaluate small regional functional changes during the rejection process.

**METHODS**

**Animals**

All rats used in the study were male inbred Brown Norway (BN) (RT1<sup>n</sup>) and Dark Agouti (DA) (RT1<sup>*</sup>) rats obtained from Harlan (Indianapolis, Indiana) with body weights between 180 and 250 g. The rats were housed individually and provided with food and water ad libitum. Animal protocols were approved by our Institutional Animal Care and Use Committee. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

In this study, 6 naïve BN rats were used for native heart controls. For the transplantation models, 20 rat transplantation pairs were used for experimental allograft hearts, and 13 rat transplantation pairs were used for isograft controls.

**Animal Model**

This study employs a novel rodent heterotopic working heart and lung model (Fig. 1B) using a DA→BN transplantation pair (10,11), with DA→DA and BN→BN transplantation as the syngeneic control. The recipient receives an additional heart and lung in the abdomen. While the native organs support life, the entire rejection process of the grafts can be investigated without serious systemic physiological alteration or mortality of the recipient from severe rejection. Although appropriate for studying immunological aspects of rejection, the single-anastomosis model (Fig. 1A) used in our earlier studies (12) is not suitable for functional investigation, due to insufficient loading, via gurigation only. The double-anastomosis working heart model (Fig. 1B) used in this study preserves intact pulmonary circulation and physiological pressure and volume loading by partially ligating the proximal end of the inferior vena cava. Although stroke volume may vary, ejection fraction is near 100%, and isografts exhibit left ventricle (LV) strains similar to native hearts (Fig. 2). The day of transplantation surgery is day 0. Histological examination (Fig. 3) shows that allografts exhibit...
grade II rejection on post-operative days 4 to 5, grade III rejection on post-operative days 5 to 6, and severe grade IV rejection on post-operative day 7.

In vivo labeling of USPIO. Dextran-coated USPIO particles are synthesized in our laboratory according to published methods (14,15) with slight modifications (16). The USPIO particles (~27 nm in size) are administered intravenously 1 day before CMR with the dosage ranging from 1.5 to 3 mg iron per animal.

In vivo CMR. Rats were first sedated with isoflurane, then intubated and ventilated. Anesthesia was maintained with a 2:1 O₂/N₂O gas mixture and 2% isoflurane. Leads were placed on both hind limbs for detecting the electrocardiogram of the transplanted heart in the abdomen. Body temperature was maintained at 36.5 ± 1°C using a rectal probe.

Figure 1. Abdominal Heterotopic Transplanted Heart and Lung Models
Schematic drawings of (A) nonloading heart model and (B) biventricular working heart model. Arrows indicate directions of the blood flow. Both models preserve all 4 heart chambers intact, yet the single-anastomosis model (A) has insufficient loading, via gurgitation only, whereas the double-anastomosis model (B) receives the proper loading via the inferior vena cava (IVC) and can pump against the abdominal aorta. AAo = recipient abdominal aorta; LA = left atrium; LV = left ventricle; RA = right atrium; Rt. Lung = donor right lung; RV = right ventricle.

Figure 2. Temporal Courses of Strains for Native and Isograft Hearts
Temporal changes of circumferential strains (Ecc, solid lines, all negative values) and radial thickening (Err, dotted lines, all positive values) strains for 3 native hearts (A, B, C) and 3 isografts (D, E, F) are plotted within a cardiac cycle. Strains from 6 short-axis slices are shown: 2 apical (Ape) slices (brown and dark red), 2 mid-level (Mid) slices (red and orange), and 2 basal (Bas) slices (yellow-orange and yellow).
and water pad with a feedback-controlled circulating water bath.

In vivo CMR was carried out on a Bruker Biospec 4.7-T/40-cm system equipped with 12-cm, 40-G/cm shielded gradients with a 5.5-cm home-built surface coil. Electrocardiogram and respiration-gated T2*-weighted images were acquired with a gradient-echo sequence, and tagging was achieved with a modified DANTE sequence. Ten short-axis slices were used to cover the entire volume of the heart. Equivalent temporal resolution for the cine loops was about 16.5 to 19.5 ms per frame, with 10 to 12 phases per cardiac cycle. Magnetic resonance images were acquired with the following parameters: repetition time: 1 cardiac cycle (~180 ms); echo time: 4 to 5 ms for tagging and 8 to 10 ms for T2*-weighted imaging; field of view: 3 to 4 cm; slice thickness: 1 to 1.5 mm; in-plane resolution: 117 to 156 μm.

