Myocardial protection from ischemia/reperfusion injury by targeted deletion of matrix metalloproteinase-9

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Abstract

Objective: Matrix metalloproteinase-9 (MMP-9) activity is up regulated in the heart subjected to ischemic insult. Whether increased MMP-9 activity contributes to acute myocardial injury after ischemia–reperfusion remains unknown. To investigate the role of MMP-9 in myocardial infarction, we utilized a MMP-9 knockout mouse. Methods and results: Standard homologous recombination in embryonic stem cells was used to generate a mouse lacking MMP-9. The left anterior descending coronary artery was occluded for 30 min followed by 24 h reperfusion, and the ischemic and infarct sizes were determined. Targeted deletion of MMP-9 protected the heart from no-flow ischemia–reperfusion-induced myocardial injury. The myocardial infarct size was reduced by 17.5% in MMP-9 heterozygotes (1/2) (P,0.01) and 35.4% in MMP-9 knockout (2/2) mice (P,0.01) versus the wild-type (1/1) mice, respectively. Analysis of MMP activity in myocardial extracts by zymography demonstrated that ischemia–reperfusion-induced expression of proMMP-9 and active MMP-9 was reduced by 77.8% (P,0.01) and 69.1% (P,0.001), respectively, in (1/2) mice compared to (1/1) mice, and was absent in (2/2) animals. The expression of TIMP-1, an endogenous inhibitor of MMP-9, was elevated 4.7-fold (P,0.05) and 21.4-fold (P,0.05) in the (1/2) and (2/2) mice, respectively, compared to (1/1) mice. Immunohistochemical analysis revealed that neutrophils were the primary cellular source of MMP-9, and less neutrophils were detected in the ischemic region of the heart following ischemia–reperfusion in (2/2) mice compared to (1/1) mice. Measurement of myeloperoxidase activity, a marker enzyme of neutrophils, demonstrated a 44% reduction in neutrophils infiltrated into the ischemic myocardium in the (2/2) mice compared to the (1/1) mice (P,0.05). Conclusion: These results suggest that MMP-9 plays an important role in ischemia–reperfusion-induced myocardial infarction and MMP-9 could be a target for prevention or treatment of acute ischemic myocardial injury.

Keywords: Ischemia; Reperfusion; Infarction; Remodeling; Extracellular matrix

1. Introduction

Matrix metalloproteinases (MMPs) are a family of structurally related, zinc-containing enzymes that degrade various components of the extracellular matrix [1,2]. Increased expression and activity of MMPs have been identified in various pathological processes such as inflam-
mation, tumor metastasis and ischemic injury. However, the correlation of particular MMPs with specific diseases remains a key question.

MMP-9 is one of the MMPs expressed in the heart. Increased myocardial MMP-9 expression or activity has been found in a variety of experimental myocardial injuries such as the permanent coronary artery occlusion model in rat and rabbit [3,4], and the reperfusion injury model in porcine [5,6]. Up regulation of MMP-9 has also been demonstrated in failing human heart, indicating a possible role of MMP-9 in cardiomyopathy [7,8]. A recent chronic study in mice showed that targeted deletion of MMP-9 attenuated permanent coronary artery ligation-induced left ventricle enlargement and collagen accumulation, suggesting that MMP-9 plays a prominent role in cardiac remodeling after ischemic injury [9]. However, whether increased MMP-9 activity contributes to acute myocardial injury (infarction) following ischemia–reperfusion remains unexplored. Such information will have a significant impact both on the understanding of the basic biology of extracellular matrix turnover in acute myocardial injury, as well as on potential avenues for pharmacological approaches to the treatment of ischemic heart diseases and failure.

The present study was undertaken in an established mouse myocardial infarction model [10] to evaluate the influence of targeted deletion of MMP-9 on acute myocardial injury induced by no-flow ischemia–reperfusion in MMP-9-knockout (−/−) mice compared to the heterozygous (+/−) littermates and wild-type (+/+ ) mice.

