Noninvasive Imaging of Myocardial Reperfusion Injury Using Leukocyte-Targeted Contrast Echocardiography

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Background—We hypothesized that myocardial contrast echocardiography (MCE) with leukocyte-targeted microbubbles could temporally and spatially characterize the severity of postischemic myocardial inflammation.

Methods and Results—In 9 open-chest dogs, either the left anterior descending or left circumflex coronary artery was occluded for 90 minutes (n=6), while the remaining dogs served as non-ischemic controls. During occlusion, MCE was performed to determine the risk area (RA) and regions supplied by collateral flow. Myocardial inflammation was assessed at 5, 60, and 120 minutes after reflow by MCE imaging of leukocyte-targeted (phosphatidylserine-containing) lipid microbubbles. The spatial extent and severity of inflammation were also assessed by radionuclide imaging of the neutrophil-avid tracer 99mTcRP517 and tissue myeloperoxidase activity. Early after reflow, MCE detected inflammation throughout the entire risk area, the extent of which decreased over time due to reduced signal in collateral-supplied regions. The spatial extent of inflammation late after reflow was similar for MCE and radionuclide imaging. The severity of inflammation in the infarct area, the noninfarcted risk area, and collateral-supplied territories determined by quantitative MCE correlated well with myeloperoxidase activity (r=0.81).

Conclusions—MCE with leukocyte-targeted microbubbles can temporally assess the severity and extent of postischemic myocardial inflammation and could be used to evaluate new treatment strategies designed to limit inflammation in acute coronary syndromes. (Circulation. 2002;105:1764-1767.)

Key Words: echocardiography inflammation ischemia

Leukocyte infiltration into the myocardium can play a detrimental role after myocardial ischemia/reperfusion by contributing to myocardial injury and necrosis1,2 and to microvascular no-reflow.3 An accurate, noninvasive method for routinely assessing inflammation is not currently available but would be valuable for further characterizing the contribution of inflammation to reperfusion injury in patients and for evaluating new treatment strategies that attenuate postischemic leukocyte recruitment.

We have recently demonstrated that inflammation can be assessed using contrast-enhanced ultrasound with microbubbles that bind to activated leukocytes adherent to the venular endothelium.4,5 Leukocyte avidity for lipid microbubbles has been greatly enhanced by incorporating phosphatidylserine (PS) into the shell, which increases microbubble retention in inflamed tissue.6 In this study, we hypothesized that the severity of myocardial inflammation after ischemia/reperfusion injury could be assessed both spatially and temporally using myocardial contrast echocardiography (MCE) with leukocyte-targeted microbubbles.

Methods

Animal Preparation
The study was approved by the Animal Research Committee at the University of Virginia. Nine anesthetized open-chest dogs (30 to 35 kg) were studied (Haycock Kennels). Ultrasound flow probes were placed around proximal portions of the left anterior descending (LAD) and left circumflex (LCx) arteries.

Leukocyte-Targeted Microbubbles
Lipid microbubbles targeted to activated leukocytes (MB-PS) were prepared by sonication of decafluorobutane gas with an aqueous dispersion of 1 mg · mL⁻¹ polyethylene glycol stearate, 2 mg · mL⁻¹ distearoyl phosphatidylcholine, and 0.3 mg · mL⁻¹ distearoyl phosphatidylserine (Avanti Polar Lipids).

Myocardial Contrast Echocardiography
MCE was performed using intermittent high-power (MI 0.9) harmonic imaging in the short-axis plane with a HDI-5000 system (Philips Ultrasound). For assessment of myocardial perfusion, a suspension (8% volume) of Optison microbubbles (Mallinckrodt Medical, Inc) was infused intravenously at 1.5 mL · min⁻¹. During coronary occlusion, the risk area was determined from background-subtracted frames acquired at a pulsing-interval (PI) of 5 cardiac cycles, whereas territories with collateral flow were determined by subsequent opacification with further prolongation of the PI to 20 cardiac cycles.3

