Matrix metalloproteinases and collagen ultrastructure in moderate myocardial ischemia and reperfusion in vivo

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The mechanism of matrix damage is believed to be the degradative actions of matrix metalloproteinases (MMPs), whose synthesis and activation may be stimulated by proinflammatory cytokines released in injured myocardial tissue, proteases produced by infiltrating neutrophils, and/or oxidant stress associated with reperfusion (5, 6, 8, 23, 29, 40, 42). Damage to the interstitial matrix may cause acute expansion of affected myocardium, manifest by increased regional myocardial dimensions in vivo and increased sarcomere length after perfusion fixation (4, 25, 26, 32). A causal relation between ischemia-induced matrix damage and postischemic myocardial expansion and contractile dysfunction has been postulated (2, 12, 24) and is supported by recent studies in mice demonstrating that treatment with an MMP inhibitor (18, 32) or targeted deletion of MMP genes (11) attenuates early ventricular enlargement and contractile dysfunction and protects against cardiac rupture after myocardial infarction (18).

To date, effects of moderate (low-flow) ischemia and reperfusion on myocardial MMPs and interstitial matrix ultrastructure have not been investigated. Moderate ischemia is a common and important clinical event, usually resulting from partial occlusion of a coronary artery and often causing significant postischemic con-
tractile dysfunction (stunning) even when infarction is minimal or absent. Our laboratory (24) has characterized a porcine model of moderate ischemia and reperfusion that does not result in irreversible injury by ultrastructural or histochemical criteria but nonetheless causes acute myocardial expansion and contractile dysfunction, raising the possibility that matrix damage is causally related. On the other hand, moderate ischemia is less likely to result in neutrophil infiltration, causes less metabolic derangement during flow reduction, and produces less oxidant stress during reperfusion (43) compared with severe ischemia. Therefore, moderate ischemia may be less likely to stimulate MMP synthesis and activation.

The goal of the current study was to determine whether moderate ischemia and reperfusion without infarction affect the content and activity of myocardial MMPs or alter myocardial collagen ultrastructure, and if so, whether such effects contribute to acute myocardial expansion and contractile dysfunction. We addressed these questions with a combination of biochemical, ultrastructural, and pharmacological techniques.

METHODS

Experimental preparation. Protocols were reviewed and approved by the institutional Animal Studies Committee and conformed to the guidelines of the American Physiological Society and the American Heart Association. A total of 23 female Yorkshire-Landrace pigs, weighing 29–35 kg, were studied. The experimental model has been described in previous communications from this laboratory (24, 35). With the pigs under α-chloralose anesthesia (100 mg/kg iv induction, 20 mg·kg⁻¹·h⁻¹ iv maintenance), a median sternotomy was performed. Fluid-filled catheters were placed in the aortic arch to measure arterial pressure and in the left atrium to measure transmural myocardial blood flow was made during the final 20 min of the 90-min ischemic period. The LAD occluder was then released, and the third set of measurements was performed during the final 20 min of the 90-min reperfusion period.

Analysis of myocardial MMPs and TIMPs (group 1). After completion of the in vivo protocol and euthanasia, full-thickness samples of myocardium were rapidly excised from the central portion of the anterior ischemic and posterior nonischemic LV free walls, frozen in liquid nitrogen, and stored at −70°C. The innermost (subendocardial) third of each tissue sample was used for analysis of MMPs and their TIMPs because this layer is most severely hypoperfused during ischemia and because this allowed correlation with subendocardial collagen ultrastructure (group 2) and regional dimensions and contractile function (groups 1 and 3).

Analysis of myocardial MMPs and TIMPs followed previously published methods (16, 49). Tissue samples weighing 100–200 mg were homogenized in 7.5 volumes of extraction buffer (0.25% Triton X-100 in 50 mM Tris·HCl, pH 7.5). The homogenate was centrifuged, the pellet was reextracted with 2.5 volumes of Triton buffer and centrifuged, and the supernatants were combined. The pellet obtained was reextracted as above with 1 M guanidine-HCl in Tris buffer. All steps were carried out at 4°C. Extracts were dialyzed against standard Tris buffer and stored in aliquots at −70°C.