2. Methods

2.1. Targeting of MMP-9 gene and generation of mutant mice

Gene targeting was performed in E14.1 ES cells by standard procedures [11], replacing exons 1–12 of the mouse MMP-9 locus (Accession X72794) with a positive selection cassette containing the neomycin phosphotransferase gene driven by the PGK promoter (Fig. 1A). Homology arms of 4.5 kb (5′) and 5.5 kb (3′) were cloned...
from a 129SVJ genomic BAC library and placed either side of the positive selection cassette. Homologous recombination in neomycin-resistant ES cells was confirmed by Southern blot of Xhol-digested genomic DNA using a 500-bp AccI–EcoRI restriction fragment as 5 prime external (which detects 11- and 8-kb bands at the wild-type and targeted locus, respectively) (Fig. 1B). Approximately 1 in 100 G418-resistant clones had undergone homologous recombination. Three targeted clones were injected into C57B16/J-derived blastocysts. Male chimeras were crossed with C57B16/J females to give N1F0 offspring, which were subsequently intercrossed to generate N1F1 offspring. Genotyping of N1F0 and N1F1 offspring was confirmed by the above Southern blot procedure. In addition, N1F0 offspring were successively back-crossed to C57B16/J females to generate N5FO mice. These were inter-crossed to create an N5F1 study population. Genotype analysis of mice during back-crossing and for the generation of the N5F1 study population was performed by PCR of tail DNA (Fig. 1C). Primers were designed to generate PCR products specific to the wild-type locus (E12, 5' primer exon 12 specific: 5'-TATCGCTTTGTCACCTTTCTG-3'; E13a and E13b, 3' primers exon 13 specific: 5'-AGCATGGAATAAGCCGTTTGCACACGT-3' or 5'-CAGTCAGGAGTGACAGTCCGAGTTAG-3' giving a product size of 1282 and 1394 bp, respectively) or targeted locus (N5'-1, neo gene specific 5' primer: 5'-TGCGGATGATGATGATCGCGGATGACGGTCAGG-3' and N3'-1, neo gene specific 3' primer: 5'-GCCATTATGGGATGACAGTCGTTC-3' giving a product of 800 bp; or N5'-2, neo gene specific 5' primer: 5'-GCCATTATGGGATGACAGTCGTTC-3' and N3'-2, neo gene specific 3' primer: 5'-GCCATTATGGGATGACAGTCGTTC-3' giving a product of 700 bp). Thirty cycles of 94°C (30s), 60°C (30s), and 72°C (90s) were used. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996. All experiments were conducted according to the requirements of the United Kingdom Animals (Scientific Procedures) Act (1986) and strictly conformed to the ethical standards of GlaxoSmithKline Pharmaceuticals.

2.2. Myocardial ischemia and reperfusion protocol

Male MMP-9 (+/+), (+/−) and (−/−) mice, ranging in age from 8 to 10 weeks, underwent myocardial ischemia–reperfusion as described in detail previously [10]. Briefly, the animals were anesthetized with pentobarbital sodium (60 mg/kg) and ventilated with a rodent ventilator. The electrocardiogram (ECG) and the body temperature of the mice were monitored throughout the experiment. Coronary artery occlusion (30 min) and reperfusion (24 h unless otherwise indicated) were induced by inflating and then deflating a nontraumatic balloon occluder that was fixed on the left anterior descending coronary artery. The successful performance of coronary occlusion and reperfusion was verified by visual inspection of color in the apex and typical ECG changes. The animals were sacrificed 24 h after reperfusion. The ischemic area was determined by negative staining with Evans blue and the infarct area was detected by triphenyltetrazolium staining, quantified by computerized videoplanimetry and expressed as a percentage of ischemic area as described previously [10].

2.3. Measurement of MMPs by zymography

The whole heart was excised from the MMP-9 (+/+), (+/−) and (−/−) mice subjected to ischemia–reperfusion or from sham-operated animals (n=4–7 per group and time point) and the vasculature, right ventricle free wall and atrial appendages were dissected away. The remaining left ventricle free wall was washed with cold PBS, snap frozen in liquid nitrogen, and stored at −80°C. Comparable samples were collected from sham-operated animals. For extraction, tissues were minced into 1-mm³ pieces and incubated with 0.5% Triton X-100 (Sigma) in PBS containing 0.01% sodium azide while gently rotating at 4°C for 18 h. The concentration of the extraction mixture for each sample was normalized to 400 mg/ml. After the extraction was complete, the samples were centrifuged at 14 000 rpm (10 min, 4°C) and the supernatants were collected. Protein concentration for each sample was determined with the BioRad DC Protein Assay kit.

MMP enzyme expression was analyzed for gelatinolytic activity by SDS–PAGE zymography as described previously [4]. Briefly, samples (100 µg) were subjected to electrophoresis, without boiling or reduction, through a gelatin affinity matrix. Gels were fixed with 40% methanol–7% acetic acid, stained with 0.1% Coomassie brilliant blue R250 and then destained with 10% methanol–7% acetic acid. Enzyme activity attributed to MMP-9 and MMP-2 was visualized as clear bands against a blue background. Standards (3 ng) of recombinant human MMP-9 and MMP-2 (Chemicon) were included on the gels for comparison and identification. Relative clearing of each sample was quantitated by determining the inverse optical density at each time point using a Kodak Image Station 440CF (NEN). Values are represented as the mean ± standard error and are presented as inverse optical density units relative to background.