For inflammation imaging, ultrasound transmission was paused and 1×10⁶ leukocyte-targeted MB-PS microbubbles were injected as an intravenous bolus. Imaging was not resumed until 15 minutes after the injection to allow microbubble retention in inflamed tissue and clearance of freely circulating microbubbles from the blood pool.5,6 Intermittent imaging was then initiated at a PI of 1 cardiac cycle for several frames. The first 2 frames, which reflect the total

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concentration of microbubbles in tissue (both retained and freely circulating).\textsuperscript{5,6} were averaged. Microbubbles within the beam were destroyed by 5 seconds of continuous imaging. Intermitting imaging was then performed at a PI of 20 cardiac cycles to derive the signal from freely circulating microbubbles alone.\textsuperscript{5,6} These frames were averaged and digitally subtracted from the initial frames to obtain a single color-coded image reflecting retained microbubbles alone. Video intensity (VI) was measured in regions-of-interest placed over the TTC-defined infarct area and portions of the noninfarcted risk area with and without collateral flow. VI values were expressed as a ratio to the normal bed. The spatial extent of inflammation on the color-coded image was planimetered by a blinded reader and expressed as a percent of the total left ventricular area.

Radionuclide Assessment of Inflammation
Approximately 30 mCi of 99m Tc-RP517 (Dupont Pharmaceuticals), which binds in vivo to the LTB$_{a}$ receptor expressed by activated neutrophils,\textsuperscript{8} was injected intravenously. Postmortem, a short-axis myocardial slice was placed on a gamma camera (Siemens Orbiter) with a high-energy collimator. A 20% energy window was centered on the 140-keV photopeak, and 110$^6$ counts were collected. The spatial extent of inflammation was planimetered similar to MCE analysis.

Tissue Myeloperoxidase Activity and Immunohistochemistry
Tissue myeloperoxidase (MPO) activity, reflecting tissue neutrophil accumulation, was measured from homogenized tissue as previously described.\textsuperscript{6} Changes in absorbance were recorded at 460 nm over 3 minutes in a spectrophotometer (SPECTRmax PLUS, Molecular Devices). Results were expressed as units of activity per mg of tissue.

Neutrophil immunostaining was performed on myocardial frozen sections using a primary mouse anti-dog monoclonal antibody (BAQ30A, VMRD Inc) against CD18 antigen expressed by neutrophils and a secondary biotinylated horse anti-mouse antibody (DAKO).

Experimental Protocol
After baseline hemodynamic measurements, either the LAD or LCx artery was occluded for 90 minutes in 6 dogs, and MCE perfusion imaging was performed. Leukocyte-targeted MCE was performed at 5, 60, and 120 minutes after reflow and at similar time intervals for nonischemic control dogs (n=3). 99mTc-RP517 was injected 60 minutes after reflow. MCE perfusion imaging was performed after the final targeted imaging stage. The dog was then euthanized and TTC staining and radionuclide imaging were performed on the short-axis slice corresponding to the ultrasound imaging plane. Samples were taken from different portions of the risk area and normal regions for immunohistology and tissue MPO.

Statistical Methods
Data are expressed as mean±SD. Comparisons between stages or techniques were made using a repeated-measures ANOVA or Bland-Altman analysis. Correlations were performed using least-squares linear regression analysis.

Results
Risk Area and Infarct Size
The mean risk area and infarct size were 30±4% and 18±4% of the total LV area, respectively. Collateral flow was present in 16±10% of the risk area. Mean coronary blood flow in the infarct-related artery progressively decreased during reperfusion (40±15, 32±11, and 27±3 mL·min$^{-1}$ at 5, 60, and 120 minutes; P<0.05).

