Matrix-Dependent Mechanism of Neutrophil-Mediated Release and Activation of Matrix Metalloproteinase 9 in Myocardial Ischemia/Reperfusion

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Background—A key component of reperfusion of myocardial infarction is an immediate inflammatory response, which enhances tissue repair. Matrix turnover is crucial to tissue repair, and matrix metalloproteinases (MMPs) are key enzymes involved in matrix degradation. The hypothesis tested is that one inflammation-based effector of tissue repair is the secretion and activation of MMP-9 by infiltrating neutrophils.

Methods and Results—Cardiac lymph and tissue were assayed for latent and active MMP-2 and MMP-9 by zymography and immunochemistry. Dual-labeling immunofluorescence determined the cellular source of MMP-9 protein. Isolated canine neutrophils were incubated with preischemic and postischemic cardiac lymph in the presence and absence of collagen-fibronectin pads, and the supernatants were assayed for latent and active MMP-9. MMP-9 increased during the first hours of reperfusion in both lymph supernatants and myocardial extracts, and this increase was of neutrophil origin. MMP-9 in the cardiac lymph remained latent but was activatable. In contrast, MMP-9 in the myocardium was in both latent and active forms. In situ zymography demonstrated that activated MMP-9 surrounded the infiltrated neutrophils. When postischemic cardiac lymph was incubated with neutrophils in vitro, MMP-9 secretion and activation occurred only in the presence of a collagen-fibronectin substrate; preischemic cardiac lymph did not induce significant secretion or activation.

Conclusions—Infiltrating neutrophils are an early source of MMP-9 after reperfusion, and a portion of MMP-9 in the myocardium is active. Infiltrating neutrophils may localize MMP-9 activation by secreting MMP-9 and as a source of activating proteases. (Circulation. 2001;103:2181-2187.)

Key Words: metalloproteinases ■ ischemia ■ reperfusion ■ blood cells
Ischemia/Reperfusion Protocol
The canine ischemia/reperfusion with lymph duct cannulation protocol has been described in detail.13,14 Briefly, healthy mongrel dogs (15 to 25 kg) of either sex were surgically instrumented with a hydraulically activated occluding device and Doppler flow probe on the circumflex coronary artery, and the cardiac lymph duct was cannulated. After 72 hours of recovery, coronary occlusion occurred for 1 hour, followed by various times of reperfusion. At 50 minutes of occlusion, radiolabeled microspheres were injected into the left atrium to quantify regional blood flow.

Total Protein Extraction
Myocardial segments were frozen and stored in liquid nitrogen until ready to use. Protein was extracted as described,16 and total protein levels were determined by Lowry assay.17

Gelatin Zymography
Samples were loaded onto nondenaturing 10% polyacrylamide gels containing 0.1% gelatin, electrophoresed, renatured, and developed as described previously.16,17,18 To determine activity levels, gels were scanned into Adobe Photoshop 4.0 (Adobe Systems, Inc) as black-and-white images and inverted, and densitometry levels were determined by use of the Scion Image (Scion Corp) gel plot 2 macro.

Histology
Cardiac tissue segments were fixed in 10% formalin or 1% paraformaldehyde, embedded in paraffin, and sectioned at 4 μm. Cells isolated from cardiac lymph were fixed in 1% paraformaldehyde, resuspended in 75% ethanol, and divided into aliquots on slides.

Immunocytochemistry was performed on lymph cells with the ABC technique. For the sheep anti–human MMP-9 antibody, a sheep kit (Pierce) that contained a biotinylated donkey anti-sheep IgG was used. For the mouse anti–dog neutrophil antibody, a mouse kit (Vector) that contained a biotinylated goat anti-mouse IgG was used. To calculate the percentage of lymph cells that were positive, random fields were scanned into Adobe Photoshop, and a minimum of 500 cells for each time point were counted with Zeiss Image.

Immunofluorescence was used on myocardial tissue sections. Both sheep anti-human MMP-9 (the binding site) and mouse anti-dog neutrophil (SG9H6) antibodies were used at a 1:100 dilution. Negative controls included using no primary antibody and isotype-matched nonimmune IgG antibodies. A donkey anti-sheep IgG conjugated with Texas Red (Jackson Immunochemicals) was used for the mouse anti–dog neutrophil antibody. No bleed-over fluorescence was observed in control sections.

