Selective spatiotemporal induction of matrix metalloproteinase-2 and matrix metalloproteinase-9 transcription after myocardial infarction

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Submitted 20 December 2005; accepted in final form 5 June 2006

Mukherjee, Rupak, Joseph T. Mingoia, James A. Bruce, Jeffrey S. Austin, Robert E. Stroud, G. Patricia Escobar, David M. McClister, Jr., Claire M. Allen, Maria A. Alfonso-Jaume, M. Elizabeth Fini, David H. Lovett, and Francis G. Spinale. Selective spatiotemporal induction of matrix metalloproteinase-2 and matrix metalloproteinase-9 transcription after myocardial infarction. Am J Physiol Heart Circ Physiol 291: H2216–H2228, 2006. First published June 9, 2006; doi:10.1152/ajpheart.01343.2005.—Myocardial remodeling after myocardial infarction (MI) is associated with increased levels of the matrix metalloproteinases (MMPs). Levels of two MMP species, MMP-2 and MMP-9, are increased after MI, and transgenic deletion of these MMPs attenuates post-MI left ventricular (LV) remodeling. This study characterized the spatiotemporal patterns of gene promoter induction for MMP-2 and MMP-9 after MI. MI was induced in transgenic mice in which the MMP-2 or MMP-9 promoter sequence was fused to the β-galactosidase reporter, and reporter level was assayed up to 28 days after MI. Myocardial localization with respect to cellular sources of MMP-2 and MMP-9 promoter induction was examined. After MI, LV diameter increased by 70% (P < 0.05), consistent with LV remodeling. β-Galactosidase staining in MMP-2 reporter mice was increased by 1 day after MI and increased further to 64 ± 6% of LV epicardial area by 7 days after MI (P < 0.05). MMP-2 promoter activation occurred in fibroblasts and myofibroblasts in the MI region. In MMP-9 reporter mice, promoter induction was detected after 3 days and peaked at 7 days after MI (53 ± 6%, P < 0.05) and was colocalized with inflammatory cells at the peri-infarct region. Although MMP-2 promoter activation was similarly distributed in the MI and border regions, activation of the MMP-9 promoter was highest at the border between the MI and remote regions. These unique findings visually demonstrated that activation of the MMP-2 and MMP-9 gene promoters occurs in a distinct spatial relation with reference to the MI region and changes in a characteristic time-dependent manner after MI.

structure; remodeling; matrix metalloproteinases

REGIONAL AND GLOBAL CHANGES in left ventricular (LV) geometry and structure, collectively termed remodeling, are common sequelae after myocardial infarction (MI) (22, 29, 34). Significant changes within the extracellular matrix (ECM) after MI can facilitate adverse myocardial remodeling (8, 9, 33, 41). Structural changes within the MI region are initiated early (within hours), whereas later phases of myocardial remodeling progress in a time-dependent manner through changes in the composition and conformation of the ECM (34). The matrix metalloproteinases (MMPs) are endopeptidases that are present within the myocardium, and changes in MMP abundance are associated with ECM and LV remodeling after MI (9, 10, 26, 28, 34, 35, 42). In previous studies, transgenic animal models or pharmacological MMP inhibition (12, 15–17, 20, 21, 26, 30, 44) was used to demonstrate a causal role for MMPs in post-MI LV remodeling. Nevertheless, it remained unknown whether these MMP species are expressed in regions of the myocardium critical to post-MI remodeling. The goal of this study was to characterize the spatial and temporal patterns of expression for certain MMP species after MI.

A number of MMP species have been identified, and the potential function of specific MMPs in post-MI LV remodeling, as well as in LV hypertrophy, is an area of active investigation (8, 10–12, 17, 21, 22, 26, 28, 29, 34, 35). Two species of MMPs, MMP-2 and MMP-9, can degrade denatured collagens (gelatins) and basement membrane components (39). Previous studies have demonstrated that mRNA and protein levels of MMP-2 and MMP-9 are increased after MI (7, 9, 15, 28, 35). The synthesis of MMP-2 and MMP-9 is highly regulated at the transcriptional level. The gene promoter region of MMP-1, MMP-3, MMP-7, MMP-9, MMP-12, and MMP-13 includes a proximal activator protein-1 (AP-1) site, which mediates an enhanced transcriptional response to a wide variety of cytokine and cellular stimuli (38). The MMP-2 promoter is notable for the absence of this proximal AP-1 site but does include discrete enhancer elements more distally, which interact with AP-1 components, as well as the YB-1 and p53 transcription factors (2, 23). Thus the promoter elements of these very similar enzymes are quite distinct, suggesting that each enzyme may operate within discrete cellular or stimulatory contexts. The present study tested the hypothesis that distinctly different spatiotemporal patterns of MMP-2 and MMP-9 expression would occur after MI, reflecting discrete transcriptional regulatory responses of the MMP-2 and MMP-9 genes to injury. To test this hypothesis, murine MMP-2 and MMP-9 reporter constructs were utilized to examine expression of these MMP types after surgical induction of MI.

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MATERIALS AND METHODS

This study was designed to examine the temporal induction of MMP-2 and MMP-9 gene promoters after MI. Transgenic mice, in which appropriate β-galactosidase (β-Gal) reporter constructs for MMP-2 or MMP-9 were inserted into the genome, were used. All animals were treated and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press, 1996), and the protocol was approved by the Institutional Animal Care and Use Committee.

