

# Role of high-mobility group box 1 protein in post-infarction healing process and left ventricular remodelling

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## KEYWORDS

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**Aims** High-mobility group box 1 protein (HMGB1) is one of the recently defined damage-associated molecular pattern molecules derived from necrotic cells and activated macrophages. We investigated clinical implications of serum HMGB1 elevation in patients with acute myocardial infarction (MI). Then, we evaluated the effect of HMGB1 blockade on post-MI left ventricular (LV) remodelling in a rat MI model. **Methods and results** Serum HMGB1 levels were examined in patients with ST-elevation MI ( $n = 35$ ). A higher peak serum HMGB1 level was associated with pump failure, cardiac rupture, and in-hospital cardiac death. Then, an experimental MI model was induced in male Wistar rats. The mRNA and protein expression of HMGB1 were increased in the infarcted area compared with those values observed in sham-operated rats. We administered neutralizing anti-HMGB1 antibody (MI/anti-H) or control antibody (MI/C) to MI rats subcutaneously for 7 days. The mRNA levels of tumour necrosis factor- $\alpha$  and interleukin-1 $\beta$  and the number of macrophages in the infarcted area were reduced on day 3 in MI/anti-H rats compared with MI/C rats. Interestingly, HMGB1 blockade resulted in thinning and expansion of the infarct scar and marked hypertrophy of the non-infarcted area on day 14. **Conclusion** Elevated serum HMGB1 levels were associated with adverse clinical outcomes in patients with MI. However, HMGB1 blockade in a rat MI model aggravated LV remodelling, possibly through impairment of the infarct-healing process. HMGB1, a novel predictor of adverse clinical outcomes after MI, may have an essential role in the appropriate healing process after MI.

## 1. Introduction

Left ventricular (LV) remodelling after myocardial infarction (MI) is the process of infarct expansion followed by progressive LV dilation and is associated with adverse clinical outcomes.<sup>1</sup> Inflammatory response and cytokine elaboration are integral components of the host response to tissue injury and play a particularly active role after MI.<sup>2–5</sup> A well-orchestrated inflammatory response after MI leads to an appropriate infarct-healing process and the formation of a scar with tensile strength, resulting in prevention of infarct expansion. Despite the importance of the inflammatory response and healing process in post-MI LV remodelling, the mechanisms that initiate and control these processes remain to be elucidated.

High-mobility group box 1 protein (HMGB1) was originally identified as a non-histone DNA-binding nuclear protein produced by nearly all cell types; it stabilizes nucleosomes and enables bending of DNA, which facilitates gene transcription.<sup>6,7</sup> Recently, it was clarified that HMGB1 is one of the damage-associated molecular pattern molecules and is released passively as an endogenous danger signal from necrotic, but not apoptotic cells.<sup>7,8</sup> Extracellular HMGB1 exhibits inflammatory cytokine-like activity and acts as a potent mediator of macrophage activation.<sup>7,9</sup> HMGB1 is also secreted extracellularly by activated macrophages in response to pro-inflammatory cytokines.<sup>7,10</sup> Therefore, HMGB1 has a unique ability to self-amplify and prolong inflammatory response and contributes to the pathogenesis of sepsis and acute lung injury.<sup>7,9–12</sup> We hypothesized that HMGB1 might be involved in post-MI inflammatory response and LV remodelling.

Previous clinical and experimental studies have demonstrated that the use of anti-inflammatory agents after MI

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leads to infarct expansion through the impairment of the healing process,<sup>13,14</sup> suggesting that the appropriate inflammatory response might be a prerequisite for the prevention of post-MI LV remodelling. On the other hand, an excessive and persistent inflammatory response could cause the myocardium to become vulnerable through additional recruitment of inflammatory cells and degradation of extracellular matrix constituents.<sup>15,16</sup> The vulnerable myocardium might be susceptible to wall stress, resulting in infarct expansion. Therefore, it is unclear whether the attenuation of the post-MI inflammatory response by HMGB1 blockade could be beneficial or harmful to the infarct-healing process and post-MI LV remodelling.

This translational study examined the involvement of HMGB1 in the pathogenesis of post-MI LV remodelling. We first examined the clinical significance of serum HMGB1 elevation in patients with MI. We then evaluated the expression level and distribution of HMGB1 in the infarcted myocardium and assessed whether blockade of HMGB1 could modulate LV remodelling in a rat MI model.

## 2. Methods

### 2.1 Patients

This clinical investigation was approved by the institutional medical Ethics Committee and conducted according to the ethical guidelines outlined in the Declaration of Helsinki. A total of 49 consecutive patients with first ST-elevation MI (STEMI) were examined. All patients were admitted to Keio University Hospital between January 2006 and December 2006. A diagnosis of STEMI was made on the basis of chest pain lasting  $\geq 30$  min, the presence of new ST-segment elevation (at least 1 mV in two or more standard leads

or at least 0.2 mV in two or more contiguous precordial leads), and an increase in a biochemical marker of myocardial necrosis [creatinine kinase (CK)-MB fraction or troponin T]. We excluded patients in whom the time elapsed from onset to admission was  $>24$  h (six patients) and those who died before the determination of the peak HMGB1 level (three patients). Patients with collagen disease, advanced liver disease, renal failure, malignancy, or any infectious disease were also excluded (five patients). Finally, 35 patients were included in this study. For comparison, blood samples were also collected from 35 patients who were admitted to the same institute with chronic stable angina (CSA). CSA was defined as the presence of known coronary atherosclerosis with typical exertional chest pain relieved by rest and/or nitrates, and without a change in the frequency or pattern for 3 months before study entry.

