Selective Matrix Metalloproteinase Inhibition Reduces Left Ventricular Remodeling but Does Not Inhibit Angiogenesis After Myocardial Infarction

Merry L. Lindsey, PhD; Joseph Gannon; Masanori Aikawa, MD, PhD; Frederick J. Schoen, MD, PhD; Elena Rabkin, MD, PhD; Lori Lopresti-Morrow, BS; Jamie Crawford; Shawn Black, PhD; Peter Libby, MD; Peter G. Mitchell, MD; Richard T. Lee, MD

Background—Broad inhibition of matrix metalloproteinases (MMPs) attenuates left ventricular remodeling after myocardial infarction (MI). However, it is not clear if selective MMP inhibition strategies will be effective or if MMP inhibition will impair angiogenesis after MI.

Methods and Results—We used a selective MMP inhibitor (MMPi) that does not inhibit MMP-1 in rabbits, which, like humans but unlike rodents, express MMP-1 as a major collagenase. On day 1 after MI, rabbits were randomized to receive either inhibitor (n=10) or vehicle (n=8). At 4 weeks after MI, there were no differences in infarct size or collagen fractional area. However, MMPi reduced ventricular dilation. The increase in end-diastolic dimension from day 1 to week 4 was 3.1±0.5 mm for vehicle versus 1.3±0.3 mm for MMPi (P<0.01). The increase in end-systolic dimension was 2.8±0.5 mm for vehicle and 1.3±0.4 mm for MMPi (P<0.05). Furthermore, MMPi reduced infarct wall thinning; the minimal infarct thickness was 0.8±0.1 mm for vehicle and 1.6±0.3 mm for MMPi (P<0.05). Interestingly, the MMPi group had increased numbers of vessels in the subendocardial layer of the infarct; the number of capillaries was increased in the subendocardial layer (46±4 vessels/field versus 17±3 vessels/field for vehicle; P<0.001), and the number of arterioles was also increased (4.0±0.8 vessels/field versus 2.0±0.4 vessels/field for vehicle; P<0.05).

Conclusions—MMP inhibition attenuates left ventricular remodeling even when the dominant collagenase MMP-1 is not inhibited; furthermore, this selective MMP inhibition appears to increase rather than decrease neovascularization in the subendocardium. (Circulation. 2002;105:753-758.)

Key Words: ventricles ■ myocardial infarction ■ metalloproteinases ■ remodeling

Left ventricular remodeling after myocardial infarction (MI) is a leading cause of congestive heart failure, and the degree of remodeling predicts morbidity and mortality.1,2 Because heart failure can occur despite the use of agents such as ACE inhibitors and β-adrenergic–blocking agents, new strategies to prevent progressive ventricular dilation are needed. One such strategy is matrix metalloproteinase (MMP) inhibition.3,4 Several lines of evidence implicate MMPs, members of a family of metalloenzymes that can degrade extracellular matrix components, as mediators of remodeling events in the myocardium. First, expression of specific MMPs increases in the myocardium after MI, in the presence or absence of reperfusion,5,6 and in nonischemic dilated cardiomyopathy.7 Second, broad pharmacological inhibition of MMPs blocks postinfarction ventricular dilation in mice and rats4,8 as well as pacing-induced dilation in pigs.3 Furthermore, deletion or overexpression of MMPs in genetically engineered mice regulates cardiac hypertrophy, function, and postinfarction remodeling.9–11 The mechanisms by which MMP inhibition regulates ventricular dilation are incompletely described. One theory is that disruption of collagen fibrils during the dilation process is necessary for cardiomyocytes to establish new matrix attachments.12 This theory is consistent with the ventricular dilation that occurs in mice with transgenic overexpression of human MMP-1, an important interstitial collagenase in humans that is not expressed postnatally in rodents.11 However, inhibition of collagen degradation may not be the sole mechanism of MMP inhibition in ventricular remodeling. For example, mice with targeted deletion of MMP-9, an enzyme that can digest a broad variety of substrates but not fibrillar collagen, have attenuated ventricular remodeling10 and decreased cardiac rupture after infarction.9