MMP-2 and MMP-9 reporter mice. MMP-2 reporter mice were generated using a 5-kb genomic fragment of the rat MMP-2 gene extending from –1686 (relative to the translational start site) to the middle of the second exon and a plasmid (pL2C) containing a polylinker 5’ to a simian virus (SV40) polyadenylation signal. The Escherichia coli β-Gal gene (lacZ) was then isolated by PCR and ligated between the MMP-2 promoter and the SV40 polyadenylation sequence to produce F8-fgal. Transgenic mice (CD-1 background strain) were generated using standard protocols and characterized by PCR screening of tail-clip DNA.

Mice with the MMP-9 reporter-lacZ construct in the CD-1 background strain were developed and described by Mohan et al. (24). As internal controls, transgene expression was ascertained by PCR screening of tail-clip DNA and β-Gal elaboration [5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal) reacted] at the cut edges of the excised tail tips.

MI induction and experimental design. To provide equivalent sex distribution, MI was surgically induced in male and female mice (108 ± 3 days of age, 35.5 ± 0.5 g body wt) in the MMP-2 and MMP-9 groups by ligation of the coronary artery, as described previously (11). This technique has been documented to cause an MI of 35 ± 4% of the LV (11). Five MMP-2 reporter mice and five MMP-9 reporter mice were included to serve as nonoperated, non-MI reference controls.

Terminal studies on the mice were performed at 1 h (acute) and 1, 3, 7, 14, and 28 days after MI. At the assigned time after MI, the mice were deeply anesthetized by inhalation of isoflurane and weighed. The thoracic cavity was opened, 0.2 ml of a 0.1 M CdCl2 solution was extracted, and washed stringently in ice-cold PBS before homogenization. Therefore, in the resultant gray-scale photographs, the region of β-Gal staining was projected as lighter areas on a darker background. The MI region was digitally planimetered for determination of the major axis of the MI region as well as the center of mass, which was then defined as the center of the MI region. A five-pixel-wide region of interest, extending from the center of the MI region to the remote myocardium along the major axis, was demarcated. With the center of the MI region defined as the origin, gray-scale intensities (0 = black, 255 = white) at 2.0 mm (border region) and 3.0 mm (remote region) were determined. Biochemistry. Before tissue processing for biochemical studies, the hearts that were frozen in OCT compound were thawed over ice, extracted, and fluorescence was measured (FLUOSTar Galaxy, BMG Labtechnologies). A calibration curve generated from a set of β-gal standards (1.6–100 ng/ml) was used to convert the fluorescence signal to nanograms per milliliter.

Gelatinase activity in the LV homogenates (20 μg total protein) was determined as a function of cleavage of a gelatinase substrate (ECM700 kit, Chemicon). Briefly, p-aminophenylmercuric acetate-reacted LV tissue homogenates and a biotinylated gelatinase substrate were added to wells of a 96-well plate. After 2 h of incubation, 100 μl of the sample/biotinylated gelatinase substrate were added to a biotin-binding plate and incubated for 30 min at 37°C. The wells were then washed, and a 1:3,000 dilution of streptavidin enzyme conjugate was added and incubated for 30 min at 37°C. After another wash cycle, the substrate solution was added, the plate was incubated at room temperature for 20 min, and the absorbance at 450 nm was recorded (Multiskan MCC, Fisher Scientific). Increasing concentrations (3–200 ng/ml) of an activated MMP-2/MMP-9 standard (catalog no. CC073, Chemicon) were included in the assay.

Levels of the endogenous tissue inhibitor of metalloproteinses (TIMP)-1 and TIMP-4 were determined by immunoblotting (17). Briefly, LV extracts (10 μg) were loaded onto 4–12% Bis-Tris gels, electrophoretically separated, and transferred to a nitrocellulose membrane. After they were blocked and washed, the membranes were incubated for 1 h with the primary antibody: Ab 8116 for TIMP-1 at 1:5,000 dilution and Ab 816 for TIMP-4 at 1:5,000 dilution (both from Chemicon). Recombinant TIMP-1 (catalog no. WBC022, R & D Systems) was used as a positive control. A calibration curve generated from a set of TIMP-1 standards (10–1,000 ng/ml) was used to convert the fluorescence signal to nanograms per milliliter.
H2218

MMP PROMOTER INDUCTION WITH MI

Systems) and TIMP-4 (catalog no. HTIMP4, Triple Point Biologics) standards were added to each respective immunoblot. 

Immunohistochemical staining. The fixed hearts previously stained for β-Gal were embedded in paraffin, and thin (4-μm) sections were obtained. The sections were placed on slides, deparaffinized, and stained with eosin or used for immunohistochemistry. The eosin-stained sections in which LV morphology was maintained were used to determine in situ LV geometrical indexes of endocardial diameter, endocardial perimeter, endocardial length of the MI segment, and LV wall thicknesses at the MI and remote regions by digital planimetry of photomicrographs (×1 objective, Zeiss Axioskop and SigmaScan version 4.0). Immunohistochemical studies on the paraffin-embedded sections were performed using primary antibodies directed against α-smooth muscle actin (catalog no. A-2547, Sigma; 1:1,000 dilution) or the AP-1 transcription factors c-fos, c-jun, FosB, Fra1, and JunB (all 1:100 dilution; Santa Cruz Biotechnologies). In addition, immunohistochemical staining for MMP-2 (Ab 809 at 1:100 dilution; Chemicon) was performed for hearts from the MMP-2 reporter mice and against MMP-9 (Ab 19047, 1:100 dilution; Chemicon) was performed for hearts from the MMP-9 reporter mice. Briefly, myocardial sections that contained the scar were blocked and then incubated for 60 min at room temperature with primary antibodies. After incubation with secondary antisera, MMP-2 and MMP-9 were detected by visualization of a 3',3'-diaminobenzidine-hydrogen peroxide substrate (Vector Laboratories). The sections were imaged on an inverted microscope (Axioskop-2, Zeiss), and the images were digitized (AxioCam MRc, Zeiss). For detection of colocalization of cell types with β-Gal staining, cryosections (5 μm) were obtained from the OTC compound-embedded samples and immunostained for β-Gal (Ab 9361, Abcam) concomitantly with antibodies for one of the following: macrophages (MAC-3, CL8943A, Cedarlane; 1:200 dilution), lymphocytes (CD4, CL012A, Cedarlane; 1:200 dilution), fibroblasts [discoïd domain receptor-2 (13), GEA4023–1, Genex; 1:250 dilution], and myofibroblasts (α-smooth muscle actin, Ab 5694, Abcam; 1:1,000 dilution). Secondary antibodies included Alexa 633-conjugated anti-chicken antibodies for β-Gal (catalog no. A21103, Molecular Probes) and Alexa 488-conjugated secondary antibodies for the other markers (catalog nos. A11006 and A11008, Molecular Probes). A laser confocal microscope (Leica SP2, Hollings Cancer Center Molecular Imaging Core Facility, Medical University of South Carolina) was used to capture low-power (×10) differential interference contrast images and high-power (×63) fluorescent images of the sections. Negative controls for the fixed- and frozen-section immunohistochemistry included substitution with nonimmune antisera. 