In situ zymography was used to determine localization of MMP-9 activity. A solution of 0.1 mg/mL gelatin–Oregon green (Molecular Probes) in 1× developing buffer (mmol/L: Tris base 50, HCl 40, NaCl 200, CaCl2 ·2H2O 5, and PMSF 50, and 0.2% [wt/vol] Brij 35) was placed onto 4-μm frozen sections. Adjacent serial sections also had 50 mmol/L EDTA or a 1:40 dilution of neutralizing mouse anti-human MMP-9 antibody (antibody 1, Calbiochem). These sections were incubated at 37°C for 3 hours, washed 3 times with water to remove unbound gelatin, and counterstained with the nuclear stain DAPI (Vector mounting media, Vector Laboratories). Sections were incubated at 37°C for 3 hours, washed 3 times with water to remove unbound gelatin, and counterstained with the nuclear stain DAPI (Vector mounting media, Vector Laboratories).

Results
Latent MMP-9 Levels Increase in Lymph Supernatants During Reperfusion
By use of gelatin zymography, lymph supernatants at various times from 11 animals were examined for MMP-2 and MMP-9 levels (Figure 1). To achieve specificity, the gels were incubated in PMSF to eliminate non-MMP activity, and MMP-2 and MMP-9 standards were loaded to confirm molecular weight sizes. To ensure that MMP enzymatic levels were in the linear range, initial gels were loaded with 1, 2.5, 5, 7.5, 10, and 20 μg total protein. An MMP-9 standard curve confirmed that the lytic band was MMP-9 and that activity remained in the linear range. A representative zymogram and its densitometry is shown in Figure 1A. None of the animals had changes in MMP 2 during the first day of reperfusion. From the 11 dogs above the collagen inserts, cardiac lymph was placed below, and the plates were incubated at 37°C in 5% CO2 for 1 hour. The neutrophils (upper fraction) were removed, centrifuged at 10,000g for 3 minutes to remove neutrophils, and assayed for MMP-9 activity.

Statistical analysis was performed with Microsoft Excel and GraphPad InStat version 3.01 (GraphPad Software).

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examined, 60 time points ranging from preocclusion to 12 hours of reperfusion were pooled into preocclusion, occlusion, 1- to 5-hour reperfusion, and 6- to 12-hour reperfusion groups and statistically analyzed (Figure 1B). Of the 11 animals examined, 6 had increases in latent MMP-9 levels after reperfusion during day 1. Incubation of the lymph samples with p-aminophenylmercuric acetate resulted in MMP-9 activation, demonstrating that MMP-9 was latent but activatable (Figure 1C). On the basis of quantification of microspheres, the 5 dogs that did not show increases in MMP-9 had normal flows in the myocardium downstream of the occluding device, indicating that collateral circulation was sufficient to compensate for the occlusion. These animals were classified as shams. The increase in MMP-9 in the lymph supernatants corresponded to a decrease in MMP-9 in the lymph cell fraction, suggesting that the source of MMP-9 was the cells in the lymph (data not shown).

Neutrophils Are the Predominant Source of MMP-9 in the Lymph

To determine which cells are positive for MMP-9, parallel time courses of lymph cells from 6 animals were immunostained with a sheep anti–human MMP-9 antibody or a mouse anti–dog neutrophil antibody. The percentage of cells that were MMP-9–positive or were neutrophils was calculated. Figure 2A shows a representative time course from 1 of these animals. When the percentages of all 6 animals (47 time points) are plotted against each other (Figure 2B), there is a significant linear correlation ($r^2=0.81$, $P<0.001$), suggesting that neutrophils are the predominant source of MMP-9 found in the cardiac lymph supernatants.

Latent and Active MMP-9 Levels Increase in the Myocardium During Reperfusion

By gelatin zymography, tissue extracts from 3 control and 5 ischemic areas from each dog were examined for MMP-9. Figure 3A shows a representative zymogram from animals undergoing 1-hour ischemia and 5-hour reperfusion. The densitometry results from 11 dogs undergoing 1-hour ischemia/5-hour reperfusion are shown in Figure 3B. For each dog, the densities from 3 control sections and 5 ischemic sections were averaged. Control, normal-flow sections did not have latent or active MMP-9 protein. The ischemic sections again demonstrate variation in the level of response, some of which is due to nonuniform reductions in flows. Of the 11 reperfused dogs, 7 had increases in latent and active MMP-9 levels, and 4 did not. Neither the reperfused nor nonreperfused groups had changes in MMP-2 levels (Figure 3B). This increase in latent and active MMP-9 in the ischemic/reperfused segments, compared with nonischemic myocardium, was statistically significant. Nonreperfused dogs with <20% collateral circulation showed no increased release or activation of MMP-9. A representative zymogram from a 6-hour ischemia/0-hour reperfusion experiment is shown in Figure 3C.