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From the Leducq Center for Cardiovascular Research (M.L.L., J.G., M.A., P.L., R.T.L.), Cardiovascular Division, Department of Medicine, and the Department of Pathology (F.J.S., E.R.), Brigham and Women’s Hospital, Harvard Medical School, Boston, Mass; and Pfizer Central Research (L.L.-M., J.C., S.B., P.G.M.), Groton, Conn.
Correspondence to Richard T. Lee, MD, Cardiovascular Division, Partners Research Facility, Room 279, 65 Landsdowne St, Cambridge, MA 02139.
E-mail rlee@rics.bwh.harvard.edu
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Thus, selective MMP inhibition that does not block fibrillar collagen degradation might reduce ventricular remodeling. This hypothesis is highly relevant because many small molecules can inhibit different MMPs, and these compounds will potentially have different pharmacological effects. For example, broad-spectrum MMP inhibition in humans can cause symptomatic tendonitis. In a previous study, a compound that did not inhibit MMP-1 activity reduced left ventricular (LV) dilation early after infarction in mice. However, because mice do not express MMP-1 postnatally, this selective MMP inhibition strategy is best tested in a species with a complement of myocardial MMPs more representative of human myocardium. This study used rabbits with experimental coronary artery occlusion to evaluate the effects of selective MMP inhibition on infarct expansion. Furthermore, because in some circumstances MMPs appear to promote endothelial migration and angiogenesis, we analyzed the impact of selective MMP inhibition on neovascularization.

Methods
All animal procedures were conducted in accordance with guidelines published in the Guide for the Care and Use of Laboratory Animals (DHEW publication NIH 85–23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, Md). All studies were approved by the Animal Research Committee of Harvard Medical School.

Pump Implantation and Coronary Artery Ligation
New Zealand White male rabbits (weight, 1.5 to 2 kg; Millbrook Breeding Labs, Amherst, Mass) were anesthetized with 16.7 mg/kg ketamine and 6.7 mg/kg xylazine (intramuscular), intubated, and mechanically ventilated with a respirator. Anesthesia was maintained with 1% isoflurane. An ear vein was cannulated with a 24-gauge catheter for saline and quinidine administration. A subcutaneous injection of buprenorphine (0.01 to 0.05 mg/kg) was given before surgery and every 12 hours for the next 48 hours and longer as needed. An Esox implantable infusion pump (V08; Access Technologies, Amherst, Mass) was implanted between the scapulae and tied down with 2-0 prolene suture. At presurgical examination, day 2, week 1, week 2, week 3, and week 4 after MI, blood was collected through the ear vein, followed by 10 mL of cardioplegic solution to arrest the heart in diastole. The heart was removed from the chest, and the right and left ventricles separated. The left ventricle was sectioned from apex to base, and both ventricles were incubated in 1% triphenyl tetrazolium in warm cardioplegic solution for 20 minutes. These slices were photographed, and infarct size was calculated through the use of image analysis software. Each image was analyzed 5 times and averaged. Wall thickness was determined by measurement of the width of the thinnest part of the infarct and the thickest part of the septum, with the use of custom-written image-analysis software. The thinning ratio is an index of the amount of wall thinning in the infarct normalized to the thickness of the septum and is calculated by dividing the infarct wall thickness with septal wall thickness. For both infarct size and wall thickness measurements, the observer was blinded to treatment group.

Western Analysis
Frozen tissue from infarcted regions was homogenized in lysis buffer (9.8 mol/L urea, 2% [wt/vol] NP40, and 100 mmol/L DTT) and centrifuged twice at 13,000g for 10 minutes. Equal amounts of protein were loaded onto 10% polyacrylamide gels and transferred onto PVDF membranes. The MMP, tissue inhibitor of metalloproteinase (TIMP), and angiotatin antibodies (Oncogene) were used at 1 μg/mL, and biots were visualized by means of chemiluminescence.

