Microvascular Structural Correlates of Myocardial Contrast Echocardiography in Patients With Coronary Artery Disease and Left Ventricular Dysfunction

Implications for the Assessment of Myocardial Hibernation

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Background—Myocardial contrast echocardiography (MCE) has been used to evaluate myocardial viability. There are no data, however, on the pathological determinants of myocardial perfusion by MCE in humans and the implications of such determinants.

Methods and Results—MCE was performed in 20 patients with coronary artery disease and ventricular dysfunction within 24 hours before myocardial biopsy at surgery using a continuous Optison infusion (12 to 16 cc/h), with intermittent pulse inversion harmonics and incremental triggering. Peak myocardial contrast intensity (MCI) and the rate of increase in MCI were quantitated. Thirty-six transmural myocardial biopsies (2 per patient) were obtained by transesophageal echocardiography. Total microvascular (<100 μm) density, capillary density and area, arteriolar and venular density, and percent collagen content were quantitated with immunohistochemistry. Peak MCI correlated with microvascular density (r=0.59, P<0.001) and capillary area (r=0.64, P<0.001) and inversely correlated with percent collagen content (r=−0.45, P=<0.01). The best relation was observed when the ratio of peak MCI in the 2 biopsied segments in each patient was compared with the ratio of microvascular density and capillary area (r=0.84 and 0.87, respectively; P<0.001). A significant overlap in microvascular density was seen between segments with and without recovery of function. The new MCE indices of blood velocity (β) and flow (peak MCI×β) better identified recovery of function compared with microvascular density and the sole use of peak MCI.

Conclusions—Microvascular integrity is a significant determinant of maximal MCI in humans. MCE indices of blood velocity and flow are important parameters that predict recovery of function after revascularization. (Circulation. 2002; 106:950-956.)

Key Words: perfusion ■ echocardiography ■ hibernation ■ ischemia ■ coronary disease

Identification of hibernating myocardium allows better selection of patients who need revascularization. Techniques evaluating hibernating myocardium have included metabolic and perfusion imaging3 and assessment of contractile reserve.2 Recently, myocardial contrast echocardiography (MCE) using intracoronary contrast administration has emerged as a modality for assessing myocardial perfusion; it has the potential for predicting myocardial viability.4 The underlying basis for assessing myocardial viability with MCE is that myocardial contrast enhancement depends on an intact microcirculation. Kloner et al5 noted that with myocardial infarction, myocyte loss is accompanied by a loss of microvasculature. Accordingly, the absence of myocardial opacification with MCE may be evidence of lack of myocardial viability. To date, there are no data on the pathological correlates of myocardial perfusion by MCE in patients with hibernating myocardium. The following study was performed (1) to evaluate the pathological and vascular correlates of intravenous quantitative MCE parameters in the hibernating myocardium and (2) to assess whether preservation of microvascular integrity and perfusion by intravenous MCE predicts recovery of function of dysfunctional, ischemic myocardium.

Methods

Patient Population

The population consisted of 20 patients with chronic, stable ischemic heart disease and left ventricular dysfunction with one or more coronary artery stenosis (≥70% diameter stenosis) who were already...
Echocardiographic Studies
Echocardiographic imaging was performed in the standard parasternal and apical views (Advanced Technology Laboratories 5000; 2 to 4 MHz transducer). Regional function was assessed using the 16-segment left ventricular model of the American Society of Echocardiography and graded from 1 (normal) to 5 (dyskinesia). Ejection fraction was quantified with the multiple diameter method. The initial and follow-up studies were interpreted without knowledge of the MCE and histopathological data. Recovery of regional function after revascularization was defined as an improvement in ≥2 grades in wall motion.

Myocardial Contrast Echocardiography
Baseline apical 4- and 2-chamber views and a long axis view of the left ventricle were obtained using pulse-inversion second-harmonic imaging. Once optimized, the gain settings remained unchanged throughout the protocol. Continuous infusion (12 to 16 cc/h) of a contrast agent (Optison) was then started using an infusion pump (Baxter, model AS50). The infusion rate was adjusted to minimize attenuation and to give the best myocardial opacification at the triggering interval of 1:4 cardiac cycles. Once steady state was achieved, repeat imaging was obtained using sequential ECG triggering at end-systole, with increasing pulsing intervals of 1:1, 1:2, 1:3, 1:4, 1:6, and 1:8. Images were captured on-line on optical disk for quantitative analysis.

