Detection of recent myocardial ischaemia by molecular imaging of P-selectin with targeted contrast echocardiography

Beat A. Kaufmann¹, Christopher Lewis², Aris Xie¹, Ayoub Mirza-Mohd², and Jonathan R. Lindner¹*

¹Cardiovascular Division, UHN-62, Oregon Health and Sciences University, 3181 SW Sam Jackson Park Road, Portland, OR 97239, USA and ²University of Virginia, Charlottesville, VA, USA

Received 22 December 2006; revised 21 March 2007; accepted 13 April 2007; online publish-ahead-of-print 26 May 2007

Aims We hypothesized that molecular imaging of endothelial P-selectin expression with targeted myocardial contrast echocardiography (MCE) could identify recently ischaemic myocardium without infarction.

Methods and results The microvascular behaviour of P-selectin-targeted (MBp) and control (MBc) microbubbles was assessed by intravital microscopy of the cremaster muscle in mice. Targeted MCE imaging with MBp and MBc was performed in mice after brief left anterior descending (LAD) occlusion and reperfusion and in open- and closed-chest controls. Regional wall motion and perfusion by MCE were assessed during occlusion and after reperfusion. On intravital microscopy, ischaemia–reperfusion produced a 10-fold increase (P < 0.01) in venular attachment for MBp. Attachment for MBc was rare. With myocardial ischaemia–reperfusion, LAD occlusion produced hypoperfusion and wall motion abnormalities that resolved after 45 min of reperfusion. At 45 min, signal enhancement in the post-ischaemic region was four-fold greater (P < 0.05) for MBp vs. MBc. MBp produced low-level enhancement in non-ischaemic myocardium in all open-chest animals, suggesting P-selectin expression from surgical cardiac exposure.

Conclusion Molecular imaging of P-selectin with targeted MCE can identify the presence of recently ischaemic myocardium in the absence of necrosis and after resolution of hypoperfusion and post-ischaemic stunning. This technique can potentially provide a method for risk stratifying patients with acute chest pain.

KEYWORDS
Contrast echocardiography; Myocardial ischaemia; Reperfusion injury; Molecular imaging

Introduction

It is thought that emerging techniques for molecular imaging will facilitate early diagnosis of disease and provide unique information to help guide therapy. One situation where better diagnostic capabilities are needed is the evaluation of patients with acute chest pain. The majority of the six million patients with chest pain who present to the emergency department annually in the United States do not have an ACS, yet many are admitted to the hospital incurring enormous health care costs.¹,² Of those with ACS, the electrocardiogram and initial serologic markers for injury are frequently non-diagnostic, leading to a delayed or even missed diagnosis.¹–⁴ Imaging of wall motion and perfusion in the emergency department has been shown to provide incremental diagnostic information to established practice.⁴,⁵ Yet even this strategy is of limited value if performed late after symptoms have resolved or in those with pre-existing abnormalities from remote events. Moreover, in patients with known severe chronic coronary artery disease, often the most important question is not if ischaemia is present, but rather how much muscle is in jeopardy.

An alternative strategy for detecting the presence and spatial extent of recently ischaemic myocardium would be to image molecular events that persist for hours after resolution of ischaemia, sometimes referred to as ‘ischaemic memory’. P-selectin is an endothelial adhesion molecule that participates in leucocyte capture and rolling on the venular endothelial surface upon inflammatory stimuli.⁶,⁷ It is attractive for purposes of imaging recent ischaemia because it is transported to the endothelial cell surface within minutes of injury,⁸–¹⁰ and can persist in a synthesis-dependent manner for hours after ischaemia–reperfusion injury.⁹ We have previously demonstrated that targeted ultrasound imaging can detect P-selectin expression in post-ischaemic renal tissue.¹¹ In this study, we hypothesized that targeted myocardial contrast echocardiography (MCE) imaging of P-selectin could be used to detect recently ischaemic but non-infarcted myocardium. The behaviour of P-selectin-targeted microbubbles (MBp) in post-ischaemic muscle was assessed with intravital microscopy, and targeted MCE was performed after brief myocardial ischaemia and reperfusion at a time when both wall motion and perfusion had returned to normal.
Methods