Histological analysis and evaluation of rejection grade.
The degree of rejection was determined by pathological examination immediately following the CMR studies. Transplanted hearts were extirpated and fixed in 10% formalin. The rejection grade of the heart grafts was determined histopathologically according to the updated International Society for Heart and Lung Transplantation criteria (17,18) in a blinded manner by the Transplantation Pathology Laboratory of the University of Pittsburgh Medical Center.

Strain and wall-motion analysis. Strains are values that quantify the extent of ventricular deformation throughout cardiac phases: stretching/elongation or compression/shortening. Strains are categorized into 2 main classes in relation to the heart axes: normal strains are defined in relation to the short-axis planes, and principal strains are defined in relation to the direction of the myocardial fiber bundles. Three orthogonal strain-tensor sets define normal strains: circumferential strains (Ecc), radial thickening (Err), and longitudinal shortening (Ell), in which strain-tensors are tangent to the epicardium surface, perpendicular to the epicardium surface toward the center of the LV, and perpendicular to the short-axis plane along the long-axis of the LV, respectively. Three orthogonal strain-tensor sets define principal strains: E1 is orthogonal to the fiber direction; E2 is parallel to the fiber direction; and E3 is perpendicular to these two. Only 4 of the 6 strain-classes are calculated for the transplanted hearts in this study because the through-plane motions are not observed by the short-axis, multi-slice, 2-dimensional-tagged CMR used in this study.

Strains were analyzed by the harmonic phase method (HARP) (19) with software obtained from Diagnosoft, Inc. (Morrisville, North Carolina). Although the harmonic phase method is capable of calculating strains in all 3 heart layers (epicardium, mid-wall, and endocardium), only the mid-wall layer values are used in this study, because the recent MESA (Multi-Ethnic Study of Atherosclerosis) study showed that the mid-wall layer yields the best interobserver and intraobserver consistency (20).
Statistical analysis. The results are presented as mean ± SD. All results were analyzed by a 2-tailed t test.

RESULTS

Tracking macrophage infiltration in vivo with USPIO labeling. Cells can be visualized with CMR by labeling them with contrast agents such as USPIO. Iron-oxide particles generate local magnetic field gradients, and the loss of water proton spin coherence can be detected as signal loss in $T_2^*$-weighted images.

One day after administration of USPIO, accumulation of labeled immune cells was identified as patches of hypointensity on $T_2^*$-weighted images of allograft hearts (Figs. 4C to 4E). Histological examination indicates that iron particles are mostly found in ED1/macrophages (9–12). The $T_2^*$-weighted images of isograft control hearts display little or no areas of hypointensity (Figs. 4A and 4B). In this in vivo-labeling scheme, both residing and circulating macrophages can ingest USPIO particles.

Macrophage-infiltrated foci, in both the left and right ventricles, are found to be spatially heterogeneous. It is, therefore, not surprising to find discrepancies in diagnosing rejection with biopsy, where the small biopsy needles are generally limited to sampling points in the anteroseptal wall of the right ventricle. Tracking immune-cell infiltration with CMR is noninvasive and provides 3-dimensional whole-volume perspectives of the overall rejection status without the pitfalls of limited sampling.

Tracking regional functional loss in vivo with tagged CMR and strain analysis. To discern if the spatial heterogeneity of acute rejection after heart transplantation is also manifested in ventricular function, tagged CMR was used during the same imaging session as the cellular imaging to detect regional wall-motion abnormality.

Tagged CMR (Fig. 5) places signal-void grids on the myocardium at end-diastole by saturating proton spins at designated planes in space before the imaging sequence. Because the rate of the signal recovery is slower than the cardiac motion, these tagged lines (signal-void grids) remain during the cardiac cycle and serve to mark the motion of the myocardium. Tagged lines in a healthy heart show maximum displacement and curvature at end-systole throughout the LV wall, as observed in native hearts (Fig. 5F) and isograft controls (Fig. 5G). In allograft hearts, although ejection fraction remains close to 95%, some tagged lines do not show similar displacement or remain straight at end-systole, revealing areas with compromised wall motion (Figs. 5H to 5J).

The extent of tissue deformation observed by tagging can be quantified by strain analysis. Four strain types were analyzed, however, only the Ecc strain (tangent to the epicardium surface) is shown in this article due to space limitations. Figure 6A shows mean Ecc values for transplanted hearts with different degrees of rejection. Although global cardiac functional parameters, such as stroke volume (Fig. 6B) or ejection fraction (Fig. 6C), do not degrade according to rejection grades very much, allografts with rejection greater than grade II all show a decrease in mean Ecc. This is indicative that strain analysis could be a more sensitive measure of function than conventional modes of evaluating cardiac function. However, mean Ecc is poor at discriminating between grade II and grade III rejection.