### 2.2 Clinical study protocol

We assessed the clinical parameters listed in *Table 1*. All patients with revascularization therapy received percutaneous coronary intervention on admission. We measured the serum HMGB1 level on admission in patients with MI and CSA, and then 6, 12, 18, 24, 72 h and 7 days after admission in patients with MI. Serum HMGB1 concentration was measured by enzyme-linked immunosorbent assay (ELISA) (Shino-Test Corporation, Sagami-hara, Japan).<sup>17</sup> We determined cumulative CK release, which was defined as the area under the time-activity curve for CK concentration over the first 24 h.<sup>18</sup> We also measured the serum C-reactive protein level on admission, and then every 24 h for at least 4 days to determine its peak value.<sup>19</sup> The incidence of in-hospital complications, including pump failure (class II or greater of Killip's classification or subset II or greater of Forrester's classification), cardiac rupture, and cardiac death was examined. Echocardiography was performed 10–14 days after MI (Sonos 5500, Phillips Medical Systems, Andover, MA, USA), and LV dimensions and fractional shortening

**Table 1** Myocardial infarcted patient characteristics and peak high-mobility group box 1 protein level

	Peak HMGB1 level (ng/mL)		P-value
	Absent	Present	
Age $\geq 70$ years	15.3 $\pm$ 10.7 (22)	27.4 $\pm$ 28.8 (13)	0.082
Male sex	23.4 $\pm$ 24.0 (8)	18.7 $\pm$ 19.0 (27)	0.56
Cigarette smoking	20.4 $\pm$ 18.2 (18)	19.0 $\pm$ 22.2 (17)	0.83
Hypertension	24.2 $\pm$ 27.7 (16)	16.0 $\pm$ 8.9 (19)	0.23
Hypercholesterolaemia	21.7 $\pm$ 26.2 (13)	18.6 $\pm$ 15.7 (22)	0.65
Diabetes mellitus	22.2 $\pm$ 22.2 (26)	12.8 $\pm$ 8.8 (9)	0.23
Pre-infarction angina	18.3 $\pm$ 16.6 (22)	22.2 $\pm$ 25.2 (13)	0.58
Anterior infarction	19.7 $\pm$ 22.9 (24)	19.9 $\pm$ 12.0 (11)	0.99
Revascularization therapy	43.2 $\pm$ 47.7 (2)	18.3 $\pm$ 17.8 (33)	0.087
Arrival $< 6$ h	14.3 $\pm$ 10.2 (7)	21.1 $\pm$ 21.6 (28)	0.42
Medication before admission			
Aspirin	19.3 $\pm$ 21.1 (30)	22.6 $\pm$ 11.1 (5)	0.74
$\beta$ -Blockers	20.4 $\pm$ 21.0 (31)	14.6 $\pm$ 7.9 (4)	0.58
ACE-inhibitors/ARBs	18.5 $\pm$ 16.6 (23)	22.1 $\pm$ 25.9 (12)	0.62
Statins	20.2 $\pm$ 22.6 (26)	18.5 $\pm$ 10.0 (9)	0.83
Calcium antagonists	20.9 $\pm$ 22.6 (26)	16.4 $\pm$ 9.1 (9)	0.56
Medication after admission			
Aspirin	31.1 $\pm$ 10.3 (2)	19.1 $\pm$ 20.3 (33)	0.42
$\beta$ -Blockers	23.7 $\pm$ 26.7 (7)	18.8 $\pm$ 18.4 (28)	0.57
ACE-inhibitors/ARBs	23.6 $\pm$ 22.6 (9)	18.4 $\pm$ 19.2 (26)	0.51
Statins	26.6 $\pm$ 23.7 (8)	17.7 $\pm$ 18.7 (27)	0.27
Calcium antagonists	21.9 $\pm$ 21.6 (28)	11.4 $\pm$ 7.6 (7)	0.22
Peak C-reactive protein level $\geq 5.5$ mg/dL	11.8 $\pm$ 7.5 (17)	24.3 $\pm$ 22.3 (18)	0.036

Data are shown as mean  $\pm$  SD (number of patients).

HMGB1, high-mobility group box 1 protein; ACE, angiotensin-converting enzyme; ARB, angiotensin type-1 receptor blocker.

were determined. We measured plasma brain natriuretic peptide (BNP) level 6 months after MI by immunoradiometric assay.<sup>20</sup>

### 2.3 Rat model of experimental myocardial infarction

Male Wistar rats, pathogen-free and weighing 200–250 g, were subjected to left coronary artery ligation or sham operation under pentobarbital anaesthesia (intraperitoneal pentobarbital 30 mg/kg).<sup>21,22</sup> To determine serial HMGB1 expression, rats were sacrificed 1, 3, 7, and 14 days after MI for RNA and protein analyses ( $n = 6$  rats per time point) and for pathological analyses ( $n = 6$  rats per time point). Serum HMGB1 level was measured using ELISA to determine serial changes of serum HMGB1 level. The LV of sham-operated rats ( $n = 6$  for RNA and protein analyses,  $n = 6$  for pathological analyses, on day 14) was collected as control. In RNA and protein analyses, LV tissue with MI was carefully divided into infarcted (infarcted zone and 1–2 mm of the border zone) and non-infarcted areas. These tissues were snap-frozen in liquid nitrogen and then preserved at  $-80^{\circ}\text{C}$ . For pathological analyses, hearts were arrested by infusion of ice-cold saturated potassium chloride, excised, and placed in ice-cold potassium chloride to achieve uniform diastolic arrest. The LV was cannulated retrogradely via ascending aorta, and the hearts were fixed with 4% paraformaldehyde at a constant intraventricular pressure. Fixed hearts with 4% paraformaldehyde were embedded in paraffin. In the HMGB1 blockade study, MI rats surviving the operation for 24 h ( $n = 80$ ) were randomly assigned to two groups: (i) neutralizing polyclonal chicken IgY anti-HMGB1 antibody (10 mg/kg/day, donated by Shino-Test Corporation)<sup>11,12,23</sup> administered subcutaneously for 7 days (MI/anti-H,  $n = 40$ ) and (ii) control chicken IgY antibody (MI/C,  $n = 40$ ). To prepare neutralizing anti-HMGB1 antibody, IgY class antibody from the egg yolk of HMGB1-immunized hens was isolated and purified.<sup>24</sup> Control IgY antibody was purified from non-immunized egg yolk. The dosage of neutralizing anti-HMGB1 antibody was determined according to the previous study.<sup>12</sup> Rats were sacrificed 3, 7, and 14 days after MI for mRNA expression ( $n = 6$  rats per time point) and pathological analyses ( $n = 6$  rats per time point). Echocardiographic (8.5 MHz linear transducer; EnVisor C, Philips Medical Systems) and haemodynamic studies (SPC-320, Millar Instruments, Houston, TX, USA) were performed 14 days after MI.<sup>21,22</sup> All procedures were performed in accordance with the Keio University animal care guidelines, which conformed 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).