**Figure 2.** Increase in MMP-9–positive cells in lymph parallels increase in neutrophils. Percentage of positive cells was determined by counting a minimum of 500 cells from random fields for each time point. A, Representative time course from 1 of 6 animals examined. B, Regression of 47 time points ranging from preocclusion to 5 days of reperfusion pooled from 6 animals.

**Figure 3.** Latent and active MMP-9 increase in ischemic myocardium after 1-hour ischemia and 5-hour reperfusion. A, Representative zymogram of 1-hour ischemia/5-hour reperfusion (1/5 h I/R). B, Cumulative data for 11 dogs, demonstrating an increase in both active and latent MMP-9 levels in ischemic myocardium after 5 hours of reperfusion. Densities were compared by Wilcoxon matched-pairs test. C, Representative zymogram from a 6-hour ischemia/0-hour reperfusion (6/0 h I) experiment demonstrating that there is no release or activation of MMP-9 in absence of reperfusion. Thus, in presence of ischemic injury but absence of neutrophil infiltration (at 6 hours of ischemia with no reperfusion), no MMP-9 protein is detected. *$P<0.05$; **$P<0.001$. 

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Active MMP-9 Is Seen Where Neutrophils Accumulate

To determine localization of the active MMP-9 within the reperfused myocardium, frozen sections were incubated with gelatin that is quenched with Oregon green, a fluorescent label. An increase in gelatinase activity is therefore visualized as an increase in fluorescence (Figure 4). To achieve specificity, all sections were incubated with PMSF to block endogenous serine protease activity. Adjacent serial sections were tested for MMP-9 activity (green fluorescence) as follows: (1) 1-hour ischemia/5-hour reperfusion, (2) + EDTA, and (3) + neutralizing MMP-9 antibody. The sections were counterstained with DAPI (blue fluorescence) to depict nuclei. Figure 5 demonstrates that MMP activity is inhibited by both EDTA and neutralizing MMP-9 antibody. No MMP activity is seen in shams or nonischemic control sections (data not shown).

The data suggest that MMP-9 activation occurs in the area in which neutrophils accumulate. To further demonstrate this association, serial sections were stained for MMP-9 activity and neutrophils. Figure 6 demonstrates that neutrophil infiltration (SG8H6-stained cells) occurs in the region of MMP activity. Figure 6A demonstrates MMP-9 activity with the quenched gelatin overlay (green fluorescence indicates activity). Figure 6B demonstrates a serial section stained for neutrophils (the black-stained cells are neutrophils). A computer-generated overlay of the neutrophil staining superimposed over the MMP-9 activity (Figure 6C) demonstrates that MMP-9 activation occurs wherever there are infiltrating neutrophils.

Postischemic but Not Preischemic Lymph Stimulates the Release of MMP-9 and Its Activator(s) From Neutrophils in the Presence of a Collagen-Fibronectin Matrix

To examine the in vitro release and activation of MMP-9, we studied neutrophils migrating into a collagen matrix in response to preischemic and postischemic cardiac lymph. Cardiac lymph was used for 2 reasons: (1) cardiac lymph is an excellent sample of the macromolecular constituents of the myocardium at any one time (1 minute lag time) and (2) latent MMP-9 but not active MMP-9 was increased in the cardiac lymph on day 1 of reperfusion (see above). Therefore, the ability of lymph to induce the release of MMP-9 and its activator would demonstrate that, as opposed to neutrophils suspended in the cardiac lymph, neutrophils stimulated in a matrix environment have the ability to activate MMP-9. The ability of postischemic cardiac lymph to induce MMP-9 activation would also provide evidence against the possibility that inhibitors in the lymph prevent activation.

