Late-phase detection of recent myocardial ischaemia using ultrasound molecular imaging targeted to intercellular adhesion molecule-1

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Aims
In this study, we attempted to detect a recent myocardial ischaemic event using ultrasound molecular imaging (UMI) with microbubbles (MB) targeted to intercellular adhesion molecule-1 (ICAM-1) in the late phase of reperfusion.

Methods and results
We created a myocardial ischaemia–reperfusion model in 60 C57/BL male mice to simulate an angina attack (ischaemia for 15 min, reperfusion for 1–24 h). The degree of myocardial inflammation and levels of ICAM-1 protein were determined by histological and immunohistochemical analyses. UMI with MB targeted to endothelial ICAM-1, as well as routine non-invasive methods including electrocardiography, echocardiography, and plasma troponin I levels, were utilized to evaluate ischaemia over the time course of reperfusion. Levels of ICAM-1 in the vascular endothelium were significantly increased over the time course of reperfusion (8–24 h) of the ischaemic myocardium. The video intensity of ICAM-1 molecular images of the ischaemic anterior wall was almost three times greater than that in the non-ischaemic posterior wall during the late phase (8–24 h) of reperfusion. In contrast, routine methods yielded only weak evidence of ischaemia.

Conclusion
UMI with MB targeted to endothelial ICAM-1 provides reliable evidence of a recent myocardial ischaemic event in the late phase of reperfusion.

Keywords
Intercellular adhesion molecule-1 (ICAM-1) • Microbubbles • Molecular imaging • Myocardial ischaemia • Contrast echocardiography

1. Introduction
Evaluation of chest pain suggestive of myocardial ischaemia, especially acute coronary syndrome, can be problematic and time consuming. Usually, symptoms have resolved for some hours or days before patients are admitted to a hospital, rendering current diagnostic tests such as electrocardiography (ECG), echocardiography, and the plasma concentration of myocardial enzymes, insufficient to detect recent myocardial ischaemic events such as angina pectoris. Further, numerous studies have shown that some patients have atypical clinical presentations, leading to a high incidence of missed or mistaken diagnoses. The development of an efficient method of evaluating myocardial ischaemic events in different phases would therefore be valuable for screening patients with chest pain for myocardial ischaemia. Fortunately, great progress has been made in recent years in ultrasound molecular imaging (UMI), which can provide sound diagnostic evidence at the molecular level for cardiovascular diseases.

It is known that progression of myocardial ischaemia–reperfusion (IR) injury is linked to endothelial activation, which is characterized by the production of various inflammatory adhesion molecules. These molecules are called the ‘inflammatory molecular memory’ because they are markedly upregulated in the vascular endothelium and their high expression levels last for a relatively long time after...
reperfusion. Previous studies identified selectins as the molecular memory for myocardial ischaemic events, and demonstrated that P-selectin provides valuable diagnostic information in the early phase of myocardial IR injury. In clinical practice, however, patients with chest pain usually do not see a physician until several hours or even days after symptoms subside. Accordingly, the time window for diagnostic molecular imaging should ideally be extended to several hours or days. We therefore trialled an alternative approach to detect myocardial ischaemic events in the late phase of reperfusion using UMI.

2. Methods

2.1 Creation of animal model of ischaemia and experimental protocol

This animal study was approved by the Animal Research Committee of the Southern Medical University and conforms with the Guide for the Care and Use of Laboratory Animals published by US National Institutes Health (NIH Publication No. 85-23, revised 1996). Mice (C57BL/6, male, weighed 25–30 g, 10–12 weeks of age) were anaesthetized with pentobarbital sodium (50 mg/kg i.p.). The left coronary artery (LCA) was tied firmly with 8-0 silk suture against a short polyethylene tubing (PE10). Ischaemia was confirmed by myocardial blanching and ECG S-T segment elevation. After ischaemia was induced for 15 min (also 20 and 30 min in the preliminary experiments), blood flow was restored by removing the ligated PE tubing (also see Supplementary materials). We divided the 60 mice into 10 equal groups as illustrated in Figure 1. Since injection of contrast can affect levels of plasma troponins (dilution), troponins measurement and targeted imaging were not performed in the same animals.

To evaluate the relation between myocardial expression of ICAM-1 and heart function, we created a chronic heart failure model in mice by transverse aortic constriction (see Supplementary materials).