### 2.4 Real-time quantitative reverse transcriptase–polymerase chain reaction

Total RNA was isolated by acid-phenol extraction in the presence of chaotropic salts (Trizol, Invitrogen, Carlsbad, CA, USA) and subsequent isopropanol–ethanol precipitation as described previously.<sup>21,22</sup>

Real-time quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) of each sample was carried out with a TaqMan RNA PCR kit and ABI Prism™ 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) as described previously.<sup>21,22</sup> Gene expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. HMGB1, interleukin-1 $\beta$  (IL-1 $\beta$ ), atrial natriuretic factor (ANF), and GAPDH were purchased as pre-optimized kits [TaqMan probe; Applied Biosystems: catalogue number Rn02377062\_g1 (HMGB1), Rn00580432\_m1 (IL-1 $\beta$ ), Rn00561661\_m1 (ANF), Rn99999916\_s1 (GAPDH)]. The primer pair and probe for tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) were as follows: forward primer: TGGGCTCCTCATCAGTT; reverse primer: TGGGCTACGGGCTTGTC; TaqMan probe: TGGCCAGACCCTCACACTCAGATC.

### 2.5 Western blotting

Frozen tissue was homogenized in cell lysis buffer (Cell Signaling, Danvers, MA, USA) containing 1% Triton X-100 and protease inhibitors. After centrifugation at 16 000 g for 30 min at  $4^{\circ}\text{C}$ , the supernatants were collected. Western blot analysis with rabbit polyclonal anti-rat HMGB1 (Pharmingen, San Diego, CA, USA) was carried out as described previously.<sup>22</sup> After probing with anti-rat HMGB1 antibody, membranes were stripped of bound immunoglobulins and re-probed with anti-rat GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to correct for protein loading.

### 2.6 Histopathological study

Tissues were stained with haematoxylin–eosin. The boundary lengths of the infarcted and non-infarcted endocardial and epicardial surfaces of all slices were traced using Image J software (version 1.38x, National Institutes of Health), and the infarct size was determined as the percentage of infarcted epicardium and endocardium of the LV.<sup>25</sup> The area of the LV cavity and total LV, and the thickness of the septum and scar in sections at the papillary muscle level were also measured, and then the expansion index was calculated as described previously: expansion index = (LV cavity area/total LV area)  $\times$  (non-infarcted septal thickness/scar thickness).<sup>26</sup>

### 2.7 Immunohistochemical study

Immunohistochemical studies were performed by immunoperoxidase methods.<sup>12,21</sup> Paraffin section-embedded specimens were cut into  $4\text{ }\mu\text{m}$ -thick sections and stained with antibodies against HMGB1 (Shino-Test Corporation) and ED-1 (for monocyte-derived macrophages; Serotec Ltd, Kidlington, Oxford, UK). The number of macrophages was quantified by counting the total number of positively stained ED-1 cells in 20 grid fields with a total area of  $0.1\text{ mm}^2$ .

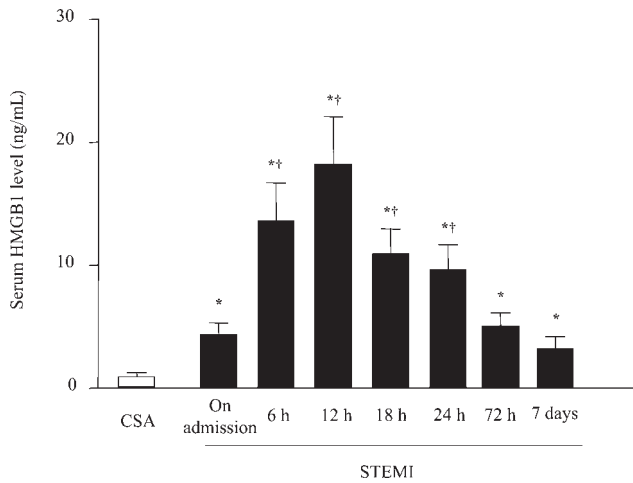
### 2.8 Statistical analyses

All continuous data were expressed as the mean value  $\pm$  SD unless otherwise stated. Comparison between groups was performed using unpaired  $t$ -test or non-parametric analysis for continuous variables and  $\chi^2$  test or Fisher's exact test for categorical variables. Statistical significance was defined as a  $P$ -value of  $<0.05$ . All statistical analyses were performed using SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

## 3. Results

### 3.1 Serum high-mobility group box 1 protein level in patients with ST-elevation myocardial infarction and chronic stable angina

Figure 1 shows serum HMGB1 level in patients with CSA and serial changes in serum HMGB1 level in patients with STEMI. Serum HMGB1 level in patients with STEMI on admission was higher than that in patients with CSA ( $4.5 \pm 5.3$  vs.  $1.0 \pm 1.6$  ng/mL,  $P = 0.0004$ ). Serum HMGB1 level significantly increased, peaking at 12 h after MI, compared with the baseline level. On day 7, serum HMGB1 level remained elevated in patients with STEMI compared with those with CSA ( $3.3 \pm 5.1$  vs.  $1.0 \pm 1.6$  ng/mL,  $P = 0.011$ ). There were no significant differences in age ( $65 \pm 13$  vs.  $64 \pm 13$  years old,  $P = 0.63$ ), sex (male/female, 27/8 vs. 29/6,  $P = 0.55$ ), smoking (49 vs. 43%,  $P = 0.63$ ), hypertension (54 vs. 51%,  $P = 0.81$ ), hypercholesterolaemia (63 vs. 60%,  $P = 0.81$ ), and diabetes mellitus (26 vs. 17%,  $P = 0.38$ ) between patients with STEMI and CSA. Among the patients with



**Figure 1** Serial changes in serum high-mobility group box 1 protein (HMGB1) level in patients with ST-elevation myocardial infarction (STEMI) compared with those with chronic stable angina (CSA). Data are mean  $\pm$  SEM. \* $P < 0.05$  vs. patients with chronic stable angina, † $P < 0.05$  vs. baseline level (on admission).

**Table 2** Clinical outcomes and peak high-mobility group box 1 protein level

	Peak HMGB1 level (ng/mL)		P-value
	Absent	Present	
Pump failure	12.8 $\pm$ 8.1 (25)	37.2 $\pm$ 29.2 (10)	0.0004
Cardiac rupture	17.9 $\pm$ 17.8 (33)	50.4 $\pm$ 37.6 (2)	0.023
Cardiac death	17.3 $\pm$ 17.7 (32)	46.4 $\pm$ 27.4 (3)	0.013

Data are shown as mean  $\pm$  SD (number of patients). HMGB1, high-mobility group box 1 protein.

STEMI, peak HMGB1 level did not significantly differ according to patient characteristics (Table 1).

