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Effect of Matrix Metalloproteinase Inhibition by Doxycycline on Myocardial Healing and Remodeling after Myocardial Infarction

Ariel Tessone [1], *Micah S. Feinberg* [1], *Israel M. Barbash* [1], *Reuven Reich* [2], *Radka Holbova* [1], *Michal Richmann* [2], *Yael Mardor* [3], *and Jonathan Leor* [1]

[1]*Neufeld Cardiac Research Institute, Tel-Aviv University, Sheba Medical Center, Tel Hashomer, Israel;* [2]*Department of Pharmacology, School of Pharmacy, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel;* [3]*Advanced Technologies Institute, Sheba Medical Center, Tel Hashomer, Israel*

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Summary. **The aim of conducting this study was to assess the clinical relevance of matrix metalloproteinase (MMP) inhibition by doxycycline, an effective MMP inhibitor, in a rat model of extensive myocardial infarction (MI) and left ventricular (LV) dysfunction. Rats ($n = 22$) were subjected to extensive anterior MI. Doxycycline (25 mg SC, daily) or saline (control) injections were started for nine days thereafter. The effect of doxycycline on MMP activity in the infarcted and remote myocardium was measured by zymography, in another subgroup ($n = 8$), nine days after MI. Echocardiography and magnetic resonance imaging (MRI) studies were performed at one and thirty days after MI to assess LV remodeling and function. After 4 weeks, hearts were fixed, and subjected to morphometric and histological analysis. Compared with control, doxycycline treatment attenuated MMP-9 and -2 activity in both infarcted and remote myocardium. Serial echocardiography studies showed that doxycycline failed to attenuate scar thinning, LV dilatation and dysfunction. MRI study showed that doxycycline impaired LV compensatory hypertrophy. Furthermore, compared with control, doxycycline reduced vessel density (/mm^2 ± SEM) in the infarcted myocardium (84 ± 16 vs. 46 ± 9/mm^2, respectively; $p < 0.05$).**

Our work suggest that effective MMPs' inhibition in the infarcted and remote myocardium by doxycycline does not prevent LV remodeling and dysfunction but impairs angiogenesis and compensatory LV hypertrophy. Our findings caution against aggressive, non-selective inhibition of MMPs in the early healing phase after MI.

Key Words. **angiogenesis, heart failure, myocytes, remodeling**

Introduction

Prevention of adverse cardiac remodeling and progressive dysfunction is a major goal after myocardial infarction (MI). However, current anti-remodeling therapies are clearly limited, as many ventricles continue to enlarge after MI and mortality and morbidity remain significantly high [1–4].

Increased expression and activation of matrix metalloproteinases (MMPs) have been identified in myocardial remodeling processes associated with myocardial infarction [5–9]. The MMPs are a family of enzymes that contribute to ventricular remodeling and heart failure by promoting extra cellular matrix (ECM) degradation [9,10]. Adverse ECM remodeling leads to impaired structural support, LV dilatation, increased wall stress, and both systolic and diastolic dysfunction [1–3]. Results from animal models have suggested that MMPs inhibition or deletion could prevent LV remodeling and dysfunction [6,8,11]. Subsequently, pre-clinical experiments have indicated the need for a safe and effective MMP inhibitors to treat cardiovascular diseases in humans [12].

The possibility of using doxycycline antibiotic as MMP inhibitor in our heart failure patients is attractive. Doxycycline is a common tetracycline antibiotic, and has a unique property of broad spectrum MMP inhibition [13] and amelioration of ischemia/reperfusion injury in the setting of MI [14]. Recent reports have showed that pretreatment—two days before MI—attenuated cardiac dilatation and improved endothelial function in rat [5,15]. The tetracycline derivative Periostat (doxycycline) is the only MMP inhibitor currently approved for clinical use, but its application is limited to periodontal disease. Treatment of coronary heart disease patients with Periostat reduced serum

Address for correspondence: Jonathan Leor, MD, Neufeld Cardiac Research Institute, Sheba Medical Center, Tel-Hashomer 52621, Israel. Tel.: 972-3-530-2614; 972-3-534 8685; Fax: 972-3-535 1139; E-mail: leorj@post.tau.ac.il

inflammatory markers as well as circulating concentrations of MMP-9 [16]. Considering its safety, efficacy, and cost, clinical trials of doxycycline in myocardial remodeling appear worth pursuing [16]. Thus, the aim of conducting this study was to test the clinical relevance of MMP inhibition by doxycycline started early after MI and its therapeutic effect upon infarct healing, scar formation, LV remodeling and dysfunction.