Animal preparation and study design

The study protocol was approved by the institutional Animal Research Committee. Male wild-type C57Bl/6 mice 6-10 weeks of age were anaesthetized with an intraperitoneal injection (12.5 μL/g) of a solution containing ketamine hydrochloride (10 mg/mL), xylazine (1 mg/mL) and atropine (0.02 mg/mL). Body temperature was maintained at 37°C with a heating pad. A jugular vein was cannulated for administration of microbubbles. Intravital microscopy was performed in nine mice to evaluate P-selectin-mediated microbubble adhesion. MCE with control microbubbles (MBc) and MBp was performed in a total of 24 mice to evaluate relative signal enhancement in post-ischaemic myocardium. For these experiments, treatment groups included left anterior descending (LAD) ischaemia–reperfusion (n = 11), open-chest sham operation (n = 4), and closed-chest controls (n = 3). MCE was also performed after LAD ischaemia–reperfusion using size-selected microbubbles that excluded microbubbles large enough to lodge non-specifically (n = 6). In three separate mice, flow cytometry was used to evaluate the presence of microbubble-platelet complexes.

Preparation of microbubbles

For perfusion imaging, lipid-shelled defluorobutametane microbubbles were prepared by sonication of a gas-saturated aqueous suspension of distearoylphosphatidylcholine and polyoxyethylene-40-stearate. For targeted imaging, biotinylated microbubbles were prepared by addition of distearoylphospholipidethanolamine-PEG(2000)biotin. Either rat anti-mouse monoclonal IgG1 against P-selectin (RB40.34 purified from hybridoma) or isotype control antibody (R3-34, Pharmingen Inc.) was conjugated to the surface of microbubbles as previously described to produce MBp or MBc. For intravital microscopy protocols, microbubbles were fluorescently labelled by the addition of either diocetadecyltetramethylammoniumcarboncaine (DiI) or diocetadecyloxacarbocyanine perchlorate (Molecular Probes Inc.) to the aqueous suspension. In selected experiments, large microbubbles were eliminated from the preparations by flotation–centrifugation at 400 g for 15 s and collection of the subnatant. Microbubble size and concentration were measured by electrozone sensing (Multisizer III, Beckman-Coulter).

Intravital microscopy

In nine anaesthetized mice, the cremaster muscle was exteriorized and prepared for intravital microscopy as previously described. Observations were made using an Axioskop2-FS microscope (Carl Zeiss, Inc.) with a saline-immersion objective (SW 40/0.8 numerical aperture). Video recordings were made with a high-resolution CCD camera (C2400, Hamamatsu Photonics) interfaced with video time-display unit (VTG-33, For-A Ltd). In four of the mice, the feeding vascular pedicle was compressed for 20 min to interrupt microvascular flow. At baseline and after reflow, microvascular shear was reassessed, and targeted MCE imaging with MBp and MBc preparations that were size-selected to exclude large microbubbles was performed by acquiring the initial frame at 10 min and then digitally subtracting subsequent averaged frames at a long pulsing interval (10 s) that were obtained after several seconds of continuous high-power imaging.

Myocardial ischaemia–reperfusion

In 21 anaesthetized mice, a tracheostomy was performed for positive pressure ventilation and a limited left-lateral thoracotomy was performed to expose the heart. A suture was secured around the left anterior descending (LAD) coronary artery with an intervening segment of polyethylene tubing to facilitate later removal of the ligature. In all but four sham-operated control mice, the suture was secured around the LAD for 10 min during which risk area by MCE and regional wall motion at the centre of the risk area were assessed. The ligature was removed and 45 min after reflow wall motion was reassessed, and targeted MCE imaging with MBp and MBc was performed in random order 10 min apart. In six of the mice undergoing ischaemia, targeted imaging was performed with MBp and MBc preparations that were size-selected to exclude large microbubbles. After targeted imaging was complete, MCE perfusion imaging was performed. The targeted imaging protocol was also performed in three closed-chest non-ischaemic control mice.