To investigate regional strain, the LV wall is divided into 6 regions (Figs. 7 and 8A), following the standard cardiology convention. Isografts (Fig. 7A) exhibit consistent Ecc throughout different subregions of the LV wall. Allografts (Figs. 7B and 7C) exhibit healthy strains in some regions, but
compromised strains in other areas, showing that the functional loss in rejection is heterogeneous. Despite having similar mean Ecc, grade III (Fig. 7C) allografts have more regions with compromised strains than grade II (Fig. 7B) allografts do and can thus be distinguished. Regional strain analysis is therefore a more sensitive way to dissect regional dysfunction and rejection grade than global functional parameters.

However, unlike coronary artery disease, rejection does not necessarily follow certain coronary artery perfusion domains, and as a result, the actual phenomenon might be lost in the conventional 6-heart-region division. Thus, starting at the interception with the right ventricle on the anterior wall, 48 probe points were placed evenly throughout the LV mid-wall to further dissect regional function. Figure 8B shows a polar plot of probe-point Ecc values at different rejection grades. The isograft control shows relatively consistent Ecc throughout the whole LV wall, whereas allografts have some stretches of probes exhibiting compromised low Ecc values.

The severity of rejection correlates with the number of compromised probe points. Figure 9A graphically shows examples of compromised Ecc probe points at different rejection grades. Probe points are scored as compromised (solid bars) if the Ecc value is lower than 1 SD from the isograft mean. Figure 9B shows the Ecc strain index (per-
centage of compromised probe points) for transplanted isograft and allograft hearts. A high score indicates poor ventricular function that can be readily correlated with rejection grade.

Simultaneously tracking regional functional loss and immune-cell infiltration in the same heart by in vivo CMR. Figure 10 shows examples of noninvasive tracking of both immune-cell infiltration (left 2 columns) and regional wall motion (right 2 columns) at the same time. Pseudocolors (Figs. 10D to 10F) are placed on top of the T$_2^*$-weighted CMR (Figs. 10A to 10C) to show regions having USPIO-labeled immune-cell infiltration. The Ecc-strain values are color coded on tagged images (Figs. 10G to 10I). In addition, Ecc values obtained by the 48 probe points are also plotted (Figs. 10J to 10L) for comparison. Although some areas with high USPIO-labeled macrophage infiltration seem to largely correlate with areas with compromised Ecc, the subnormal areas with the 2 modalities do not seem to match completely.

Although the signal- and contrast-to-noise ratios from T$_2^*$-weighted images do not always exhibit a positive correlation with rejection grades, the areas identified as having USPIO-laden macrophage infiltration (Fig. 11A) do increase up to a moderate rejection grade, with no further increase observed from moderate rejection to severe rejection. Although both the volume with USPIO-laden macrophage infiltration and Ecc values do not correlate perfectly with rejection grades, they do correlate in the same heart (Fig. 11B). Regardless of the rejection grades, the hearts with higher infiltration volume exhibit lower Ecc values in most cases.

**DISCUSSION**

Monitoring acute allograft rejection with CMR has the advantage of being noninvasive and providing 3-dimensional information on the whole heart, whereas biopsy is restricted to limited tissue samples. Our 2-pronged approach of monitoring both immune-cell infiltration and organ function simultaneously can better diagnose episodes of acute allograft rejection.

It is known that discrepancies are observed clinically among biopsy, rejection, and organ dysfunction, even with the updated and improved pathological grading system (4). Variation could be due to subjective interpathologist interpretation. Alternatively, one may argue that mononuclear-cell infiltration may not be the only mechanism of acute cardiac rejection (8). Interestingly, our data reveal a different observation and explanation, namely that the infiltration of immune cells is highly heterogeneous, even in grade III rejection. The mononuclear-cell infiltration can be expected to be even more sparse and inhomogeneous at earlier rejection phases (10). Therefore, biopsy with finite sampling sizes can miss the infiltrated sites, resulting in false diagnosis and a discrepancy between biopsy, actual rejection, and organ dysfunction. Because CMR can cover the whole volume of the heart, monitoring immune-cell infiltration with CMR could reveal overall distribution of mononuclear cells. Our approach of monitoring mononuclear-cell infiltration in vivo with CMR is not only noninvasive, but also potentially can provide a more accurate diagnosis. In combination with automated algorithms for detecting USPIO-laden mononuclear-cell infiltration foci (21),
our approach may make accurate and objective diagnosis of cellular rejection possible.