### 3.2 Relationships among peak C-reactive protein level, cumulative creatine kinase release, and peak high-mobility group box 1 protein level in patients with ST-elevation myocardial infarction

The mean peak C-reactive protein level was  $7.2 \pm 5.2$  mg/dL (0.7–23.2 mg/dL, median 5.5 mg/dL) and cumulative CK release was  $41\,205 \pm 26\,117$  h IU/L (7347–102 893 h IU/L, median 37 070 h IU/L) in patients with STEMI. The mean elapsed time from the onset of MI to the C-reactive protein peak was  $3 \pm 1$  days. The peak C-reactive protein level ( $r = 0.49$ ,  $P = 0.003$ ), but not cumulative CK release ( $r = 0.25$ ,  $P = 0.15$ ), showed a significant positive correlation with the peak HMGB1 level. Patients with peak C-reactive protein level greater than or equal to the median value had a higher peak HMGB1 level than those without (Table 1).

### 3.3 Clinical significance of serum high-mobility group box 1 protein elevation in patients with ST-elevation myocardial infarction

Peak HMGB1 level was higher in patients with pump failure ( $P = 0.0004$ ), cardiac rupture ( $P = 0.023$ ), and in-hospital cardiac death ( $P = 0.013$ ) compared with those without

(Table 2). There was no statistically significant correlation between peak serum HMGB1 level and LV dimensions (LV end-diastolic dimension,  $r = -0.47$ ,  $P = 0.81$ ; LV end-systolic dimension,  $r = 0.11$ ,  $P = 0.54$ ) or fractional shortening ( $r = -0.22$ ,  $P = 0.24$ ) 2 weeks after MI. The mean plasma BNP level 6 months after MI was  $85.9 \pm 104.5$  pg/mL (6.2–518.0 pg/mL, median 48.9 pg/mL). Peak serum HMGB1 level was positively correlated with plasma BNP level 6 months after the onset ( $r = 0.44$ ,  $P = 0.016$ ). Patients with plasma BNP level greater than or equal to the median value had a higher peak HMGB1 level than those without ( $24.3 \pm 23.3$  vs.  $10.2 \pm 6.8$  ng/mL,  $P = 0.037$ ).

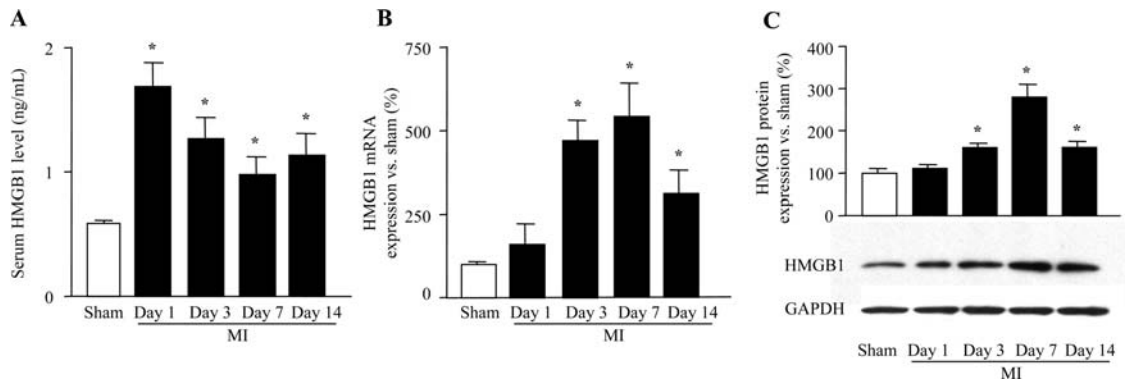
### 3.4 Serum and myocardial high-mobility group box 1 protein levels in experimental myocardial infarction

Serum HMGB1 level was significantly increased in MI rats compared with sham-operated rats during the observation period (Figure 2A). The mRNA expression of HMGB1 in the infarcted area was elevated on day 3, peaked on day 7 up to  $\sim 5.4$ -fold compared with that in sham-operated rats, and remained elevated on day 14 after MI (Figure 2B). The mRNA expression of HMGB1 in the non-infarcted area of MI rats was not elevated compared with that in sham-operated rats during the observation period (day 1;  $110 \pm 14\%$ , day 3;  $105 \pm 6\%$ , day 7;  $102 \pm 18\%$ , day 14;  $100 \pm 10\%$  mRNA expression vs. sham). Western blotting showed that the level of HMGB1 protein in the infarcted area was increased on day 3, peaked on day 7, and remained elevated on day 14 after MI (Figure 2C).