2.4. Western blot analysis for MMP-9 and TIMP-1

Samples (100 µg, n=4–7 per group) were resolved by
electrophoresis through a 10 or a 12% polyacrylamide gel under reducing conditions and then transferred to a nitrocellulose membrane for MMP-9 and TIMP-1 analysis, respectively. After blocking overnight at 4°C with 5% nonfat powdered milk in 1.5 M NaCl, 0.5% Triton X-100, 0.1 M Tris–HCl, pH 8.0 (TBST buffer), the blot was incubated with a rabbit anti-MMP-9 (0.4 μg/ml, #AB19047, Chemicon) or mouse anti-TIMP-1 antibody (1 μg/ml, #IM32L, Oncogene Research), washed with TBST and then incubated with a goat anti-rabbit or mouse IgG secondary antibody conjugated to horseradish peroxidase (1:5000; Gibco-BRL). The blot was developed using the enhanced chemiluminescence method (Amersham) according to the manufacturer’s instructions. Relative levels of TIMP-1 expression were quantitated by determining the optical density units of each band using a Kodak Image Station 440CF (NEN).

2.5. Immunohistochemical analysis

Immunohistochemical staining for MMP-9 was performed using a rabbit anti-mouse MMP-9 polyclonal primary antibody (0.7 μg/ml, Chemicon). Prior to this, antigen retrieval was performed by incubating the tissue sections with proteinase K (Dako) for 3 min at room temperature. Immunohistochemical staining for neutrophils was performed using an adsorbed rabbit anti-mouse PMN polyclonal primary antibody (0.75 µg/ml, Inter-Cell Technologies). As a negative control, serial sections were incubated with rabbit IgG (0.7 μg/ml, Vector). For macrophage analysis, a rat anti-murine pan macrophage antibody (#T-2006, 1:100, BMA) and rat IgG (2 μg/ml, Sigma), as a negative control, were used. The sections were then incubated with a biotinylated goat anti-rabbit IgG secondary antibody. Additional sections were also stained with hematoxylin and eosin (H&E) using standard histological methods.

2.6. Measurement of myeloperoxidase (MPO) activity in cardiac tissue

MPO activity, an enzyme that is specific for neutrophils, was determined in ischemic cardiac tissue by the method of Bradley et al. [12] as described previously [13], and was used as an index of neutrophil accumulation. Briefly, cardiac tissue samples were homogenized in 0.5% hexadecyltrimethyl ammonium bromide (Sigma) and dissolved in 50 mmol/l potassium phosphate buffer (pH 6). Homogenates were centrifuged at 14 600 rpm and 4°C for 30 min. The supernatants were then collected and reacted with 0.167 mg/ml of o-dianisidine dihydrochloride (Sigma) and 0.0005% H₂O₂ in 50 mmol/l phosphate at pH 6.0. The change in absorbance at 460 nm was measured using a microplate reader (Molecular Devices, SPECTRAmax 190). One unit of MPO was defined as that quantity of enzyme that hydrolyzed 1 mmol of peroxide per minute at 25°C.
2.7. Statistical analysis

Data are expressed as mean±S.E.M. and analyzed by one-way ANOVA with subsequent post hoc paired comparison (Bonferroni) or by unpaired student’s t-test. Differences with a value of \( P<0.05 \) were considered statistically significant.

