Leukocyte-Targeted Myocardial Contrast Echocardiography Can Assess the Degree of Acute Allograft Rejection in a Rat Cardiac Transplantation Model

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Background—Repetitive endomyocardial biopsies are necessary to monitor the effects of immunosuppressants after cardiac transplantation. Contrast ultrasound with microbubble targeting of leukocytes detects acute leukocyte infiltration. We examined whether leukocyte-targeted myocardial contrast echocardiography (MCE) could provide for the quantitative assessment of acute cardiac rejection.

Methods and Results—Hearts from Brown Norway rats or Lewis rats were transplanted into other Brown Norway rats. Isografts and groups of allografts either untreated or treated with cyclosporin A (CsA) at a low dose (3 mg · kg⁻¹ · d⁻¹) or high dose (10 mg · kg⁻¹ · d⁻¹) from 3 days before transplantation were compared at posttransplantation day 3. Echocardiography-derived left ventricular wall thickening was comparable among the 4 groups. Myocardial blood flow assessed with MCE, relating pulsing intervals with signal intensity (SI), was slightly decreased only in untreated allografts. However, myocardial SI (in gray levels) obtained after a 10-minute period allowing microbubble–leukocyte interactions after contrast injection exhibited a clear gradient in these groups (12±2 in untreated allografts, 9±5 in allografts treated with low-dose CsA, 6±3 in allografts treated with high-dose CsA, and 2±1 in isografts, P<0.001). The pattern of difference in SI among the groups agreed well with that in ED-1–positive cell (macrophage) count (25±7, 12±4, 5±3, and 1±0 cells per high-power field, respectively, P<0.001), which correlated with CD3-positive cell (T lymphocyte) count (33±5, 22±5, 9±4, and 1±0 cells per high-power field, respectively, P<0.001).

Conclusions—Leukocyte-targeted MCE can noninvasively assess the degree of rejection in transplanted hearts by directly revealing the magnitude of intramyocardial infiltration of macrophages and T lymphocytes. (Circulation. 2004;109:1056-1061.)

Key Words: rejection ■ leukocytes ■ cyclosporine ■ echocardiography

Allograft rejection remains a major complication after transplantation, causing graft loss and increased mortality in the recipients, although cyclosporin A (CsA)–based immunosuppression has contributed to allograft survival.1 Endomyocardial biopsy is the “gold standard” routinely used to diagnose cardiac rejection and monitor immunosuppression therapy.3 However, this procedure is invasive and is associated with some risk of morbidity and mortality,4 and rejection that is focal or of patchy distribution is subject to false-negative results because of limited sampling sites.5 Therefore, many attempts6–11 have been made to develop noninvasive approaches to globally assess cardiac rejection, none of which are in widespread clinical use.

Although T cells play a central role in graft rejection,12 infiltration of T cells is accompanied by monocyte/macrophage infiltration11–15 because of their close interactions.10,15 Agents that affect primarily T cells,12 including CsA,16,17 can also limit monocyte/macrophage infiltration.10,16 Indeed, the extent of macrophage infiltration as assessed by MRI with a magnetic contrast agent targeted to those cells was shown to allow detection of acute cardiac rejection in rats.10

Some microbubble agents initially introduced as blood flow tracers for myocardial contrast echocardiography (MCE) are adhesive to and phagocytosed by leukocytes, including macrophages.18,19 The microbubbles interacting with leukocytes remain acoustically active and provide for ultrasound imaging of inflammation.19–22 Therefore, in the present study, we sought to clarify whether leukocyte-targeted MCE can provide for the noninvasive detection of acute cardiac rejection and enables quantitative assessment of the magnitude of infiltration of monocyte/macrophages and T cells and the histological severity of acute cardiac rejection.
Methods

Cardiac Transplantation

The study protocol was approved by the Animal Research Committee of Kagawa University School of Medicine. Fifty-two male Brown Norway (BN) rats and 30 male Lewis rats (250 to 300 g; Seac Yoshitomi, Fukuoka, Japan) were used. For isograft transplantation (n = 11), the donor heart was harvested from a BN rat and transplanted to a donor BN rat. For allotransplantation, a graft harvested from a Lewis rat was transplanted to a BN rat. These allotransplanted rats were divided into 3 groups of 10 rats each, which were either untreated or treated with a low dose (3 mg · kg$^{-1}$ · d$^{-1}$) or a high dose (10 mg · kg$^{-1}$ · d$^{-1}$) of subcutaneously administered CsA from 3 days before transplantation.

