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Early Activation of Metalloproteinases after Experimental Myocardial Infarction Occurs in Infarct and Non-infarct Zones

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The collagen matrix of the heart forms a network linking muscle fibers, muscle bundles, and intramyocardial blood vessels. Collagen turnover in the heart is normally a dynamic process that involves both collagen synthesis and degradation. Collagen breakdown generally involves its chemical digestion by matrix metalloproteinases (MMPs) which are activated in tissue repair, wound healing, and myocardial ischemia. We studied activation of MMPs by zymography in infarct (anterolateral wall) and non-infarct (septum) zones of rat hearts following coranary artery ligation, as well as in sham operated rats. Rats were sacrificed at 30 minutes, 1 hour, 2 hours, 4 hours, and 24 hours post infarction (six hearts for each time period). MMP activity was detected at different molecular weights, with bands at 54 kDa (MMP-1), 62 kDa (MMP-2), and 92 kDa (MMP-9) being the most prominent. MMP activities were indexed by densitometer optical reading. Activity was detected as early as 1 hour post infarct in the MI and remote zones at the 54 kDa (MMP-1) ($p < 0.01$) and 62 kDa bands (MMP-2) ($p < 0.001$), and at 2 hours post infarct in the infarct zone only at 92 kDa (MMP-9) ($p < 0.05$). MMPs are activated early after infarction both in the infarct and importantly, non-infarct zones. This may contribute to collagen breakdown, infarct expansion, and left ventricular remodeling, known to occur early after infarction in experimental and clinical settings. *Cardiovasc Pathol 1998;7:307–312* © 1998 by Elsevier Science Inc.

The myocardium contains cellular and extracellular compartments (1). The extracellular matrix consists of a structural protein network composed largely of type I and type III fibrillar collagens (2). This collagen matrix forms a complex network which mechanically links muscle fibers, muscle bundles, and intramyocardial blood vessels (3,4). Collagen turnover in the heart is normally a dynamic process with collagen synthesis and degradation (5).

Collagen breakdown generally involves its chemical digestion by matrix metalloproteinases (MMPs) (6,7), a family of enzymes referred to collectively as collagenases. MMPs are synthesized by several cell types, including fibroblasts and monocytes, and exist in an inactive form in the extracellular matrix. They are activated by cleavage of an n-terminal peptide which occurs in settings such as tissue repair, wound healing, and myocardial ischemia. Tissue inhibitors of metalloproteinases (TIMP) have also been identified in the normal heart (8,9), and their role in the regulation of MMPs is under investigation.

Acute myocardial ischemia can result in collagen damage. The exact time course, nature, and mechanism of this damage is not clear. It has been suggested that the onset of collagen damage is early and extensive (10). Morphologic and biochemical evidence has been presented to indicate that collagenolysis begins within a few hours after myocardial ischemia and infarction (11–13). The loss of the fibrillar collagen integrity may be one factor which allows myocyte slippage, sarcomere overdistension and wall thinning, which characterize infarct expansion (14–16). Activation of MMPs has been shown to occur two days after infarction in rat hearts, increasing until day 7 and declining thereafter, but only in the infarcted myocardium (17).

Infarct expansion is known to occur early in the infarcted zone. Remodeling has been reported to begin early in the

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non-infarcted region (15). We postulated that MMP activation occurs early after coronary artery ligation both in the infarct and the non-infarct zones. We studied activation of MMPs by zymography in infarct (anterolateral wall) and non-infarct (septum) zones of rat hearts between 30 minutes and 24 hours following coronary artery ligation.

Materials and Methods

Animals

Adult male Sprague Dawley rats, weighing 250–300 g were used. Animals were housed and experiments performed according to the guidelines for care and use of laboratory animals approved by our institution's animal care committee. All rats were given standard rat chow and water.

A total of 44 rats were operated, with a survival rate of 68%. This resulted in 15 infarcted and 15 sham operated animals that were included in the present study, with six animals per time point.