3. Results

3.1. Expression of MMP-9, MMP-2 and TIMP-1 in myocardium subjected to ischemia–reperfusion

Fig. 2A,B show the time-course of MMP-9 protein expression following myocardial ischemia–reperfusion in the MMP-9 (+/+ ) mice (n=4–7 per time point). Significant expression of proMMP-9 and active MMP-9 was detected after 30 min of ischemia and further upregulated following reperfusion. MMP-9 expression was absent in the sham (basal) control samples. ProMMP-2 was detected in all samples and was elevated significantly at 4 and 24 h after reperfusion (Fig. 2A,C). As shown in Fig. 3A, the expression of proMMP-9 and active MMP-9 was markedly attenuated (reduced 77.8±6.6% (\( P<0.01 \)) and 69.1±7.2% (\( P<0.001 \)), respectively) in (+/+ ) mice compared to (+/+ ) mice, and was absent in (−/−) mice in samples collected after 24 h of reperfusion (n=4–7 per group). The expression profile of MMP-9 in the three groups of mice was also confirmed by Western blot (Fig. 3B). Expression of proMMP-2 was comparable in the three groups of mice (Fig. 2A). It should be noted, though, that an intermediate between pro and active MMP-2 was also observed in the (+/+ ) mice. Further, TIMP-1, an endogenous MMP-9 inhibitor, was shown to be expressed in MMP-9 (+/+ ) mice after ischemia–reperfusion and was up-regulated (4.7±2.0-fold, \( P<0.05 \)) in (+/+ ) and (21.4±5.8-fold, \( P<0.05 \)) in (−/−) mice (Fig. 4A,B).

3.2. Neutrophils express MMP-9 in ischemic myocardium

Immunohistochemical analysis demonstrated that, based on morphological criteria and immunostain, MMP-9 was expressed in infiltrating neutrophils in the ischemic myocardium (Fig. 5). Immunostaining was also performed for macrophages to determine co-localization with MMP-9. Analysis was performed over the entire time-course (30-min ischemia and up to 24-h reperfusion), but macrophages were not detected during this acute phase of (data not shown).
Fig. 5. Immunohistochemical analysis of MMP-9 and a marker for PMNs in the heart from MMP-9 (+/+) mice subjected to ischemia–reperfusion (24 h). (A,B) MMP-9; (C,D) PMN; (E,F) IgG-negative control; (G,H) H&E stain. Original magnification: ×10 in left panels; ×20 in right panels. Arrowheads indicate enlarged region shown on right.
3.3. Targeted deletion of MMP-9 protects myocardium from ischemia–reperfusion injury

As shown in Fig. 6A left side, all three groups of mice displayed a similar-sized ischemic area, indicating that a comparable degree of ischemic jeopardy existed in (+/+), (+/−) and (−/−) mice. However, the myocardial infarct size (percent of ischemic area) was reduced from 65.3±1.6 in the (+/+) group (n=10) to 53.9±2.1 in the (+/−) group (17.5% reduction, n=9, P<0.01). The myocardial infarct size was further decreased in (−/−) group to 42.2±2.8 (35.4% reduction, n=8, P<0.01 versus both the (+/+) and (+/−) group) (Fig. 6A, right side, and B).

3.4. Targeted deletion of MMP-9 reduces neutrophil infiltration in the heart after ischemia–reperfusion injury

Immunohistochemical analysis for neutrophils indicated less neutrophils in the ischemic region of the heart following ischemia–reperfusion (24 h) in (−/−) mice compared to (+/+) mice (Fig. 7A). Specific quantitation of neutrophil infiltration was performed by biochemical analysis of MPO expression, a marker for neutrophils. The results demonstrated that MPO activity in the ischemic myocardium from (−/−) mice was reduced by 44% (P<0.05; n=8) compared to that from (+/+) mice (n=7), further indicating a significant reduction in neutrophils infiltrated into myocardium subjected to ischemia–reperfusion (Fig. 7B). The basal level of MPO activity was very low in the non-ischemic myocardium in both (−/−) and (+/+) groups (data not shown).

4. Discussion

Several recent studies which focused on chronic cardiac remodeling have demonstrated that MMP activation might contribute to the changes in left ventricular geometry and

Fig. 6. Targeted deletion of MMP-9 reduces ischemia–reperfusion-induced myocardial infarction. (A) Left side shows the ischemic areas, expressed as percent of left ventricle, of the three groups of mice. Right side is the infarction sizes, expressed as percent of ischemic area, of the three groups of mice. *P<0.01 versus MMP-9 (+/+ ) group; #P<0.01 versus MMP-9 (+/+ ) group (n=8–10). (B) Representative transverse heart sections with the ischemic area consisting of both the red and pale/white regions. The ischemic area was determined by negative staining with Evans blue and the infarct area was detected by triphenyltetrazolium staining as described in Section 2. Note that the area of infarction is the pale/white zone, indicative of dead tissue. The area not at risk (non-ischemic) is stained blue.