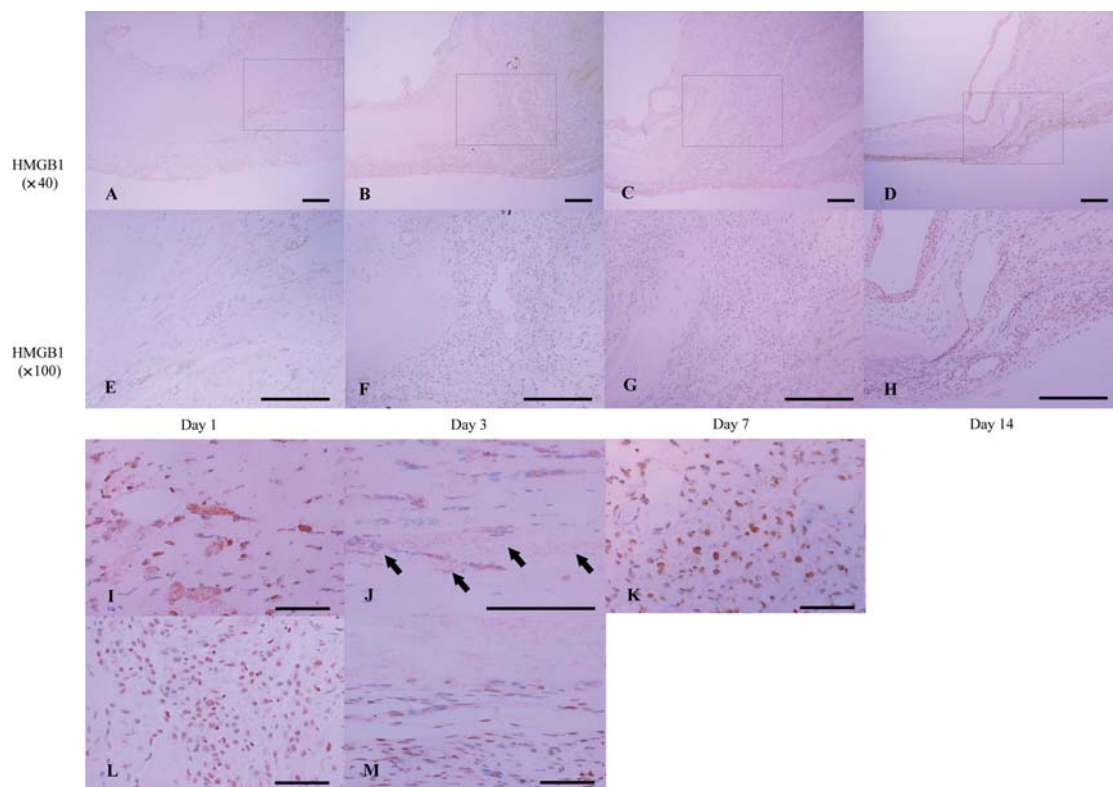
Immunohistochemical staining demonstrated that HMGB1-positive nuclear staining of myocytes was present in the sham-operated rat myocardium. In MI rats, immunohistochemical staining of HMGB1 was enhanced in the infarcted and border zone myocardium (Figure 3A–H). At 24 h after MI induction, HMGB1 expression was detected in the cytoplasm of degenerated cardiomyocytes (Figure 3I) and extracellular fields (Figure 3J) in the infarcted area. HMGB1 immunoreactivity was also seen in infiltrating inflammatory cells 24 h after the induction of MI (Figure 3K). During the observation period, HMGB1 staining was most intense on day 3 and was expressed mainly in infiltrating inflammatory cells (Figure 3L). On day 7, HMGB1 staining extended over a larger area but weaker labelling was visible. The number of inflammatory cells with HMGB1 immunostaining was decreased on day 14, and HMGB1 immunostaining was mainly present in fibroblasts (Figure 3M).

### 3.5 Effect of high-mobility group box 1 protein blockade in experimental myocardial infarction

To determine whether HMGB1 is involved in post-MI inflammatory response and LV remodelling, we treated MI rats with neutralizing anti-HMGB1 antibody or control antibody. Neutralizing anti-HMGB1 antibody treatment diminished MI-induced mRNA expression of TNF- $\alpha$  and IL-1 $\beta$  in the infarcted area on day 3 (Figure 4A and B). HMGB1 mRNA expression in the infarcted area was significantly lower in MI/anti-H than in MI/C on days 3, 7, and 14 (Figure 4C). In the non-infarcted area, the expression of TNF- $\alpha$ , IL-1 $\beta$ , and HMGB1 was not different between MI/C and MI/anti-H during the observation period (Figure 4D–F). The increased



**Figure 2** High-mobility group box 1 protein (HMGB1) level in serum and infarcted myocardium in rat myocardial infarction (MI) model. (A) Quantified data of serum high-mobility group box 1 protein level by enzyme-linked immunosorbent assay. (B) Quantified data of high-mobility group box 1 protein mRNA expression in the infarcted area by real-time quantitative reverse transcriptase–polymerase chain reaction. (C) Quantified data of high-mobility group box 1 protein expression in the infarcted area by western blotting. Data are mean  $\pm$  SEM. \* $P < 0.05$  vs. sham-operated rats.



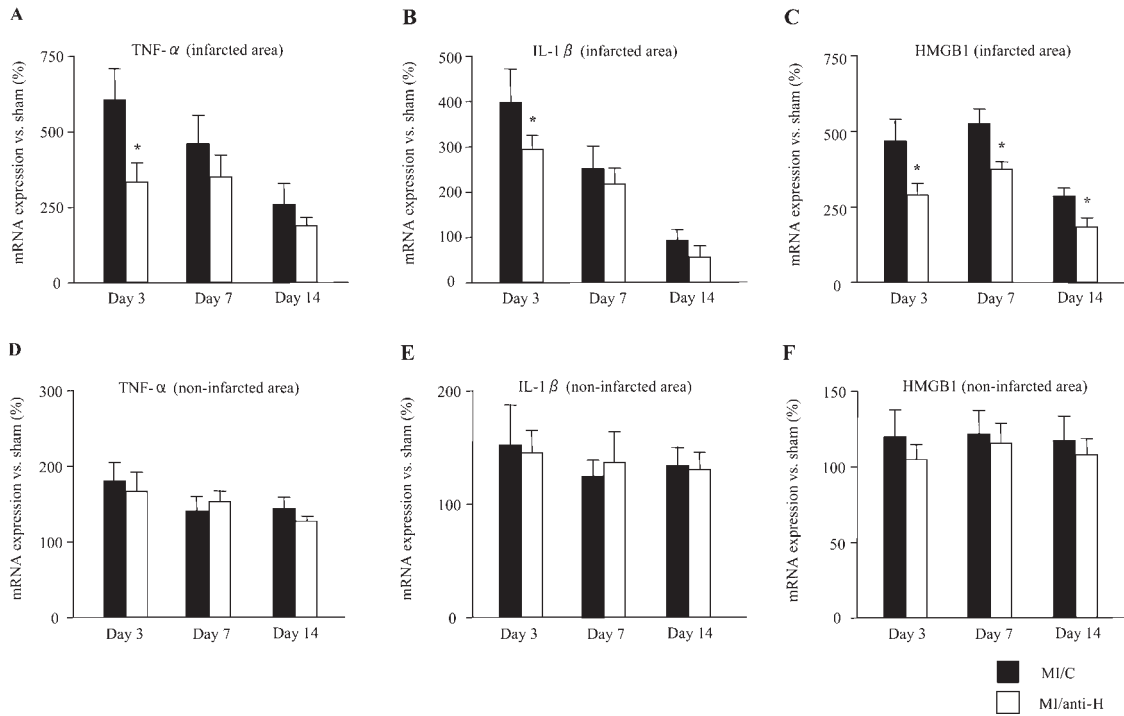
**Figure 3** High-mobility group box 1 protein (HMGB1) immunostaining in the infarcted area. Representative low-power micrographs showing high-mobility group box 1 protein immunohistochemical staining in the infarcted and border zone areas (A–D). The parts of the border zone area indicated by boxes are shown at a higher magnification in panels (E–H). In the infarcted area, high-mobility group box 1 protein was seen clearly in degenerated myocytes (I), extracellular fields (black arrows) (J), and inflammatory cells (K) on day 1. High-mobility group box 1 protein was mainly expressed in inflammatory cells on day 3 (L), and in fibroblasts on day 14 (M). Scale bar indicates 500  $\mu$ m (A–H) and 100  $\mu$ m (I–M).

number of ED-1-positive macrophages in the infarcted area on day 3 was attenuated by the blockade of HMGB1 (Figure 5A–C).

