Ultrasound Imaging of Acute Cardiac Transplant Rejection With Microbubbles Targeted to Intercellular Adhesion Molecule-1

Gregory E.R. Weller, PhD; Erxiong Lu, MD; Melissa M. Csikari, BS; Alexander L. Klibanov, PhD; David Fischer, BS; William R. Wagner, PhD; Flordeliza S. Villanueva, MD

Background—Noninvasive techniques for detecting acute cardiac transplant rejection are limited. We hypothesized that ultrasound contrast microbubbles targeted to the endothelial cell (EC) inflammatory marker intercellular adhesion molecule-1 (ICAM-1) would selectively bind to rejecting versus nonrejecting myocardium and that myocardial contrast echocardiography can therefore detect acute rejection.

Methods and Results—Lipid-based microbubbles were conjugated to anti-rat ICAM-1 (MB_ICAM) or isotype control antibody (MB_Control). In vitro MB_ICAM adhesion to cultured rat ECs, as assessed in a parallel plate flow apparatus, was greater to inflammatory versus normal ECs (11±3 versus 3±2 microbubbles/EC, \(P<0.005\)). In vivo abdominal heterotopic heart transplantation was performed in rats (rejection group: Brown Norway to Lewis strain; control group: Lewis to Lewis or Brown Norway to Brown Norway). Triggered myocardial contrast echocardiography was performed during intravenous MB_ICAM or MB_Control (2.5×10^6) injection on postoperative day 5. Myocardial videointensity from adhered MB_ICAM was significantly higher in rejecting (n=8) versus control (n=7) rats (10±4 versus 1±4 U, \(P=0.01\)). Postmortem histology showed normal myocardium in control rats, whereas allograft myocardium demonstrated grade III to IV rejection and strong immunohistochemical ICAM-1 staining.

Conclusions—Preferential adherence of ICAM-1–targeted microbubbles to rejecting versus nonrejecting rat cardiac transplant myocardium can be detected ultrasonically. Targeted microbubbles may thus offer a noninvasive ultrasound imaging technique for the detection of acute cardiac transplant rejection and other processes characterized by endothelial dysfunction. (Circulation. 2003;108:218-224.)

Key Words: rejection ■ echocardiography ■ contrast media ■ cell adhesion molecules ■ transplantation

Acute rejection is a leading cause of mortality in heart transplant recipients, responsible for 20% of deaths in the first posttransplantation year and up to 15% thereafter. Presently the "gold standard" for the diagnosis of rejection is endomyocardial biopsy, which commits posttransplantation patients to repetitive invasive procedures over the course of their lives. Previously reported2–4 noninvasive tools to diagnose rejection have limitations that preclude their widespread use, and the development of a sensitive noninvasive method for detecting rejection remains an elusive goal.

A pivotal event in the pathogenesis of organ transplant rejection is endothelial dysfunction, which also occurs in a variety of cardiovascular diseases, including cardiomyopathy, hypertension, and atherosclerosis.5–7 Endothelial dysfunction is characterized by the inflammatory upregulation of leukocyte adhesion molecules (LAMs) that arrest blood-borne leukocytes to the vascular wall for extrusion into the inflamed tissue.8 To the extent that endothelial dysfunction is a hallmark of allograft transplant rejection, a technique that offers direct, noninvasive clinical assessment of the inflammatory status of the endothelium may allow diagnostic evaluation of these disease processes.

Myocardial contrast echocardiography (MCE) is an ultrasound imaging technique that uses intravenously injected, gas-filled, acoustically reflective microbubbles that act as red blood cell tracers as they pass unimpeded through the microcirculation.9 Unlike inert microbubble blood tracers, targeted microbubbles are designed to adhere to specific endothelial surface epitopes to allow ultrasonic detection of these epitopes.