Flow cytometry

Flow cytometry was performed in three separate mice undergoing ischaemia–reperfusion to quantify interactions between MBp and circulating platelets in vivo. Platelets were labelled in vivo by intravenous injection of 1.5 mg rhodamine-6G (Sigma) 10 min prior to withdrawal of 450 μL of whole blood. Dio-labelled MBp microbubbles were administered 1 min prior to blood withdrawal. Blood was mixed with 50 μL 3% acid-citrate dextrose (20 mM citric acid, 110 mM sodium citrate, 5 mM dextrose) and 500 μL EDTA–HEPES [150 mM NaCl, 10 mM HEPES (pH 7.6), 1 mM EDTA], and centrifuged for 15 min at 300 g. The entire plasma fraction containing platelets and microbubbles was obtained for flow cytometry (FACS Calibur, Becton-Dickinson). Samples of plasma and microbubbles alone were vortexed, and flow cytometry of the samples was performed.

where \( r_t \) is the number of rolling leucocytes observed over 1 min, \( d \) is vessel diameter, and \( C_L \) is the systemic blood leucocyte concentration measured by haemocytometry. At 45 min, \( 5 \times 10^5 \) Dio-labelled MBp and Dio-labelled MBc were injected simultaneously by intravenous route. Twenty random non-overlapping optical fields were observed with dual fluorescent epi-illumination (469–500 or 530–560 nm excitation filters) to determine the number of microbubbles retained and the mechanism for attachment.
using red and green fluorescent channel gates that were placed at the 99% exclusion gate for microbubbles and platelets, respectively.

**Statistical methods**

Data were analysed on RS/1 (Domain Manufacturing Corp.). Groups were compared using paired or unpaired Student’s t-test (two sided). For each outcome on intravital microscopy, we used a Bonferroni correction to adjust for multiple comparisons between groups and treatments. Intravital microscopy data that were not normally distributed were compared using a Mann–Whitney test. For MCE data, multiple comparisons according to microbubble type and according to region were made for each treatment group. Differences were considered significant at $P < 0.05$. Symbols denoting statistical significance were stratified according to degree of significance.

**Results**

**Targeted microbubble attachment in post-ischaemic muscle**

Intravital microscopy of the cremaster muscle was used to directly observe microbubble attachment in post-ischaemic tissue. Exteriorization of the cremaster without ischaemic injury resulted in leucocyte rolling in all observed venules (Table 1) which has been shown previously to be mediated by endothelial P-selectin expression. In the group undergoing cremasteric ischaemia, leucocyte rolling velocity and flux fraction prior to vascular pedicle occlusion were similar to that found in non-ischaemic control muscle. After ischaemia and reperfusion, mean rolling velocity decreased and leucocyte flux fraction increased, consistent with further expression of endothelial selectins. Ischaemia and reperfusion also produced a small decrease in venular blood velocity and a corresponding decrease in shear rate.

Cremasteric microvascular retention of MBc was infrequent in all animals (Figure 1A) and was mediated almost exclusively by their attachment to adherent leucocytes. Retention of MBp was greater than for MBc in both non-ischaemic and ischaemic muscle. MBp retention was markedly increased by ischaemia–reperfusion, almost all of which was mediated by direct attachment to the venular endothelium (Figure 1B).