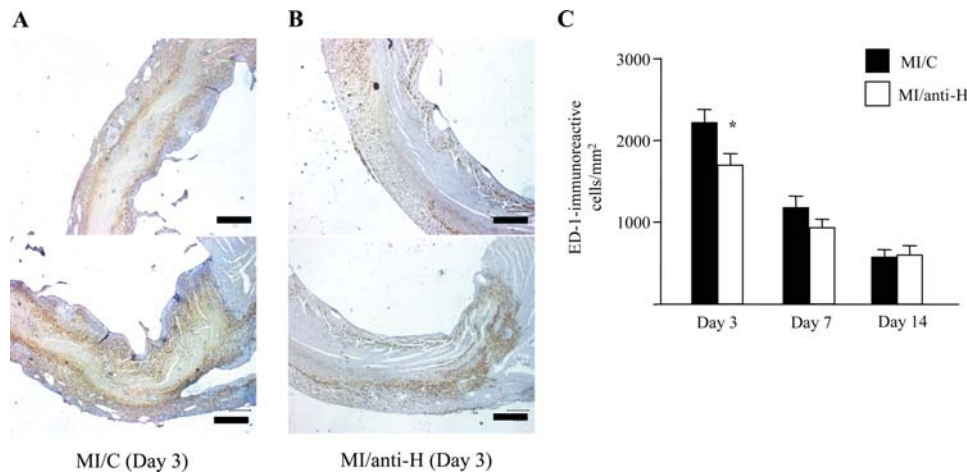
As shown in Table 3, haemodynamic measurement showed LV systolic pressure, maximum rate of isovolumic pressure development ( $+dP/dt_{max}$ ), and minimum rate of isovolumic pressure decay ( $-dP/dt_{min}$ ) to be lower and LV end-diastolic pressure (LVEDP) higher in the MI/C rats than in sham-operated rats. Echocardiographic findings indicated that LV dimensions increased while LV fractional shortening decreased in the MI/C rats compared with those in sham-operated rats. No differences existed in left and

right ventricular weight per body weight, LV systolic pressure, and heart rate on day 14 between MI/C and MI/anti-H. Rats in MI/anti-H exhibited significantly lower LV fractional shortening and higher LV dimensions compared with those in MI/C on day 14. LV  $+dP/dt_{max}$  and  $-dP/dt_{min}$  were lower and LVEDP was higher in MI/anti-H than in MI/C on day 14.

LV remodelling was also assessed by LV histomorphometric analysis. There was no difference in infarct size between MI/C and MI/anti-H on days 7 ( $52 \pm 2$  vs.  $53 \pm 4\%$ ,  $P = 0.83$ ) and 14 ( $50 \pm 3$  vs.  $50 \pm 2\%$ ,  $P = 0.99$ ). Blockade of HMGB1 exaggerated thinning of the infarcted LV wall on days 7 and 14.



**Figure 4** Real-time quantitative reverse transcriptase-polymerase chain reaction analyses of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ : A and D), interleukin-1 $\beta$  (IL-1 $\beta$ : B and E), and high-mobility group box 1 protein (HMGB1: C and F) gene expression in the infarcted and non-infarcted areas in myocardial infarcted rats treated with control (MI/C) or neutralizing anti-high-mobility group box 1 protein antibodies (MI/anti-H). Data are mean  $\pm$  SEM. \*  $P < 0.05$  vs. MI/C.



**Figure 5** Immunohistochemical staining with ED-1 antibody in the infarcted area of myocardial infarcted rats treated with control (MI/C: A) or neutralizing anti-high-mobility group box 1 protein (HMGB1) antibodies (MI/anti-H: B) on day 3. (C) Graph shows ED-1-immunoreactive cells/mm<sup>2</sup>. Scale bar indicates 500  $\mu$ m. Data are mean  $\pm$  SEM. \*  $P < 0.05$  vs. MI/C.

Myocardial hypertrophy of the non-infarcted LV wall was promoted by HMGB1 blockade on day 14 (Figure 6A–D). The expansion index was significantly higher in MI/anti-H than in MI/C on days 7 and 14 (Figure 6E).

In parallel with the non-infarcted myocardial hypertrophy, ANF expression in the non-infarcted area significantly increased in MI/anti-H compared with MI/C on day 14 (Figure 6F).

#### 4. Discussion

We demonstrated that patients with STEMI had a markedly increased serum HMGB1 level, peaking at 12 h after MI, and that a higher peak serum HMGB1 level was associated

with pump failure, cardiac rupture, and in-hospital cardiac death. In an experimental study using a rat MI model, we found increased myocardial HMGB1 expression in the infarcted area, which persisted for 14 days after MI. Blockade of HMGB1 by a neutralizing anti-HMGB1 antibody attenuated the post-MI inflammatory response; however, it resulted in marked infarct scar thinning and exaggerated LV remodelling. These findings suggested that HMGB1 could be a predictor of adverse clinical outcomes after MI, but also be a prerequisite for an appropriate healing process and for preserving the structural integrity of the infarcted heart.

Accumulated evidence indicates that an excessive post-MI inflammatory response is associated with aggravated LV

remodelling and poor clinical outcomes.<sup>2,3,15,19–21</sup> It is known that HMGB1 is quickly released extracellularly after ischaemic injury.<sup>27,28</sup> Because extracellular HMGB1 induces the expression of pro-inflammatory cytokines and adhesion molecules as an inflammatory mediator,<sup>7,9</sup> we hypothesized that serum HMGB1 level could be a predictor of poor clinical

outcomes in patients with MI. Here, we demonstrated that a marked HMGB1 elevation after MI was associated with pump failure, cardiac rupture, and in-hospital cardiac death. Peak serum HMGB1 level was positively correlated with plasma BNP level 6 months after MI. These findings suggest that serum HMGB1 level could be a predictor of adverse clinical outcomes and late-phase LV dysfunction after MI. We previously reported that an increased peak serum C-reactive protein level can predict pump failure, cardiac rupture, LV aneurysm, and 1 year cardiac death after MI;<sup>19</sup> however, peak C-reactive protein occurs 2–3 days after MI, which is not practically useful. Because C-reactive protein is mainly produced in hepatocytes through stimulation by pro-inflammatory cytokines,<sup>29</sup> it is plausible that HMGB1 might be an upstream regulator of serum C-reactive protein production. We demonstrated that serum HMGB1 level peaked at 12 h after admission, and peak HMGB1 level correlated positively with peak serum C-reactive protein level. Serum HMGB1 level measurement could be a useful early predictor of worse clinical outcomes after MI.