We have previously shown that ultrasound microbubbles targeted to the LAM intercellular adhesion molecule-1 (ICAM-1) via conjugation with anti–ICAM-1 antibody preferentially bind to inflammatory versus resting cultured human
coronary artery endothelial cells (ECs).10,11 Because acute organ transplant rejection is associated with endothelial ICAM-1 overexpression, we hypothesized that MCE using ICAM-1–targeted microbubbles can detect acute heart transplant rejection. We tested this hypothesis using a 2-step approach. Because our previous data examined microbubble adhesion to cultured human ECs, we first conducted in vitro experiments to prove that our ICAM-1–targeted microbubbles would bind to inflammatory rat ECs under shear flow conditions. Second, to determine if we could ultrasonically detect microbubble binding in vivo, we performed ultrasound imaging of our targeted contrast agent in a rat abdominal heterotopic heart transplantation model. Our report describes selective in vitro binding of our ICAM-1–targeted microbubbles to inflammatory rat endothelium and their in vivo ultrasonic detection in the setting of acute cardiac allograft rejection.

**Methods**

**Microbubble Preparation**

Phospholipid-based, perfluorobutane-filled microbubbles (diameter 3.4±1.2 μm) were synthesized as previously described.12 The biotinylated shell was conjugated to either mouse anti-rat ICAM-1 monoclonal antibody (clone 1A29, Pharmingen) (MBICAM) or non-specific mouse antibody (Caltag) (MBControl) via multistep avidin-biotin bridging.11,12 By quantitative flow cytometry, 11 antibody biotinylated shell was conjugated to either mouse anti-rat ICAM-1 antibody or control antibody (as used above) at a molar ratio of 10:1 (i.e., 11 antibodies per 103 microbubbles).

**In Vitro Studies**

**Cell Culture and ICAM-1 Expression**

Rat heart microvessel ECs (passages 3 to 5; VEC Technologies) were grown to confluence on glass coverslips and inflamed using interleukin-1β (IL-1β, 100 U/mL for 5 hours) (Pierce Endogen).

Endothelial ICAM-1 expression was quantified via flow cytometry11 using the same biotinylated anti-rat ICAM-1 antibody as used for MBICAM, followed by PE-labeled streptavidin (Pharmingen) (n=5 per condition).

**In Vitro Perfusion Protocol**

A previously described rectangular parallel plate perfusion chamber was used to quantify microbubble adhesion to cultured ECs.12 Coverslips of normal or IL-1β-activated ECs (n=3 to 6 per condition) were mounted in the chamber and perfused with a suspension of microbubbles (3.33×106 microbubbles/mL) in culture medium at a flow rate corresponding to a wall shear rate of 100 s⁻¹ for 3 minutes, followed by a 3-minute microbubble-free wash at 100 s⁻¹. The chamber was mounted on an epifluorescent microscope (Zeiss), 20 randomly selected fields were digitally acquired (×1000), and the number of ECs and adhered microbubbles was quantified.

**In Vivo Experiments**

**Heterotopic Heart Transplantation Model**

Acute cardiac transplant rejection was modeled using male Brown Norway (BN) (150 to 190 g) and Lewis (L) (280 to 310 g) rats (Harlan, Indianapolis, Ind) undergoing abdominal heterotopic heart transplantation. These 2 strains of rats are widely used for acute transplantation. These 2 strains of rats are widely used for acute transplant rejection studies because of their strong immunogenic response.14

Preliminary dosing studies demonstrated that a bolus of 2.5×10⁸ Optison resulted in visually strong, reproducible opacification of the donor myocardium. Accordingly, rats were given an initial intravenous bolus of 2.5×10⁸ Optison during intermittent triggered MCE imaging to permit optimization of image settings and to verify uniform perfusion of the donor heart. After complete Optison washout, an intravenous bolus of 2.5×10⁸ MBICAM or MBControl in 0.05 mL saline was administered followed by a 0.2-mL saline flush during MCE imaging. Each rat received randomly ordered, paired injections of MBICAM and MBControl.