**Targeted imaging of post-ischaemic myocardium**

In mice undergoing LAD ischaemia for 10 min and reperfusion, the perfusion defect measured by MCE during arterial occlusion varied in size from 18–45% (median 32%) of the myocardial short-axis area. After 45 min of reflow, myocardial perfusion in the risk area was normal without residual perfusion defect. A wall motion abnormality was present in the centre of the ischaemic zone during LAD occlusion, reflected by a severe reduction in percent wall thickening during systole (Figure 2). After 45 min of reflow, the wall motion abnormality had recovered with no significant difference in percent wall thickening for the ischaemic and remote zones. Illustrated in Figure 3 are MCE images from a single mouse demonstrating a large anterior perfusion defect during LAD occlusion (Figure 3A). After 45 min of reperfusion, targeted imaging with MBp produced signal enhancement that was

---

**Table 1** Venular haemodynamic parameters and leucocyte rolling and adhesion data

<table>
<thead>
<tr>
<th></th>
<th>Control muscle (n = 5)</th>
<th>Baseline (n = 4)</th>
<th>Post-reperfusion (n = 4)</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Venular shear rate (1/s), median (IQ range)</td>
<td>1901 (405)</td>
<td>2236 (600)</td>
<td>1456 (397)</td>
<td>0.04</td>
</tr>
<tr>
<td>Leucocyte rolling velocity (μm/s), median (IQ range)</td>
<td>38.1 (30.1)</td>
<td>40.5 (31.6)</td>
<td>20.6 (12.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Leucocyte rolling flux fraction, median (IQ range)</td>
<td>0.12 (0.6)</td>
<td>0.11 (0.06)</td>
<td>0.21 (0.14)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*P-values are for comparisons between baseline and post-reperfusion states. IQ, interquartile range.

---

**Figure 1** Intravital microscopy results. (A) Mean (±SEM) number of P-selectin-targeted and control microbubbles retained in the microcirculation of control and post-ischaemic cremaster muscle during intravital microscopy. *P < 0.05 vs. control microbubbles; †P < 0.05 vs. non-ischaemic control muscle. (B) Two separate images illustrating fluorescently labelled P-selectin-targeted microbubble attachment in post-capillary venules. Scale bars = 10 μm.

**Figure 2** Mean (±SEM) percent systolic wall thickening measured in the centre of the anterior risk area and the non-ischaemic posterior wall during coronary occlusion (Isch) and after 45 min after reperfusion (Rep). *P < 0.05 vs. reperfusion and vs. the posterior region during ischaemia.

---

Downloaded from https://academic.oup.com/eurheartj/article-abstract/28/16/2011/492797 by guest on 12 February 2018
selectively greater in the previously ischaemic LAD perfusion territory (Figure 3B). Figure 4 depicts data for targeted MCE with microbubble preparations that were not size-separated. These preparations had a mean diameter that ranged from 2.2 to 2.6 μm. In animals undergoing LAD ischaemia-reperfusion, MBc produced a small amount of signal enhancement in both the post-ischaemic anterior myocardium and remote posterior region. Signal enhancement for MBp was greater than that for MBc in both the anterior and posterior wall. MBp signal was greater in the post-ischaemic anterior risk area compared with the remote posterior non-ischaemic territory. In open-chest sham-operated control animals, the signal for MBp was greater than for MBc in both the anterior and posterior regions. The degree of relative signal enhancement for MBp in both these regions was similar to that found in the remote territory in post-ischaemic animals. In closed-chest animals, there was equal signal enhancement for MBp and MBc, the degree of which was similar to MBc signal in all other animals.