At the dosages used clinically, USPIOs have been shown to possess low toxicity, and the iron is metabolized by incorporating into normal endogenous iron pools (22). Immune cells, mainly macrophages, are readily labeled by direct intravenous administration of USPIO, with no need for cell isolation or culture. This in vivo labeling strategy also avoids possible unwanted activation of immune cells, if cells are isolated, labeled ex vivo, and reintroduced into patients. This in vivo labeling approach is simple and makes clinical translation much more feasible than the ex vivo cell-labeling methods. It is commonly accepted that T and B lymphocytes are the dominant cells responsible for the rejection and inflammatory processes. However, macrophages are present abundantly when rejection occurs. We have previously found that the numbers of both macrophages and T cells increase during the rejection process (12,13). Thus, macrophages are a good target for in vivo imaging for detecting rejection. This study and our earlier studies have shown that macrophage infiltration is indeed a useful index for acute rejection (9–13), possibly even for chronic rejection (23).

CMR tagging is a powerful noninvasive tool for monitoring cardiac wall motion. Our results have shown that regional loss of function is

**Figure 8. Local Ecc for Different Heart Locations**

(A) Drawing of 6 heart regions. R1 = anterior; R2 = anterolateral (LCX); R3 = lateral; R4 = inferior; R5 = infero-septal (RCA); and R6 = anterio-septal (LAD). (B) 48 probe-points are placed throughout the LV wall: probes 1 to 8 (R1), probes 9 to 16 (R2), probes 17 to 24 (R3), probes 25 to 32 (R4), probes 33 to 40 (R5), probes 41 to 48 (R6). |Ecc| = absolute values of Ecc are plotted without negative signs. Black line = averaged Ecc for isografts; yellow triangle = Grade I allograft; green circle = Grade II allograft; blue square = Grade III allograft; red diamond = Grade IV allograft.
highly heterogeneous, which is not easy to achieve with other functional and imaging modalities. These results are consistent with clinical observations and emphasize that the correlation of organ dysfunction with acute allograft rejection is a better indicator of rejection than biopsy alone (6). Detailed local strain analysis provides a sensitive way to detect small regional wall motion abnormality. The harmonic phase method provides an easy and fast way for strain analysis, which can facilitate high-throughput strain analysis for clinical applications.

Although very informative, the strain maps are not straightforward for discerning mildly defective hearts, because even healthy hearts possess some variability in strains throughout different regions of LV myocardium. By using probe points for analysis, areas with compromised...
strains can readily be identified; furthermore, the percentage of probe points having compromised Ecc strain correlates well with rejection grades. One standard deviation from mean isograft Ecc values is used here as the criteria to distinguish “compromised” and “healthy” probe points. However, this criterion obtained from our heterotopic model may not be readily applicable for human patients with orthotopic transplantation. Additional human studies to determine criteria for transplant patients will be necessary to translate this approach to the clinical setting.

In this study, although 4 different strain types were analyzed, only Ecc results are shown due to space limitations. There are no obvious differences in all 4 strain types based on our analysis. Further detailed analysis could be interesting to see whether a specific strain class is affected more by the rejection and could be used as the most sensitive functional parameter for staging rejection.

Coupling cellular imaging of immune-cell infiltration and functional parameters provides additional power for detecting and grading rejection. Regional loss in the contractile function appears to correlate with the areas of the myocardium found to have significant USPIO-labeled macrophage infiltration; however, some mismatch was found. This mismatch could be due to mechanical, physiological, or electrical reasons. For example, myocytolysis or degenerated myocardium could physically affect contractile function of the neighboring myocardium; the contractile defect from 1 necrotic region could radiate to larger remote areas through connecting myocardial fiber; or electrical conductivity could be hindered or interrupted by the necrotic regions. Some mismatch found here may also result from pixel mismatch between the 2 methods. Better computational algorithms for precise pixel-by-pixel analysis and a larger sampling group will be beneficial for understanding the more detailed biomechanics of rejection.

Signal- and contrast-to-noise ratios correspond with the amount of contrast agent found in the tissue; however, the number of iron-labeled cells that have accumulated in an image voxel is inherently difficult to quantify by T2*-contrast. In addition, the degree of immune-cell labeling by direct in vivo injection of contrast agent is not well controlled. These drawbacks, however, do not limit our method, because our results indicated that the absolute amount of cell infiltration may not be important for staging rejection by CMR. We have found out that the percentage of contrast-containing-volume may be very useful for discerning areas with labeled cell infiltration. Furthermore, although the extent of labeled cell infiltration and Ecc strain reduction themselves show some variations, the transplanted hearts with more labeled cell infiltration exhibit poorer Ecc strains in most cases. This further testifies the advantage of using a 2-parameter diagnosis.

CONCLUSIONS

Our results suggest that myocardial rejection is spatially highly heterogeneous, for both cellular infiltration and myocardial function. The immune-
REFERENCES


Key Words: cardiac magnetic resonance • noninvasive detection of acute cardiac rejection • cardiac transplantation • biopsy.