MCE images were analyzed quantitatively using a prototype software (HDI Laboratory, Advanced Technology Laboratories). Background-subtracted, end-systolic myocardial contrast intensity (MCI) was measured at all pulsing intervals in each myocardial segment. For each segment, plots of MCI versus pulsing intervals were constructed and fit to an exponential function $y = A \times (1 - e^{-bt})$, as described by Wei et al. The plateau or peak MCI (A), the slope of the ascending curve of MCI (b), and the product (peak MCI×b) were calculated (Figure 1). Because of variability in blood pool concentration of microbubbles among patients, Peak MCI of a biopsied segment (n) was also normalized to the segment with the highest peak MCI (max) as follows: peak MCI/peak MCI max.

Quantitative Coronary Angiography
Selective coronary angiography was performed in multiple views using the Judkins technique. The coronary angiograms were analyzed and quantitated by an independent observer using the Cardiovascular Angiography Analysis System (Pie Medical Instruments). The degree of stenosis was expressed as the percent reduction of the internal luminal diameter in relation to the normal reference. Collateral vessels were assessed visually and scored as present or absent.

Transmural Left Ventricular Biopsies
Transmural myocardial biopsies were obtained with a 20 mm, 14-gauge Tru-cut biopsy needle at the time of bypass surgery but before cardioplegia. Transesophageal echocardiography was used to direct the biopsy to the selected myocardial segments. Two biopsies were acquired per patient in 2 different coronary distributions: one from a dysfunctional segment and another from a normal segment to be used as a control. However, when none of the segments that could be biopsied were normal (n=10), 2 dysfunctional segments were biopsied.

Quantitation of the Microvasculature and Fibrosis
Heart biopsy samples were fixed in B5 fixative and embedded in paraffin. Immunohistochemical studies were performed as previously described using samples fixed in B5 fixative, which ensures optimal antigenic survival. Sequential 3- to 5-μm sections were cut by microtomy. Immunostaining was performed using the ELITE mouse kit (Vector Laboratories). Briefly, sections were pretreated with a solution of 3% hydrogen peroxide to inhibit endogenous peroxidase activity and incubated with 2% horse serum to block nonspecific protein binding. Subsequently, they were incubated with the primary antibody for 2 hours at room temperature. After rinsing with phosphate-buffered saline, the slides were incubated for 30 minutes with the secondary antibody. The slides were rinsed with phosphate-buffered saline and incubated for 30 minutes in avidin-biotin-peroxidase complex reagent. Peroxidase activity was detected using diaminobenzidine with nickel. Slides were counterstained with eosin.

The following primary monoclonal antibodies were used for immuno-histochemistry: anti-CD31 antibody (Dako) to label endothelial cells, and anti-α-smooth muscle actin antibody (Sigma) to identify smooth muscle cells. Stained sections were photographed with a Leica MicroLumina digital camera mounted on a Zeiss microscope. Multiple digital images were taken and stored for each sample. Staining was analyzed by the Zeiss image-analysis software.

Total microvessel density was calculated as the number of CD31-positive vessels in the section divided by the total area. Arteriolar density was calculated by analyzing sections stained for α-smooth muscle actin, which labels the medial smooth muscle cells. Venular density was calculated by counting the number of microvessels with a minor axis >15 μm without defined media. Capillary density was then derived as follows: total microvascular density−arteriolar density−venular density. Capillary area was calculated as total lumen area of capillaries, expressed as a percentage of the total area of the section.

Serial sections were stained for collagen with picrosirius red to demonstrate areas of fibrosis. Total collagen staining was analyzed using the Zeiss image analysis system and expressed as a percentage of total area.

Statistical Analysis
Continuous data are presented as mean±SD. Linear regression analysis was used to correlate microvascular parameters with fibrosis and MCE indices. Stepwise regression analysis was performed to assess the determinants of MCE parameters. ANOVA was used to compare microvascular density in segments with normal resting function and dysfunctional segments with and without recovery of function after revascularization. An unpaired t test was applied for...
the comparison of different contrast parameters in segments with intact microcirculation, with and without recovery of function. A paired t test was used to compare the preoperative and postoperative ejection fraction. Significance was at \( P \leq 0.05 \).