Surgery

Animals were anesthetized with chloral hydrate (0.05 gr/100 gr weight) and ventilated by positive pressure through an endotracheal tube attached to a small animal respirator (HARVARD rodent ventilator model 683). Following left thoracotomy and incision of the pericardium, the left descending coronary artery was ligated by silk ligature (16,19,20). After closing the chest the animal was returned to its cage. The sham procedure consisted of left thoracotomy without ligation.

Collection

After 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours, 6 rats (3 infarct and 3 sham) at each time point were again anesthetized with chloral hydrate. The chest was opened with midline thoracotomy and the heart rapidly excised. Immediate viewing of the heart allowed us to recognize the ischemic-infarcted (pale) zone. The left ventricle was divided into infarct zone (anterolateral wall) and to non-infarct zone (septum). To facilitate this process methylene blue was injected retrograde into the cut end of the aorta and was used to identify the risk region supplied by the occluded artery.

The heart was sliced transversely parallel to the A-V groove, in 2-mm sections from apex to base. The infarct and non-infarct zones were separated, and the border zone (the 1–2-mm region between the clearly demarcated zones) was discarded. Samples were frozen and stored at −70°C.

Zymography

Proteolytic enzyme activity was determined by zymography (6,21,22,25).

Sample preparation. Samples were cut into small pieces, homogenized in Tris buffer (50 mM Tris-HCL pH 7.5, 2M NaCL, 5 μM PMSF, 0.02% Na2N3) and centrifuged at 12000 $\times g$ for 15 minutes. The pellet was resuspended in Tris buffer (50 mM Tris-HCL pH 7.5, 2 M NaCL, 10 μM PMSF, 0.05% Brij-35, 0.02% Na2N3) and homogenized. The homogenate was centrifuged at 12000 $\times g$ for 15 minutes. The pellet was then resuspended a second time in a Tris buffer (50 mM Tris-HCL pH 7.5, 10 μM PMSF, 1M NaCL, 0.02% NaN3, 2M urea), homogenized, and centrifuged at 12000 $\times g$ for 45 minutes. The supernatant was dialyzed against 50 mM Tris buffer (pH 7.9) overnight. The protein concentration of tissue extract was measured using Bio-Rad dye binding assay kit.

Electrophoresis. 15 μg protein of each sample was loaded in each gel line. The samples were electrophoresed on a 8% SDS-polyacrylamide gel containing 0.1% gelatin. Following electrophoresis, gels were washed twice with 2.5 % Triton-100 using 100 ml each time for 15 minutes, rinsed with distilled water twice, and incubated with 50 mM Tris HCL pH 7.9, 5 mM CaCl2 at 37°C, with shaking overnight. Gels were stained with 20% TCA (w/v), 0.25% Coumassie Blue R-250 (w/v). Enzyme activity was indicated by zones of lysis after destaining in 15% methanol (v/v) and 7.5% acetic acid (v/v). The molecular weight of each band was determined by comparison to standards (Bio-Rad Company, prestained SDS-PAGE, low range: 107 kDa, 76 kDa, 52 kDa, 36.8 kDa).

Densitometeric Analysis of Enzyme Activity

The gels were scanned with a densitometer (Computing Densitometer Model 300A, Molecular Dynamics Company), and the images were quantitated using ImageQuant Program (Molecular Dynamics Company). Quantification of specific lysis bands was accomplished by computer scanning of gels and then expressing band intensities in arbitrary units relative to the background reading (set to a value of 0) and the reading of the brightest band present on the gel (set to a value of 1).

Statistics

All grouped data (MI, remote, sham) were expressed as mean ± standard deviation. Differences between MI, remote, and sham were analyzed using ANOVA test. Post hoc analysis of between group differences was performed using Student's *t*-test with Bonferroni correction. The level of significance was taken at $p < 0.05$.

Results

Proteolytic enzyme activity was detected at different molecular weights with bands at 54 kDa, 62 kDa, and 92 kDa being most prominent, corresponding to MMP-1, MMP-2, and MMP-9, respectively.