Most tissues contain MMP inhibitory activity due to TIMPs and/or macroglobulins. These can be destroyed without affecting MMPs by reduction and alkylation with 2 mM dithiothreitol and 5 mM iodoacetamide (49). Tissue extracts were assayed for collagenase activity after reduction/alkylation using 3H-labeled acetylated type I rat skin collagen substrate and measuring the appearance of radioactivity in the soluble fraction (49). Activity was corrected for the amount detected in blanks set up with 1,10-phenanthroline, which chelates zinc and inactivates collagenase. To determine both active and total (active plus latent) collagenase activity, measurements were made before and after activation of latent (proenzyme) collagenase with p-aminophenylmercuric acetate (APMA).

Gelatin zymography was performed to determine tissue content of other MMPs. A modification of the procedure of Herron et al. (17) was used to detect picogram quantities of MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa) and nanogram quantities of other MMPs. SDS-PAGE was performed in 7.5% or 10% polyacrylamide containing 0.33 mg/ml gelatin. The gels were then rinsed twice in 0.25% Triton X-100 for 20 min at 21°C and incubated for 18 h at 37°C in assay buffer (16). Gels were stained with Coomassie blue R-250 and scanned in an imager. The images were analyzed using Gelbase/GelBlot Pro software (UVP, Upland, CA) to generate peaks for the lanes on the gel corresponding to latent and active forms of each MMP. To calculate myocardial enzyme content in units of nanograms enzyme per gram wet weight tissue, experimental peaks were calibrated from peaks obtained with MMP standards that were added to each zymogram gel and bracketed a linear range of assay.
TIMPs were analyzed using a kit (University Technologies International, Alberta, Canada) that incorporates gelatin substrate and conditioned cell medium containing MMPs into an acrylamide mixture. Tissue extracts were fractionated by SDS-PAGE using 12.5% acrylamide and 0.75 mg/ml gelatin solution. After being washed with 2.5% Triton X-100, gels were stained with Coomassie blue solution to reveal cleared and uncleared areas of the gelatin in the gel. Uncleared blue areas are revealed only if TIMPs are present. This method reveals quantities of TIMPs as small as 2 ng (16). TIMP levels were graded on a five-level semiquantitative (arbitrary) scale from 0 (undetectable) to 4 (strongly positive) using Gelbase/GelBlot software.

Assessment of collagen ultrastructure (group 2). Fibrillar collagen ultrastructure was examined using the cell maceration/scanning electron microscopy technique. At the conclusion of the in vivo protocol, the heart was perfusion-fixed in situ (24) using cold phosphate-buffered 4% paraformaldehyde (400–800 ml). Transmural tissue blocks were removed from the anterior (ischemic) and posterior (nonischemic) LV, adjacent to (but not including) the crystal insertion sites. The innermost (subendocardial) third of each block was stored in fixative at 4°C until preparation for scanning electron microscopy.

Assessment of fibrillar collagen ultrastructure by conventional scanning electron microscopy suffers from significant limitations: collagen architecture may be partially obscured by cellular elements, and the collagen weave may be torn and disrupted during the process of tissue fracture. To minimize these problems, we employed the cell maceration/scanning electron microscopy technique originally described by Ohtani et al. (27). This technique involves prolonged treatment of tissues with NaOH solution to remove cellular elements while leaving the collagen framework remarkably intact. In preliminary experiments, we found that this technique provided much more consistent and uniform visualization of myocardial fibrillar collagen ultrastructure than conventional scanning electron microscopy without cell maceration.

Fixed myocardiun was cut into 3-mm cubes that included an endocardial face for orientation. After immersion in 10% NaOH for 3 days at room temperature, the pieces were rinsed in distilled water for 1 or 2 days. They were then treated with 1% aqueous tannic acid for 2–3 h and rinsed in distilled water for several hours. They were postfixed in a 1% aqueous solution of osmium tetroxide for 1–2 h and dehydrated in graded alcohols according to standard methods. The tissue was then frozen in liquid nitrogen and cracked with a cooled razor blade; the resulting pieces were placed in acetone before being transferred to tetramethylsilane for 10 min. Subsequently, the tissue was air dried at room temperature. At least three dried specimens from each region of each heart were oriented and mounted on aluminum holders and sputter-coated with gold-palladium.