Histology
The midcavity section was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 5 μm. Sections were stained with picrosirius red and Masson’s trichrome stain to evaluate collagen deposition. Immunohistochemistry was performed with the use of Vector kits that use the ABC technique. The DAB chromogen was used to visualize positive staining, and the sections were counterstained with hematoxylin and/or eosin. An antibody that selectively recognizes the C-terminal neopeptite generated by collagenase (MMP 1, MMP 8, MMP 13, or MT1-MMP) cleavage of collagen I, II, or III (9A4 antibody) was used at a concentration of 14 μg/mL. Endothelial cells were detected by biotinylated Grifonia (Bandeiraea) Simplicifolia Lectin-I (GSL, Vector Laboratories; 100 μg/mL). For quantitative analysis of immunoreactive cleaved collagen, measurements were performed with the use of Image-Pro software (Media Cybernetics) to calculate the percentage of total area stained positive. GSL staining was quantified manually by counting the number of vessels per field from the infarcted region, scanned at ×25 magnification. For both, quantification was performed in a blinded manner by using a minimum of 5 sections for each animal. All animals were used for the analysis.

Statistical Analysis
Data are presented as mean±SEM. The echocardiographic data were analyzed by repeated-measures ANOVA; comparisons between

Echocardiographic Imaging
Echocardiographic acquisition and analysis were performed by an echocardiographer blinded to treatment group. Echocardiographic studies were performed under light anesthesia with spontaneous respiration with intramuscular ketamine and xylazine. The heart rate for a normal conscious rabbit is 200 to 300 bpm; all images were taken at a heart rate >150 bpm to minimize effects of anesthesia. Images were acquired with the use of a Sonos 5500 (Agilent Technologies) and a 12-MHz transducer and were analyzed at the time of acquisition. Measurements were taken from long-axis 2-dimensional views with end-diastolic and systolic short-axis measurements at the center of the ventricle. Echocardiographic studies were performed at baseline (18 to 24 hours after MI) and weekly for 4 weeks. For each measurement, 3 consecutive cardiac cycles were averaged. The average standard deviation for the baseline measurements (n=18) was 2.5% and 3.7% for end-diastolic and systolic measurements, respectively.

Tissue Collection and Infarct Characterization
A lethal dose of ketamine and xylazine was injected through the ear vein, followed by 10 mL of cardioplegic solution to arrest the heart in diastole. A left thoracotomy was performed, the pericardium was cut, and the large marginal branch of the circumflex coronary artery was catheterized to promote endothelial migration and angiogenesis. Furthermore, because in some circumstances MMPs appear to promote endothelial migration and angiogenesis, we analyzed the impact of selective MMP inhibition on neovascularization.

Drug Administration and Plasma Assay
CP-471,474 (Pfizer Inc, MW 3689) is a previously described MMP inhibitor. CP-471,474 was dissolved in 30% methylcellulose for a final concentration of 30 g/L. The pumps were refilled with freshly prepared drug or vehicle alone every 1 to 2 days. Flow rates were calculated on the basis of the remaining volume in the pumps. The final dosage of MMP inhibitor was ~120 mg/d.

At presurgical examination, day 2, week 1, week 2, week 3, and week 4 after MI, blood was collected through the ear vein in heparinized tubes. The tubes were centrifuged for 5 minutes at 12 000g, and plasma was stored at −80°C until use. Plasma concentrations were analyzed by liquid chromatographic assay with mass spectroscopy detection monitoring for an ion characteristic of CP-471,474. For this assay, the range of detection was <25 ng/mL to >5 μg/mL.
individual groups were made with the use of a Student’s t test. A 2-tailed value of $P<0.05$ was considered statistically significant.