Methods

Our study was approved by Tel-Aviv University Ethic Committee and 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).

Rat model of MI

Our MI model was previously described [17]. Male Sprague-Dawley rats (~250 g) were anesthetized with a combination of 40 mg/kg ketamine and 10 mg/kg xylazine, intubated and mechanically ventilated. The chest was opened by left thoracotomy, the pericardium was removed and the proximal left coronary artery was permanently occluded with an intramural stitch.

Experimental groups

Rats were treated with either subcutaneous injections of doxycyclin hydrochloride (Sigma-Aldrich D9891) 12.5 mg b.i.d. (total 25 mg) or saline (control) 0.5 ml bi.d. at the first day after induced MI and continued for 9 days. The dose of doxycycline and mode of delivery was selected based on previous report [13], and found to inhibit MMP activity in a pilot study. The time of doxycycline administration was based on temporal activity of MMPs in the infarcted myocardium of rat [18].

Tissue MMP activity by zymography

The inhibitory effect of doxycycline on MMP activity in the infarcted and remote myocardium was assessed in a separate group of rats ($n = 8$) on substance impregnated gels as previously described [19]. Nine days after MI, the treated and control rats were killed with overdose of phenobarbital, the hearts were excised and the infarcted and remote viable areas were quickly dissected and frozen. Hearts from 3 normal rats (without MI) served as a reference group for MMP activity. Samples were separated on gelatin-impregnated (1 mg/ml, Difco, Detroit, MI), SDS-8% polyacrylamide gels under non-reducing conditions, followed by 30 min shaking in 2.5% Triton X-100 (BDH, UK). The gels were then incubated for 16 h at 37°C in 50 mM Tris, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35 (w/v) at pH 7.6. At the end of the incubation, the gels were stained with 0.5% Coomassie G 250 (Bio-Rad, Richmond, CA) in methanol/acetic acid/H_2O (30;10;60). The intensity of the various bands was determined on a computerized densitometer (Molecular Dynamics type 300A). MMP activity is presented as a multiplication of normal MMP activity in intact heart: Normal heart MMP activity is expressed as one unit.

Echocardiography to evaluate remodeling and function

Transthoracic echocardiography was performed on all animals one day after MI, before randomization (baseline echocardiogram), and 4 weeks after. Previous reports have demonstrated the accuracy and reproducibility of transthoracic echocardiography in rats [17]. Echocardiograms were performed with a commercially available echocardiography system equipped with 12-MHz phased–array transducer (Hewlett Packard, Andover, Massachusetts) as previously reported [17]. We measured LV anterior wall thickness, maximal LV end-diastolic dimension; minimal left ventricular end-systolic dimension in M-mode and 2-D imaging; and fractional shortening (FS) as a measure of systolic function which was calculated as FS (%) = [(LVIDd−LVIDs)/LVIDd]×100, where LVID indicates LV internal dimension, s is systole, and d is diastole. Index of change in LV area (%) was calculated as [(EDA−ESA)/EDA]×100 where EDA indicates LV end diastolic area, ESA indicates LV end systolic area [11]. All measurements were averaged for 3 consecutive cardiac cycles and were performed by an experienced technician who was blinded to the treatment group.

Magnetic resonance imaging

Interventional magnetic resonance imaging (iMRI) studies were performed on 14 animals one day after MI (before randomization) and four weeks after MI. Our method was previously described and has good correlation with echocardiography [20]. Briefly, the rats were anesthetized with a combination of 50 mg/kg ketamine and 10 mg/kg xylazine and. iMRI data was obtained using the 0.5T GE iMRI with a specially constructed animal probe. In these experiments, the T1-weighted images were averages of heart systole and diastole that provided excellent details and morphology. We studied the whole LV area (mm^2), LV cavity area (mm^2), and LV muscle area (mm^2).