Each specimen was examined in a JEOL 840 scanning electron microscope. The endocardial aspect of each piece was identified, and the adjacent tissue searched for regions that were cut parallel to the long axis of the myofibers. Each such area was photographed at ×1,000 for an overview and at ×7,500 to demonstrate fine collagen architecture. Representative areas where the tissue had been cut in cross section were also photographed. Electron micrographs were analyzed by a cardiac pathologist (P. C. Ursell) blinded to the region (ischemic vs. nonischemic) from which the tissue was taken.

The cell maceration method was also used to determine total collagen content in myocardial specimens from ischemic and nonischemic regions. After fixation, subendocardial tissue samples (~300 mg) were oven-dried at 50°C for 72 h and weighed to obtain dry tissue weight. The specimens were then treated with NaOH, distilled water, and tannic acid as described above, redried, and reweighed to obtain dry collagen weight. Collagen content was expressed as percent dry weight.

Ultrastructure of cardiac myocytes and myocardial basal laminae was examined by transmission electron microscopy using subendocardial tissue from ischemic and nonischemic regions of seven additional hearts that had been subjected to the same in vivo protocol of ischemia and reperfusion. These hearts underwent in situ perfusion fixation with buffered 2.5% glutaraldehyde-4% paraformaldehyde and were prepared for transmission electron microscopy as described previously (24).

Treatment with intracoronary MMP inhibitor (group 3). Pigs in group 3 were treated with an intracoronary infusion of the MMP inhibitor GM-2487 (Glycomed, Alameda, CA). Pigs in group 1 served as the controls for these experiments. GM-2487 is a water-soluble peptide hydroxamic acid (FW 501) that is effective in vivo after parenteral administration (22). Its structure and formula correspond to compound 6w (as listed in Ref. 21). GM-2487 was dissolved in 0.9% NaCl to a concentration of 1.3 mM and infused at a rate of 0.75 ml/min. In group 1 pigs, the saline vehicle alone was infused at the same rate. Infusion of drug or vehicle was begun after baseline measurements. An additional set of hemodynamic and microsphere measurements was performed after 30 min of drug infusion but before the onset of ischemia to determine any direct effects of GM-2487. Treatment was continued throughout the ischemic and reperfusion periods. LAD flow rates averaged ~20 ml/min at baseline and 10 ml/min during ischemia. Therefore, the dose of GM-2487 resulted in local arterial blood concentrations of ~50 μM under nonischemic conditions and ~100 μM during ischemia. These blood concentrations are 103 to 105 times greater than the published K values of GM-2487 for MMP-9 (1.9 nM), MMP-2 (0.71 nM), MMP-3 (1.3 nM), and collagenase (0.39 nM).

Hemodynamic data collection and analysis. Hemodynamic data were collected and analyzed as previously described (24). Regional function of both anterior (ischemic) and posterior (nonischemic) zones was assessed from LV pressure versus segment-length loops recorded under steady-state conditions and during brief occlusion of the venae cavae. Five to six sets of data were averaged under each condition in each pig.

Regional diastolic function was assessed from LV end-diastolic pressure (EDP) versus segment length (EDL) relations. Using an iterative curve-fitting routine, EDP versus EDL data were fit to a monoexponential relation. This relation was used to calculate regional EDL at EDP ranging from 0 to 10 mmHg. EDL at near-zero EDP reflects minimally loaded regional myocardial dimension. Myocardial expansion was inferred from an increase in EDL at near-zero LV pressure (24).

Regional systolic function was assessed from LV pressure segment-length loops. Steady-state regional external work was determined from the area of loops recorded without occlusion of the venae cavae. Regional preload-recruitable stroke work (PRSW) relations were derived by plotting the area of each pressure segment-length loop (an index of regional external work) against its corresponding EDL during brief occlusion of the venae cavae. Slopes and intercepts of PRSW relations were determined by linear regression.
RESULTS

Regional myocardial blood flow (groups 1 and 3). As expected, partial coronary occlusion caused a nonuniform transmural distribution of myocardial blood flow with moderate subendocardial and milder subepicardial ischemia. Anterior LV subendocardial blood flow fell from 1.3 ± 0.2 ml·g⁻¹·min⁻¹ at baseline to 0.4 ± 0.1 ml·g⁻¹·min⁻¹ during ischemia, and subepicardial blood flow declined from 1.4 ± 0.2 ml·g⁻¹·min⁻¹ at baseline to 0.8 ± 0.2 ml·g⁻¹·min⁻¹ during ischemia. With reperfusion, transmural blood flow recovered to values similar to baseline. Myocardial blood flow did not differ between experimental groups.