We performed heterotopic cardiac transplantation according to the Ono-Lindsey method. Briefly, after anesthesia with pentobarbital (35 mg/kg IP), the donor rats were heparinized (500 U/kg IV). A thoracotomy was then performed, and the heart was exposed. The superior and inferior venae cavae and the pulmonary veins were ligated. The aorta and pulmonary artery were cut, and the explanted heart was immediately immersed in cold saline. The recipient rats were similarly anesthetized. The abdomen was opened by a midline incision, and segments of the aorta and the vena cava were isolated and occluded with small-vessel forceps. The aorta and pulmonary artery of the donor heart were anastomosed end to side to the abdominal aorta and the vena cava of the recipient rat, respectively. The abdominal wall via an acoustic coupler to record short-axis images of the left ventricle of the graft. An ECG of the transplanted heart was selectively recorded by placing the surface electrodes at the base and apex of the transplanted heart. 2D images were recorded with the fundamental mode on S-VHS videotapes. Systolic thickening was evaluated in the anterior and posterior walls as %thickening = 100X (end-systolic thickness − end-diastolic thickness)/end-diastolic thickness.

Myocardial Contrast Echocardiography

With the same ultrasound system and transducer position as those for the functional assessment, we performed 2 types of MCE: perfusion imaging and leukocyte-targeted imaging. We used a phospholipid-stabilized sulfur hexafluoride microbubble agent, BR1 (Bracco Research SA), supplied as powder (25 mg/vial). The vial was shaken vigorously for 20 seconds after addition of 5 mL saline to obtain microbubble solution, which was further diluted with saline to a final concentration of 2X10$^{6}$ microbubbles/mL. We used a mechanical perfusion imaging, the ultrasound emission was suspended while microbubbles and leukocytes and the clearance of freely circulating microbubbles from the blood pool. Subsequently, the imaging was resumed on end systole at a rate of 30 frames/sec for 2 seconds, in which the initial frame depicted signals from both retained and freely circulating microbubbles and subsequent frames destroyed microbubbles in the beam. Then the PI was increased to 1:80 to allow complete replenishment of the beam elevation with blood containing freely circulating microbubbles. The initial image and those with a PI of 1:80 were digitized. Seven precontrast frames were averaged and subtracted from the initial frame and from the averaged frame obtained at a PI of 1:80. The resultant images, “Initial” and “PI(1:80),” respectively, were color-coded with shades of red, orange, yellow, and white for increasing VI for display. The intensity of signals originating from only the retained microbubbles was obtained as the difference in the background-subtracted VIs of Initial over PI(1:80) frames.

Histological Analysis

After MCE was completed, rats were killed, and the transplanted hearts were subjected to histopathology. Hematoxylin-eosin staining was performed to determine rejection grade according to the International Society for Heart and Lung Transplantation (ISHLT) criteria1 for each graft. Immunohistochemistry was performed with a commercial kit (Simple Stain MAX PO, Nitirei) and monoclonal antibodies (Serotec Ltd). On deparaffinized sections, anti-rat macrophage antibody ED-1112,13 and anti-rat CD3 antibody were used as the primary antibodies for macrophage lineage cells and T cells, respectively, and anti-granulocyte antibody HIS-4826 was used as the primary antibody for neutrophils on frozen sections. The magnitude of respective cell infiltration was quantified by counting positively stained cells in at least 10 high-power fields randomly selected for each section.

Statistics

Data are expressed as mean ± SD. Differences in measured values and incidence among the groups were analyzed by Kruskal-Wallis test with Dunn’s multiple comparison test and χ$^2$ test, respectively. A level of P<0.05 was considered statistically significant.

Results

Wall Thickness and Systolic Function of Grafts

All transplanted hearts were beating well on palpation at postoperative day 3. The anterior wall thickness was significantly greater only in the untreated allografts compared with isografts. The wall thickening was comparable both for the anterior and posterior walls independently of isograft transplantation or allotransplantation and CsA doses (Table 1).