Thirty minutes post coronary ligation there was no significant MMP-1 (54 kDa), MMP-2 (62 kDa), and MMP-9 (92 kDa) activity at any region. One hour post coronary ligation MMP-1 (54 kDa) and MMP-2 (62 kDa) activity were increased in the infarct and in the remote zones compared to sham operated rats (Figure 1). There was no significant MMP-9 (92 kDa) activity at any region at this time point. Two hours post coronary ligation MMP-1 and MMP-2 activity were increased in the infarct and remote zones compared to sham operated rats. An increase in MMP-9 activity was detected in the infarct zone only (Figure 2). Four hours post coronary ligation MMP-1 and MMP-2 activity decreased in both infarct and non-infarct zones compared to earlier time. At this time point MMP-9 activity was significant in the infarct zone but not in the non-infarct zone. Twenty-four hours post coronary ligation MMP-1 and MMP-2 were increased in the infarct and remote zones compared to sham operated rats (Figure 3). Absolute activity in the infarct zone at 24 hours in the 54 kDa and 62 kDa bands was higher than at 2 and 4 hours post coronary ligation.

Absolute activity in the remote zone at 24 hours in the 54 kDa bands and 62 kDa bands was higher than at 4 hours but lower than at 2 hours post coronary ligation. This may represent a bimodal time curve with lesser activity at 4 hours. Very prominent MMP-9 activity was demonstrated in the infarct zone compared to remote zone and sham operated rats (Figure 3).

MMP activities indexed by densitometer optical reading were significantly increased at 1 hour post coronary ligation in the infarct and non-infarct zones at the 54 kDa (MMP-1) ($p < 0.01$) and 62 kDa (MMP-2) bands ($p < 0.001$), and at 2 hours post coronary ligation in the infarct zone only at 92 kDa (MMP-9) ($p < 0.05$) (Table 1).

Discussion

Acute myocardial ischemia and infarction can result in collagen damage. The exact time course, nature, and mechanism of this damage is not clear. In cases of sustained ischemia, it has been suggested that the onset of collagen damage is early and extensive (10). Unresolved issues include the time of initiation, mechanism, the severity of the process, and whether it involves only the infarcted area or the non-infarct area as well. The relation between collagen degradation and left ventricular remodeling needs to be defined.

Sato et al. (23) studied the collagen network in pig hearts after coronary artery ligation with scanning and transmission electron microscopy. They showed that at 40 minutes after coronary occlusion the collagen network became irregular in arrangement. At 2 hours after occlusion collagen fibrils and microfibrils between cardiomyocytes became separated from the basement membrane, the banded pattern of collagen disappeared, and collagen fibrils, elastic fibers, and microfilaments were broken down and decreased in content. Takahashi et al. (11) demonstrated that 1, 2, and 3 hours after coronary artery ligation in rats total collagen content was decreased in the infarct zone by 25 ± 8%, 35 ± 7%, and 50 ± 10%, respectively (mean ± SD). Collagenase, neutral proteinase, and lysosomal serine proteinase activities were increased two- to three-fold, suggesting that the increase in these degrading enzymes may be responsible for the rapid degradation of the extracellular matrix collagen after coronary ligation. These changes in the myocardial collagen content and in the degradative enzymes were noted only in the infarct zone and not in the non-infarct tissue.

Cardiomocyte necrosis is not the major factor in enhancing collagenase activity. In stunned myocardium, after repeated occlusion and reperfusion in dogs, no cellular damage is observed by scanning and transmission electron microscopy (12). The extracellular collagen matrix, however, is changed. The collagen weave surrounding myocytes completely disappears and collagen struts, the interconnection between myocytes or myocytes and capillaries, become sparse or completely absent. Enhanced collagenolytic activ-