**Histological Analysis**

Transplanted hearts were harvested postmortem for H&E histology. Blinded observers assessed rejection grade using International Society for Heart and Lung Transplantation criteria.15

Myocardial specimens from additional isografts and allografts (n=3 each) were harvested on postoperative day 5 and stained using an immunoperoxidase kit (Dako) and the same anti-rat ICAM-1 antibody as above. Endothelial ICAM-1 staining was scored semiquantitatively by blinded observers on a scale of 0 (minimal staining consistent with constitutive ICAM-1 only) to 4 (intense microvascular staining).

**Myocardial Contrast Echocardiography**

Ultraharmonic MCE was performed using send/receive frequencies of 1.3/3.6 MHz (Sonos 5500, Phillips Corp). Gain settings, depth, and focus (mid-field) were initially optimized and maintained throughout the experiment. Five short-axis ECG-triggered images (mechanical index, 1.6) of the donor heart were digitally acquired at baseline (preinjection), 3 minutes, and 3 minutes 20 seconds after microbubble injection. Images were analyzed offline for average pixel videointensity (VI) in left ventricular myocardial regions of interest.16

Our approach to discriminating between adhered and circulating microbubbles was based on previously described principles of ultrasound-induced microbubble destruction and replenishment16,17 and our observation in pilot studies using Optison (Amersham Health) that myocardial contrast was no longer detectable by 3 minutes after injection. Based on these considerations, myocardial backscatter at 3:00 should derive predominantly from adherent microbubbles and less so from any few remaining circulating microbubbles present in and destroyed by the ultrasound beam. Therefore myocardial contrast at 3:20 should be attributable to any persistently circulating microbubbles replenishing the beam in the intervening 20 seconds, the VI difference between the 3:00 and 3:20 frames should be attributable to microbubble adhesion only. An additional index, VIₚ₀₋ₚ₂₀, was calculated as the mean VI difference for MBICAM injections minus the VI difference for MBControl injections in the same rat, resulting in a measure that reflected only the acoustic signal attributable to specific adhesion of ICAM-1–targeted microbubbles.

**Experimental Protocol**

On postoperative day 5, animals were anesthetized, intubated, and ventilated as above. The right jugular vein was cannulated for contrast administration. Anesthesia was maintained with pentobarbital (15 mg/kg IV, every 15 to 30 minutes). The animal was positioned prone on a scaffold with a cutout under the abdomen for MCE imaging.

Rats were anesthetized with pentobarbital (50 mg/kg IP), intubated, and ventilated. Donor rats were heparinized (1000 U/kg IV), and the anterior rib cage was opened to expose the heart. The inferior vena cava (IVC), superior vena cava, and pulmonary veins were ligated and divided, the great vessels were transected, and the explanted heart was immersed in 4°C saline.

In recipient rats, a midline abdominal incision was made, the donor aorta was anastomosed to the recipient abdominal aorta, and the donor pulmonary artery was anastomosed to the recipient abdominal IVC. The heart was reperfused, the abdomen was closed, and the rats were allowed to recover.
Statistical Analysis

Results are expressed as mean±SD. Data were analyzed with 2-tailed Student’s t tests and 2-way ANOVA. Statistical significance was defined as \(P<0.05\).

Results

In Vitro Experiments

Adherence of MBControl was minimal to both normal and inflammatory rat ECs (0.2±0.1 and 0.3±0.1 microbubbles/EC) (Figure 1). Adherence of MBICAM was significantly greater to IL-1β–activated ECs than normal ECs (10.6±3.2 versus 3.4±1.6 microbubbles/EC, \(P<0.005\)). Figure 2 shows bright-field micrographs of coverslips of activated and normal ECs perfused with both species of microbubbles. MBICAM adhered preferentially to inflamed (left top) versus normal (right top) endothelium, whereas adhesion of control microbubbles was minimal (bottom panels).

MBICAM adhesion to inflammatory ECs was paralleled by an 8-fold increase in ICAM-1 expression. By flow cytometry, IL-1β activation increased the ICAM-1 signal on cultured rat ECs from a baseline of 22±14 to 180±65 intensity units.