Spatial Extent of Inflammation
In postischemic dogs, myocardial enhancement during MCE with MB-PS was similar in location to 99mTc-RP517–labeled leukocyte accumulation and extended beyond the region of infarction (Figure 1A). Immunohistology demonstrated perivascular and interstitial accumulation of leukocytes throughout the risk area. The spatial extent of MCE enhancement 5 minutes after epicardial artery reflo w was similar to the total risk area (Figure 1B) and encompassed collateral-supplied regions. Over the 2 hour reperfusion period, the spatial extent of enhancement decreased due to progressive reduction in signal from collateral-supplied regions and peripheral portions of the noninfarcted risk area. Illustrated by the arrows in Figure 1A, microvascular no-reflow in portions of the infarct zone occasionally precluded MB-PS entry into tissue late (≥1 hour) after reflow at a time when hyperemic blood flow had abated. The extent of inflammation by MCE at 120 minutes was similar to that obtained by radionuclide imaging of 99mTc-RP517–labeled leukocyte accumulation (Figure 1C).

Quantification of Inflammation
Background-subtracted VI from MB-PS in control dogs was very low and similar to that in remote, nonischemic myocardium in dogs undergoing ischemia/reperfusion (11±6 versus 14±8). VI from MB-PS at 5 minutes after reflow was similar for the infarct zone and noninfarcted portions of the risk area. In the infarct zone, VI peaked 60 minutes after reflow (normalized units of 1.7±0.8, 2.3±0.9, and 1.8±0.5 at 5, 60, and 120 minutes, respectively). In collateral-supplied regions, VI tended to decrease after reflow (normalized units of 1.6±0.6, 1.3±0.4, and 1.3±0.3 at 5, 60, and 120 minutes, respectively). The background-subtracted VI in different regions of the risk area by MB-PS at 120 minutes correlated with both tissue MPO and 99mTc-RP517 activity (Figures 2A and 2B).

Discussion
In this study, we have demonstrated that myocardial inflammation after ischemia/reperfusion can be characterized non-invasively by MCE imaging of leukocyte-targeted microbubbles. Targeting was achieved by incorporating PS in the lipid shell, which enhances complement-mediated microbubble attachment to activated leukocytes adherent to the venular endothelium.\textsuperscript{4,6} Because microbubbles are confined to the vascular compartment, targeted MCE provides unique information on the active intravascular recruitment of leukocytes rather than total tissue burden. In this study, the spatial extent of inflammation by MCE was similar to that measured by 99mTc-RP517, which was administered late after reperfusion in order to minimize signal from extravasated 99mTc-labeled neutrophils. Myocardial VI from retained MB-PS, which parallels the number of adherent leukocytes within the inflamed microcirculation,\textsuperscript{4} correlated moderately well with both quantitative 99mTc-RP517 data and tissue MPO. Because MPO reflects total leukocyte accumulation in tissue over the entire reperfusion period, the relative degree of inflammation was consistently less for MCE.
The capability of performing sequential targeted imaging provided unique temporal information on postischemic inflammation within the same animal. Our findings corroborate postmortem histological studies that have demonstrated a time-dependent shift in the location of leukocytes in postischemic myocardium from intravascular (which peaks about 1 hour after reflow) to interstitial (which peaks >5 hours after reflow). Using MCE perfusion imaging with nontargeted microbubbles to assess collateral perfusion within the risk area, we were able to uniquely determine that leukocyte recruitment was attenuated but not completely prevented by collateral flow. These results support previous demonstration of an inverse relation between myocardial blood flow and postischemic inflammation.

We anticipated that targeted microbubble imaging in the setting of ischemia/reperfusion would be problematic because microbubble entry into portions of the infarct zone would be prevented by low- or no-reflow. In the first several hours after reflow, however, there is marked heterogeneity of microvascular perfusion within risk area and hyperemia within the infarct region, which should facilitate microbubble entry even within necrotic tissue. In the present study, microvascular no-reflow was uncommon, thereby permitting sufficient entry and retention of MB-PS to produce contrast enhance-
ment. We could not, however, entirely exclude the possibility of MB-PS attachment to entrapped leukocytes at the capillary or precapillary level in small regions of no-reflow.

Results of this study indicate that site-targeted MCE has potential for investigating new therapeutic strategies with specific antiinflammatory agents in acute coronary syndromes. We believe that MCE is well-suited for this purpose because of its ability to detect active vascular recruitment of leukocytes, which allows very early responses to be studied, and the short protocol duration, which allows serial assessment of the response to therapy.

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