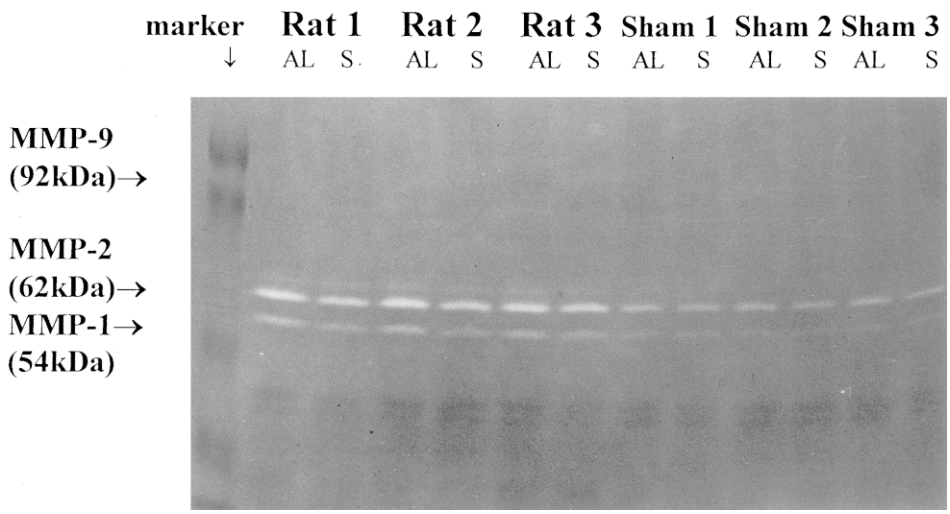

Figure 1. Detection of collagenase activity by zymography 1 hour post infarction. Pairs of specimens from the infarct (AL) zone and from remote, uninfarcted septal region (S) from rats with left coronary artery ligation are presented (rats 1–3). Pairs of specimens from the anterolateral (AL) wall and from the septum (S) from sham operated rats are presented (sham 1–3). Increase in MMP-1 (54 kDa) and MMP-2 (62 kDa) activity is shown in the infarct (AL) and in the remote zones (S) compared to sham operated rats. There is no significant MMP-9 (92 kDa) activity in any region. (AL = anterolateral, S = septum.)

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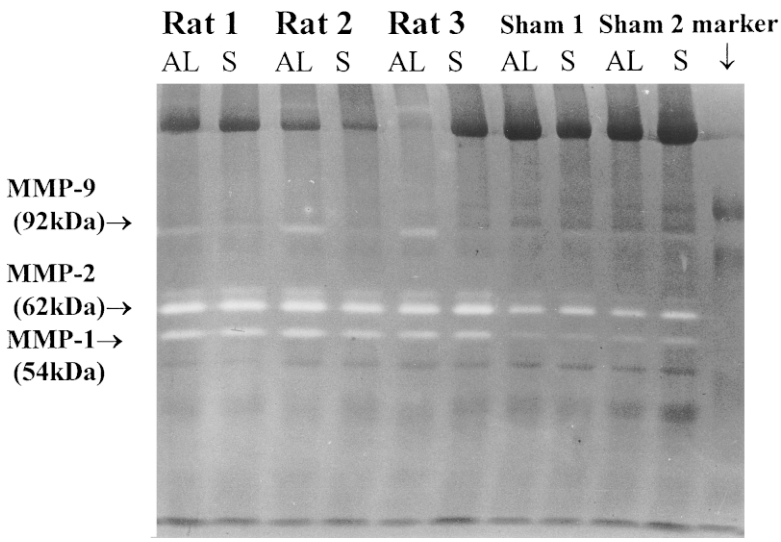

Figure 2. Detection of collagenase activity by zymography 2 hours post infarction. Pairs of specimens from the infarct (AL) and from remote region (S) from rats with left coronary artery ligation are presented (rats 1–3). Pairs of specimens from the anterolateral (AL) wall and from the septum (S) from sham operated rats are presented (sham 1–2). Increase in MMP-1 (54 kDa) and MMP-2 (62 kDa) activity is shown in the infarct (AL) and remote zones (S) compared to sham operated rats. Increase MMP-9 activity (92 kDa) is shown in the infarct zone only (rat 1-AL, rat 2-AL, rat 3-AL) compared to the remote zone and sham operated rats. (AL = anterolateral, S = septum.)

ity after myocardial infarction can lead to fibrillar collagen degradation and a loss of structural integrity.