Myocardial MMPs and TIMPs (group 1). Gelatin zymography demonstrated the presence of MMP-9 (gelatinase B) in both latent and active forms. As shown in Table 1, total (latent plus active) MMP-9 content was 70% higher in ischemic than in nonischemic regions. The increase in total MMP-9 content was due primarily to an increase in the active form of the enzyme in ischemic regions (48 ± 3 ng/g wet wt vs. 13 ± 2 ng/g wet wt, P < 0.001). In addition, zymography revealed a high molecular mass band at 220 kDa, probably corresponding to dimerized MMP-9 (20), that was more prominent in ischemic regions. Each heart demonstrated a strong band of gelatinolysis corresponding to dimerized MMP-9 (20), that was more prominent in ischemic regions. Each heart demonstrated a strong band of gelatinolysis corresponding to the latent form of MMP-2 (gelatinase A) and a faint band corresponding to the active form of MMP-2 (representing 5–11% of total MMP-2). Neither MMP-2 band differed discernibly between ischemic and nonischemic regions. No evidence of the presence of MMP-3 (stromelysin) or MMP-7 (matrilysin) was observed when heart extracts were analyzed using substrate transferrin zymograms.

Myocardial collagenase was determined after destruction of inhibitors by reduction and alkylation. No significant active form of collagenase was observed in extracts from either ischemic or nonischemic myocardium. Collagenase activity was only observed when reduced and alkylated extracts were treated with APMA. This indicates that collagenase was present almost entirely in latent form, even after ischemia and reperfusion. Nonetheless, latent collagenase content was significantly higher in extracts from ischemic regions (48 ng/g wet wt) compared with nonischemic regions (25 ng/g wet wt, P < 0.001).

Analysis of TIMPs by reverse zymography revealed only the presence of TIMP-2 and TIMP-3. Quantification of TIMPs from reverse zymograms is difficult due to other protein bands that are found around the TIMPs; therefore, TIMP-2 and TIMP-3 bands were graded visually on a semiquantitative scale (16). No differences between ischemic and nonischemic regions were observed. TIMP-1 levels were below the threshold for detection in both ischemic and nonischemic regions.

In summary, the data indicate that moderate myocardial ischemia and reperfusion cause prompt increases in active MMP-9 and latent collagenase without detectable changes in other MMPs or TIMPs.

Ultrastructure (group 2). Light microscopy of samples of myocardium from ischemic areas as well as nonischemic areas showed normal histology. Importantly, no neutrophilic infiltration was identified in any region of myocardium from any heart. Presumably, therefore, the MMPs in these tissues were not of neutrophil origin. Light microscopy demonstrated no evidence of hemorrhage or infarction in any region. Transmission electron micrographs revealed no features of irreversible injury or infarction such as contraction bands, sarcoplasmic edema, mitochondrial swelling, or disruption of cristae. Examination of the basal laminae of cardiac myocytes revealed no qualitative differences between ischemic and nonischemic regions. In all regions examined, the basal laminae appeared intact with normal, uniform thickness and electron density.

Under scanning electron microscopy, each piece of tissue was oriented by the presence of the smooth endocardial surface. Subject to the endocardial surface, a thin layer of epimysial collagen was distinguished by a dense network of thick collagen cables and sparse collagen fibrils. Appearing identical in ischemic and nonischemic regions, epimysial collagen was not the object of this study.