In Vivo Experiments

Myocardial Contrast Echocardiography

Of the 23 recipient rats, 1 died during surgery and 4 died from anesthetic complications before complete MCE data collection. Three rats were excluded from the study because of absent perfusion localizing to coronary territories of the donor myocardium as qualitatively visualized during the initial Optison injection, most likely attributable to perioperative epicardial coronary artery injury.

Figures 3 and 4 show ultraharmonic images from the same rejecting (top panels) and control (bottom panels) rats at 3:00 after injection of MBICAM (Figure 3) or MBControl (Figure 4). At 3 minutes after MBICAM injection, there is intense myocardial opacification in the rejecting rat (Figure 3A) that is not seen in the control rat (Figure 3C). At 3 minutes after MBControl injection, there is mild contrast in the rejecting myocardium (Figure 4A), but this is much less than that seen after MBICAM injection in this same rat (Figure 3A). Additionally, there is minimal contrast at 3 minutes after MBControl injection into the control rat (Figure 4C). At 3:20 after each injection, there was no significant myocardial opacification (Figures 3B, 3D, 4B, 4D).
and 4D), confirming that any contrast enhancement at 3:00 was attributable predominantly to adhered rather than circulating microbubbles. Figure 5 summarizes the data for all 15 analyzed rats. The mean \( V_{\text{targeted}} \) for the control isograft transplants was not significantly different from zero. The mean \( V_{\text{targeted}} \) for the rejecting allograft transplants was significantly higher than zero (\( P < 0.01 \)) and the isograft \( V_{\text{targeted}} \) (\( P < 0.01 \)).

**Histological Analysis**

As seen in Figure 6, isograft myocardium (panel A) showed normal histology, whereas allograft myocardium (panel B) showed histological evidence of International Society for Heart and Lung Transplantation grade III to IV acute rejection, including lymphocytic infiltration, edema, fibrosis, and myocyte damage.\(^{15}\)

On postoperative day 5, rejecting allografts had a significantly higher ICAM-1 score than normal isografts (3.7 ± 0.6 versus 0.7 ± 0.6, \( P < 0.005 \)). As shown in Figure 7, there was intense ICAM-1 staining (red-brown) in rejecting myocardium (Figure 7B), which was absent in the nonrejecting tissue (Figure 7A).

**Discussion**

The main finding of this study is that MCE using microbubbles targeted to ICAM-1 is capable of identifying acute rejection of the transplanted heart. The in vitro data suggest that this capability is attributable to microbubble adhesion to dysfunctional endothelium overexpressing ICAM-1, which ultimately manifests as persistent contrast enhancement on ultrasound imaging. This study is the first description of an ultrasound approach for detecting acute cardiac allograft rejection using a targeted contrast agent and has major implications for the noninvasive diagnosis of heart transplant rejection in particular and organ rejection in general.

**Microbubble Adhesion to Rat Endothelium**

We have previously demonstrated that ultrasound contrast microbubbles specifically targeted to ICAM-1 via conjugation with anti–ICAM-1 antibodies adhere preferentially to

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**Figure 5.** Graph of mean \( V_{\text{targeted}} \) (see text for definition) for normal isograft versus rejecting allograft hearts (mean±SD).

**Figure 6.** H&E-stained isograft myocardium (A) is histologically normal, whereas allograft myocardium (B) shows edema, lymphocytic infiltration, and myocyte damage. Scale bar=50 μm.

**Figure 7.** Micrographs of isograft (A) and allograft (B) myocardium stained for ICAM-1 (reddish-brown), demonstrating upregulation of endothelial ICAM-1 in the microcirculation of the rejecting heart. Scale bar=50 μm.
Activated cultured human ECs overexpressing ICAM-1 and that such adhesion depends on the microbubble antibody density and local shear conditions. In the present study, an important prerequisite to pursuing the in vivo studies was to establish that ICAM-1–targeted microbubbles are capable of binding to rat endothelium. Hence, in vitro studies were performed using a parallel plate chamber that provided a uniform controllable shear field across the experimental surface and that has been widely used to study particle adhesion to endothelium.