Cleutjens et al. (17) examined collagen degradation in the myocardium after infarction in rat hearts 6 hours to 28 days after coronary artery ligation. After coronary artery ligation, a transient increase in collagenolytic activity of MMP-1 was found only in the infarcted myocardium and not in non-infarcted tissue or sham operated controls. Collagenolytic activity was transiently increased from day 2 until day 7 following ligation, declining thereafter. In contrast to the studies of Sato et al. (23) and Takahashi et al. (11), no increase in MMP-1 activity was determined before day 2 after infarction. Collagenolytic activity of gelatinases MMP-2 and MMP-9 followed a similar time course. Transcription of TIMP mRNA was observed at 6 hours in the infarcted ventricle, peaked on day 2 after MI, and slowly decreased thereafter. No changes in TIMP mRNA expression were observed at remote sites or in sham operated controls (17).

In our study, MMP activity following coronary artery ligation in rats was detected at different molecular weights, with bands at 54 kDa (MMP-1, 62 kDa (MMP-2), and 92kDa (MMP-9) being the most prominent. We demonstrated MMP-1 (54 kDa) and MMP-2 (62 kDa lysis band) activity as early as 1 hour post coronary ligation both in the infarct and in the non-infarct zones (compared to sham rats). MMP-9 activity (92 lysis band) was detected as early as 2 hours post coronary ligation but only in the infarct zone. In the non-infarct zone, quantitative assessment of MMP-9 activity demonstrated a trend toward increased activity (p = 0.07) at 24 hours post coronary ligation.

At 30 minutes of coronary artery occlusion in the rat a subendocardial infarct results. Therefore, even though we discarded the "border zone," data obtained at 30 minutes

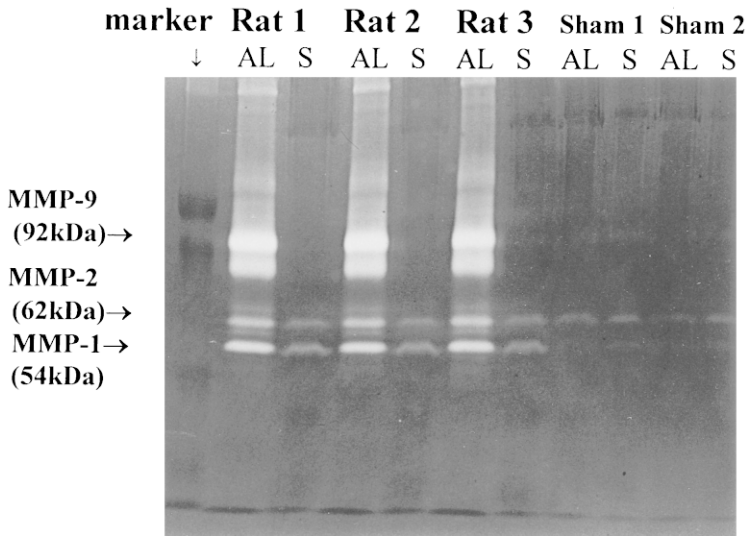

Figure 3. Detection of collagenase activity by zymography 24 hours post infarction. Pairs of specimens from the infarct (AL) and from the remote region (S)from rats with left coronary artery ligation are presented (rats 1–3). Pairs of specimens from the anterolateral (AL) wall and from the septal (S) wall from sham operated rats are presented (sham 1–2). Increase in MMP-1 (54 kDa) and MMP-2 (62 kDa) activity is shown in the infarct (AL) and in the remote zones (S) compared to sham operated rats. Very prominent MMP-9 activity (92 kDa) is shown in the infarct zone only (rat 1-AL, rat 2-AL, rat 3-AL) compared to remote zone and sham operated rats. (AL = anterolateral, S = septum.)