**Results**

**Drug Levels**

Mean drug levels were assayed from plasma samples. The mean plasma concentration ranged from 100 ng/mL (270 nmol/L) to 350 ng/mL (945 nmol/L) for the duration of the study (Figure 1). The IC$_{50}$ values for CP-471,474 are 1170 nmol/L for MMP-1; 0.7 nmol/L for MMP-2; 16 nmol/L for MMP-3; 13 nmol/L for MMP-9; and 0.9 nmol/L for MMP-13. CP-471,474 is ≈90% plasma protein bound in most species. Therefore, although the plasma concentration at the 4-week time point approached the IC$_{50}$ for MMP-1, it is unlikely that MMP-1 was inhibited at the tissue level. These data indicate that rabbits allocated to the treatment group received therapeutic levels of inhibitor at all time points examined.

**Mortality and Infarct Sizes**

Of the 23 rabbits that survived MI surgery, 18 survived the entire 4-week protocol. The 5 deaths occurred between 5 and 20 days after MI (total mortality rate, 22%), and there was no difference in mortality rate between the MMP inhibitor (MMPi) and vehicle groups ($n=3$ and $n=2$, respectively). There was also no difference in infarct size. The vehicle group had an average infarct size of 26±2% (range, 17% to 31%), whereas the MMPi group had an average infarct size of 30±3% (range, 16% to 44%; $P=0.27$).

**Echocardiographic Measurements**

Baseline measurements are listed in Table 1. Infarcted rabbits allocated to MMPi had significantly smaller increases in end-diastolic dimensions (EDD) and end-systolic dimensions (ESD) compared with vehicle (Figure 2). At 3 weeks, the increase in EDD from day 1 after MI was 2.1±0.4 mm for vehicle and 1.6±0.4 mm for MMPi; the increase in vehicle EDD was significantly higher than week-1 values ($P<0.01$). The change in vehicle ESD was 2.8±0.5 mm for vehicle and 1.3±0.4 mm for MMPi. The increase in vehicle ESD was significantly higher than week-1 values ($P<0.01$) and MMPi week-4 values ($P<0.01$). The change in ESD was 2.8±0.5 mm for vehicle and 1.3±0.4 mm for MMPi. The increase in vehicle ESD was significantly higher than week-1 values ($P<0.01$) and MMPi week-4 values ($P<0.05$). At 4 weeks, there was no correlation between individual differences in plasma MMPi levels and EDD ($r^2=0.03; P=0.68$) or ESD ($r^2=0.05; P=0.58$), indicating that rabbits with higher plasma MMPi levels did not have additional effects on LV dilation. Although these data demonstrate that LV dilation is reduced in rabbits treated with MMPi despite lack of MMP-1 inhibition, we cannot exclude the possibility that some degree of MMP-1 inhibition caused by higher MMPi plasma levels at week 4 affected remodeling.

**Collagen, Wall Thickness, and MMP Levels**

To determine if selective MMPi reduced collagen accumulation in the infarct area, a midcavity section was stained with Masson’s trichrome, and the percentage of area occupied by collagen was calculated. The entire section was scanned at low power and used for quantification. The vehicle group had 31±4% collagen fractional area, whereas MMPi had 26±4% collagen fractional area ($P=0.37$). There were also no differences in infarct total collagen by picrosirius red staining or collagen I, collagen III, MMP-1, MMP-3, MMP-8, and TIMP-1 levels by Western analyses (Table 2). MMP-7 and

![Figure 1](Image)

**Figure 1.** Therapeutic MMP inhibitor levels in plasma. Plasma levels were collected at day 2 (24 hours after drug infusion was initiated), week 1, week 2, week 3, and week 4 after MI. Dotted lines indicate IC$_{50}$ for MMP-3 (6 ng/mL) and MMP-1 (433 ng/mL). IC$_{50}$ for MMPs 2, 9, and 13 are all <6 ng/mL.