Data analysis. From the in situ measurements of LV morphometrics, relative MI size was computed as the ratio of the endocardial length of the MI segment to LV endocardial perimeter. A wall thickness ratio was computed as the ratio of the thickness at the MI region to the maximum thickness remote from the MI (18). Changes in heart mass indexes, β-Gal staining, and β-Gal activity were compared between the MMP-2 and MMP-9 reporter mice using two-way analysis of variance, where the two main blocks were reporter group and time after MI. Pairwise comparisons were performed by Bonferroni’s adjusted t-test. For analysis of β-Gal activity, gelatinase activity, and TIMP-1 and TIMP-4 levels, values obtained from the non-MI reference control samples were averaged and used to normalize values obtained in the MI groups as well as in the non-MI group. Therefore, changes in these biochemical indexes were expressed as percent difference from non-MI values and compared with normalized non-MI control values using a two-sided t-test. Statistical tests were performed using BMDP (version 7.0) or Stata (Stata Intercooled, version 8.0) software packages. Values are means ± SE. P < 0.05 was considered to be statistically significant. 

RESULTS

MI was induced in a combined total of 124 mice that elaborated the reporter construct for MMP-2 and MMP-9 gene promoters. A total of 13 [9 male and 4 female, P = 0.143 (by χ2 analysis)] mice died in the post-MI period: 6 died at 3 days after MI, 2 at 5 days after MI, 1 at 6 days after MI, 2 at 7 days after MI, and 1 each on 9 and 13 days after MI. The final sample size of mice at each time after MI is provided in Table 1. When indexed to body weight, heart mass was increased from acute values by 1 day after MI and peaked between 7 and 14 days after MI for the MMP-9 and MMP-2 reporter groups (Table 1). Echocardiographic determinations of LV geometry and function were determined in a subset of mice from the MMP-9 reporter group. Representative long-axis two-dimen-

Table 1. Time-dependent changes in heart mass indexes and intensity of positive β-Gal staining in (MMP-2 and MMP-9 reporter mice after MI) 

<table>
<thead>
<tr>
<th>Days After MI</th>
<th>0 (Acute)</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
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<tbody>
<tr>
<td><strong>Heart mass, mg</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MMP-2 reporter</td>
<td>162±14</td>
<td>176±14</td>
<td>210±9b</td>
<td>215±14b</td>
<td>235±14bc</td>
<td>229±10be</td>
</tr>
<tr>
<td>MMP-9 reporter</td>
<td>185±11</td>
<td>183±13</td>
<td>208±14</td>
<td>245±11bc</td>
<td>252±12bced</td>
<td>235±10bced</td>
</tr>
<tr>
<td><strong>Heart mass/body wt, mg/g</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>MMP-2 reporter</td>
<td>4.5±0.1</td>
<td>5.4±0.2b</td>
<td>5.9±0.4b</td>
<td>6.1±0.2b</td>
<td>6.4±0.3b</td>
<td>5.6±0.2b</td>
</tr>
<tr>
<td>MMP-9 reporter</td>
<td>5.1±0.2b</td>
<td>6.1±0.2a,b</td>
<td>7.0±0.3#*#</td>
<td>8.4±0.6abcd</td>
<td>7.1±0.5bce</td>
<td>7.4±0.4b,cde</td>
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<tr>
<td><strong>LV epicardial area, mm²</strong></td>
<td></td>
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<tr>
<td>MMP-2 reporter</td>
<td>32.7±2.3</td>
<td>37.8±1.6</td>
<td>45.4±2.8bc</td>
<td>44.8±2.8b</td>
<td>45.9±2.4bc</td>
<td>46.9±2.7bc</td>
</tr>
<tr>
<td>MMP-9 reporter</td>
<td>37.9±2.6</td>
<td>41.7±2.8</td>
<td>35.6±2.8bc</td>
<td>41.2±2.9</td>
<td>44.5±2.0bc</td>
<td>42.3±3.8bc</td>
</tr>
<tr>
<td><strong>Area of β-Gal staining, mm²</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2 reporter</td>
<td>0.3±0.3</td>
<td>4.7±2.2bc</td>
<td>16.1±2.9c</td>
<td>27.4±3.2bcde</td>
<td>28.0±3.6bcde</td>
<td>20.5±2.6bcde</td>
</tr>
<tr>
<td>MMP-9 reporter</td>
<td>0.4±0.3</td>
<td>0.2±0.2</td>
<td>7.1±2.9bc</td>
<td>20.5±2.1bcde</td>
<td>13.2±2.5bcde</td>
<td>11.1±1.6bcdef</td>
</tr>
<tr>
<td><strong>Intensity of β-Gal staining, ×10⁶ pixels · luminosity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-2 reporter</td>
<td>84±84</td>
<td>1.291±0.630</td>
<td>3.225±0.573bc</td>
<td>6.898±0.409bced</td>
<td>7.911±2.21bced</td>
<td>4.527±5.13bcedf</td>
</tr>
<tr>
<td>MMP-9 reporter</td>
<td>114±80</td>
<td>71±53</td>
<td>1.949±0.729bc</td>
<td>7.106±9.16bced</td>
<td>4.133±3.29bcde</td>
<td>3.138±0.486bcdf</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of mice. β-Gal, β-galactosidase; MMP, matrix metalloproteinase; MI, myocardial infarction; LV, left ventricular. *P < 0.05 vs. MMP-2 reporter. bP < 0.05 vs. acute. #P < 0.05 vs. 1 day. aP < 0.05 vs. 3 days. cP < 0.05 vs. 7 days. dP < 0.05 vs. 14 days.
sional-mode echocardiographic images from a mouse at baseline and 28 days after MI are provided in Fig. 1. In these mice, LV end-diastolic diameter increased from 4.2 ± 0.1 mm before MI to 5.8 ± 0.2 mm 28 days after MI (P < 0.05). Concomitantly, LV free wall thickness decreased from 0.77 ± 0.01 mm before MI to 0.56 ± 0.02 mm (P < 0.05) 28 days after MI. End-diastolic volume increased from 59 ± 2 μl before MI to 164 ± 17 μl (P < 0.05) 28 days after MI, and LV ejection fraction decreased from 53 ± 2% before MI to 13 ± 3% (P < 0.05) 28 days after MI.