Myocardial Perfusion

No apparent myocardial opacification was noted at a PI of 1:1, but VI was gradually increased according to the increase in PI in all grafts. Although the untreated allografts exhibited a lower rate of VI rise (β) and peak plateau VI (A) and hence lower myocardial blood flow (A×β) than other grafts, allografts treated with high- or low-dose CsA showed results similar to those in isografts. Thus, perfusion imaging could detect the abnormality only in the untreated allografts, but allografts treated with CsA showed normal perfusion.

Leukocyte-Targeted Imaging

Figure 1 depicts representative images in leukocyte-targeted myocardial imaging. In the initial frames shown on top, although homogeneous and dense opacification was exhibited in an untreated allograft (left), the opacification was attenuated in another allograft that received high-dose CsA, and nearly no signals were detected in an isograft (right). The lack
of opacification at the PI of 1:80 in all grafts indicated that nearly no free microbubbles remained in the blood pool at this time point after microbubbles infusion (bottom images). The VI originating from microbubbles retained in the myocardium was highest in untreated allografts, second highest in low-dose CsA–treated allografts, second lowest in high-dose CsA–treated allografts, and lowest in isografts (Table 1).

### Immune Cell Infiltration and Severity of Rejection
Marked and diffuse infiltration of both ED-1–positive cells (Figure 2, A-1) and CD3-positive cells (Figure 2, E-1) was exhibited in an untreated allograft. Although the majority of these cells were seen in the perivascular interstitial space, some of them were observed in small vessels, as shown in the higher-power photographs (Figure 2, A-2 and E-2). Infiltration of these cells was less extensive in an allograft treated with low-dose CsA (Figure 2, B and F). An allograft treated with high-dose CsA exhibited only focal infiltration of both ED-1–positive (Figure 2C) and CD3-positive (Figure 2G) cells. Infiltration of these cells was absent in an isograft (Figure 2, D and H). HIS-48–positive cells were sparse in all groups (Figure 2, I-L). Thus, marked infiltration of both macrophages and T-cell lymphocytes was induced in allotransplanted hearts, which was significantly suppressed by CsA treatment in a dose-dependent manner. Neutrophil infiltration was limited in each group (Table 2). In addition, there was a close correlation between the cell counts of ED-1–positive cells and CD3-positive cells (r=0.96, P<0.0001). The severity of rejection by ISHLT grade was highest in untreated allografts, suppressed dose-dependently by CsA in treated groups, and nearly absent in isografts (Table 2).

### Table 1. Summary of Echocardiographic Analyses of Left Ventricle

<table>
<thead>
<tr>
<th></th>
<th>Allograft-CsA(0)</th>
<th>Allograft-CsA(3)</th>
<th>Allograft-CsA(10)</th>
<th>Isograft</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=10)</td>
<td>(n=11)</td>
</tr>
<tr>
<td>End-diastolic wall thickness, mm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior wall</td>
<td>1.14±0.11†‡</td>
<td>1.07±0.11</td>
<td>1.04±0.05</td>
<td>1.02±0.10</td>
</tr>
<tr>
<td>Posterior wall</td>
<td>1.13±0.12</td>
<td>1.09±0.09</td>
<td>1.07±0.08</td>
<td>1.05±0.05</td>
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<tr>
<td>Wall thickening, %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anterior wall</td>
<td>82±12</td>
<td>85±14</td>
<td>92±7</td>
<td>101±7</td>
</tr>
<tr>
<td>Posterior wall</td>
<td>87±18</td>
<td>91±10</td>
<td>94±9</td>
<td>104±9</td>
</tr>
<tr>
<td>Myocardial perfusion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (gray levels)</td>
<td>15.3±0.7†</td>
<td>17.0±0.8</td>
<td>17.5±0.5</td>
<td>17.7±0.6</td>
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<tr>
<td>β, s⁻¹</td>
<td>0.55±0.03†</td>
<td>0.65±0.02</td>
<td>0.66±0.01</td>
<td>0.67±0.01</td>
</tr>
<tr>
<td>A×β</td>
<td>8.4±0.7†</td>
<td>11.1±0.6</td>
<td>11.5±0.4</td>
<td>11.8±0.4</td>
</tr>
<tr>
<td>Leukocyte-targeted imaging</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Videointensity</td>
<td>12±2*</td>
<td>9±5†</td>
<td>6±3‡</td>
<td>2±1</td>
</tr>
</tbody>
</table>

Allograft-CsA(0) indicates allograft untreated with CsA; Allograft-CsA(3), allograft treated with 3 mg · kg⁻¹ · d⁻¹ of CsA; Allograft-CsA(10), allograft treated with 10 mg · kg⁻¹ · d⁻¹ of CsA; and Isograft, untreated isograft.

