Near-Infrared Fluorescent Imaging of Matrix Metalloproteinase Activity After Myocardial Infarction

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Background—We used a molecular probe activated by protease cleavage to image expression of matrix metalloproteinases (MMPs) in the heart after myocardial infarction.

Methods and Results—We synthesized and characterized a near-infrared fluorescent (NIRF) probe that is activated by proteolytic cleavage by MMP2 and MMP9. The NIRF probe was injected into mice at various time points up to 4 weeks after myocardial infarction induced by ligation of the left anterior descending coronary artery. NIRF imaging of MMP activity increased in the infarct region, with maximal expression at 1 to 2 weeks, persisting to 4 weeks. Zymography and real-time polymerase chain reaction analysis showed that MMP9 expression is increased at 2 to 4 days, and MMP2 expression is increased at 1 to 2 weeks. Dual-label confocal microscopy showed colocalization of NIRF imaging with neutrophils on day 2, and flow cytometric analysis confirmed that NIRF signal is associated with leukocytes in the infarct zone.

Conclusions—This study demonstrates that the activity of MMPs in the myocardium may be imaged by use of specific activity–dependent molecular probes. (Circulation. 2005;111:1800-1805.)

Key Words: matrix metalloproteinases ■ myocardial infarction ■ imaging ■ gelatinase ■ remodeling

Myocardial infarction (MI) can lead to complex structural alterations in the heart, including dilation, contractile dysfunction, and congestive heart failure.1–4 Matrix metalloproteinases (MMPs) play important roles in the structural and functional sequelae of cardiac ischemia and infarction. In the heart, as in other tissues, MMPs participate in degradation of the extracellular matrix (ECM). In addition, MMPs have additional effects independent of ECM breakdown, including degradation of troponin I5 and modulation of platelet aggregation6,7 and vascular tone.8,9

The expression and activities of MMPs increase in the heart after MI.10–13 Specifically, the gelatinases MMP2 and MMP9 and the tissue inhibitors of matrix metalloproteinases (TIMPs) have been implicated in the cardiac response to ischemia and infarction. Gene knockout of MMP214 or MMP915 decreases left ventricular remodeling and limits ventricular dilation after MI. Loss of TIMP4 leads to enhanced MMP activity in the heart after ischemia,16 and gene knockout of TIMP-1 exacerbates ventricular remodeling.17 Pharmacological blockade of MMP activity leads to decreased ventricular remodeling.18–21

Noninvasive methodology to optically image MMP activity would be of use to follow the temporal and spatial patterns of MMP activity after MI and in assessing treatment approaches. We hypothesized that we could monitor the myocardial remodeling process by imaging MMP activity. We used a novel, long-circulating, quenched near-infrared fluorescent (NIRF) probe that is activated by proteolytic cleavage22,23 by MMP2 and MMP9. Our studies indicate that specific molecular probes can be used to image MMP activity during left ventricular remodeling. This approach complements existing methods for after MMP levels and activity, such as zymography, immunohistochemistry, Western blotting, and scintigraphy.24

Methods

MI Model

All procedures were approved by and performed in accordance with the Institutional Animal Care and Use Committee of the Massachusetts General Hospital. Male C57BL6J mice, ranging in age from 8 to 12 weeks and weighing 25 to 30 g, underwent left anterior descending coronary artery (LAD) ligation to induce MI.25 Animals were anesthetized intraperitoneally with ketamine 0.065 mg/g body wt, acepromazine 0.001 mg/g, and xylazine 0.013 mg/g. Animals were intubated and ventilated with a rodent respirator. After thoracotomy, the LAD was ligated with a 7-0 silk suture at a location 3 to 4 mm from the tip of the left atrium. Successful ligation of the LAD was verified by visual inspection of the left ventricular apex. The chest was closed with continuous 6-0 silk suture, and the skin was
closed with 4-0 silk sutures. All mice were given water and food ad
libitum.

**MMP-Sensitive NIRF Probe**

The NIRF probe used in this study was synthesized by use of a previously published method,\textsuperscript{26} except that a peptide sequence recognized by MMP2 and MMP9 was used. This sequence, SGKG-PRQITA, was identified by screening a phage library of random sequences using MMP9 and is cleaved between the Gln and Ile residues. It has a $k_{cat}/K_m$ of 188 000 mol/L$^{-1}$·s$^{-1}$, which is 12- to 14-fold higher for MMP9 than for MMP-13 and MMP-7.\textsuperscript{27} This sequence was used with the linking residues GGPRQITAGK(Fitc)C to attach the fluorochrome Cy5.5 to a pegylated poly-L-lysine backbone.