Deep to the epimysial layer, there were no remaining cellular elements (myocytes, fibroblasts, or endothelial cells), only an extensive collagen framework suggestive of the architecture of the myocardium. The orientation of digested muscle fibers in the long axis was inferred from the presence of collagen-bounded grooves (Fig. 1A). The orientation of digested muscle fibers in cross

<table>
<thead>
<tr>
<th>Table 1. Myocardial MMPs and TIMPs</th>
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<tr>
<td>Ischemic Zone (Anterior LV)</td>
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<tr>
<td>Collagenase</td>
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<tr>
<td><strong>Active</strong></td>
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<tr>
<td>Latent</td>
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<tr>
<td>Total</td>
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<tr>
<td>MMP-2</td>
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<tr>
<td>(gelatinase A)</td>
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<tr>
<td>Active</td>
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<tr>
<td>Latent</td>
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<tr>
<td>Total</td>
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<tr>
<td>MMP-9</td>
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<tr>
<td>(gelatinase B)</td>
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<tr>
<td>Active</td>
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<tr>
<td>Latent</td>
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<tr>
<td>Total</td>
</tr>
<tr>
<td>High molecular weight gelatinase (MMP-9 dimer)</td>
</tr>
<tr>
<td>TIMPs (scale 0–5)</td>
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<tr>
<td>TIMP-1</td>
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<td>TIMP-2</td>
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<td>TIMP-3</td>
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All values are means ± SE; n = 9. MMPs, matrix metalloproteinases. Units for collagenase and gelatinases are ng protein/g wet weight tissue. Units for high molecular weight gelatinase are arbitrary. Tissue inhibitors of metalloproteinases (TIMPs) are graded from reverse zymograms on a semiquantitative visual scale (0 = not detectable to 5+ = strongly positive. NS, not significant. ND, not detectable. LV, left ventricle.
section was inferred from a honeycomb-like arrangement of orifices of collagen tubes (Fig. 1B). The cross-sectional views provided by the scanning electron microscope included the first 5–10 μm of each tube.

The collagen grooves (or tubes) were formed predominantly by a fine network of individual collagen fibrils (weave), as well as scattered larger bundles of collagen fibrils. There was no qualitative difference in fibrillar collagen ultrastructure between ischemic (anterior LV) and nonischemic (posterior LV) regions of the same heart. In particular, there was no evidence of rupture or fraying of collagen fibrils in either region. Although the amount of collagen in each region could not be quantified from the photographs, there was no apparent difference in the density of the collagen fibrils in ischemic, compared with nonischemic, tissue. This was confirmed by direct measurement of myocardial collagen content: collagen constituted 3.9 ± 0.4% of dry tissue weight in ischemic regions and 3.7 ± 0.2% in nonischemic regions (P = NS).

Regional ventricular function and effects of treatment with MMP inhibitor GM-2487 (group 3). Table 2 shows hemodynamics and regional LV function in pigs treated with GM-2487 (group 3) and untreated control pigs (group 1). In both groups, regional systolic function was depressed during ischemia (external work ~35% of baseline) with only partial recovery after reperfusion. In most experiments, sonomicrometry demonstrated early systolic bulging of the anterior LV during ischemia and reperfusion.

Persistent expansion of the ischemic region was manifest by increases in minimally stressed end-diastolic myocardial dimensions, as reflected by increased EDL at EDP near 0 mmHg (Table 2). Values of EDL during ischemia and reperfusion were normalized to the preischemic baseline value; therefore, values greater than unity indicate expansion. EDL versus EDP relations did not differ between groups 1 and 3, indicating that myocardial expansion was not prevented or attenuated by treatment with GM-2487.

Fig. 1. Scanning electron micrographs from anterior (ischemic) and posterior (nonischemic) regions of the left ventricle of a representative heart after cell maceration. A: longitudinal sections; B: transverse sections. No qualitative differences in collagen architecture between ischemic and nonischemic regions are apparent.
Table 2. Hemodynamics and regional LV function