*P<0.001, †P<0.01, ‡P<0.05 vs isograft.

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**Figure 1.** Background-subtracted color-coded ultrasound images at posttransplantation day 3. Initial frames acquired 10 minutes after injection of microbubbles (top) and images subsequently obtained at a PI of 80 beats (bottom) are shown. A 256-level color scale for background-subtracted VI is illustrated at bottom of each image. Untreated allograft (left) showed homogeneous opacification on initial frame (top). Opacification in initial frame was decreased in allografts treated with CsA dose-dependently and was lowest in isograft (right). CsA(0) indicates untreated; CsA(3), treated with 3 mg · kg⁻¹ · d⁻¹ of CsA; CsA(10), treated with 10 mg · kg⁻¹ · d⁻¹ of CsA.
Histology Versus Leukocyte-Targeted Imaging

Close correlations existed between the VI from the retained microbubbles and both ED-1-positive cell count ($r=0.87$, $P<0.0001$) (Figure 3A) and CD3-positive cell count ($r=0.81$, $P<0.0001$) (Figure 3B). By contrast, neutrophil counts were too low to be correlated with the VIs. Compared among grafts with various ISHLT grades, the VI from the retained microbubbles exhibited a clear correlation with the severity of rejection (Figure 4).

Discussion

In this study, we propose a novel application of leukocyte-targeted MCE for the quantitative assessment of acute cardiac rejection. We have shown that delayed ultrasound imaging allowing a period of microbubble-leukocyte interaction after microbubble injection reveals the extent of immune cell infiltration in the grafts and therefore the severity of rejection at an early stage at which no apparent deterioration of myocardial function or perfusion is manifested.

Because allograft rejection remains a critical concern for transplant recipients, an accurate method to diagnose rejection has been pursued. Because the functional deterioration does not occur until moderate to severe rejection is histologically manifested, functional assessment by echocardiography in a previous study yielded only a low sensitivity. Although the anterior wall thickness was increased only in the untreated allografts compared with isografts, which might suggest acute rejection, wall thickening was preserved at normal levels in all groups with varying degrees of histological abnormalities until at least posttransplantation day 3 in our study. Although

TABLE 2. Summary of Histological Analyses

<table>
<thead>
<tr>
<th>ISHLT rejection grade, No. of rats§</th>
<th>Allograft-CsA(0) (n=10)</th>
<th>Allograft-CsA(3) (n=10)</th>
<th>Allograft-CsA(10) (n=10)</th>
<th>Isograft (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>I</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>III</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IV</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ISHLT indicates International Society of Heart and Lung Transplantation; cell counts of ED-1, CD3, and HIS-48 represent those of macrophages, T cells, and neutrophils, respectively. Other abbreviations as in Table 1.

* $P<0.001$, † $P<0.01$, and ‡ $P<0.05$ vs isograft; § $P<0.001$ by χ² test.
diastolic dysfunction assessed with Doppler echocardiography preceding systolic dysfunction would be more sensitive in detecting the functional deterioration, atrial dysfunction secondary to surgical anastomosis and asynchronous contraction of the native and donor atrial tissue may limit its usefulness.  

A study in rats demonstrated that myocardial blood flow assessed by the hydrogen-clearance method recovered during the first day after transplantation in both allografts and isografts but decreased again in allografts 3 days after transplantation, which was followed by systolic dysfunction at day 5. 27 In the present study, MCE based on destruction-replenishment dynamics 25 revealed a significant reduction of myocardial perfusion in the untreated allografts but not in the allografts treated with high- or low-dose CsA. Thus, perfusion imaging was insensitive to any abnormalities in allografts treated with CsA at posttransplantation day 3.