2.2 Histological examination

Myocardial infarction was confirmed using 1% triphenyl tetrazolium chloride (TTC) staining at 37°C for 20 min. All the murine hearts after UMI were harvested and the ventricles were cut into five pieces. At the left ventricular (LV) papillary level of each heart, one piece was TTC stained to confirm whether infarction occurred or not. Samples with any detectable necrosis were excluded from this study. The remaining ventricular tissue was fixed in 4% paraformaldehyde, and embedded in paraffin in 4–6 μm sections. Haematoxylin and eosin staining was utilized to evaluate histological changes of the myocardium, and immunohistochemical assays were performed to examine ICAM-1 expression, using anti-mouse CD54 antibody (BD Pharmingen, USA), in vascular endothelium of myocardium as previously described.

2.3 Evaluation of ischaemic events using routine methods

ECG, echocardiography, and levels of plasma troponin I (TnI) were measured at different reperfusion time points. M-mode transthoracic echocardiography was performed to evaluate cardiac function at each reperfusion time point (1, 8, and 24 h) using a 15L8 transducer (Sequoia 512, Siemens, Germany). Mice were immobilized without anaesthesia. Two-dimensional short-axis views of the LV were obtained for guided M-mode measurement of the LV end-diastolic and -systolic diameters (LVEDd, LVESd), and the corresponding LV posterior wall diastolic and systolic thickness (LVPWd, LVPWs). LV fractional shortening (FS) was calculated as follows: $\text{LVFS} = \frac{\text{LVEDd} - \text{LVESd}}{\text{LVEDd}} \times 100\%$.

After harvesting blood from the right ventricle, plasma TnI concentrations were measured using a mouse Troponin I ELISA kit (R&D Systems, Minnesota, MN, USA) in accordance with the manufacturer’s instructions.

2.4 Preparation and evaluation of targeted microbubbles

Phospholipid-based perfluorobutane-filled microbubbles (MB) were synthesized as previously described with some modifications. Briefly, the biotinylated shell was conjugated to either rat anti-mouse ICAM-1 monoclonal antibody (BD, MBICAM) or non-specific rat antibody (BD, MBNS).
MB_{BCG}) via multistep avidin–biotin bridging (Supplementary methods and Supplementary material online, Figure S1). After elimination of large-size MB by flotation centrifugation, MB_{ICAM} and MB_{BCG} were measured using a Coulter counter (Beckman M3). The targeted MB were subjected to fluorescent-targeted secondary antibodies (DAKO, Denmark) against the targeting moiety, and were observed under fluorescence microscopy to evaluate the efficiency of targeted antibodies on the surface of agents.

A parallel plate flow chamber (GlycoTech, America) was used to measure the binding and retention rates of targeted MB to the antigen ICAM-1 immobilized on a culture dish. Shear stress of the micobubble fluid was fixed in the physiological condition of microcirculation at 0.6 dyn/cm² as previously reported, whereas 1000 ng/mL ICAM-1 substrates were incubated on a dish to simulate inflammatory molecular expression. To evaluate the sensitivity of antigen-antibody binding, different concentrations of ICAM-1 were added into the flow chamber and the binding rates to ICAM-1 MB were assessed, respectively.

### 2.5 Myocardial contrast echocardiography

Myocardial contrast echocardiography was performed using an ultrasound system with a 15L8 transducer and multi-pulse protocol (Contrast Pulse Sequencing, Sequia 512, Siemens, Germany) at a frequency of 7 MHz and frame rate of 30–40 per second to obtain images of myocardial perfusion and targeted contrast-enhanced ultrasound (CEU). The transducer was positioned at the fourth intercostal space to obtain a short-axis image of mid-LV, the depth was set at 2 cm, gain settings were optimized and held constant, and the dynamic range was set at 55 db. For assessment of myocardial perfusion, a suspension of ultrasound MB (SonoVue, Bracco Diagnostics Inc., Italy) was infused intravenously.

Targeted myocardial contrast echocardiography imaging of the mice was performed with a caudal intravenous injection of 5 × 10⁶ MB_{ICAM} or MB_{BCG} in random order. Probe position, compression, gain settings, and depth were initially optimized and machine settings were maintained between animals. The key points of the targeted myocardial contrast echocardiography imaging were low (0.18) mechanical index (MI) imaging 5 min after injection for maximum contrast imaging (signal from adhered MB and circulating MB), 2–3 s of high (1.9) MI imaging that destroys MB, followed by low MI imaging 30 s later for background contrast imaging (signal from residual circulating MB). The average pixel video intensity (VI, grey level) of the maximum contrast imaging subtracted from background in the LV myocardial region was quantified to yield the signal attributable to the adherent MB using quantitative imaging analysis software (MCE2.7 Yabko, LLC 2002, USA). After setting the grey scale at 255, and selecting the anterior wall of the LV as the region of interest, the pixel VI on each frame was automatically transformed from a logarithmic-compressed scale to a linear scale with random values using the known dynamic range and range of intensity values. After the 5.5 min frame was digitally subtracted from the 5 min frame, the resulting image was colour coded.