Histology

Rats were sacrificed, after four weeks, with an overdose of pentobarbital followed by KCl to ensure maximal myocardial relaxation. The hearts were then perfused with formaldehyde (30 mmHg) for 30 min and then embedded in paraffin, sectioned into 5 μm slices 5 mm from the heart's apex and stained for hematoxylin & eosin, Masson's trichrome or immunolabelled with α-smooth muscle actin antibodies.

Slices immunolabelled for α-smooth muscle actin were used to identify pericytes and arterioles in the scar tissue. We averaged the number of small blood vessels per 1 mm^2 in nine random fields from the scar area in each heart.

Statistical analysis

All values are shown as mean ± SE. Because each rat in both groups was used as its own control, changes in echocardiography or MRI measurements between baseline and 4 weeks in treated and control groups were assessed with paired t tests (InStat, Version 3.01; GraphPad Software Inc.). Differences in vessel density between treated and control groups were compared by unpaired t test. All tests were 2 tailed and significance was accepted at $p < 0.05$.

Results

The immediate mortality rate associated with the surgical procedure to induce MI was 31% (17 of 55). Overall, 38 rats were included in the final analysis. Of the 38 rats that survived the operation, 24 (63%) had MI based on echocardiography, gross macroscopic and histological examinations (14 rats had no evidence of MI and were not included in the analysis). The survival rate after 1 month was similar in the doxycycline group compared to control (8 of 9 vs. 6 of 7; $p = 0.8$).

The echocardiograpic and MRI studies were performed in 16 rats. The remaining 11 rats were part of study to determine MMP activity in the infarcted and remote myocardium.

Myocardial MMP activity

Compared with remote viable myocardium, activity of MMP-9 and MMP-2 was significantly higher in the infarcted tissue in both control and treated groups (Figs. 1–3). Doxycycline inhibited MMP-9 activity in both infarcted tissue and remote myocardium (Fig. 2, 2.03 ± 0.09 vs. 3.02 ± 0.15 AU; $p = 0.006$ and 0.83 ± 0.09 vs. 1.37 ± 0.15 AU; $p = 0.02$; respectively). In addition, doxycycline inhibited significantly MMP-2 activity in the infarcted area (Fig. 3; $p = 0.04$) and to lesser extent in the viable myocardium ($p = 0.06$).

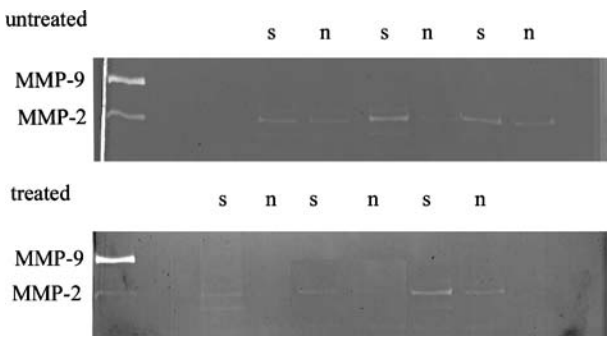

Fig. 1. *Representative MMP activity by zymography in infracted (marked 'S'; scar tissue) and remote (marked 'N'; normal tissue) myocardium. Doxycycline inhibited MMP-9 and MMP-2 in both infarcted and remote myocardium.*

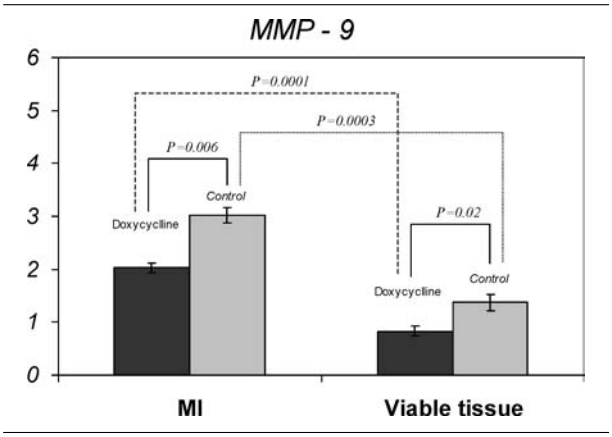

Fig. 2. *Relative MMP-9 activity, by zymography, at nine days after myocardial infarction in the infarcted and remote myocardium. Doxcycycline decreased MMP-9 activity in both the infarcted and remote myocardium.*