LV geometry was measured from in situ sections. In the MMP-2 reporter mice, endocardial dimension at the base of the LV was increased from acute values of 3.0 ± 0.2 mm to 4.2 ± 0.4 mm 7 days after MI (P < 0.05) and remained increased (4.9 ± 1.0 mm) 28 days after MI (P < 0.05 vs. acute). Similarly, in the MMP-9 reporter mice, LV endocardial dimension increased from acute values of 3.2 ± 0.2 mm to 4.6 ± 0.4 mm 7 days after MI (P < 0.05) and remained increased (5.2 ± 0.5 mm) 28 days after MI (P < 0.05 vs. acute). However, there were no differences in any of the in situ morphometric measurements between the MMP-2 and MMP-9 reporter groups. Because the background strain (CD-1) of these mice was the same and there was no difference in the morphometric measurement between the two groups, these data were pooled, and the results are summarized in Table 2. The endocardial length of the MI segment was measured by determination of the extent of myocyte necrosis. In the sections obtained 1 h after MI (acute), myocyte necrosis was not readily observed, and MI size could not be computed. MI size was increased by 3 days after MI and increased further by 7 days after MI. LV wall thickness at the MI region was lower than acute values from 7 days after MI, whereas wall thickness diametrically across the MI region was increased from acute values by 7 days after MI. Consequently, the ratio of wall thickness at the MI to wall thickness at remote regions (18) decreased from acute values of 0.86 ± 0.06 to 0.69 ± 0.05 by 7 days after MI (P < 0.05) and further decreased to 0.39 ± 0.05 28 days after MI (P < 0.05 vs. acute and 7 days).

MMP-2 and MMP-9 promoter activation was assayed as the extent of elaboration of the β-Gal reporter. In the non-MI hearts from either group, β-Gal staining could not be visually detected on the LV. Representative photographs of β-Gal-stained hearts at each time after MI are shown in Fig. 2. β-Gal-Gal staining increased through 7 and 14 days after MI in the MMP-9 and MMP-2 reporter groups, respectively. Quantitative values for the area of positive β-Gal staining are provided in Table 1. β-Gal-staining area normalized to LV epicardial area in the MMP-2 reporter group was increased by 1 day after MI, increased further through 7 days after MI, and fell thereafter (Fig. 3, top). MMP-9 promoter activation increased from acute and 1-day post-MI values by 3 days and peaked 7 days after MI. β-Gal activity was biochemically determined to provide a sensitive assay for detection of post-MI MMP-2 and MMP-9 promoter activation. Compared with non-MI reference values, β-Gal activity in the MMP-2 reporter group increased by 1 day after MI, further increased by 7 days after MI, and remained elevated thereafter. In the MMP-9 reporter group, β-Gal activity was increased from reference control and acute values by 3 days and peaked 7 days after MI. At 7 days after MI, β-Gal staining was higher in the MI and border regions than in the remote region for the MMP-2 and MMP-9 reporter groups (Fig. 3, bottom). However, in the MMP-2 reporter group, there was no difference in β-Gal staining between the MI and border regions. In contrast, β-Gal staining in the post-MI hearts from the MMP-9 reporter group demonstrated higher staining intensity at the border region than in the MI region 7 days after MI (Fig. 3, bottom).