Because HMGB1 was released passively from necrotic cells and secreted actively by inflammatory cells,<sup>7,8,10</sup> there might be two possible sources of HMGB1 in MI: necrotic myocardium and infiltrating inflammatory cells. In contrast to the early elevation of serum HMGB1 level after MI, myocardial HMGB1 mRNA level in the rat MI model did not increase on day 1. However, immunohistochemical study showed that HMGB1 immunoreactivity was observed in degenerated myocytes in the infarcted area on day 1 and rapidly disappeared thereafter, suggesting that the early serum HMGB1 elevation was mainly due to its passive release from necrotic myocytes. We demonstrated that the elevation of serum HMGB1 was sustained for 14 days after MI. In addition, HMGB1 mRNA level in the infarcted area in the rat MI

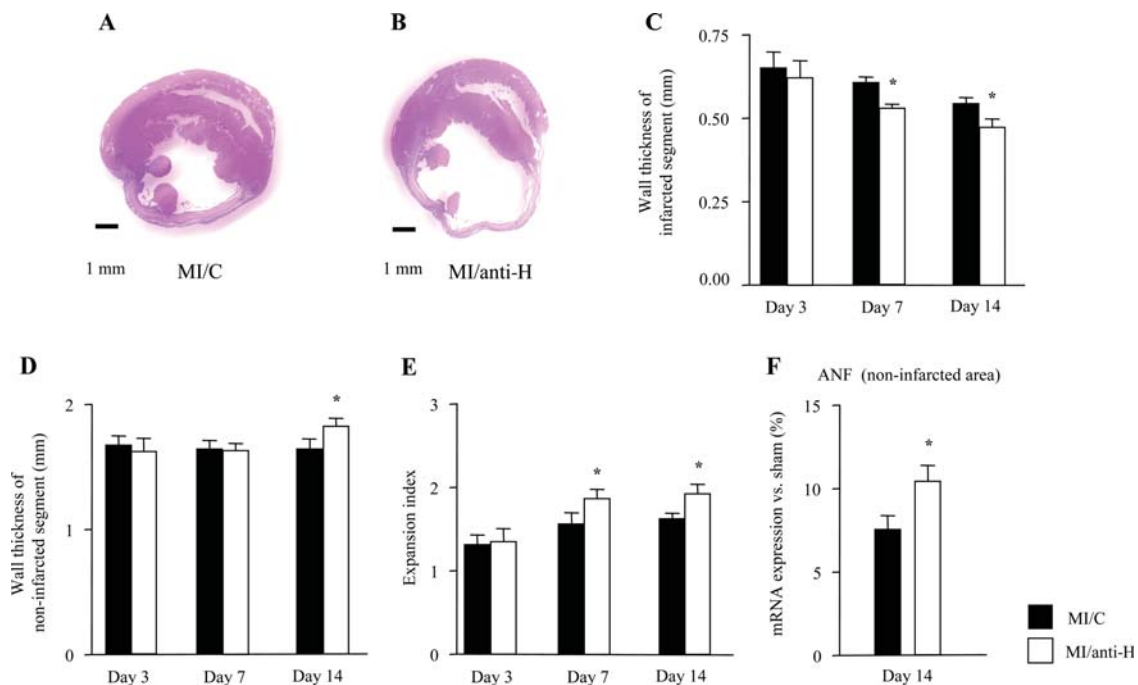
**Table 3** Heart weight, haemodynamics, and echocardiographic data on day 14 after myocardial infarction

	Sham	MI/C	MI/anti-H
LVW/BW (mg/g)	1.98 ± 0.02	2.19 ± 0.09	2.29 ± 0.03*
RVW/BW (mg/g)	0.52 ± 0.03	0.66 ± 0.04*	0.75 ± 0.05*
LVSP (mmHg)	148 ± 7	94 ± 4*	97 ± 3*
HR (b.p.m.)	419 ± 11	377 ± 12*	383 ± 4*
LVEDP (mmHg)	1 ± 1	10 ± 1*	16 ± 2**†
LV +dP/dt <sub>max</sub> (mmHg/s)	11284 ± 781	6094 ± 364*	5099 ± 161**†
LV -dP/dt <sub>min</sub> (mmHg/s)	7967 ± 559	3748 ± 111*	3218 ± 166**†
LVEDD (mm)	6.7 ± 0.3	7.6 ± 0.2*	8.3 ± 0.3**†
LVESD (mm)	3.6 ± 0.4	6.7 ± 0.2*	7.5 ± 0.2**†
FS (%)	46 ± 4	13 ± 1*	9 ± 1**†

Data are shown as mean ± SEM.

MI, myocardial infarction; Sham, sham-operated rats; MI/C, MI rats treated with control antibody; MI/anti-H, MI rats treated with neutralizing anti-HMGB1 antibody; LVW, left ventricular weight; BW, body weight; RVW, right ventricular weight; LVSP, left ventricular systolic pressure; HR, heart rate; LVEDP, left ventricular end-diastolic pressure; LV +dP/dt<sub>max</sub>, left ventricular maximum rate of isovolumic pressure development; LV -dP/dt<sub>min</sub>, left ventricular minimum rate of isovolumic pressure decay; LVEDD, left ventricular end-diastolic dimension; LVESD, left ventricular end-systolic dimension; FS, fractional shortening.

\**P* < 0.05 vs. Sham, †*P* < 0.05 vs. MI/C.