<table>
<thead>
<tr>
<th>Hemodynamics</th>
<th>Baseline (Vehicle)</th>
<th>Baseline (GM-2487)</th>
<th>Ischemia (Vehicle)</th>
<th>Ischemia (GM-2487)</th>
<th>Reperfusion (Vehicle)</th>
<th>Reperfusion (GM-2487)</th>
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</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>103 ± 3</td>
<td>104 ± 6</td>
<td>102 ± 4</td>
<td>105 ± 5</td>
<td>103 ± 4</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>Mean aortic pressure, mmHg</td>
<td>78 ± 4</td>
<td>78 ± 9</td>
<td>70 ± 2</td>
<td>73 ± 5</td>
<td>70 ± 6</td>
<td>72 ± 5</td>
</tr>
<tr>
<td>LV systolic pressure, mmHg</td>
<td>90 ± 5</td>
<td>91 ± 9</td>
<td>81 ± 4</td>
<td>78 ± 5</td>
<td>80 ± 6</td>
<td>84 ± 4</td>
</tr>
<tr>
<td>LV end-diastolic pressure, mmHg</td>
<td>5 ± 2</td>
<td>5 ± 2</td>
<td>9 ± 3*</td>
<td>10 ± 3*</td>
<td>9 ± 2*</td>
<td>8 ± 3*</td>
</tr>
<tr>
<td>Anterior LV subendocardial blood flow, ml·g⁻¹·min⁻¹</td>
<td>1.37 ± 0.18</td>
<td>1.24 ± 0.20</td>
<td>0.38 ± 0.08*</td>
<td>0.40 ± 0.07</td>
<td>0.95 ± 0.23</td>
<td>1.00 ± 0.21</td>
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Regional systolic function (fraction of baseline)
- Steady-state EDL: 1.05 ± 0.03 vs. 1.03 ± 0.02
- Steady-state external work: 0.35 ± 0.05* vs. 0.36 ± 0.04*
- PRSW slope: 0.50 ± 0.05* vs. 0.55 ± 0.04*
- PRSW intercept: 1.10 ± 0.04* vs. 1.12 ± 0.03*

EDL at given end-diastolic pressures (fraction of baseline)
- EDL at 0 mmHg: 1.14 ± 0.06* vs. 1.12 ± 0.07*
- EDL at 1 mmHg: 1.10 ± 0.06* vs. 1.08 ± 0.06*
- EDL at 2 mmHg: 1.08 ± 0.04* vs. 1.06 ± 0.06*
- EDL at 5 mmHg: 1.02 ± 0.03 vs. 1.03 ± 0.04
- EDL at 10 mmHg: 1.01 ± 0.04 vs. 1.00 ± 0.03

Values are means ± SE. Group 1, n = 9; group 3, n = 7. PRSW, preload recruitable stroke work relation; EDL, end-diastolic segment length. Regional systolic and diastolic function were measured in the anterior LV subendocardium. Indexes of regional systolic and diastolic function are normalized to preischemic (baseline) values in each pig to eliminate variability due to intercrystall distance. *P < 0.05 vs. baseline. Myocardial expansion is inferred from increases in EDL at low end-diastolic pressures.

Table 2 also shows that regional systolic function (steady-state external work, expressed as a fraction of the preischemic baseline value) declined during ischemia and remained depressed during reperfusion. Ischemic zone PRSW relations were characterized by reduced slope during ischemia and increased intercept during ischemia and reperfusion. Again, no differences were apparent between groups 1 and 3, indicating that treatment with GM-2487 did not attenuate the severity of ischemic or postischemic contractile dysfunction.

DISCUSSION

New findings of this study. The current study provides the first demonstration that myocardial MMP content and activity are altered by moderate ischemia and reperfusion, even when irreversible myocyte injury and inflammatory cell infiltration are absent. The content and activity of myocardial MMP-9 increased, and collagenase increased in latent form. However, no structural or functional consequences of the changes in MMPs could be discerned: cell maceration/scanning electron microscopy revealed no changes of collagen ultrastructure, and treatment with an MMP inhibitor did not prevent or attenuate early myocardial expansion or contractile dysfunction of ischemic-reperfused myocardium. Thus although previous data have suggested that the action of MMPs contributes to chronic ventricular expansion and remodeling after myocardial infarction, the present data indicate that this mechanism is not responsible for acute myocardial expansion and dysfunction in a model of stunning.

Changes in myocardial MMP content and activity with moderate ischemia and reperfusion. Several factors known to stimulate MMP synthesis may have operated to increase MMP-9 and collagenase content in this study. Even brief episodes of myocardial ischemia may increase levels of nuclear transcription and inflammatory cytokines (5, 6) that, in turn, stimulate expression of MMPs (29). It is also possible that excessive mechanical stretch of ischemic myocardium increased MMP expression. Passive stretch stimulates MMP expression by cardiac fibroblasts in vitro (41) and might produce similar effects in vivo when a hypercontractile, nearly passive region of ischemic myocardium is subjected to high systolic stress by contraction of surrounding nonischemic myocardium.