Few methods have directly targeted the immunological alterations of the graft, such as the endothelial adhesion molecule overexpression 11 and immune cell infiltration, until recently. 10,11,13 In acute transplant rejection, T cells are known to play a critical role in the initiation of the rejection process. 12 However, macrophages are also recruited via chemokines, including interferon-γ derived from T cells. More recently, interferon-γ has been shown to stimulate macrophages to release monokine, which directs T cells into the allograft. 15 Thus, both T cells and macrophages are involved in the process of acute rejection, 14 with mutual interactions. Although CsA, a calcineurin inhibitor, exerts its immunosuppressant actions primarily by inhibiting T cells, 17 CsA was found to be effective in macrophage activation syndrome, 16 probably because of their close relationship to T cells. In fact, there was a close correlation between macrophage cell counts and T-cell counts among the rats subjected to various doses of CsA in our study.

Lindner et al 18–20 recently showed that lipid microbubbles are retained within the microcirculation of inflamed tissue because of their attachment to and phagocytosis by activated leukocytes adhering to the venular wall in the inflamed site. These retained microbubbles maintain their acoustic properties, which provide for ultrasound imaging of leukocytes recruited by reperfusion injury in the kidney 20 and heart. 22 We have extended the application of this method to the evaluation of immune cell infiltration in allograft rejection in which macrophages as well as T cells infiltrate. Our results are in agreement with those by Kanno et al, 10 who used a contrast medium targeted to macrophages and detectable with MRI. They also observed a clear difference in signal intensity alterations among allografts, allografts treated with CsA, and isografts, which paralleled the intragroup difference in ED-1-positive cell count. It is possible that immune cells other than macrophages, such as lymphocytes and granulocytes, may also play a role in the retention of microbubbles in the rejecting grafts, either by directly trapping the microbubbles on their surface or by stimulating adhesion molecules on endothelial cells. However, because the efficiency of microbubble targeting may be less for T cells incapable of phagocytosis than for macrophages and because the majority of recruited cells in acute cardiac rejection in the early stage are T cells and macrophages 10 but not neutrophils, as shown in this study, it is likely that the signal intensity obtained by our method also reflects primarily macrophage-related changes in graft rejection.

Weller et al 11 were the first to apply MCE to detection of allograft rejection. By using anti–intercellular adhesion molecule-1 antibody–conjugated microbubbles, they demonstrated increased microbubble retention in grafts undergoing severe rejection. However, it remains unknown whether the intercellular adhesion molecule-1 expression or microbubble attachment to the molecules parallels the severity of rejection. 11 By contrast, in our study, leukocyte-targeted imaging was applied to grafts with varying severities of rejection and was shown to provide for a quantitative assessment of immune cell infiltration and hence the severity of acute rejection.

Study Limitations
Macrophages are involved not only in allograft rejection but also in other antigen-nonspecific pathological conditions,
including reperfusion injury. In addition, neutrophils may be recruited in the reperfusion site and may produce opacification during leukocyte-targeted MCE early after transplantation. However, because the signal intensity was very low in isografts that were subjected to reperfusion injury at the time of surgery similarly to other opacified groups and because HIS-48-positive cells were sparse in all groups, it is likely that the effect of reperfusion subsided by postoperative day 3 in our model.

Because the microbubbles are delivered by blood flow to the immune cells, sufficient perfusion is essential for the present method. In this regard, myocardial perfusion was preserved intact or sufficient to deliver microbubbles in the present study. Other methods based on functional assessments may be available in determining the rejection at an advanced stage when the blood flow is deteriorated.

We used only a single combination of microbubble agent and ultrasound condition. Modification of the microbubble shell for a higher affinity to macrophages or directly to T cells would improve the sensitivity and accuracy of this method. We used a high carrier frequency of ultrasound to ensure a resolution sufficient to image the grafts in rats. Although the wide band responsiveness of this transducer (5 to 12 MHz) may allow it to cover both fundamental and harmonic signals from microbubbles, other more sensitive modalities, such as power-pulse inversion or power modulation, that use lower carrier frequencies may be available and be a promising application in patients.

Finally, we did not apply our method to the detection of chronic rejection. It is possible that our method is feasible also in assessing chronic rejection, in which T cells and macrophages are known to be the most abundant leukocytes in the infiltrate. Nevertheless, further studies are needed to clarify whether a sufficient amount of microbubbles can be delivered to macrophages in chronic rejection manifested by coronary arteriosclerosis.

Conclusions

The leukocyte-targeted MCE can directly image immune cell infiltration, which precedes the deterioration in myocardial perfusion or function. This method has the potential to provide an accurate and noninvasive evaluation of allograft rejection at its early stage and thereby may be useful to monitor immunosuppression after cardiac transplantation.

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References


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