**Figure 6** Effects of high-mobility group box 1 protein (HMGB1) blockade on myocardial histological characteristics and atrial natriuretic factor (ANF) mRNA expression in the non-infarcted area. Representative haematoxylin-eosin-stained cross-sections of infarcted hearts treated with control (MI/C: A) or neutralizing anti-high-mobility group box 1 protein antibodies (MI/anti-H: B) on day 7. Effect of high-mobility group box 1 protein blockade as measured by the thickness of the infarcted and non-infarcted segments (C and D) and expansion index (E). (F) Quantified data of atrial natriuretic factor mRNA expression in the non-infarcted area on day 14 by real-time quantitative reverse transcriptase-polymerase chain reaction. Data are mean ± SEM. \**P* < 0.05 vs. MI/C.

model was significantly increased on day 3, peaked on day 7, and remained elevated on day 14 after MI, and its expression was detected mainly in infiltrating inflammatory cells. Thus, the sustained serum HMGB1 elevation after MI might be caused by active secretion from infiltrating inflammatory cells. Whereas our clinical findings implicate HMGB1 as an early mediator of the post-MI inflammatory response, HMGB1, released from activated inflammatory cells, acts late as a downstream mediator of inflammation, and its expression increases after the initial rise in TNF- $\alpha$  and IL-1 $\beta$  and persists after the disappearance of these pro-inflammatory cytokines.<sup>7,10,23</sup> We revealed that the kinetics of HMGB1 mRNA expression in the infarcted area is also delayed relative to that of TNF- $\alpha$  and IL-1 $\beta$ , as reported previously.<sup>5,22</sup> These findings led to speculation that passive HMGB1 release from necrotic myocardium might initiate the post-MI inflammatory response, and subsequent active release of HMGB1 from infiltrating inflammatory cells might contribute to the prolongation of the post-MI inflammatory response.

An excessive and persistent inflammatory response after MI could be unfavourable, leading to LV aneurysm, cardiac rupture, or chronic LV dilatation.<sup>3,15,19–21</sup> We have reported that peripheral monocytosis after reperfused MI was associated with LV dysfunction and LV aneurysm,<sup>20</sup> and that granulocyte-macrophage colony-stimulating factor induction in a rat MI model resulted in exaggerated LV remodelling with increased monocyte-derived macrophage infiltration and impaired reparative fibrosis in the infarcted area.<sup>21</sup> We initially hypothesized that the attenuation of the post-MI inflammatory response by HMGB1 blockade might prevent LV remodelling. However, HMGB1 blockade failed to demonstrate any beneficial effect on post-MI LV function. On the contrary, it resulted in the exaggeration of post-MI LV remodelling in association with an inhibited inflammatory response. Although neutralizing anti-HMGB1 antibody inhibited the HMGB1 self-amplification pathway and reduced pro-inflammatory cytokine expression and macrophage infiltration in the infarcted myocardium, the alteration of the inflammatory response by HMGB1 blockade might be harmful to infarct healing.

Infarct healing can be divided into three overlapping phases: inflammatory phase, proliferative phase, and maturation phase.<sup>3</sup> Infiltrated macrophages during the inflammatory phase contribute to the removal of necrotic tissue, release of proteolytic enzymes, and production of fibrinogenic and angiogenic mediators that are important for the formation of granulation tissue, leading to the proliferative and maturation phases of infarct healing. van Amerongen *et al.* demonstrated that macrophage depletion impairs wound healing and increases LV remodelling after myocardial injury in mice, suggesting that macrophages play an important role in the infarct-healing process.<sup>30</sup> HMGB1 is a potent stimulator of macrophages, and its effect on macrophages includes the promotion of pro-inflammatory cytokine secretion.<sup>9</sup> A recent study demonstrated that a lower tissue HMGB1 level in diabetic skin was associated with impaired wound healing, and this defect was overcome by topical application of HMGB1.<sup>31</sup> It is possible that specific blockade of HMGB1 might impair the post-MI healing process, resulting in adverse LV remodelling. HMGB1 during the early phase of MI might be a key mediator of the healing process, which preserves the structural integrity of the

infarcted tissue and protects against increased dilatation of the LV.

Recent experimental studies enhance our understanding regarding the role of HMGB1 in post-MI LV remodelling. Exogenous administration of HMGB1 in the peri-infarcted LV might have therapeutic potential for the attenuation of LV remodelling in a permanent MI model through a mechanism that involves the activation of stem/progenitor cells.<sup>32,33</sup> Kitahara *et al.* reported that HMGB1 enhances angiogenesis and restores cardiac function in a permanent MI model using transgenic mice with cardiac overexpression of HMGB1.<sup>34</sup> These findings demonstrated that HMGB1 has beneficial effects on the heart after MI through biological responses related to healing process. Because HMGB1 is also associated with post-MI inflammatory response, the caution might be needed when applying these results to practical use. Multiple points of control may exist to ensure that the inflammatory response is contained (both topographically and temporally) according to the area and time of injury.<sup>35,36</sup> Because an excessive and persistent inflammatory response after MI could be unfavourable, HMGB1 administration during the delayed phase might have different results. It is also expected that modest HMGB1 blockade in a situation with an excessive inflammatory response might have some therapeutic potential. The inflammatory response is much more severe in reperfusion injury than in permanent MI. In fact, Andrassy *et al.* recently showed that systemic administration of HMGB1 causes an increase in the inflammatory responses and worsens LV remodelling after myocardial ischaemia-reperfusion injury. Conversely, HMGB1 inhibition with a functional antagonist of HMGB1 conferred protection against ischaemia-reperfusion injury.<sup>37</sup> HMGB1 might act as a double-edged sword in post-MI inflammatory response and have bidirectional effects on LV remodelling depending on the site, extent, and timing of HMGB1 modulation.<sup>38</sup>

The present study has some limitations. First, the number of study participants and events was limited; therefore, it is likely that the statistical power is too low. To confirm the clinical significance of serum HMGB1 elevation, a large prospective clinical cohort study is needed. Second, in the experimental study, we did not evaluate the exact level of HMGB1 blockade in serum and myocardium. Because the results could be altered with the extent of HMGB1 blockade as discussed earlier, further studies including the quantification of HMGB1 blockade are needed to clarify the exact significance of HMGB1 in post-MI LV remodelling.

## 5. Conclusions

We demonstrated that the elevation of serum HMGB1 level is associated with pump failure, cardiac rupture, and in-hospital cardiac death, in association with an increased serum C-reactive protein level. These findings suggest that the overexpression of HMGB1 is associated with poor clinical outcomes through an excessive inflammatory response in patients with MI. However, blockade of HMGB1 in a rat MI model resulted in worsening of LV remodelling through impaired infarct healing and marked scar thinning. These findings indicate that HMGB1, a novel predictor of adverse clinical outcomes after MI, might have an essential role in the appropriate healing process and in preserving the structural integrity of the infarcted LV.



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