Table 1. Densitometric Optical Reading in the MI (Anterolateral Wall) and Remote (Septum) Zones Compared to Sham at 54 kDa (MMP-1), 62 kDa (MMP-2), and 92 kDa (MMP-9)

Time	MI	Remote	Sham
54 kDa			
30 minutes	0.026 ± 0.004	0.026 ± 0.030	0.035 ± 0.012
1 hour**	0.093 ± 0.019†	0.074 ± 0.009††	0.023 ± 0.010
2 hours***	0.257 ± 0.015††	0.250 ± 0.023††	0.135 ± 0.114
4 hours*	0.083 ± 0.007	0.088 ± 0.005	0.054 ± 0.020
24 hours***	0.410 ± 0.023††	0.233 ± 0.013††	0.139 ± 0.006
62 kDa			
30 minutes	0.109 ± 0.012	0.074 ± 0.002	0.115 ± 0.054
1 hour***	0.159 ± 0.008†††	0.126 ± 0.004††	0.069 ± 0.008
2 hours**	0.314 ± 0.037†	0.303 ± 0.040†	0.159 ± 0.019
4 hours*	0.099 ± 0.006	0.096 ± 0.006	0.066 ± 0.020
24 hours***	0.349 ± 0.014†††	0.171 ± 0.031†	0.057 ± 0.013
92 kDa			
30 minutes	0.018 ± 0.002	0.013 ± 0.008	0.013 ± 0.014
1 hour	0.024 ± 0.004	0.021 ± 0.005	0.020 ± 0.011
2 hours*	0.107 ± 0.020†	0.062 ± 0.037	0.032 ± 0.011
4 hours***	0.232 ± 0.022††	0.035 ± 0.011	0.044 ± 0.036
24 hours***	0.555 ± 0.029†††	0.106 ± 0.036§	0.021 ± 0.018

The sample size (n) is 3 for each time point and each myocardial region.
***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ using ANOVA.
$^{\dagger\dagger\dagger}p < 0.001$, $^{\dagger\dagger}p < 0.01$, $^{\dagger}p < 0.05$, $^{\S}p = 0.07$ (compared to sham), using t-test with Bonferroni Correction.

and to some extent 1 hour post coronary ligation, will represent a mixture of infarcted and ischemic tissue. This could "dilute" early MMP activity if necrosis is required for activation or if there is a difference in activation due to ischemia versus cell death.

To the best of our knowledge, our study is the first one to show "early" activation of MMPs in the infarct and in non-infarct zones post coronary artery ligation. Interestingly, MMP-1 (54 kDa) and MMP-2 activity (62 kDa) were found to decrease at 4 hours post coronary ligation and reappeared later at 24 hours post infarction, while MMP-9 continued to increase over time. We do not have a clear explanation for this apparent pattern, nor for the difference.

To summarize, our study provides evidence for early activation of metalloproteinases in experimental acute myocardial infarction in the infarct and non-infarct regions of the left ventricle. This is in contrast to the finding of Cleutjens et al. (17) who showed no increase in proteolytic activity in the remote area early after myocardial infarction. The reason for this discrepancy is not entirely clear. Differences in the two studies includes: use of optical densitometry in our study, and examination of different time points with only 24 hours in common.

Our study is limited in that we did not evaluate collagen content, therefore could not correlate MMP activity and collagen content. Also, the number of hearts examined at each time point were relatively small (3). Only the infarcted rats had a needle passed through the myocardium and this maneuver cannot be excluded as a stimulus for MMP activation.

Implications

This early collagenolytic activity after myocardial infarction may lead to fibrillar collagen degradation which may result in a loss of structural integrity including myocyte slippage (15) and wall thinning (18). This process may play a role in remote remodeling with lengthening of the non-infarcted zone. In addition, early collagenolytic activity resulting in collagen degradation may be important in early cardiac rupture, where absence of silver stainable struts has been reported (24).

Thus, MMPs are activated very early after infarction both in the infarcted and importantly, in the non-infarct zones. Further studies are needed to assess whether this leads to collagen breakdown, infarct expansion, and remote remodeling, known to occur early after infarction in experimental and clinical settings.