There are several potential mechanisms for the selective activation of MMP-9 in these experiments. A reduction in myocardial TIMP content may disinhibit MMPs. In particular, TIMP-1 exerts its greatest inhibitory activity against MMP-9; therefore, a reduction in TIMP-1 in ischemic myocardium (1) could lead to selective activation of MMP-9. The likelihood of this mechanism is diminished in the present experiments because TIMP-1 was undetectable by reverse zymography in both ischemic and nonischemic myocardium; however, we cannot exclude the possibility that a decrease in the level of TIMP-1 below our threshold for detection led to activation of MMP-9. Another potential mechanism of MMP-9 activation involves a cascade of events beginning with increased expression of tissue plasminogen activator in ischemic myocardium and increased plasmin generation (50). Plasmin may then activate MMP-3, which is a potent activator of MMP-9 (28). Whereas transferrin zymography was not sufficiently sensitive to detect MMP-3 in the present experiments (threshold tissue concentration for detection is ~6 μg/g wet wt), this does not exclude the possibility that a lower concentration of activated MMP-3 led to MMP-9 activation in the ischemic region.
Active MMP-9 plays an important role in normal and pathological connective tissue remodeling, including wound healing, tumor invasion, and chronic inflammatory disorders. It is increased in chronic ischemic and idiopathic dilated cardiomyopathy (15, 39, 40) and after myocardial infarction (31), suggesting a role in the pathogenesis of ventricular enlargement in those conditions. This is supported by the finding that LV dilation after infarction is attenuated in transgenic mice with targeted deletion of the MMP-9 gene, compared with wild-type mice (11).

In prior studies of severe ischemia due to permanent, prolonged, or repetitive complete coronary occlusion, activation of MMP-9 was demonstrated concurrently with activation of collagenase and/or MMP-2 (3, 7–10). In contrast, in the present study moderate ischemia and reperfusion caused activation of MMP-9 without activation of collagenase or MMP-2. Differences in the pattern of MMP activation among these studies may be related to the severity of ischemia: when irreversible myocardial injury results from severe ischemia, intense expression of cytokines may activate MMPs and infiltrating inflammatory cells may release MMPs as well as proteases that activate MMPs. Differences in MMP activation may also be attributable to greater oxidant stress and free radical generation with reperfusion after complete coronary occlusion, compared with reperfusion after partial coronary occlusion (23, 43).

We found increases in MMP content and activity after 90 min of reperfusion. Our data do not allow us to determine whether increases had already occurred during the antecedent ischemia. Prior data indicate that there may be a delay between the onset of ischemia and a rise in MMPs (9); however, once MMP activity has risen it is unlikely to decline again for several days (19, 33). Therefore, although it is possible that MMP content and activity had already increased during the ischemic period in the present experiments, it is unlikely that any such increases were greater than those detected after reperfusion. Furthermore, the oxidative stress associated with reperfusion is a potentially important factor in MMP activation. Therefore, we chose the end of the reperfusion period as the single time point most likely to demonstrate MMP activation in this experimental protocol.

Our measurement of collagenase activity does not distinguish between that contributed by MMP-1 (interstitial), MMP-8 (neutrophil), or MMP-13 (collagenase-3). Cardiac fibroblasts are a likely source of increased MMP-1 and MMP-9 in the present experiments. Whereas inflammatory cells were not detected on histological or ultrastructural examination, it is possible that small numbers of infiltrating polymorphonuclear or mononuclear leukocytes were the source of MMP-8 and MMP-9 (31), or of lysosomal proteases that may activate interstitial MMPs or act directly upon matrix components. Ischemia may also induce degranulation of resident cardiac mast cells, causing release of cytokines (14), which in turn increases expression and activation of MMPs.