Activation of the probe (0.2 μmol/L) was studied by incubating with 1 μg recombinant MMP9 or MMP2 (Oncogene) in 50 mmol/L Tris HCl, pH 7.5, 10 mmol/L CaCl$_2$, 100 mmol/L NaCl, and 0.005% Brij-35 at 37°C. The fluorescence signal changes were monitored at the specific excitation (675 nm) and emission (694 nm) wavelengths of Cy5.5 by use of a fluorescence plate reader (SPECTRAmax Gemini, Molecular Devices).

**NIRF Imaging**

Four nmol NIRF probe was injected into the tail vein of mice 0 days, 2 days, 1 week, 2 weeks, 3 weeks, and 4 weeks after MI. This dose was chosen on the basis of previous experience with other enzyme-activatable probes in mice. The hearts were excised 48 hours later. NIRF imaging (2-minute acquisition) was performed with a 12-bit monochrome CCD camera (Kodak) equipped with an f/1.2 12.5- to 75-mm zoom lens and an emission long-pass filter at 700 nm (Omega Optical). The NIRF signal was determined as mean signal intensity (SI) from either the infarct region or remote myocardium. Target-to-background ratios (TBR) were calculated as follows: $TBR = SI_{(infarct\ area)}/SI_{(remote\ area)}$.\textsuperscript{28} All results are presented as mean±SEM. Statistical analysis was conducted by use of ANOVA, with values of $P<0.05$ being considered significant.

**Left Ventricular Tissue Extracts**

Hearts were excised and washed in cold PBS. The infarct region and remote myocardium were separated under a dissecting microscope. Tissues were snap-frozen in liquid nitrogen and homogenized in 0.5 to 1.0 mL of ice-cold lysis buffer. The Western blot lysis buffer contained 50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L PMSF, 5 ng/mL aprotinin, 5 ng/mL leupeptin, 1% Triton X-100, 1% sodium deoxycholate, and 1% SDS. The homogenates were centrifuged for 10 minutes at 4°C at 20 000 g, and the supernatant was transferred to clean tubes. Protein concentration was determined by use of a protein assay (Bio-Rad) using BSA as standard. The extracted samples were stored at −80°C until use.

**Zymography**

Protein samples containing 25 μg protein were adjusted to 40 μL, mixed with an equal volume of 2× loading buffer (126 mmol/L Tris-HCl, 20% glycerol, 4% SDS, 0.05% bromophenol blue, pH 6.8), and incubated for 10 minutes at room temperature before loading to 10% gelatin-ready zymogram gel (Bio-Rad, 161-1167). Then, 10 μL. Precision protein standards (Bio Rad, 161-0372) were used as molecular weight standards, and 0.5 ng recombinant human MMP9 protein (Oncogene, PF024) and 0.5 ng purified MMP2 (Chemicon, cc071) were loaded as positive controls. Gels were run at 80 V for 2 hours. The gels were incubated in 2.5% Triton X-100 for renaturation for 30 minutes. They were then equilibrated in fresh...
developing buffer (50 mmol/L Tris-HCl, 0.2 mmol/L NaCl, 5 mmol/L CaCl₂, 0.02% Brij 35) and incubated at 37°C overnight. Gels were stained with 0.5% Coomassie Blue G (40% methanol, 10% acetic acid) for 2 hours and destained with methanol:acetic acid:water (5:1:4) for 30 minutes. Areas of protease activity appear as clear bands against a dark blue background where the protease has digested the gelatin substrate.

Quantitative Reverse Transcription–PCR
MMP9 and MMP2 mRNA levels were measured by quantitative real-time polymerase chain reaction (PCR) of cDNA. Total RNA was extracted from infarct regions by use of Trizol (Gibco-BRL) and reverse-transcribed to cDNA by use of Superscript II RT (Gibco-BRL) using oligo (dT) as primers. Real-time PCR was performed by use of an ABI Prism 7000 Sequence Detection system using SYBR Green. Primers were designed by use of Primer Express software. The primers for β-actin were TGGAAATCCTGTGGCATCGATGAAAC (forward) and TAAAACGCAGCTCAGTAACAGTCCG (reverse). The primers for MMP2 were GACATACATCTTTGAGACAAG (forward) and TCTGCGATGAGCTTAGGGAAA (reverse). The primers for MMP9 were CCTGGAACTCACACGACTTC (forward) and TGGAAACTCACACGCCAGAA (reverse). For each primer set, a standard curve was generated between the threshold cycle number and cDNA concentration. MMP2 and MMP9 mRNA levels were expressed as a ratio normalized to β-actin. Melting curves of the PCR products and agarose gel electrophoresis confirmed the specificity of the reaction.