### 2.6 Examination of tumour necrosis factor α-induced ICAM-1 expression and permeability

Isolation and culture of neonatal rat cardiac microvascular endothelial cells (ECs) were performed as described previously with modifications. Tumour necrosis factor α (TNFα 50 ng/mL) was added to the ECs for 24 h to stimulate the expression of ICAM-1 (detected by western blot). Permeability of ECs in response to TNFα stimulation was measured as described elsewhere.

### 2.7 Statistical analysis

Data were expressed as mean ± SD and analysed using SPSS 13.0 (Chicago, IL, USA). For plasma TnI and LVFS data, differences between groups were compared using analysis of variance with the post hoc analysis using Student–Newman–Keuls exact probability test. For myocardial contrast echocardiography data, the paired t-test was used for comparisons between targeted and control MB, and between the anterior and posterior walls of the same heart. The least-squares method was used to assess linear correlations between selected variables. A value of P < 0.05 was considered statistically significant.

### 3. Results

#### 3.1 Fifteen minutes of coronary occlusion caused serious inflammation without obvious necrosis

To simulate serious ischaemia without obvious necrosis, in preliminary experiments we tested different ischaemic durations. We performed 2,3,5-TTC staining on hearts following 15, 20, or 30 min of coronary occlusion. The results showed different degrees of necrosis in hearts subjected to 20 and 30 min coronary occlusion (Supplementary material online, Figure S2A and B), while there was no obvious necrotic myocardium in the 15 min ischaemic hearts (Supplementary material online, Figure S2C).

Compared with the non-ischaemic posterior wall (Figure 2B), swollen cardiomyocytes and leucocytosis were seen in the ischaemic anterior wall in all the IR groups (Figure 2A), indicating the existence of an inflammatory imprint in the ischaemic myocardium even without the occurrence of necrosis.

Immunohistochemical examination demonstrated that, although some physiological expression of ICAM-1 molecules was seen in the sham and IR 1 h groups (Figure 2C and D), high levels of ICAM-1 expression were identified in the endothelium of capillaries and larger vessels at 8 and 24 h after reperfusion in the ischaemic area (Figure 2E and F), indicating the existence of molecular memory in the late phase of reperfusion. Since myocardial ischaemia and subsequent reperfusion may induce an inflammatory state of the myocardium and consequently activate ECs, ICAM-1 can be upregulated even when no infarction occurs. The above findings suggest that in vivo detection of molecular memory of ICAM-1 can provide diagnostic evidence for myocardial ischaemic events in the late phase.

#### 3.2 Routine methods were ineffective in late phase detection of myocardial ischaemia

ST segments were significantly elevated after LCA ligation and returned to baseline as soon as reperfusion started. In comparison with sham animals, there were no significant ECG differences in animals with different durations of reperfusion (Figure 3A).

IR mice underwent a transient suppression of the systolic heart function in the early phase of reperfusion, but recovered to normal after 24 h of reperfusion (Figure 3B). Compared with the corresponding sham group, LVFS was markedly decreased in both the IR 1 h and IR 8 h groups. However, no significant difference was seen between the IR 24 h and sham 24 h groups (Figure 3E). The relative indices of LVEdD and LVEsd are shown in Figure 3C and D.

No significant differences were seen in plasma Tnl concentrations between the IR and sham groups at each time point. However, in both the sham and IR groups, the concentration of Tnl in the 1 h group was greater than that in the 8 and 24 h groups, possibly caused by open chest surgery rather than myocardial ischaemia (Figure 3F).

These results indicate that neither ECG nor plasma Tnl concentrations provide sufficient evidence for ischaemic events after
reperfusion, whereas M-mode echocardiography only provided ischaemia-associated findings within 8 h after reperfusion.

3.3 Targeted MB showed stable performance

MBICAM subjected to fluorescent-targeted secondary antibodies shown with a bright green colour under fluorescence microscopy, suggesting that MB were conjugated with anti-ICAM-1 antibody with high efficiency (Figure 4A and B).

After 5 min of continuous injection, parallel plate flow chamber examination showed that under physiological shear stress (0.6 dyn/cm²), there were 168 ± 8 MBICAM per microscope field binding to 1000 ng/mL ICAM-1 antigen, whereas only 8 ± 2 MBISO were observed under the same conditions (Figure 4C). The binding rates in response to different concentrations of ICAM-1 are shown in Figure 4D. These findings confirmed that the targeted MB prepared in the present study have stable physical characteristics with fine specificity and sensitivity.