**Results**

**Patient Population**
The patient population consisted of 20 patients (18 men) with a mean age of 65 years (range, 51 to 73 years) and left ventricular ejection fraction of 29\%\%. Nine patients had a history of previous myocardial infarction, 15 had symptoms of heart failure, and 11 had stable angina before surgery. Complete revascularization was performed with a total of 1 to 4 grafts per patient; none developed postoperative ischemic or cardiac events. Late after surgery (>3 months), regional function improved in 38% (96 of 255) of severely dysfunctional segments. After bypass surgery, ejection fraction increased from 29\%\% to 36\%\% (\( P = 0.03 \)).

**Myocardial Function of Biopsied Segments**
A total of 36 segments were biopsied. In 2 patients, myocardial biopsies could not be obtained: one patient became unstable during the induction of anesthesia and in another, the biopsy was not taken due to technical problems. In 2 patients, the biopsy specimens were small and only limited histological studies could be performed. Eight of the 36 segments had a normal resting function, 20 segments were severely hypokinetic at rest, and 8 were akinetic. Of these 28 dysfunctional segments, 12 (43\%) recovered after revascularization.

**Relation of Microvascular Density to Fibrosis**
The total microvascular density in the biopsied segments (inclusive of capillaries, arterioles, and venules) averaged 1133±337 microvessels/mm² (range, 312 to 1795 cells/mm²). Capillaries constituted the majority of the microvasculature (mean, 97.5\%; range, 77\% to 99\%), with a mean density of 1125±342 capillaries/mm² (range, 282 to 1790 capillaries/mm²). The total capillary area averaged 6.1±1.9\% of the myocardial biopsy areas (range, 1.63\% to 9.9\%). Both arterioles and venules had very small densities compared with capillaries (\( P < 0.0001 \)), with a mean of 8.6±8.4 capillaries/mm² and 6.6±5.0 venules/mm², respectively.

The microvasculature related significantly to the extent of collagen content. An inverse correlation was found between percent collagen content and total microvascular density (\( r = -0.73, P < 0.001 \)), capillary density (\( r = -0.72, P < 0.001 \); Figure 2), and capillary area (\( r = -0.73, P < 0.001 \)). However, an increase in arteriolar density was noted with increasing extent of collagen content (\( r = 0.69, P < 0.001 \); Figure 2). The density of arterioles, however, was still small (Figure 2). No correlation was found between venular density and collagen content (\( r = 0.03, P < 0.88 \)).

**Relation of MCE Parameters to Microvascular Density and Fibrosis**

**Peak MCI**
In the biopsied segments, absolute and normalized peak MCI averaged 14.9±3.9 dB and 0.81±0.02 dB, respectively. Peak MCI, an index of myocardial blood volume, related significantly to total microvascular density, capillary density, and capillary area and was inversely related to arteriolar density and percent collagen content (\( r = -0.51 \) to 0.38). No relation was seen with venular density. Overall, normalized peak MCI had stronger relations to histological parameters than absolute peak MCI (microvascular density: \( r = 0.59, P < 0.001 \); capillary density: \( r = 0.61, P < 0.001 \); capillary area: \( r = 0.64, P < 0.001 \); percent collagen, \( r = -0.45, P < 0.01 \); Figure 3). The best relations were observed when the ratio of peak MCI of the 2 biopsied segments per patient was compared with the respective ratio of histological parameters (microvascular density: \( r = 0.84, P < 0.001 \); capillary density: \( r = 0.81, P < 0.001 \); capillary area: \( r = 0.87, P < 0.001 \); Figure 3). Total microvascular density was the main histological predictor of absolute peak MCI, and capillary area was the main predictor of normalized peak MCI by stepwise regression analysis.

**β and Peak MCI×β**
The rate of rise of MCI (\( β \)) averaged 0.45±0.42 in the biopsied segments. No significant linear correlations were
found between $\beta$ and any of the microvascular parameters or collagen content ($r = -0.18$ to $0.12$; $P = 0.11$ to 0.7). Similarly, no correlations were observed between the peak MCI×$\beta$ and any of the histological parameters ($r = -0.26$ to 0.18; $P > 0.05$). Segments with <30% collagen content and, thus, preserved microvascular density (>800 microvessels/mm²) had a wide range of $\beta$ (0.03 to 1.5; mean, 0.50±0.45) and peak MCI×$\beta$ values (0.49 to 26.6; mean, 7.83±7.86; Figure 4). In contrast, segments with increased collagen content (>30%) had a narrow range of low $\beta$ values (0.15 to 0.34; mean, 0.25±0.08) and peak MCI×$\beta$ values (1.88 to 3.7; mean, 2.48±1.3; $P = 0.05$ and $P = 0.03$ versus respective values for collagen content <30%; Figure 4).