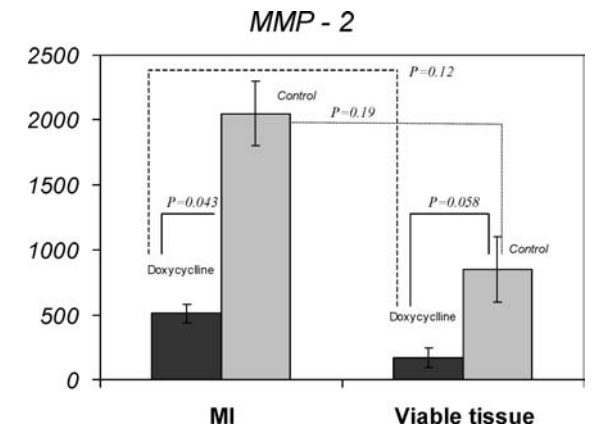

Fig. 3. *MMP-2 activity, by zymography, at nine days after myocardial infarction, in the infarcted and remote myocardium. Doxcycycline decreased MMP-2 activity in the infarcted myocardium and the remote tissue.*

Echocardiography functional study

Serial echocardiograpy studies revealed that the control group displayed a typical course of LV remodeling. We observed a significant increase in LV diastolic and systolic internal dimensions and scar thinning (Table 1). LV end-diastolic and systolic cavity areas were increased significantly by 67% and 50% respectively (Table 1). This course is similar to that observed in untreated human patients after extensive anterior MI.

In treatment group, doxycycline did not attenuate LV remodeling. Furthermore, compared with control, remodeling parameters in doxycycline-treated animals showed greater relative deterioration from baseline (Table 1), e.g. LV end diastolic and systolic dimension increased by 39% vs. 28% and 44% vs. 28%,

Table 1. *Results of echocardiography study*

	Doxycycline ($n = 8$)			Control ($n = 6$)		
Mean ± SEM	Day 1	Day 30	p	Day 1	Day 30	p
Heart rate (mean ± SD)	341 ± 28	272 ± 40	0.004	300 ± 22	246 ± 27	0.06
LV AW dia cm	0.08 ± 0.01	0.07 ± 0.02	0.3	0.08 ± 0.01	0.07 ± 0.01	0.2
LV AW sys cm	0.11 ± 0.012	0.09 ± 0.02	0.1	0.10 ± 0.02	0.09 ± 0.02	0.6
LVEDD cm	0.67 ± 0.09	0.93 ± 0.07	<0.0001	0.75 ± 0.08	0.96 ± 0.1	<0.01
LVESD cm	0.5 ± 0.1	0.72 ± 0.11	<0.0001	0.6 ± 0.1	0.77 ± 0.09	<0.01
LVED area (cm^2)	0.36 ± 0.09	0.61 ± 0.1	<0.0001	0.42 ± 0.1	0.7 ± 0.17	<0.01
LVES area (cm^2)	0.2 ± 0.08	0.4 ± 0.1	<0.0001	0.3 ± 0.1	0.46 ± 0.13	0.01
LV FS %	26 ± 10	23 ± 8	0.3	18 ± 7	20 ± 5	0.4
LV mass (g)	0.4 ± 0.035	0.54 ± 0.02	<0.05	0.39 ± 0.03	0.68 ± 0.08	<0.05

Doxycycline treatment does not attenuate left ventricle remodeling one month after myocardial infarction. left ventricle end diastolic, end systolic dimension and area are significantly increased both in the control group and in the treatment group.
LVESD—left ventricle end systolic dimension; LVEDD—left ventricle end diastolic dimension; FS—fractional shortening; AW—anterior wall.

respectively. The only exception was cardiac mass which was increased in controls more than doxycycline—treated group: 74% vs. 35%. In addition, MMP inhibition did not prevent scar thinning (Table 1).

MRI remodeling study

Table 2 compares MRI data between groups at day one and 30 days after MI. In both groups, we found a significant LV cavity dilatation 30 days after MI. However, while there was a significant compensatory LV hypertrophy, indicated by muscle area, in the control group ($p = 0.006$, Fig. 4), it was attenuated in doxycycline group.