The finding of consistent low-level signal enhancement from MBc in all animals and a similar degree of MBp in control closed-chest animals suggested the presence of non-specific myocardial retention of microbubbles. This finding may represent non-specific microbubble retention that can occur in the murine heart from entrapment of intravenously administered microbubbles larger than the capillary dimension (＞5 μm) that transit to the systemic circulation via pulmonary arteriovenous shunts. Accordingly, targeted MCE experiments were performed in six separate mice undergoing ischaemia-reperfusion with microbubble preparations that were size-segregated to exclude large microbubbles (Figure 5). The mean diameters for MBc and MBp after size segregation were 2.0 ± 0.4 μm and 2.1 ± 0.4 μm, respectively, and ≤0.1% of microbubbles were ＞5 μm compared with 3–6% prior to separation. In animals undergoing LAD ischaemia-reperfusion, signal enhancement from size-segregated MBc was negligible in both the anterior and posterior myocardial territories (Figure 6). However, size-segregated MBp still produced signal enhancement that was selectively greater in the previously ischaemic anterior LAD perfusion territory. In these studies, the relatively low acoustic intensities were reflective of the conversion from log-compressed to linear scale, which was performed for relative analysis. The on-screen video intensity enhancement for MBp in the ischaemic territory prior to log-linear transformation was 35 ± 10.
To address limitations of state-of-the-art practice, methods for rapid imaging of post-ischaemic tissue are being developed. Echocardiographic detection of post-ischaemic stunning at the bedside in the emergency department represents the simplest form of ischaemic-memory imaging. This strategy can differentiate ischaemia from infarction when combined with MCE perfusion imaging. Yet this strategy is limited when imaging is performed late or in those with pre-existing abnormalities. The identification of abnormal molecular processes that persist long after resolution of ischaemia is a potential alternative. One promising strategy is the detection of abnormalities in myocardial fatty acid metabolism by SPECT imaging of an iodinated branched fatty acid ($\beta$-methyl-$p$-[125I]iodophenyl-pentadecanoic acid) that persists after resolution of ischaemia. Interest in developing an echocardiographic strategy for ischaemic memory imaging is based on practical issues such as cost, availability, ability to do bedside studies, and rapid imaging protocols. Unlike radionuclide agents, targeted microbubble agents are confined to the vascular space and are not able to probe intracellular events. The solution proposed in this study was to target an endothelial cell adhesion molecule that is up-regulated with ischaemia.

P-selectin is a member of a family of glycoproteins that participate in leucocyte capture and rolling on the venular endothelial surface and has been implicated in the post-ischaemic inflammatory response of many organs, including the myocardium. P-selectin is an attractive target for ischaemic memory imaging for several reasons. It is pre-stored in the Weibel–Palade bodies and therefore will not be recognized by targeted intravascular tracers such as microbubbles until its translocation to the endothelial cell surface, which occurs within minutes of stimulation. P-selectin surface expression can also persist in a synthesis-dependent manner for several hours after ischaemia–reperfusion injury. It is ideal for targeted microparticle attachment by virtue of the number of extracellular consensus repeat segments that project the lectin domain well away (40 nm) from the cell surface.

As an initial step in this study, intravital microscopy was used to determine whether MBp were selectively retained in muscle undergoing brief ischaemia–reperfusion. Intravital microscopy does not, however, provide for any true 'non-injured' control. Surgical preparation of the cremaster muscle for microscopy stimulates surface P-selectin expression on the endothelium, resulting in rapid leucocyte rolling within minutes of muscle exteriorization. It was, therefore, not surprising that retention of MBp was greater than that for MBc even in non-ischaemic muscle. Ischaemia and reperfusion produced a marked increase in the fraction of rolling leucocytes and a decrease in rolling velocity. These changes are characteristic for increased P-selectin expression, although it is also likely that enhanced expression of other adhesion molecules that participate in
slow rolling such as E-selectin and α4-integrins may have also contributed.6,17 It should also be recognized that the ischaemic duration chosen for these studies is unlikely to exactly mimic the degree of myocardial injury caused by brief LAD occlusion. Nonetheless, the selective attachment for MBp in post-ischaemic tissue confirmed that a P-selectin targeting strategy could potentially be used to detect recent ischaemia and that almost all selective retention was secondary to direct endothelial attachment.

Imaging studies with targeted MCE were performed after brief myocardial ischaemia that did not result in evidence for infarction by wall motion or perfusion imaging. Imaging with MBp produced selective contrast enhancement in the risk area. MBp also produced some selective signal enhancement in the non-ischaemic territory, albeit less than in the ischaemic territory. When open-chest sham-operated animals were studied, there was a small amount of selective enhancement from MBp in both the anterior and posterior regions. The degree of enhancement was similar to that in the remote territory in post-ischaemic mice. Hence, we believe that P-selectin signal in the remote zone after ischaemia–reperfusion was due, at least in part, to thoracotomy and cardiac exposure. It has also been demonstrated that myocardial territories remote from the ischaemic territory can undergo activation and P-selectin expression.10 Only closed-chest ischaemic models will be able to determine the relative contribution of this response.