Table 2. Time-dependent changes in LV morphometrics pooled from MMP-2 and MMP-9 reporter mice after MI

<table>
<thead>
<tr>
<th>Days after MI</th>
<th>0 (Acute)</th>
<th>1</th>
<th>3</th>
<th>7</th>
<th>14</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV diameter at base, mm</td>
<td>3.0±0.2</td>
<td>3.2±0.4</td>
<td>3.7±0.4</td>
<td>4.4±0.2</td>
<td>4.7±0.4</td>
<td>5.1±0.4</td>
</tr>
<tr>
<td>LV endocardial perimeter, mm</td>
<td>19.5±1.2</td>
<td>22.8±0.9</td>
<td>29.1±1.4</td>
<td>32.8±2.1</td>
<td>33.9±2.1</td>
<td>38.4±3.7</td>
</tr>
<tr>
<td>MI size, %</td>
<td>20±4</td>
<td>26±2</td>
<td>33±2</td>
<td>36±3</td>
<td>37±3</td>
<td></td>
</tr>
<tr>
<td>Wall thickness, mm</td>
<td>0.70±0.09</td>
<td>0.67±0.08</td>
<td>0.64±0.05</td>
<td>0.59±0.06</td>
<td>0.52±0.06</td>
<td>0.48±0.04</td>
</tr>
<tr>
<td>Remote</td>
<td>0.81±0.07</td>
<td>0.82±0.09</td>
<td>0.92±0.10</td>
<td>1.10±0.06</td>
<td>1.32±0.08</td>
<td>1.62±0.18</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>9</td>
<td>15</td>
<td>23</td>
<td>16</td>
<td>15</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, number of mice. *P < 0.05 vs. acute. **P < 0.05 vs. 1 day. ***P < 0.05 vs. 3 days. ****P < 0.05 vs. 7 days. *****P < 0.05 vs. 14 days.
Fig. 2. Regions of positive β-galactosidase (β-Gal) staining in hearts from matrix metalloproteinase (MMP)-2 and MMP-9 gene promoter reporter mice 1 h (acute) and 1, 3, 7, 14, and 28 days after MI induction. In MMP-2 reporter mice, areas of β-Gal staining were evident by 1 day after MI and increased further 3 and 7 days after MI. In MMP-9 reporter mice, β-Gal staining was observed after 3 days and peaked at 7 days after MI. RV, right ventricle; LA, left atrium. Scale grid represents four 1 mm \times 1 mm squares.

Fig. 3. Top: area of positive β-Gal staining normalized to LV epicardial area. Sample sizes for MMP-2 and MMP-9 reporter mice at each post-MI time point are presented in Table 1. Bottom: spatial localization of MMP-2 and MMP-9 gene promoter activation with respect to the center of the MI region 7 days after MI. In MMP-2 and MMP-9 reporter groups, β-Gal staining was higher in MI and border regions than in respective MI regions. In MMP-2 reporter group, there was no difference in β-Gal staining between the border region and the MI region. β-Gal staining was higher at the border region than within the MI region for MMP-9 gene promoter reporter group. #P < 0.05 vs. acute (1 h after MI). +P < 0.05 vs. 1 day after MI. *P < 0.05 vs. 3 days after MI. ^P < 0.05 vs. 7 days after MI. *P < 0.05 vs. MMP-2 reporter. *P < 0.05 vs. remote region. *P < 0.05 vs. border region.
The gelatinase assay used provided a summation of all gelatinase activity, including that of MMP-2 and MMP-9. In the MMP-2 and MMP-9 reporter groups, low levels of gelatinase activity were detected in the non-MI reference control samples (5.8 ± 1.2 and 5.1 ± 0.8 ng/ml, respectively, \(P = 0.33\) between groups). Although gelatinase activity increased after MI in both groups, there was no difference between groups at any time after MI (lowest \(P = 0.19\) at 3 days after MI). Therefore, the results were pooled and are summarized in Fig. 4 (top). Gelatinase activity was increased from non-MI reference control values and acute post-MI values by 1 day after MI (17.9 ± 6.6 ng/ml, \(P < 0.05\)) and further increased 7 days after MI (44.1 ± 5.8 ng/ml, \(P < 0.05\)). At 28 days after MI, gelatinase activity (30.1 ± 4.2 ng/ml) remained higher than non-MI values (\(P < 0.05\)) but was lower than values recorded 7 days after MI (\(P < 0.05\)).

Robust immunoblotting signals (Fig. 4, bottom) for TIMP-1 and TIMP-4 were detected at all times after MI and in the non-MI reference control samples. In the non-MI samples or at any time after MI, there were no group-dependent differences in TIMP-1 or TIMP-4 levels. Accordingly, the data at each time after MI were pooled between the groups, and the results are summarized in Fig. 4 (bottom). TIMP-1 levels were increased compared with non-MI values 3 days after MI but were lower than non-MI and 3-day post-MI values 14 and 28 days after MI. TIMP-4 levels were consistently lower than non-MI or acute values at all times after MI.