Histology and Immunohistochemistry
Frozen sections (8 μm) were used for histology (hematoxylin and eosin staining) or immunohistochemistry for MMP9 and markers for macrophage and neutrophils. The following antibodies were used: (1) mouse anti-human MMP9 IgG (1:100, Chemicon, MAB 13415); (2) rat anti-mouse Ly-6G (Gr-1) IgG-FITC (1:50, PharMingen, 553126); (3) rat anti-mouse Mac3 IgG-FITC (1:50, PharMingen, 01784D); and (4) donkey anti-mouse IgG-Texas Red (1:50, Jackson, 715-075-150). The staining procedures have been described in detail elsewhere. Confocal imaging was performed by use of a confocal microscope (Leica TCS NT4D) with the use of a BP530/30 filter for FITC and an LP590 filter for Texas Red. When the FITC and Texas Red signals colocalize, a yellow signal is seen.

Flow Cytometric Analysis
Mice with infarcted hearts or control mice were injected with NIRF probe or vehicle control. Twenty-four hours after probe administration, animals were euthanized by CO₂ asphyxiation, and their hearts were immediately excised and washed in Hank's balanced salt solution (HBSS). Infarct and remote heart tissues were separated. All tissues were then minced finely and digested in collagenase (Worthington Biochemical, 2 mg/mL wt/vol in HBSS) at 37°C for 45 minutes. The resulting cell suspensions were filtered through 100-μm pore-size filters, pelleted, and resuspended in RPMI-1640 containing 5% FCS. Cells were then stained with anti-mouse CD45-FITC conjugated antibody (BD Pharmingen). Each population was analyzed on a BDFACSCalibur flow cytometer for the presence of CD45 and the NIRF probe. A minimum of 10 000 cells per sample were analyzed.

Results
NIRF Probe Detects MMP2 and MMP9 Activity
The NIRF probe used in this study was similar to those used previously for imaging other proteases. The probe contains multiple NIRF Cy5.5 dye molecules attached to the recognition peptide sequence, which is positioned on a pegylated poly-L-lysine backbone. In the intact probe, the Cy5.5 molecules are in close proximity, and fluorescence resonance energy transfer results in efficient quenching. In the presence of MMP2 or MMP9, the peptide sequence is cleaved, resulting in liberation of the Cy5.5 dye molecules from the probe.

We tested the activation of the NIRF probe by MMP2 and MMP9 (Figure 1). At the zero time point, there was only background fluorescence activity. Both MMP2 and MMP9 caused time-dependent increases in fluorescence, with roughly equivalent kinetics. Overall, either protease resulted in 200-fold signal amplification. Therefore, the NIRF probe is equally sensitive to MMP2 and MMP9.

Figure 3. Time course of NIFR signal after MI. NIRF signal intensity in infarct region (closed circles) and remote myocardium (open circles) is shown over time. Error bars show SEM.
To image MMP2 and MMP9 activity in the heart after MI, we performed LAD ligation in mice, resulting in an apical infarct. Figure 2 shows the appearance of the heart 2 days and 1 week later. The area of infarction is pale compared with surrounding tissue. At 1 week, the left ventricular apex showed thinning. The MMP2- and MMP9-sensitive probe was injected, and NIRF imaging was performed. In the control animal, infarcted area had a signal similar to that of normal tissue, with a TBR of 1.08±0.11. In animals receiving the probe, the TBR increased to 3.34±0.43 at 1 week after MI, and areas of infarction were intensely bright (Figure 2).

We imaged MMP2 and MMP9 activity in different cohorts of animals to establish a time course over a 4-week period. The NIRF signal in the infarct zone rises and peaks at 1 to 2 weeks after MI (Figure 3). It then decreases over time, but was still significantly elevated at 4 weeks compared with baseline values or values from remote myocardium. Quantification of the absolute NIRF signal, as well as the ratio of the signal between the infarct zone and a remote area, shows the same pattern.

**MMP9 and MMP2 Levels in the Myocardium After MI**

To correlate the NIRF imaging results with expression of MMP2 and MMP9, we performed zymography and quantitative reverse transcription–PCR analysis. Zymography showed that MMP9 activity is increased in the infarct zone at 2 and 4 days after MI, with levels dropping subsequently (Figure 4). Of interest, MMP9 activity is increased in the remote area as well at 4 days, although less than in the infarct zone. In the infarct region, MMP2 activity increases by 1 week and reaches a maximum at 2 and 3 weeks. MMP2 activity is present in the remote area at low but detectable levels. Multiple molecular species are seen for MMP9 and MMP2.