Histological and immunohistological analysis

Area of fibrosis, as indicated by Masson Trichrome blue staining, was 17.2 ± 2% in treated group and 22 ± 2% in control group (Fig. 5; $p = 0.1$). Surprisingly, compared with control, doxycycline decreased significantly vessel density (vessels/mm^2 ± SE.)- in the infarcted myocardium (84 ± 16 vs. 46 ± 9/mm^2, respectively; $p < 0.05$).

Discussion

The main new finding of the present study suggests that effective inhibition of MMPs' activity by doxycycline does not prevent LV remodeling and dysfunction after MI. Contrary to our expectations, doxycycline impairs angiogenesis and compensatory LV hypertrophy in the infarcted hearts as compared with controls. Thus, our findings **suggest** a critical role of MMPs in infarct repair and caution against aggressive, and nonselective inhibition of MMPs in the early healing phase after MI.

Comparison with previous reports

It has been suggested that MMPs play a role in myocardial remodeling and dysfunction [5–9]. Increased MMP activity and consequent ECM degradation favors myocyte slippage, with reduced myocyte-to-myocyte mechanical coupling and dilation.

Studies in animals have investigated the effects of MMP inhibition with pharmacological agents or by gene deletion in transgenic animals in models of cardiac injury and failure [5–8]. For example, Rohde et al. showed that a nonselective pharmacological MMP inhibitor (CP-471 474) reduced LV dilatation four days following surgical infarction in mice [21]. However, follow-up at later time intervals (15 days) post-MI in MMP-9-deficient mice showed defective wound healing with diminished collagen accumulation in the infarct zone and decreased infiltration of macrophages compared to wild type [22].

MMP-2 is prominently over expressed after MI and in heart failure and target deletion of MMP-2 resulted in better outcome. Hayashidani et al. [11] have reported significantly better survival rate after MI

Table 2. *Results of iMRI study at one month after myocardial infarction*

	Doxycycline ($n = 8$)			Control ($n = 6$)		
Mean ± SEM	Day 1	Day 30	p	Day 1	Day 30	p
LV cavity area (mm^2)	39 ± 3	66 ± 4	<0.0001	43 ± 2	76 ± 10	0.01
LV muscle area (mm^2)	102 ± 3	103 ± 3	0.5	92 ± 6	112 ± 6	0.01

Doxycycline does not attenuate left ventricular dilatation, both groups exibit the same pattern of left ventricle lumen enlargement. However doxycyclline does prevent compensatory hypertrophy as seen in the treatment group.

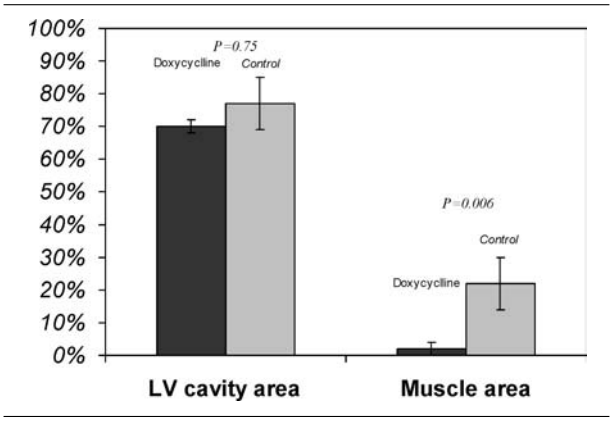

Fig. 4. *Changes in left ventricle cavity and muscle area by MRI study at one month after myocardial infarction. Compared with control, doxycycline attenuated the increase in left ventricle muscle area.*

in MMP-2 knockout (KO) mice as compared with wild type mice. Notably, despite similar infarct size, the MMP-2 KO mice had a significantly lower incidence of LV rupture, less LV cavity dilatation and improved fractional shortening after MI.

Doxycycline has been shown to inhibit MMPs activity in a number of pathological scenarios *in vivo* [5,14,15,23]. In this study, the dose of doxycycline and mode of delivery were selected based on previous report [13], and found to inhibit effectively MMP activity in the infarcted heart. The time of doxycycline administration was based on temporal activity of MMPs in the infarcted myocardium of rat [18].