Representative photomicrographs of eosin-stained sections 7 and 14 days after MI are provided in Fig. 5. Distribution of β-Gal in the MMP-2 reporter mice was detected within the MI region as well as viable myocardium. In the MMP-9 reporter mice, β-Gal staining was localized at the border between the MI and viable myocardial regions. For localization of β-Gal elaboration to specific myocardial cell types, as well as nuclear activation factors (Figs. 6–9), immunoblotting was performed on paraffin-embedded or frozen sections. The specificity of each of the primary antibodies was determined by 1) substitution with nonimmune antisera from the same species in which the primary antibody was raised and 2) immunohistochemical staining of known cell types in non-MI samples. For example, a strong immunohistochemical signal against α-smooth muscle actin (Fig. 6) was detected in smooth muscle cells around blood vessels in non-MI samples but not when the primary antibody was substituted with nonimmune antisera. In the post-MI sections from the MMP-2 reporter group, positive staining for α-smooth muscle actin was detected around some cells, presumably myofibroblasts, that colocalized with β-Gal staining (Fig. 5). A similar colocalization of β-Gal and α-smooth muscle actin staining was not readily evident in the post-MI sections from the MMP-9 reporter group. Immunohistological staining for MMP-2 protein in myocardial sections from the MMP-2 reporter mice and staining for MMP-9 protein in the MMP-9 reporter mice could be localized adjacent to areas of β-Gal staining 7 and 14 days after MI (Fig. 7). Colocalization of staining for β-Gal and markers for specific cell types was undertaken in double-immunolabeled sections 7 and 14 days after MI (Fig. 7). Because β-Gal staining peaked 7 days after MI for the MMP-2 and MMP-9 reporter groups, colocalization of β-Gal staining and AP-1 activation factors was examined 7 days after MI (Fig. 9). A minimum of 20 randomized, but separate, high-power fields (HPF) were qualitatively assayed for coincident staining of the cell marker or AP-1 activation factor with β-Gal. Coincident staining was graded as follows: absent (in <20% of HPF), possible (in 20–80% of HPF), and definite (in >80% of HPF). A summary of cell types and AP-1 activation factors that colocalized with β-Gal staining 7 days after MI is provided in Table 3. In the...
7. Areas of important findings of the present study are twofold. Using transgenic reporter constructs (2, 24). The unique and MMP-2 and MMP-9 gene promoters after MI was examined in mice. Moreover, MMP-2 or MMP-9 promoter activation was higher in the border region than in the MI region in the MMP-9 reporter group (Fig. 9). Areas of β-Gal staining colocalized with nuclei that stained positive for FosB, Fra1, and JunB in the MMP-2 reporter group and with nuclei that stained positive for c-jun, c-fos, and FosB in the MMP-9 reporter group (Fig. 9).

**DISCUSSION**

In the present study, the time-dependent activation of MMP-2 and MMP-9 gene promoters after MI was examined using transgenic reporter constructs (2, 24). The unique and important findings of the present study are twofold. 1) Post-MI LV remodeling was associated with distinct patterns of MMP-2 and MMP-9 promoter activation. Specifically, although induction of the MMP-2 and MMP-9 gene promoters peaked 7 days after MI, MMP-2 promoter induction remained higher than MMP-9 promoter induction from 7 days after MI. Moreover, there were distinct differences in the spatial activation of MMP-2 and MMP-9 gene promoters with respect to the localization in the post-MI myocardium. Specifically, distribution of the reporter protein was similar in the border region in the MMP-2 and MMP-9 reporter mouse, whereas MMP-9 promoter activation was higher in macrophages and lymphocytes but was also detected in fibroblasts. These findings provide important insight into the post-MI induction of the MMP-2 and MMP-9 gene promoters.

In the present study, induction of MMP-2 and MMP-9 promoters was assayed as a function of β-Gal elaboration. Therefore, interpretation of findings is premised on the synchronous transcription of lacZ and the MMP genes. There are at least two reasons for the parallel transcription of lacZ and endogenous MMP-2 and MMP-9. 1) The design of the targeting vectors did not disrupt the MMP-2 or MMP-9 promoter regions, in that only the open reading frames were replaced by the lacZ cassette (2, 24). 2) In vitro induction of MMP-9 caused parallel increases in β-Gal elaboration and endogenous MMP-9 levels (24). Cells derived from mice were capable of activating the rabbit MMP-9/β-Gal promoter and the endogenous MMP-9 promoter identically (24). Consistent with these findings, the post-MI localization of MMP-2 and MMP-9 proteins occurred at areas of β-Gal localization in the MMP-2 and MMP-9 reporter mice, respectively. Thus changes in β-Gal levels likely provided a reliable surrogate index of de novo MMP-2 or MMP-9 transcription after MI.

The gelatinases MMP-2 and MMP-9 have been uniformly demonstrated to contribute to post-MI remodeling, and changes in mRNA and protein levels of MMP-2 and MMP-9 occur after MI (7, 9, 28, 32, 35). Importantly, causal roles for MMP-2 and MMP-9 in post-MI LV remodeling have been established (12, 15, 16, 21). For example, Ducharme et al. (12) demonstrated that post-MI LV dilation was attenuated in MMP-9-deficient mice, and Hayashidani et al. (15) and Matsumura et al. (21) reported a reduction in post-MI mortality in MMP-2-deficient mice. However, MMP levels and activity change as a function of post-MI duration, and unique temporal profiles of protein and mRNA expression after MI have been identified for several MMP types (7, 9, 15, 28, 35). Consistent with the time-dependent changes in gelatinase activity observed in the present study, Cleutjens et al. (9) reported that total gelatinase zymographic levels were highest in rat hearts 7 days after MI. However, it must be recognized that a number of proteolytic systems within the myocardium possess gela-
tinolytic activity. These gelatinolytic systems include the gelatinases MMP-2 and MMP-9, other MMPs, and members of the "disintegrin and metalloproteinase" family (31, 39). To determine relative contributions of MMP-2 and MMP-9 in post-MI remodeling, Tao et al. (35), using gelatin zymography, reported that although MMP-9 zymographic levels were increased by 1 day and achieved a peak at 2 days, MMP-2 zymographic levels did not increase until >4 days after MI. In the present study, MMP-2 promoter activation preceded the detection of MMP-9 promoter activation. There are several reasons for the apparent disparity between the results of the present study and those reported by Tao et al. 1) Because sample processing required for zymography results in unfolding of the proform, as well as the active form, of the enzymes, the zymographic levels reported by Tao et al. likely reflected changes in total MMP-2 and MMP-9 protein levels, in contrast to "recruitable" gelatinase activity measured in the p-amino-phenylmercuric acetate-activated samples in the present study. The zymographic technique, which allows for the electrophoretic separation of MMPs on the basis of molecular weight, can differentiate between the heavier proform and the active form. Such a separation would not be readily appreciated in the gelatinase assay employed in the present study. 2) In the study by Tao et al., the early post-MI appearance of MMP-9 protein was coincident with the appearance of neutrophils (35) that release preformed MMP-9 (19). Therefore, it is possible that the early post-MI increase in MMP-9 zymographic levels was due to release from existent stores, and not to activation of the MMP-9 gene promoter. Moreover, the myocardial ECM can also serve as a store for latent MMPs (25, 37), which may be activated in the early post-MI period. Thus activation of these latent MMPs, which would not require immediate synthesis, may be a potential explanation for the apparent temporal lag between the early post-MI increase in zymographic MMP levels (35) and the MMP gene promoter activation (present study). 3) MMP-2 is constitutively expressed in myocytes and myocardial fibroblasts (2, 40), and an early post-MI upregulation of MMP-2 transcription from these myocardial cell types cannot be ruled out. 4) As documented by Peterson et al. (28), a temporal discordance between mRNA expression and protein formation may have occurred after MI. These differences notwithstanding, the present study builds on the existing knowledge of post-MI MMP regulation in two important ways. 1) This study demonstrated that at least one cause of changes in MMP levels is activation of MMP gene promoters. 2) The findings of this study demonstrate distinct spatial differences with respect to induction of the MMP-2 and MMP-9 gene promoters after MI.