![Figure 2](Image)

**Figure 2.** Changes in EDD (ΔEDD) and ESD (ΔESD) from day 1 after MI in MMPi and vehicle groups. MMPi attenuated remodeling at 3 and 4 weeks after MI. *$P<0.05$ and **$P<0.01$, vehicle at 1 week vs vehicle at 3 weeks or 4 weeks; †$P<0.05$ and ††$P<0.01$, vehicle vs MMPi.

| Table 1. Baseline Echocardiographic Measurements of Infarcted Rabbits |
|------------------------|---------------------|
|                        | MMPi                | Vehicle              | $P$    |
| n                      | 10                  | 8                    |        |
| Body weight, kg         | 3.3±0.1             | 3.3±0.1              | 0.996  |
| Heart rate, bpm         | 256±11              | 240±9.54             | 0.29   |
| EDD, mm                 | 15.3±0.5            | 15.0±0.3             | 0.54   |
| ESD, mm                 | 11.3±0.3            | 11.4±0.4             | 0.78   |
| FS, %                   | 26.2±1.9            | 23.8±1.67            | 0.38   |

Data are expressed as mean±SEM. EDD indicates end-diastolic diameter; ESD, end-systolic diameter; and FS, fractional shortening. There was no significant difference between the groups at baseline.
MMP-13 levels were increased in the infarcted regions of the MMPi group.

Although MMPi did not change total collagen, it did reduce postinfarct wall thinning (Figure 3). The average wall thickness at the infarct was 0.8±0.1 mm for vehicle and 1.6±0.3 mm for MMPi (P<0.05). There was a trend toward more compensatory hypertrophy in the vehicle group (5.2±0.6 mm for vehicle; 4.2±0.3 mm for MMPi), although this did not reach statistical significance (P=0.056). The average thinning ratio was 0.17±0.03 for vehicle and 0.40±0.08 for MMPi (P<0.05).

MMP Inhibition Selectivity

To verify ongoing collagen cleavage after infarct despite selective MMPi by an independent measure, immunohistochemistry was performed with the use of the 9A4 antibody that recognizes the C-terminal neoepitope generated by collagenase (MMP 1, MMP 8, MMP 13, or MT1-MMP) cleavage of collagen I, II, or III (9A4 antibody).22–24 For each animal, the area stained was quantified by averaging 10 random high-power fields from the infarct area. There was no difference in the areas of cleaved collagen between vehicle and MMPi-treated groups by image analysis, indicating that fibrillar collagen cleavage was not inhibited by MMPi (Figure 4).

Neovascularization

Experimental evidence indicates that MMPs participate in angiogenesis, and therefore MMP inhibition may potentially impair beneficial neovascularization after MI. However, MMPs may also generate inhibitors of angiogenesis, so that MMP inhibition could potentially promote neovascularization.25–28 In infarcted rabbits treated with MMPi, there were significant differences in vessel numbers in the infarct region.

**TABLE 2. Western Analyses**

<table>
<thead>
<tr>
<th></th>
<th>MMPi (n)</th>
<th>Vehicle (n)</th>
<th>Change in MMPi Group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen I</td>
<td>11920±1529 (3)</td>
<td>13199±1185 (3)</td>
<td>↔</td>
<td>0.545</td>
</tr>
<tr>
<td>Collagen III</td>
<td>10774±900 (3)</td>
<td>11485±797 (3)</td>
<td>↔</td>
<td>0.586</td>
</tr>
<tr>
<td>Sirius red</td>
<td>23375±4775 (3)</td>
<td>21240±411 (3)</td>
<td>↔</td>
<td>0.679</td>
</tr>
<tr>
<td>MMP 1</td>
<td>3738±735 (6)</td>
<td>2928±1243 (5)</td>
<td>↔</td>
<td>0.573</td>
</tr>
<tr>
<td>MMP 3</td>
<td>13746±1376 (3)</td>
<td>9094±1429 (3)</td>
<td>↔</td>
<td>0.079</td>
</tr>
<tr>
<td>MMP 7</td>
<td>12876±1189 (3)</td>
<td>7082±594 (3)</td>
<td>↑</td>
<td>0.012</td>
</tr>
<tr>
<td>MMP 8</td>
<td>12062±1267 (3)</td>
<td>8606±616 (3)</td>
<td>↓</td>
<td>0.070</td>
</tr>
<tr>
<td>MMP 13</td>
<td>7736±320 (6)</td>
<td>5531±674 (6)</td>
<td>↑</td>
<td>0.012</td>
</tr>
<tr>
<td>TIMP 1</td>
<td>10557±1783 (3)</td>
<td>14671±819 (3)</td>
<td>↔</td>
<td>0.104</td>
</tr>
</tbody>
</table>

Densitometry (arbitrary units) are expressed as mean±SEM. The number of hearts used for each experiment is given in parentheses. ↔ indicates no change; and ↑, increased.