MCE Parameters and Angiographic Findings
Coronary artery percent diameter stenosis averaged 84±4%. No correlation was observed between peak MCI and severity of the coronary stenosis ($r = -0.27$, $P = 0.11$) or the presence of collaterals by angiography ($P > 0.05$). A weak inverse correlation was found between $\beta$ and the severity of coronary stenosis ($r = -0.3$, $P = 0.09$), and no relation was found with the presence of collaterals ($P > 0.05$).

Microvascular Density: Relation to Myocardial Function and Prediction of Functional Recovery
Microvascular and histological indices in segments with normal function and in those with myocardial dysfunction, with and without recovery after revascularization, are depicted in the Table, with corresponding examples in Figure 5. Microvascular density, capillary density, and capillary area were highest in segments with normal function, lowest in dysfunctional segments without recovery, and intermediate in segments with recovery of function. An opposite trend was seen for arteriolar density and collagen content. Overall, a significant overlap was observed in microvascular density (Figure 6). Segments with low microvascular density (<800 microvessels/mm²) generally did not recover function after revascularization. In contrast, a preserved microvascular density did not necessarily imply recovery of function at follow-up (Figure 6).

Quantitative MCE Parameters: Relation to Myocardial Function and Prediction of Functional Recovery
Quantitative MCE parameters in segments with normal function and in those with myocardial dysfunction, with and without recovery of function, are shown in Figure 7. Peak

Figure 3. Relation of normalized peak MCI to total microvascular density and capillary area. The lower panels depict the relation of the respective ratios of these parameters in the 2 biopsied segments for each patient (see text).

Figure 4. Relation of normalized peak MCI, $\beta$, and peak MCI×$\beta$ to percent collagen content in the corresponding biopsied segments.
Comparison of Histological Parameters Among Various Myocardial Segments

<table>
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<th>Dysfunctional Segments With Recovery</th>
<th>Dysfunctional Segments Without Recovery</th>
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<td>Capillary density, n/mm²</td>
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<td>Capillary area, %</td>
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<td>% Collagen</td>
<td>11.1±2.48</td>
<td>20.2±7.2*</td>
<td>31.8±21.99*</td>
</tr>
</tbody>
</table>

n indicates number of respective microvessels.

*P<0.05 vs normal segments.

MCI, β, and the product of peak MCI×β were highest in segments with normal function. Segments with recovery of function had intermediate values, whereas those without recovery had the lowest MCE parameters. This differentiation was most pronounced for β and the product of peak MCI×β (Figure 7, top). Using receiver operator curves, the area under the curve for prediction of recovery was highest for peak MCI×β and β (0.77 and 0.76, respectively) and lowest for normalized peak MCI (0.62) and microvascular density (0.54).

The new contrast indices of blood velocity and flow also improved prediction of recovery of function in dysfunctional segments with preserved microvascular density (>800 microvessels/mm²; n=22; Figure 7, bottom). Although peak MCI was similar in segments with (n=12) and without recovery of function, β and peak MCI×β were significantly higher in segments that recovered function. Using receiver operator curves, the area under the curve for prediction of recovery was highest for peak MCI×β and β (0.77 and 0.76, respectively) and lowest for normalized peak MCI (0.55).

Interobserver and Intraobserver Variability

The interobserver variability for quantitation of peak MCI and β was 8% and 17%, respectively. The intraobserver variability was 7% for quantitation of peak MCI and 13% for β.

Discussion

The present study demonstrates for the first time the histological correlates of MCE parameters in humans and their implications in the setting of suspected myocardial hibernation. First, peak MCI, an index of myocardial blood volume, correlates with microvascular density and capillary area and inversely with collagen content. The relation is strongest when these parameters are compared within each patient. Second, contrast indices of myocardial blood velocity and flow are reduced in the presence of high collagen content and low microvascular density, but they are quite variable when the microvasculature is preserved. Finally, an intact microvasculature alone does not necessarily predict recovery of function. MCE parameters of myocardial blood flow and velocity in these segments help differentiate hibernating myocardium from myocardium with irreversible dysfunction.