In general, activation of the promoter region for a particular gene should serve as a surrogate marker of mRNA transcription of that gene. In the present study, activation of the MMP-9 gene promoter, assayed as a function of the elaboration of the β-Gal marker protein, was detected 3 days after MI and peaked 7 days after MI. In contrast, Chen et al. (7) reported that MMP-9 mRNA levels peaked 2 days after MI. However, there may be several reasons for the discordant transcription/translation of a reporter gene construct and mRNA expression for the protein of interest. 1) The two gene products, the promoter reporter construct and the native gene, are distinctly different, and the end-point measurements for transcription of these two gene products were different. Specifically, Chen et al. determined the message signal as a readout for MMP-9 transcription, whereas levels of a surrogate marker protein were measured in the present study. 2) It must be recognized that the surrogate protein marker must be transcribed and translated. Therefore, posttranscriptional/posttranslational regulation of the reporter gene product could potentially introduce latency, i.e., a time delay, with respect to detection of MMP-9 promoter activation. 3) The MMP-9 reporter construct used in the present study, albeit functional, was truncated at ~522 bases from the start of the transcription site (24). Therefore, it is possible that transcription factors that bind at more distal sites may be responsible for the rapid transcription of endogenous MMP-9 mRNA. In light of the differences between the findings of the present study and those reported by Chen et al., a
study that examines the relation between MMP-9 mRNA expression and elaboration of the β-Gal marker after MI is warranted.

Because MMP transcription generally occurs “on demand” (24), transcriptional regulation represents an important step in MMP synthesis. The promoter regions of many of the MMP genes contain binding sites for a number of transcription factors, including AP-1, Sp-1, and NF-κB (2, 24). Activation of these transcription factors likely initiates the synthesis of a number of proteins, including the MMPs. Yoshiyama et al. (45) reported that, in a rat MI model, activities of AP-1, Sp-1, and NF-κB remained similar to control values 1 day after MI, progressively increased through 5 days after MI, and remained elevated through 14 days after MI. In the present study, MMP-2 and MMP-9 promoter activation followed a time course similar to that described for the transcription factors by Yoshiyama et al. Therefore, it is likely that transcription factor induction and post-MI activation of the gene promoters for these MMPs were temporally coincident. However, this issue remains speculative, and a study characterizing the role of transcription factor activation in post-MI MMP-2 or MMP-9 promoter activation would be appropriate.

Despite similar substrate specificities, MMP-2 and MMP-9 are separate gene products, and synthesis of these similar enzymes is likely dictated by a differential activation of transcription factors. Specifically, the structure of the MMP-2 promoter differs from that of MMP-9, in that it lacks a proximal AP-1 binding site, previously shown to interact with the AP-1 components c-fos and c-jun (2). Consequently, until recently, MMP-2 was considered to be an AP-1-unresponsive gene. However, a recent in vitro study demonstrated that MMP-2 transcription was inducible by neurohormones and cytokines and that this response was magnified by hypoxia (2). Promoter mapping studies localized the hypoxia response to a distal AP-1 site that binds FosB/JunB heterodimers (2). In the present study, post-MI MMP-2 promoter activation colocalized with the AP-1 factors FosB, Fra1, and JunB, whereas MMP-9 promoter activation colocalized with c-fos, c-jun, and FosB. Thus the temporal differences between MMP-2 and MMP-9 promoter activation in the early post-MI period were likely due to differential activation of specific AP-1 factors and stimulation of cell-specific signaling cascades. Future studies using transgenic AP-1 binding site deletion constructs are required to elucidate potential differential effects of AP-1 activation of the MMP-2 and MMP-9 promoters.