**Implications and limitations of ultrastructural findings.** Prior studies that employed models of prolonged or repetitive complete coronary occlusion demonstrated profound degradation of the collagen weave surrounding individual cardiac myocytes (34, 52). In contrast, the alterations in MMPs in the present study were not associated with detectable changes in ultrastructure of fibrillar collagen. Although active MMP-9 can degrade fragments of fibrillar collagen, this occurs only after initial degradation by collagenase. Therefore, our findings of uniform ultrastructural integrity of fibrillar collagen and equal collagen content in ischemic and nonischemic regions are consistent with the absence of active collagenase. However, normal collagen ultrastructure could not have been assumed from biochemical data alone. Rather, ultrastructural examination was necessary because fibrillar collagen may be damaged by excessive passive ventricular dilatation, even in the absence of active collagenase (13). By analogy, mechanical collagen damage might have occurred in the present experiments when the hypocontractile ischemic region was subjected to high systolic wall stress generated by surrounding nonischemic myocardium (e.g., during the observed early systolic bulging of the ischemic region). Nonetheless, our examination revealed no evidence of frayed, disrupted, or broken collagen fibers. In the present experiments, the absence of discernible damage to fibrillar collagen or of an effect of treatment with GM-2487 suggests that activation of MMP-9 did not contribute to the early myocardial expansion or dysfunction that resulted from moderate ischemia and reperfusion. However, there are several important limitations to the present data: whereas the cell maceration/scanning electron microscopy technique allowed us to examine fine collagen architecture with a degree of detail and consistency not achievable with conventional scanning electron microscopy or optical techniques, there are also limitations to this technique. Our assessment of myocardial collagen ultrastructure was qualitative, and we cannot exclude the possibility that ischemia and reperfusion caused subtle changes in collagen fiber orientation. Whereas the cell maceration technique provides excellent visualization of the collagen weave enveloping individual myocytes, visualization of collagen struts that connect adjacent myocytes may be compromised by the removal of cellular elements. However, because the diameter of the struts is greater than that of the weave (30), the latter would be expected to be more susceptible to enzymatic damage. We cannot exclude the possibility that active MMP-9 acted upon matrix components that were not specifically examined in this study (e.g., elastin, proteoglycans). We also cannot exclude the possibility that MMP-9 caused basement membrane damage that was not detected by transmission electron microscopy but would have been revealed by other techniques, such as immunohistological examination (45). It is also possible that MMP-9 caused a functional dissociation between myocytes and basement membrane components, including type IV collagen or laminin (51). Finally, it is
possible that effects of MMP activation on matrix ultrastructure might have become evident after a longer period of reperfusion; however, the duration of the present experiments was limited by the stability of an anesthetized, open-chest model.

Effect of treatment with MMP inhibitor. Synthetic MMP inhibitors have been shown to attenuate ventricular enlargement and contractile dysfunction following experimental myocardial infarction (32) and heart failure (36). In the current model, the finding that treatment with GM-2487 did not prevent or attenuate myocardial expansion or contractile dysfunction after ischemia and reperfusion supports the conclusion that the action of MMPs is not responsible for these changes. However, several limitations of these data must be considered. Whereas the estimated concentration of GM-2487 in LAD blood (50–100 μM, calculated from the rates of drug infusion and LAD blood flow) exceeded the K_i of GM-2487 for MMP-9 by a factor of 10^4 and the K_i for other MMPs by a factor of 10^5–10^6, there were no established methods for measuring the concentration of GM-2487 in tissue. Nor was it possible to use zymography of tissue extracts from GM-2487-treated hearts to verify that the drug inhibited MMPs in vivo, because the reversible inhibitor is readily released from MMPs during preparation of extracts and by SDS, revealing the underlying activity of the MMPs. Despite these limitations, the likelihood that GM-2487 inhibited myocardial MMPs in the present experiments is supported by previous studies that demonstrated inhibition of the effects of metalloproteinases in vivo after parenteral administration of GM-2487 (22) or its congener GM-6001 (37).

Implications. Moderate myocardial ischemia and reperfusion, neither prolonged nor severe enough to result in irreversible injury, nonetheless cause rapid increases in content and activity of myocardial MMPs. Whereas the current data do not indicate that changes in MMPs are responsible for acute myocardial expansion or contractile dysfunction, future studies may determine whether these early changes in MMPs lead to later alterations of myocardial structure and function.

REFERENCES