A consistent low-level signal enhancement occurred from MBp when using preparations that had not been size-segregated. This finding was likely due to pulmonary arterial-venous shunts that are present in mice and permit passage of microbubbles sufficiently large to be entrapped in myocardial capillaries.13 Since these entrapped microbubbles are by definition large, even a small number in tissue would be expected to contribute a disproportionate amount of signal during targeted imaging.18 To address this issue, MCE was repeated in separate animals using microbubble preparations that had undergone flotation–centrifugation in order to eliminate microbubbles >5 μm in diameter. This modification eliminated non-specific retention of microbubbles resulting in a relative eight-fold higher degree of signal enhancement for MBp vs. MBc in the post-ischaemic zone. It must be noted that for all of our quantitative data, log-linear transformation resulted in a relative scale with low values compared with the actual on-screen video intensity. On-screen intensity before log-linear transformation was robust even for the studies where size-segregated microbubble populations were used (enhancement of 35 video intensity units for MBp in the ischaemic zone).

There are several limitations of this study. Most importantly, we did not study the relation between the duration of either ischaemia or reperfusion and targeted P-selectin signal. Clinical applicability of this targeted ultrasound strategy in humans will rely on the ability to detect P-selectin signal for several hours after an ischaemic period. Although in mice P-selectin is expressed for several hours after ischaemia or hypoxia,9,10 we chose not to study multiple post-reperfusion time intervals because our goal was to test feasibility and because the spatial-temporal pattern of P-selectin expression is likely to differ between small animal models and humans. A systematic study of how the variables of ischaemic duration and reflow time influence P-selectin signal will need to be performed in an animal model closer to humans in order to determine the potential clinical utility of this technique. In a related issue, brief coronary occlusion does not necessarily recapitulate the pathophysiology of acute coronary syndromes. Immunohistology for P-selectin was not performed due to our previous experience with polyclonal primary staining where differentiating stored from endothelial surface expression has been difficult. P-selectin is also stored in the alpha granules of platelets and can be expressed on the surface upon activation.19 However, attachment of microbubbles to adherent platelets or leucocyte–platelet complexes, which has been observed in cytokine-stimulated tissue,15 was rare on intravital microscopy. Additionally, flow cytometry demonstrated that the vast majority of circulating microbubbles in post-ischaemic animals were not associated with circulating platelets. The flow cytometry data was also important for demonstrating that platelet attachment to microbubbles is not a significant impediment to MBp adhesion to activated endothelial cells. Last, we must also note that small regions of persistent myocardial hypoperfusion would be difficult to detect despite using relatively high-frequency perfusion imaging.

We conclude that P-selectin-targeted imaging with MCE can detect post-ischaemic myocardium in the absence of necrosis. These results are encouraging that molecular imaging of endothelial cell adhesion molecules may provide a unique opportunity for early detection of ischaemia and risk stratification in patients presenting with chest pain, or possibly to diagnose ischaemia provoked by diagnostic stress testing.15 The practical benefits of this ultrasound-based strategy are the ability to obtain diagnostic information rapidly at the bedside and the technical ease of execution that involves a single injection and delayed imaging. The potential uses for P-selectin-targeted imaging will probably extend to other myocardial inflammatory processes such as transplant rejection and myocarditis.20

Acknowledgements

J.R.L. is supported by grants RO1-HL-078610 and RO1-DK-063508 from the National Institutes of Health, Bethesda, Maryland. B.A.K. is supported by research grants from the Novartis Foundation, the Swiss National Science Foundation, and the Lichtenstein Foundation. We thank Dawn Peters, for her assistance with statistical methods.

Conflict of interest: none declared.

References