Our finding contradict several previous reports about the therapeutic potential of broad inhibition of MMPs. Villaarreal et al. [5] have shown that pretreatment (48 h before MI) with doxycycline prevent LV remodeling and dysfunction in rat model. These conflicting findings could be explained by several differences between experimental designs. Villaarreal et al. [5] gave doxycycline orally, in two doses of 30 mg/kg per day, starting 48 h before and continued up to 36 h after MI. Our protocol might be more aggressive: we gave doxycycline at total dose of 25 mg IP, started after MI, for nine days. They assessed LV function by isolated heart system and remodeling was assessed by post mortem morphometry and vessel density was not reported. We assessed LV remodeling and function by serial echocardiography studies, MRI and post mortem morphometry.

Potential risk of MMP inhibition after MI

A significant finding of our work is lower vessel density in the treated hearts compared with controls. This disappointing finding suggests that MMP inhibition by

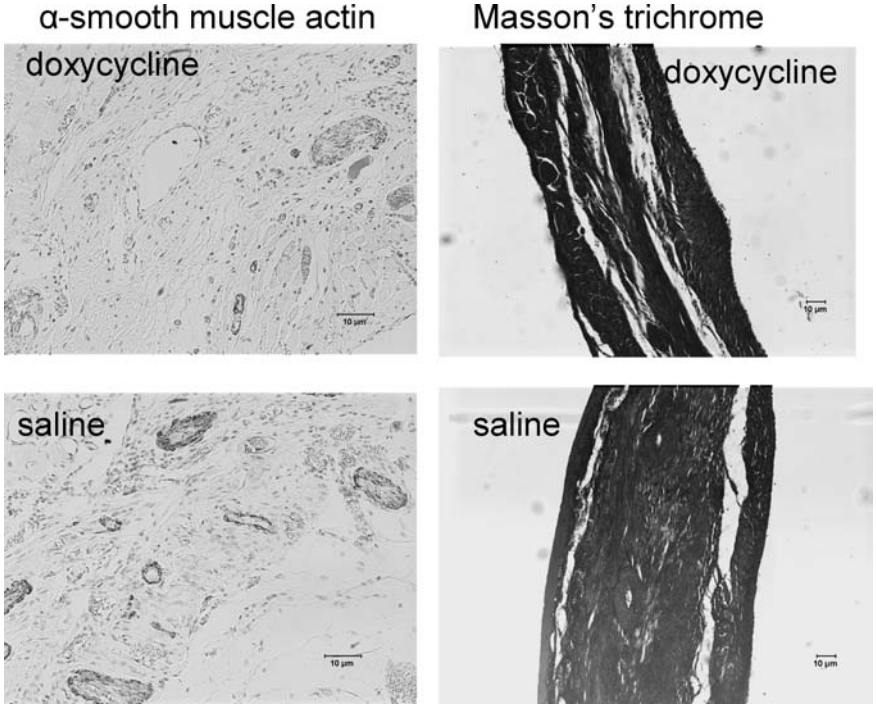

Fig. 5. *Representative left ventricle myocardial sections. Tissue samples were taken from mid-myocardium transverse section (5 mm from the apex) and contain mid scar area from parallel regions in the left ventricle wall of saline and doxycycline-treated hearts. Parallel slides were processed for smooth muscle α-actin immunostaining (×200) and Masson Trichrome staining (×100). The collagen stained blue and the host myocardium stained deep red. Blood vessels are stained positive (brown color) by smooth muscle α-actin antibodies. Compared with control, doxycycline reduced blood vessel density.*

doxycycline may have harmful effect on angiogenesis (formation of capillaries and arterioles) and tissue healing and repair, and might partially explain the overall negative results in the treated group. Lamparter et al. [24] have shown that doxycycline exerts an inhibitory effect on tissue formation in a tissue repair model in rat. The inhibition is mediated by MMP-2 inhibition and through its attenuation of angiogenesis and modulations of collagen turnover.

MMP-9 is crucial for adequate healing of injured tissues. Angiogenesis is a critical step in healing, remodeling and regeneration after MI. MMPs are proangiogenic [25,26] and promote endothelial migration and angiogenesis [27,28]. Furthermore, MMP-9 is involved in bone-marrow stem cell mobilization and mediated matrix alterations that may create a favorable tissue environment for stem cell migration [26,29]. MMP-9 also plays documented roles in stromal derived factor (SDF)-1, a stem cell chemokine, degradation and stem cell mobilization from the marrow. In the setting of liver injury, MMP-9 is associated with increased CXCR4 expression on CD34+ cells, and MMP inhibitors reduce homing of these cells to the liver [30]. Johnson et al. [31] have showed that matrix MMP-9 is critical for adequate angiogenesis of ischemic tissues. Recent studies performed in mice have demonstrated that early disruption of MMP expression in an evolving MI may be deleterious to the normal wound-healing process [32,33]. Taken together, MMP-9 seems to have a significant role in healing and regeneration after MI and therefore, broad MMP inhibition might interfere with healing and regeneration [34].