Myocardial Contrast Echo Parameters and Microvascular Integrity

Myocardial contrast echo agents are intravascular tracers that have similar rheology to red blood cells in the microcirculation. In acute animal models, peak MCI correlates with myocardial blood volume and the blood pool concentration of microbubbles, whereas the rate of increase in contrast intensity (β) and their product (peak MCI×β) reflect myocardial blood velocity and blood flow, respectively. To the best of our knowledge, there are no previous reports of the histological correlates of myocardial contrast echo parameters in man. With increasing collagen content, a decrease in microvascular density and its main component, capillary density, were observed. Arteriolar density increased slightly with more fibrosis, suggesting that arterioles are more prominent in areas of scar and may be more resistant to the ischemic process. No changes in venular densities were observed. Capillary area related best to normalized contrast intensity by stepwise regression analysis. This index accounts in part for the variable degree of capillary closure that may occur distal to severe coronary stenosis. The comparatively low number of arterioles and venules may explain the insignificant contribution of these vessels to peak contrast.

Figure 6. A plot showing the microvascular density in segments with normal resting function and in dysfunctional segments with and without recovery of function.

Figure 5. Biopsy specimens from 3 different segments with CD31 staining. A, In a segment with normal resting function, the microcirculation is preserved. B, In a dysfunctional segment that improved after revascularization, the microvascular density is normal. C, Low capillary density and significant fibrosis are seen in a dysfunctional segment that did not recover function.
intensity but accounts for some of the contrast intensity observed even in areas of chronic infarction.

The parameters $\beta$ and peak MCI$\times\beta$ did not relate linearly to histological findings. These observations are not totally unexpected because myocardial blood velocity and flow are dynamic parameters, particularly in the presence of coronary artery disease. In segments with intact microvasculature, a wide range of myocardial blood velocity and flow was seen, whereas in segments with high collagen content and reduced microvascular density, only reduced values of these parameters were noted. Accurate validation of these parameters in humans would require further studies evaluating regional myocardial blood flow with techniques such as positron emission tomography.

**Microvascular Integrity, Contrast Echocardiography, and Recovery of Function**

MCE is effective in predicting myocardial viability in patients after acute myocardial infarction. However, few studies have evaluated the role of MCE in myocardial hibernation. DeFilippi et al and Nagueh et al showed the effectiveness of intracoronary MCE in suspected myocardial hibernation. Using maximal MCI and fundamental imaging, both studies demonstrated a high sensitivity and moderate specificity for predicting recovery of function after revascularization. The predictive accuracy was similar to rest-redistribution TI-201 scintigraphy.

The present study, the first to use intravenous contrast for the assessment of myocardial hibernation, demonstrates that the use of the new MCE parameters $\beta$ and peak MCI$\times\beta$ further refine the prediction of recovery of function compared with the traditional peak MCI parameter and with histological indices of microvascular integrity. The collagen content of biopsy specimens was similar to those in previous studies on viability involving positron emission tomography. A significant overlap was noted in microvessel integrity between segments with and without recovery of function. However, $\beta$ and peak MCI$\times\beta$ were significantly higher in segments with recovery, implying a higher blood velocity and flow, albeit one that was lower than those of normal segments (Figure 7). These results indicate that although peak MCI is an index of viability, indices of myocardial blood velocity ($\beta$) and flow (peak MCI$\times\beta$) are more important in predicting myocardial hibernation. In light of the several mechanisms proposed to account for the depressed resting function in myocardial hibernation, it is conceivable that the higher blood flow measured with the new MCE parameters in segments with similar degrees of cellularity, fibrosis, and microvessel density maintained a higher level of viability, as demonstrated by recovery of function after revascularization.

**Figure 7.** Top, Comparison of normalized peak MCI, $\beta$, and peak MCI$\times\beta$ in biopsied myocardial segments with normal function (NL) and those with depressed function, with and without recovery after revascularization. Bottom, similar comparison in myocardial segments with depressed function and preserved microvascular integrity (total microvascular density >800 microvessels/mm$^2$).
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