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Fig. 7. Analysis of neutrophil infiltration in MMP-9 (+/+ ) and (−/−) mice. (A) Immunohistochemical analysis of neutrophil infiltration (anti-PMN) in the heart in mice subjected to ischemia–reperfusion (24 h). (A,B) (+/+ ) mice; (C,D) (−/−) mice. Original magnification: ×10 in left panels and ×20 in right panels. Arrowheads indicate enlarged region shown on right. (B) Quantitative results of neutrophil infiltration based on biochemical analysis of MPO activity in heart samples collected from (+/+ ) mice (n=7) and (−/−) mice (n=8) subjected to ischemia–reperfusion (24 h). *P<0.05.
dysfunction that occur with the progression of dilated cardiomyopathy [14–16]. However, which MMP plays a role was not clear as broad-spectrum MMP inhibitors were used in these studies. In a mouse heart failure model, Ducharme et al. [9] reported that deletion of MMP-9 decreased end-systolic and -diastolic ventricular dimensions at 1–2 week after permanent ligation of coronary artery, suggesting a role for MMP-9 in chronic cardiac remodeling. However, the role of MMP-9 in acute myocardial injury is still unknown. The present study, using targeted gene deletion, has demonstrated that MMP-9 plays an important role in ischemia–reperfusion-induced myocardial infarction. To our knowledge, this is the first study that demonstrates the pivotal role of MMP-9 in acute myocardial injury.

As shown in Fig. 6, there was no difference in ischemic area, expressed as percent of left ventricle, among the three groups of mice, indicating that the animals in the three groups received a comparable degree of ischemic jeopardy. Therefore, the significant reduction of infarct size in the (−/−) and (+/−) mice compared with the (+/+)) mice was due to the targeted deletion of MMP-9. This conclusion was further supported by the fact that MMP-9 expression levels correlated with the myocardial infarct size among the three groups of mice (Fig. 6). The results clearly indicate that deficiency of MMP-9 provides a protective effect against ischemia–reperfusion-induced myocardial infarction.

Expression of MMP-9 in the heart has been demonstrated in previous studies [5–8]. However, the cellular source of MMP-9 in the heart is not clear. Our study has demonstrated that infiltrating neutrophils are the major source of MMP-9 in the reperfusion-injured heart (Figs. 5 and 7). Our data are in accordance with a recent study published while this manuscript was in preparation [17]. The study, conducted in canine, indicated neutrophils as the primary source of MMP-9 in ischemia–reperfusion-injured myocardium. The expression and secretion of MMP-9 by neutrophils would aid in their migration, contribute to tissue destruction and activate inflammatory mediators that occur following reperfusion. It is conceivable that attenuation of neutrophil function is an important mechanism for myocardial protection by targeted deletion of MMP-9. In our acute model, we were unable to detect significant macrophage infiltration. However, it has been reported by Ducharme et al. [9] that macrophages infiltrate the heart tissue at about 4 days of reperfusion. It is probable that co-localization of MMP-9 with macrophages was not evident because only early time-points relevant to an acute situation were studied.

In addition to showing changes in MMP activity, the results demonstrated that the expression of an endogenous tissue inhibitor of MMP-9, TIMP-1, was up-regulated 24 h post-ischemia–reperfusion in the heart tissue of the (+/−) and (−/−) mice relative to the (+/+)) mice. These results suggest that the tissue protection observed in the (+/−) and (−/−) mice may also be due, in part, to the elevated expression of TIMP-1 in addition to the lack of MMP-9. In diseases where matrix degradation and inflammatory cell influx occur, the balance between MMPs and TIMPs is often offset resulting in an overall net increase in MMP activity. Changes in MMP expression and the imbalance between proteinases and proteinase inhibitors have been noted in cardiovascular diseases including ischemic cardiomyopathy [18], cardiac dilation and heart failure [7,19,20], acute coronary syndromes [21], and cardiac rupture after acute myocardial infarction [22]. Of importance, in the (−/−) mice the balance is offset in favor of proteolytic inhibition, thus contributing to the positive effect observed. Notably, our study was designed to investigate the role of MMP-9 in the acute phase of myocardial ischemia and reperfusion. Indeed, targeted deletion of MMP-9 and the up-regulation of TIMP-1 resulted in reduced infarct size when analyzed 24 h following ischemic injury. Similarly, others have demonstrated that targeted deletion of MMP-9 and up regulated TIMP-1 are also beneficial in chronic myocardial injury [9]. Conversely, expression of other MMPs such as MMP-2 have been shown to be important in myocardial healing that occurs in the later phases after injury [9,22].

In conclusion, our results suggest that MMP-9 plays an important role in ischemia–reperfusion-induced acute myocardial infarction. Attenuation of neutrophil activity and proteolytic-mediated tissue damage by targeted deletion of MMP-9 could contribute to this protection. MMP-9 may be a target for prevention or treatment of acute ischemic myocardial injury.

References

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