3.4 Molecular image of ICAM-1 obtained from targeted contrast-enhanced ultrasound

Perfusion images of the myocardium obtained from myocardial contrast echocardiography showed an obvious perfusion defect in the infraction-related anterior wall (Figure 5A). After reperfusion, the perfusion defect in the risk area for ischaemia disappeared (Figure 5B).

Representative pictures of ICAM-1-targeted contrast-enhanced ultrasound in the IR 1 h, 8 h, 24 h, and sham groups are shown in Figure 5C–J. Since no significant alterations in histological findings or ICAM-1 targeted contrast-enhanced ultrasound were seen in sham-operated mice from 1 to 24 h after surgery, we showed only the data of sham 1 h group. Signals obtained using MBISO and MBICAM showed no obvious enhancement in the sham group (Figure 5C and D). In the IR 1 h group, colour-coded contrast-enhanced ultrasound images of both MBICAM and MBISO presented similar moderate non-specific enhancement of the anterior wall (Figure 5E and F). Contrast-enhanced ultrasound imaging of MBICAM after 8 and 24 h of reperfusion demonstrated significant selective enhancement in the anterior wall (Figure 5H and J), although only slight myocardial MBISO opacification was seen in the same location (Figure 5G and I). Quantitative analysis of ICAM-1 expression, estimated using the VI of the contrast-enhanced ultrasound images of the anterior and posterior walls, is depicted in Figure 5K. In all the animals undergoing LCA occlusion and reperfusion, MBISO produced a small amount of signal enhancement in the post-ischaemic anterior region. In the IR 8 h and IR 24 h groups, signal enhancement for MBICAM in the post-ischaemic anterior wall was significantly greater than that for MBISO, and was also significantly greater than the VI of MBICAM in the non-ischaemic posterior territory.

We evaluated whether there was any association between cardiac expression levels of ICAM-1 and systolic function. In mice with myocardial IR or sham operation, no significant correlation was found between VI of ICAM-1 MB in LV anterior wall and the LVFS (see Supplementary material online, Figure S3, \( r = -0.113, P > 0.05 \)). In a chronic murine model of LV pressure overload or sham operation, however, a significant reverse correlation between cardiac expression levels of ICAM-1 and LVFS was noted (\( r = -0.706, P < 0.02 \), see Supplementary material online, Figure S4). These results suggest that ICAM-1 molecular imaging may reflect only inflammatory processes occurring within the vascular compartment but not necessarily the changes in the physiological function.

3.5 TNFα increases both endothelial ICAM-1 expression and permeability

It was reported that acute coronary syndrome would enhance the production of cytokines, such as TNFα.26 Here we checked whether TNFα might increase ICAM-1 expression and endothelial permeability. We found that TNFα stimulation for 24 h significantly
increased ICAM-1 expression in ECs (Figure 6A) and that TNFα stimulation increased permeability of ECs dose- and time-dependently (Figure 6B and C). We speculated that the induction of endothelial ICAM-1 expression and permeability by TNFα should be one of the mechanisms for infiltration of leucocytes and for the subsequent cardiac function damage when the ischaemic stress persists.

4. Discussion

In this study, we confirmed that ECG, M-mode echocardiography, and plasma TnI concentration were non-diagnostic for myocardial ischaemic events without necrosis when reperfusion lasted for 24 h, findings in agreement with the clinical situation where a considerable proportion of patients with chest pain visit hospital in the late phase of reperfusion.27

One of the solutions to this issue is the combination of myocardial contrast echocardiography with special vascular endothelial molecular imaging. Two pioneering studies demonstrated the feasibility of UMI of P-selectin in early reperfusion phase assessment of myocardial ischaemia.8,9 Although application of this strategy will rely on further characterization of the optimal time frame of selectin imaging, thus far no reports have addressed the utility of UMI in the late phase of myocardial reperfusion. In this study, we used the UMI techniques similar to those employed previously in evaluation of the incremental diagnostic value of UMI in the setting of IR.8,9 In contrast, however, this study selected ICAM-1 as a molecular target, was the first to extend the time frame of molecular imaging of endothelial ICAM-1 from 1 to 24 h of reperfusion, made a comparison with conventional diagnostic tools, and investigated potential mechanism. Expression of ICAM-1 is reported to increase and last for a relatively
long period under pathological conditions in cardiovascular tissues such as the vascular endothelium of the pressure-overloaded murine heart, interleukin-1 beta-activated human coronary artery ECs, and in a rat abdominal heterotopic heart transplantation model. Variations in the time course of myocardial endothelial adhesion molecules expression during acute coronary syndrome are largely unknown and need to be clarified for the purpose of providing various targeted molecules for UMI.