Our data add strength to the findings of Heymans et al. [33] and of Creemers et al. [32] who showed that disruption of plasminogen activity (which in turn could activate MMP enzymes) attenuates healing after MI. Long- term inhibition of *u-PA* gene function, that regulate MMP-9 activation, impaired infarct healing, predisposed to cardiac failure under adrenergic stress and prevented myocardial angiogenesis. However, temporary PA/MMP inhibition by adenoviral gene transfer prevented rupture without aborting infarct healing and therefore may constitute an alternative strategy. [33] Subsequently, Lindsey et al. [25] have showed, in rabbit MI model, that selective MMP inhibitor, that does not inhibit MMP-1, can reduce LV remodeling while promoting angiogenesis. Yarbrough et al. [35] suggest that MMP-1 and MMP-7 have protecting effects on the post MI myocardium, and that post MI MMP inhibition should spare these two MMPs. We did not measure MMP-7, however our findings suggest that selective inhibition is warranted.

Another unexpected finding is that doxycycline prevents compensatory hypertrophy as indicated by echocardiography (cardiac mass) and MRI (muscle area) study. Remodeling involves myocyte hypertrophy, fibrosis and alterations in ventricular architecture to distribute the increased wall stress more evenly to stabilize the distending forces and prevent further cardiac deformation. Reduction in LV mass and thickness after MI might increase LV wall stress and LV dysfunction. It is possible that doxycycline-induced impairment in angiogenesis and healing contributes to impaired compensatory hypertrophy and cardiac dysfunction.

The results of the our study are supported by the preliminary results of the Prevention of MI Early Remodeling (PREMIER) trial, presented at the American College of Cardiology 2005 Scientific Sessions [36]. The MMP inhibitor PG-116800, an MMP inhibitor with high affinity for MMPs 2, 3, 8, 9, 13 and 14, did not reduce remodeling in post MI patients with heart failure and LV dysfunction. In the PREMIER trial, 250 patients with ST-elevation MI were randomized to treatment with PG-116800 or placebo 48 h after reperfusion therapy. Patients underwent echocardiography at baseline and at 90 days to assess remodeling. The primary end point was change in left ventricular end diastolic volume index at 90 days after MI. Results showed that this was not different between the two groups, and there was no signal of any difference in clinical events [36].

Limitations

One may speculate that lower doxycycline dose or shorter treatment period could produce favorable results. However, other reports have suggested that higher doxycycline dose and longer or sustained broad inhibition of MMPs resulted in therapeutic effect [5,15,37,38]. The optimal therapeutic dose of doxycycline is uncertain and it is possible that different dose would yield better outcome. Our results show that doxycycline treatment may have negative effect on both muscle mass and vessels density. We suggest that the inhibitory effect of doxycycline on MMP activity explains these findings. However, it is possible that other properties of doxycycline, such as inhibition of protein and collagen synthesis [39], may have adverse effect on LV remodeling. Finally, broad spectrum MMP inhibition may increase MMP-9 production as a part of a feedback regulation [12,40]. Thus, earlier measurement of MMP-9 levels could demonstrate more effective inhibition.

Summary implications and future research

The present study suggest that broad inhibition of MMPs, by doxycycline, in the early phase after MI might impair angiogenesis, compensatory LV hypertrophy and does not attenuate LV dilatation and dysfunction. Our work adds to the evidence that MMPs are involved in angiogenesis and infarct healing and are compatible with the unfavorable preliminary results of a recent clinical trial on post MI MMP inhibition.

The unfavorable findings could be related to the specific dose and timing of doxycycline administration. Still, there are many questions to be answered, such as

which MMP to target and the optimal timing of MMP inhibition. Based on our findings, caution is advised when applying aggressive non-selective MMP inhibition early after MI.

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