Figure 3. Selective MMP inhibition reduces infarct thinning. A, Representative hearts stained with hematoxylin and eosin from MMPi and vehicle groups. RV indicates right ventricle; and LV infarct, left ventricle infarct. As shown in B, there was significantly less wall thinning in the group treated with MMPi. C, Thinning ratio was significantly different between the 2 groups. *P<0.05.

Figure 4. Collagen cleavage is not impaired in MMPi group. Sections were immunostained with 9A4 Ab (brown stain), which recognizes collagen that has been cleaved and counterstained with hematoxylin and eosin. Top, Representative images taken at ×10 magnification (bar=400 μm); bottom, higher magnification (×40) of same field (bar=100 μm). Quantitative image analysis showed no difference in area of staining, as indicated by graph.
as demonstrated by staining with biotinylated GSL-I. Control animals had a distinct paucity of vascularization in the subendocardium, with the majority of vessels present in the midmyocardium. In contrast, MMPi had a more uniform distribution of vessels (Figure 5). One potential mediator of this effect is angiostatin, an inhibitor of angiogenesis that is generated by MMPs. To determine if this difference could be due to the generation of angiostatin by MMPs, angiostatin levels were measured in plasma samples by Western analysis. No differences were observed between the 2 groups (Figure 6). Angiostatin levels were also unchanged in myocardial samples (data not shown).

Discussion

The primary finding of this study is that selective MMP inhibition attenuated LV remodeling after MI. EDD and ESD progressively increased in rabbits receiving the vehicle, whereas MMPi reduced dilation. At 4 weeks after MI, MMPi did not increase total collagen levels in the infarct area but reduced wall thinning. This suggests that a noncollagenolytic mechanism is critical in regulating ventricular remodeling and highlights the potential importance of noncollagen MMP substrates. At 4 weeks, the chronic healing phase has commenced and is characterized by reactive fibrosis and hypertrophy in the remote myocardium.29–32 The decrease in infarct wall thickness and thinning ratio in the MMPi group suggest a protective effect in preserving wall thickness in the scar and preventing compensatory hypertrophy in the septum, thus preserving tissue homogeneity. This finding is similar to results shown by Heymans et al.,9 who demonstrated a reduction in ventricular aneurysms in MMP-9 null mice after MI.

A surprising finding of this study is that angiogenesis was not decreased by MMPi. The lack of vessels in the subendocardium in control animals is a hallmark of ischemic cell death and is related to the transmural gradient of collateral blood flow, which is preferentially shunted to preserve the subepicardial region.33 That the MMPi group had more vessels in the subendocardial layer raises the hypothesis that MMPs in vivo generate angiostatic factors that may prevent vessel formation in this area of high metabolic demand. This finding again suggests a potential importance for non–matrix MMP substrates. Although MMPs can directly stimulate angiogenesis, MMPs can also generate inhibitors of angiogenesis such as angiostatin.25,28,34,35 In these experiments, we could not demonstrate a change in angiostatin in either the plasma or myocardium of these animals. However, the compensatory increase in MMPs 7 and 13 suggests a potential role for these MMPs in regulating either neovascularization or remodeling.

In summary, selective MMP inhibition prevented progressive LV dilation in a rabbit model of ventricular remodeling. This reduction in dilation was not due to changes in collagen levels, suggesting that factors other than absolute collagen levels are responsible. One such effect may be increased angiogenesis.

Acknowledgments

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References


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