It has been well established that activated coronary vascular ECs play an important role in the development of cardiac tissue damage during IR and the extent of IR injury is dependent on the number of infiltrating leucocytes. ICAM-1 has now been shown to lead to activation of proinflammatory cascades that can perpetuate an inflammatory response. However, a transient upregulation of ICAM-1 after a resolved ischaemic event may not necessarily induce a detectable functional deficit. But it has a potential diagnostic value in that CEU may image an inflammatory process by detecting a transient upregulation of ICAM-1. Although myocardial necrosis also induces an upregulation of myocardial ICAM-1 expression, imaging ICAM-1 may not be of incremental diagnostic value for acute myocardial infarction because ECG and plasma myocardial enzyme are sufficient to confirm the diagnosis. On the other hand, detection of an increase of circulating ICAM-1 is not sufficient to diagnose a myocardial ischaemic event because the increase of plasma ICAM-1 may occur in patients with non-cardiac diseases.

In vivo molecular imaging in the late reperfusion phase (8–24 h) demonstrated persistent myocardial contrast enhancement of MBICAM in the risk area for ischaemia, in concordance with the upregulated ICAM-1 expression observed with immunohistochemical staining. It should be noted that small numbers of MBICAM also adhered to the posterior myocardium in the IR 8 h and IR 24 h groups, which may be attributable to low levels of ICAM-1 expression activated by inflammation. In addition, consistent with the previous report that ICAM-1 was not upregulated in 2 h after an acute ischaemic stimulus, no significant difference in opacification between MBICAM and MB ISO was seen in the IR 1 h group. Moreover, the low level of enhancement in the anterior wall of MB ISO-injected IR groups was non-specific and may be attributable to the phagocytosis of phospholipid-based MB by activated endothelium-adherent leucocytes. On the other hand, no significant opacification was seen in the sham group, because neither inflammation nor significant expression of ICAM-1 was present in the endothelium.

It should be noted that there are several limitations to this study. It is known that <10 min of coronary occlusion in rodents merely causes endothelial disturbance without obvious inflammation. Therefore, an inherent limitation of adherent molecules is that they are not
ideal diagnostic targets for mild or moderate myocardial ischaemia in rodents, although myocardial ischaemia persisting for <15 min is more frequently encountered in clinical practice. However, there is evidence that brief ischaemia in patients with coronary heart disease can increase adhesion molecules immediately,32,33 providing basis for future evaluation of the diagnostic value of UMI targeted to adhesion molecules in patients with mild or moderate myocardial ischaemia.

Another limitation of the present study is the lack of evaluation of the LV regional function. It is usually difficult to evaluate the LV regional function in mice using routine echocardiography. The third limitation of this study is that ICAM-1 molecular imaging is able to detect inflammation, but is not specific to ischaemia. Other insults to vascular tissues also trigger the response of binding labelled MB, for example hypertension,10 heart failure,11 and atherosclerosis.12 However, local myocardial ischaemia provokes regional inflammation, a helpful point in making the differential diagnosis. ICAM-1 was reported to increase in several cardiovascular diseases such as hypertension, chronic atherosclerosis, and heart transplantation.6 Using molecular imaging of rat heterotopic cardiac rejection with ICAM-1-targeted MB, Weller et al.29 demonstrated selective ICAM-1 signal enhancement in the strain-mismatched heart allografts. Therefore, we should keep in mind that accompanied cardiovascular diseases might enhance the intensity of ICAM-1 MB signals. We believe that establishment of optimal animal models would help clarify whether accompanied cardiovascular diseases influence the diagnosis of myocardial ischaemic events using UMI targeted to ICAM-1.

Contrast UMI has various potential clinical applications in the diagnosis and therapeutic evaluation of cardiovascular diseases, such as transplant rejection,29 IR injury,8,9 atherosclerosis,34 angiogenesis,35 and thrombus formation.36 Molecular imaging using various targets holds great promise in assisting the diagnosis of recent myocardial ischaemia. Because different adhesion molecules remain on the endothelial surface for different durations after resolution of ischaemia, it may be possible to evaluate myocardial ischaemic events using P-selectin imaging in the early phase,8,9 and ICAM-1 imaging in the late phase, of reperfusion. However, it should be noted that there is a long way to go to transfer the contrast molecular imaging technique to a clinical tool.

In conclusion, our findings in this study indicate that UMI with MB targeted to endothelial ICAM-1 can provide reliable detection of recent myocardial ischaemic events in the late phase of reperfusion.
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Supplementary material

Supplementary material is available at Cardiovascular Research online.

References