We used real-time PCR analysis of cDNA reverse-transcribed from cardiac mRNA to quantify MMP2 and MMP9 expression (Figure 5). In the infarct region, MMP9 mRNA levels were highest at 2 and 4 days after MI and decreased subsequently. MMP2 levels, in contrast, rose by 4 days, peaked at 1 to 2 weeks after MI, and were still significantly increased at 4 weeks. Taken together, zymography and real-time PCR results were in agreement that MMP9 levels were increased at 1 and 3 days after MI and that MMP2 levels increased at 1 to 4 weeks.

**MMP9 Is Leukocyte Derived in the Infarcted Myocardium**

Comparison of microscopic NIRF imaging with hematoxylin and eosin staining of an adjacent section shows that the...
fluorescence signal correlates with regions of leukocyte infiltration (Figure 6). Confocal microscopy with dual labeling for cell-specific markers and for MMP9 confirmed colocalization of MMP9 with a neutrophil marker at 1 day (Figure 7). To verify that MMP9 was leukocyte derived in the infarcted myocardium, we made use of the NIRF probe to perform flow cytometric analysis on cells isolated from the heart. We used NIRF to detect the gelatinase probe and immunoreactivity to the leukocyte common antigen CD45 to detect leukocytes. In the noninfarcted heart tissue, the majority of cells that display NIRF are CD45-negative. In the infarct region, however, there is an increase in the number of CD45-positive cells that display NIRF (Figure 8). These results confirm our macroscopic observations and point to infiltrating leukocytes as the main source of the increase in MMP9 activity after MI.

Discussion

The MMPs are a family of zinc-containing metalloproteinases that play important roles in ECM degradation. MMPs have been divided into subgroups on the basis of substrate specificity and domain structure. MMP2 and MMP9 are of particular interest because they break down collagen, elastin, and basement membrane components. They share a common domain structure that includes type II fibronectin-like domains, differentiating them from other MMPs. Both MMP2 and MMP9 also have effects that seem to be independent of ECM breakdown. MMP2 degrades troponin I, a mechanism important to its effects on the heart after ischemia. Other MMP targets affect vascular tone and platelet aggregation.

Several lines of evidence point to the importance of MMP2 and MMP9 in cardiac remodeling after MI, as well as in vascular remodeling after injury and during atherogenesis. Gene knockout of either MMP2 or MMP9 results in attenuation of LV dilation after MI. Similarly, gene knockout of the MMP inhibitor TIMP-1 worsens ventricular dilation and remodeling after MI. Furthermore, pharmacological blockade of MMP activity results in attenuation of ventricular dilation.

Our results indicate that MMP9 and MMP2 activity show different time courses after MI. MMP9 increases by days 2 to 4, whereas MMP2 activity increases between 1 and 2 weeks. Other studies show similar time courses for MMP2 and MMP9 after MI. Despite their shared substrate, MMP2 and MMP9 may play distinct roles after tissue injury, as shown in vascular remodeling. In terms of cellular source, early after MI, NIRF activity colocalizes with areas of leukocytic infiltration (Figure 6) and with neutrophils (Figure 7). Flow cytometry confirms that CD45-positive leukocytes account for the increase in NIRF cells in the heart after MI (Figure 8). These results indicate that infiltrating neutrophils are the likely cellular source of MMP9 activity early after MI.

Monitoring of MMP activity is complicated by overlapping substrate specificities of the MMPs and by the fact that there are multiple species, including pro-MMP zymogens, and multiple levels of regulation by TIMPs and other MMP inhibitors. In addition, a recent report demonstrates that MMPs can be activated by S-glutathiolation in the presence of peroxynitrite. Here, we show that an optically detectable probe that is activated by MMP2 and MMP9 can be used to image the activities of these proteases in the heart after MI. The use of such enzyme-activated probes would allow monitoring of the spatial and temporal patterns of MMP activity over time in the heart and may facilitate the study of cardiac remodeling. They also provide a high degree of molecular specificity, because their fluorescence is based on activation by enzyme activity. However, the NIRF probe described here, like gelatin zymography, does not distinguish between MMP2 and MMP9.

In summary, we have shown that enzyme-activated NIRF probes can be used to image MMP activity in the myocardium after MI, providing both spatial and temporal information on activity. These probes may be useful to complement other techniques used to study MMPs, including zymography and scintigraphy. Our results also indicate that these probes can be used to monitor enzyme activity on a microscopic level and can be coupled with flow cytometry. Similar NIRF probes have been coupled with optical tomography methods for in vivo imaging.

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References


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