A positive relationship was found between ICAM-1 expression and MBICAM adhesion in vitro. The observed (low) adherence of MBICAM to normal cells is likely attributable to the constitutive expression of ICAM-1 on resting ECs. Non-specific control microbubbles showed minimal adherence to both cell types, suggesting that neither the microbubble lipid shell nor the attached immunoglobulins were responsible for the adhesion.

These in vitro studies established that the adhesion of ICAM-1–targeted microbubbles to rat ECs depends on the expression level of ICAM-1, targeted microbubbles can bind to rat EC ICAM-1 under physiologically relevant shear conditions, and MBICAM adhesion is attributable to specific binding interactions of the antibody with ICAM-1. These findings permit us to etiologically link our MCE findings to the phenomenon of specific microbubble adhesion.

### MCE Detection of Acute Rejection

The MCE data demonstrating persistent myocardial contrast enhancement unique to rejecting rats receiving MBICAM are consistent with microvascular MBICAM adhesion in vivo. The upregulated ICAM-1 expression observed on immunohistochemical staining of these hearts is the likely basis for preferential binding of MBICAM versus MBControl to rejecting hearts, resulting in high VITargeted. Conversely, the control myocardium showed little difference between MBICAM and MBControl adhesion, which is consistent with low ICAM-1 expression observed on isograft myocardium. Interestingly, as seen in Figure 3C and the VITargeted results, there was at least some MBICAM adherence to nonrejecting control myocardium; this was likely attributable to low levels of constitutive ICAM-1 expression, as was seen in the in vitro studies as well. Additionally, Figure 4A suggests that there may have been some adhesion of MBControl to rejecting myocardium. This would not be unexpected, because microbubble attachment to activated, endothelium-adherent leukocytes has been previously shown in acute inflammation.

The degree of myocardial contrast resulting from such attachment (Figure 4A), however, is much less than that resulting from MBICAM attachment to ICAM-1 (Figure 3A), perhaps reflecting either greater numbers of ICAM-1 sites relative to leukocytes available for binding microbubbles or greater retention of microbubbles bound via specific antibody/antigen interaction. Our data thus favor a targeted approach over reliance on nonspecific microbubble adhesion for detecting the rejection process.

Immunohistochemical ICAM-1 findings paralleled the histological rejection status: isografts showed low ICAM-1 expression and normal histological findings, whereas allografts demonstrated high ICAM-1 expression and classic features of acute rejection. This association between rejection status, ICAM-1 expression, and targeted ultrasound signal suggest that ICAM-1–targeted MCE can successfully detect acute cardiac transplant rejection.

As shown in Figure 3, in some cases the pattern of adhesion appeared patchy, possibly attributable to either inhomogeneous myocardial ICAM-1 overexpression, reflecting the patchiness of acute rejection, or regional differences in capillary volume, leading to inhomogeneous tissue concentration of MBICAM. This qualitative patchiness was generally not observed in the Optison injections, but this finding does not affirm a particular mechanism for the patchiness. We did not specifically quantify capillary volume, which would have required continuous nontargeted microbubble infusion.

### Comparison With Previous Studies

Targeted ultrasound has been used to detect other disease markers using a variety of agents. Thrombus has been ultrasonically imaged using a fibrinogen-targeted microbubble and a biotinylated microbubble injected after avidin precoating of thrombus. Inflammation has been ultrasonically imaged by targeting microbubbles to activated leukocytes in mouse cremaster muscle and postischemic canine myocardium and to P-selectin in inflammatory mouse kidney.

For the noninvasive detection of acute rejection, imaging approaches have used scintigraphic methods to target apoptosis, major histocompatibility complex antigens, and ICAM-1 but are limited in spatial resolution and by the requirement for radioactive tracers. Other investigational, nontargeted, radiologic imaging methods such as MRI and PET measure morphological and metabolic changes, respectively, associated with rejection but require instrumentation limited to tertiary care centers.

Our study is unique in several ways. First, we targeted microbubbles to adhesion molecules in the microcirculation of the heart. Second, we used ultrasound, a highly available clinical tool, to detect ICAM-1 in vivo. Finally, by identifying cellular expression of ICAM-1 with echocardiography, this is the first study to detect the phenomenon of acute transplant rejection with targeted ultrasound.