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References

1. Cleutjens JPM, Weber K. Collagen degradation after myocardial infarction. In: Weber K, ed. Wound Healing in Cardiovascular Disease. Armonk, NY: Futura Publishing Company Inc., 1995;169–177.
2. Weber K. Cardiac interstitium in health and disease. J Am Coll Cardiol 1989;13:1637–1652.
3. Robinson TF, Factor SM, Capasso JM. Morphology, composition, and function of struts between cardiac myocytes of rat and hamster. Cell Tissue Res 1987;249:247–255.
4. Caulfield JB, Borg TK. The collagen network of the heart. Lab Invest 1979;40:364–372.
5. Laurent GJ. Dynamic state of collagen: pathways of collagen degradation in vivo and their possible role in regulation of collagen mass. Am J Physiol 1987;252:C1–C9.
6. Sodek J, Overall CM. Matrix degradation in hard and soft connective tissues. In: Davidovitch Z, ed. The Biological Mechanisms of Tooth Eruption and Root Resorption. Birmingham: EBSCO Media, 1988;303–311.
7. Tyagi SC, Ratajska A, Weber KT. Myocardial matrix metalloproteinases: localization and activation. Mol Cell Biochem 1993;126:49–59.
8. Kodama S, Kishi J, Obata K, Iwata K, Hayakawa T. Monoclonal antibodies to bovine collagenase inhibitors. Coll Relat Res 1987;7:341–350.
9. Chakraborty A, Eghbali M. An immunoassay for qualitative estimation of collagenase activity in mammalian tissues. Clin Biochem 1979;24:455–461.
10. Whittaker P. Unraveling the mysteries of collagen and cicatrix after myocardial infarction. Cardiovasc Res 1995;29:758–762.
11. Takahashi S, Barry AC, Factor SM. Collagen degradation in ischemic rat hearts. Biochem J 1995;265:233–241.
12. Zhao M, Zhang H, Robinson TF, Factor SM, Sonnenblick EH, Eng C. Profound structural alterations of the extracellular collagen matrix in postischemic dysfunctional ("stunned") but viable myocardium. J Am Coll Cardiol 1987;10:1322–1334.
13. Cannon RO, Butany JW, McManus BM, et al. Early degradation of collagen after acute myocardial infarction in the rat. Am J Cardiol 1983;52:390–395.
14. Whittaker JPM, Boughner DR, Kloner RA. Role of collagen in acute myocardial infarct expansion. Circulation 1991;84:2123–2134.
15. Weisman HF, Bush DE, Mannisi JA, Weisfeldt ML, Healy B. Cellular

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November/December 1998:307–312

mechanisms of myocardial infarct expansion. Circulation 1988;78:186–201.

16. Hochman JS, Bulkley BH. Expansion of acute myocardial infarction: an experimental study. Circulation 1982;65(7):1446–1450.
17. Cleutjens JPM, Kandala JC, Guarda E, Guntaka RV, Weber KT. Regulation of collagen degradation in the rat myocardium after infarction. J Mol Cell Cardiol 1995;27:1281–1292.
18. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction;experimental observations and clinical implications. Circulation 1990;81:1161–1172.
19. Fishbein MC, Maclean D, Marco PR. Experimental myocardial infarction in the rat. Am J Pathol 1978;90:57–70.
20. Pfeffer MA, Pfeffer JM, Fishbein MC, Fletcher PJ, Spadaro J, Kloner RA, Braunwald E. Myocardial infarct size and ventricular function in rats. Circ Res 1979;44:503–512.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
22. Heussen C, Dowdle EB. Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Annal Biochem 1980;102:196–202.
23. Sato S, Ashraf M, Millard RD. Connective tissue changes in early ischemia of porcine myocardium: an ultrastructural study. J Mol Cell Cardiol 1983;15:261–275.
24. Factor SM, Robinson TF, Cho S. Alteration of the myocardial skeletal framework in acute experimental and clinical infarction with and without ventricular rupture. J Am Coll Cardiol 1985;5:401.
25. Newman KM, Malon AM, Shin RD, Scholes JV, Ramey WG, Tilson MD. Matrix metalloproteinases in abdominal aortic aneurysm characterization, purification, and their possible sources. Connective Tissue Research 1994;30:265–276.