Neutrophils were placed above collagen and fibronectin inserts, and 10%, 20%, or 40% dilution of cardiac lymph was placed below the collagen and fibronectin inserts. Negative controls included PBS. After a 1-hour incubation, the supernatants were collected, centrifuged to remove the unadhered neutrophils, and assayed for MMP-9. Preischemic and post-
ischemic lymph controls were also analyzed to confirm that the levels assayed were neutrophil-derived. As shown in Figure 7, postischemic cardiac lymph stimulated the release and activation of MMP-9 from the neutrophils. This activation was attended by release of the primary granule marker, myeloperoxidase. The increase in latent and active MMP-9 was statistically significant. Preischemic cardiac lymph induced a small, statistically insignificant, increase in latent MMP-9 but no activation of MMP-9 and no release of myeloperoxidase. Saline controls initiated no release or activation. In contrast, when postischemic lymph was used to stimulate neutrophils in suspension (n=6 experiments), there was no increase in either latent or active MMP-9 or myeloperoxidase release (data not shown).

### Discussion

#### MMP-9 Levels In Vivo

MMP-9 levels in cardiac lymph increase in the first hours of reperfusion, an increase that parallels neutrophil numbers and is accompanied by a loss of MMP-9 from neutrophils in the lymph sample. This suggests that MMP-9 in the lymph is secreted by neutrophils in the lymph rather than by neutrophils in the myocardium. As with the lymph, MMP-9 increases in the myocardium during the first hours of reperfusion, much earlier than reported previously in studies examining MMP-9 gene induction by in situ hybridization. Because MMP-9 protein is synthesized in the neutrophil early in its development and is stored in tertiary granules, very little mRNA for MMP-9 is seen in the neutrophil once it enters the circulation. In contrast to the MMP-9 in the lymph, MMP-9 is activated in the myocardium, as demonstrated by both gelatin zymography and in situ zymography. The neutrophil is also the source of early MMP-9 appearance in the myocardium. The data localize potential matrix-degrading capability to the area of injury and introduce the idea that the neutrophil may also be important in controlling MMP-9 activation. Because MMP-9 activity surrounds the neutrophil, it is possible that neutrophil proteases contribute to MMP-9 activation. The presence of more classic MMP activators, such as plasmin and, perhaps, proteases released from injured tissue, suggest that activation may have multiple components.

Conceptually, neutrophil-derived activation of MMP-9 has potential biological advantages. Owen and Campbell suggested that pericellular proteolysis during injury and repair would be desirable to prevent more uncontrolled global proteolytic degradation. At least in early reperfusion, MMP-9 activation could be localized to the perineutrophil area and might be initiated by neutrophils adhering to the extracellular matrix. Thus, neutrophils can easily be activated by chemoattractant factors to secrete MMP-9 from the tertiary granules. Additional or greater stimulation is required for degradation of neutrophil primary granules, which contain the neutrophil proteases that might activate MMP-9. A greater sensitivity to chemoattractant factors occurs when neu-
trophils are adherent, perhaps due to cytoskeletal rearrangement, which is necessary for full degranulation to occur. This is compatible with the data in Figure 7, in which we demonstrate that posts ischemic cardiac lymph would not initiate neutrophil-derived activation of MMP-9 unless the cells were adherent to a fibronectin/collagen matrix.

Functional Roles of MMP-9

The potential beneficial and potential deleterious aspects of MMP-9 activity on myocardial injury and repair overlap to a great extent. Potential deleterious effects of MMP-9 include stimulating inappropriate extracellular matrix degradation, activating inflammatory mediators, and/or increasing capillary permeability. Potential beneficial effects of early MMP-9 activation include removing matrix and necrotic myocytes, releasing growth factors and cell surface receptors, remodeling the extracellular matrix for scar formation, processing inflammatory mediators such as interleukin-1α, and influencing angiogenesis. An increase in MMP-9 that occurs within hours after reperfusion could serve a proactive function, with the overall result being an accelerated healing. The more focused secretion and activation of MMP-9 postischemic cardiac lymph would not affect the infarct size at 4 days reperfusion at 6 hours did not affect the infarct size at 4 days.

Reperfusion may control the timing, localization, and levels of matrix degradation to optimize events of remodeling. During the healing phase, damaged collagen must first be degraded and removed before necrotic myocytes can be resorbed and new collagen generated to form a scar. Reperfusion may control the timing of these steps by initiating matrix degradation and myocyte resorption and allowing new collagen deposition at a much earlier time course (within hours versus several days).

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