### Limitations

Our data demonstrated ultrasonic detection of fulminant cardiac allograft rejection. For this proof-of-principle study, our observations of microbubble adhesion and contrast enhancement were limited to the extremes of normal and fulminant disease. It is unknown if similar differential adhesion of targeted microbubbles would occur with lesser degrees of inflammation, as seen in lower-grade rejection, where ICAM-1 expression is lower. In vitro EC perfusion studies in which the magnitude of ICAM-1 expression was experimentally modulated over a 20-fold range, we found a
positive, linear relationship between the degree of endothelial ICAM-1 expression and MBICAM adhesion (unpublished data, 2002), suggesting that the extent of targeted microbubble adhesion can indeed track the extent of inflammation. Additional studies are needed to determine if our imaging method can discern this range of difference in microbubble adhesion so that it can differentiate normal from inflamed tissue at lower ICAM-1 levels, as would be required to monitor inflammation in response to therapy. This would be of particular importance in view of our observation of constitutive endothelial ICAM-1 expression by normal ECs.

The microbubble signals detected at 3:00 may not have been solely attributable to EC-adherent microbubbles. It is conceivable that MBICAM did not adhere exclusively to endothelium but rather to other cells that express low levels of ICAM-1, including erythrocytes, activated lymphocytes, and macrophages. However, the fact that the V1targeted was much higher in allografts than isografts suggests that interaction with blood cells was minimal. The lack of strong contrast enhancement at 3:20 indicates that neither persistently circulating free microbubbles nor microbubbles adherent to blood elements were likely responsible for the measured backscatter. Additionally, our previous observation of absent myocardial opacification at 3:00 using Optison at similar doses suggests that this time frame is ample to allow washout of unadhered microbubbles.

A destructive imaging protocol that includes a 3-minute delay may be difficult to apply clinically, because precisely locating the heart before the first destructive pulse may be challenging. For clinical MCE, sonographers have already been trained to initially locate an image and, with the patient lying still, fix the transducer on the chest such that after a period of blinded waiting, the next image can still be accurately registered. Additionally, ultrasound systems are being developed that may offer high mechanical index (nondestructive) displays, allowing transducer adjustment during the microbubble injection protocol. We did not quantify donor myocardial perfusion, which could affect microbubble delivery. The small rat heart size precludes use of radiolabeled microsphere technology to measure blood flow. Although continuous infusions of an agent such as Optison during MCE imaging could have been used to quantify perfusion, the infusion volume required, in addition to the MBICAM and MBControl injections, would likely have been cumulatively too high to maintain a physiological preparation. Instead, each experiment began with a single injection of Optison to rule out gross hypoperfusion or infarction, which appeared as contrast defects, and resulted in exclusion of 3 rats. Furthermore, the V1targeted index minimized any flow dependence of adhesion by making direct comparisons between the VI attributable to MBICAM and MBControl in the same rat.

Finally, left ventricular systolic function of the graft was not examined in detail. In particular, ejection fraction, a measure that has limited significance when applied to an unloaded heart such as an abdominal cardiac graft, was not examined. Functional measurements were limited to the preliminary Optison imaging used to rule out gross abnormalities.

Clinical Implications

Acute rejection remains a major cause of morbidity and mortality in patients undergoing heart transplantation for end stage cardiomyopathy. Presently, the cornerstone of diagnosis and monitoring of treatment is endomyocardial biopsy, which is invasive. Ultrasonic detection of acute rejection using a contrast agent targeted to an endothelial marker that is upregulated in rejection could be a powerful noninvasive tool for diagnosing and following the course of allograft rejection. Furthermore, to the extent that these pathophysiologic events similarly underlie acute rejection of other organs such as the kidney, this imaging approach may lend itself to the surveillance of other transplanted organs and may also have applications in other fields such as ischemic heart disease, oncology, and targeted drug delivery.

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References

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