In the present study, localization of MMP-9 promoter activation, compared with that of the MMP-2 promoter, occurred at the border region of the MI. It must be recognized that the cellular sources for MMP-2 and MMP-9 are different. For example, MMP-2 has been reported to be expressed in cardiac fibroblasts, myofibroblasts, and myocytes, and MMP-9 is mainly expressed in inflammatory cells, including macrophages and leukocytes (12, 20, 35). The present study builds on the findings of these previous reports by demonstrating post-MI activation of the MMP-2 or MMP-9 gene promoters in the same cell types previously shown to express MMP-2 or MMP-9 proteins. Because fibroblasts and myofibroblasts are present within the MI region at later phases of post-MI remodeling (8, 10, 34), the persistence of MMP-2 promoter activation may have been due to the continual activation by fibroblasts and myofibroblasts within the MI region and myocytes in the viable remote myocardium. The early post-MI expression of MMP-9 is considered to play a crucial role in facilitating cellular migration to the MI region (10). Specifically, Heymans et al. (16) reported a reduction in the number of infiltrating leukocytes 7 days after MI in MMP-9-deficient mice. The
relative reduction in MMP-9 promoter activation during the later post-MI periods likely reflects the resolution of the inflammatory response within the myocardium (35). Thus the distinct spatiotemporal patterns for MMP-2 and MMP-9 promoter activation after MI were likely due to dynamic changes with respect to the cellular composition of the MI and peri-infarct regions.

The endogenous TIMPs are important regulators of MMP-mediated tissue remodeling (10, 11, 17, 28, 39). Of the four TIMP types identified, TIMP-1 remains the best characterized, whereas the distribution of TIMP-4 has been demonstrated to occur at higher concentration in the myocardium than in other tissues (14). Therefore, post-MI alterations in TIMP-1 and TIMP-4 levels were examined in the present study. Changes in the levels of TIMP-1 and TIMP-4 have been reported within the post-MI myocardium (26, 28, 42). Moreover, this laboratory has reported exacerbated post-MI LV dilation in TIMP-1-deficient mice (17), suggesting a key role for the TIMPs in attenuating post-MI remodeling. In the present study, an early increase in TIMP-1 levels followed by a reduction to below non-MI values was observed in the MMP-2 and MMP-9 reporter groups. TIMP-4 levels were consistently lower than non-MI or acute values at each subsequent time after MI. The significance of these findings is twofold. 1) The similar time-dependent changes in TIMP-1 and TIMP-4 levels in the MMP-2 and MMP-9 reporter groups suggest that the genomic insertion of the reporter constructs did not idiosyncratically alter the post-MI remodeling phenotype. 2) The concomitant increase in gelatinase activity at the later post-MI time points suggests that a stoichiometric imbalance between MMPs and TIMPs, due to the persistent activation of the MMP-2 and MMP-9 promoters and a loss of TIMP-mediated inhibition of the MMPs, contributed to the progression of post-MI LV remodeling. Nevertheless, TIMP levels were determined from LV homogenates, and a spatial pattern for the distribution of these TIMP types or cellular sources could not be determined. Future studies are needed to examine colocalization of TIMP-1, TIMP-4, and the other TIMPs with MMP-2 or MMP-9 promoter activation.

Limitations and conclusions. Several limitations of the present study must be recognized. 1) The transgenic reporter mouse lines were useful in detecting the spatiotemporal induction of MMP-2 or MMP-9 transcription vs. MMP-2 or MMP-9 functionality. In addition, despite the existence of
an associative relation between AP-1 activation and changes in MMP-2 and MMP-9 levels in humans (1), there may be species-dependent differences in MMP transcriptional regulation between humans and mice. 2) In the present study, post-MI changes in total gelatinase activity were determined. In light of the findings of this study, a future study that differentiates between the temporal changes in MMP-2 and MMP-9 activity and relates these changes to post-MI MMP-2 or MMP-9 gene promoter activation would be appropriate. 3) Male and female mice were included in the present study. Although more male than female mice died after MI, the sex-based difference in post-MI mortality did not attain statistical significance. Nevertheless, sex differences with respect to post-MI remodeling and rupture rates are becoming increasingly apparent (3–6, 43). Cavasin et al. (4–6) reported a greater degree of post-MI LV dilation in male mice. In addition, Bridgman et al. (3) recently demonstrated that sex-based differences in post-MI LV remodeling were associated with higher post-MI levels of atrial natriuretic peptide in the female mice. Because sample sizes at each time after MI are relatively small in the present study, subset analyses to determine sex-based differences with respect to post-MI activation of the MMP-2 or MMP-9 gene promoters would likely be underpowered and were not performed. 4) Colocalization of MMP-2 and MMP-9 promoter activation was examined in only a specific subset of cell types and at specific times after MI. Because cellular sources for MMP-2 and MMP-9 may differ during the course of post-MI LV remodeling, a study in which MMP-2 and MMP-9 promoter activation is examined in a more complete portfolio of cell types during the early and late phases of post-MI remodeling is warranted. Nevertheless, this study provided unique visual evidence for post-MI induction of the MMP-2 and MMP-9 gene promoters in a region- and time-specific manner. Specifically, MMP-2 promoter activation increased through 7 days after MI and remained elevated through 28 days after MI. Moreover, MMP-2 promoter activation was not limited to the MI region but was also identified in the remote myocardium. In contrast, MMP-9 promoter activation peaked 7 days after MI and was detected most prominently at the border between the MI and the remote regions. Taken together, these unique findings demonstrate that activation of the MMP-2 and MMP-9 gene promoters occurs in distinct spatial relation with reference to the MI region and follows characteristic time-dependent patterns after MI.

ACKNOWLEDGMENTS

The authors acknowledge the assistance of Sarah S. Camens, Brooke S. Kaplan, Amy H. Leonardi, Julie E. McLean, I. Matthew Mains, and Stuart M. Saunders.

GRANTS

This study was supported by National Institutes of Health Grants HL-66029 (R. Mukherjee), HL-45024, HL-97012, and PO1-HL-48788 (F. G. Spinale), EY-12651 and EY-14801 (M. E. Fini), and PO1-HL-68738 and DK-29776 (D. H. Lovett). M. E. Fini also received grant funding from the Walter G. Ross Chair Foundation.
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