Effects of HMGB1 on Ischemia-Reperfusion Injury in the Rat Heart

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Background: Coronary ischemia-reperfusion (I/R) injury causes cardiomyocyte necrosis in a multi-step process that includes an inflammatory reaction. A recent study has suggested that high-mobility group box 1 (HMGB1) is a late mediator of lethal sepsis and an early mediator of inflammation and necrosis following I/R injury. In the present study a neutralizing monoclonal antibody (mAb) for HMGB1 was used to clarify the role of HMGB1 in cardiac I/R injury.

Methods and Results: Rats underwent 30 min of left coronary artery occlusion followed by 60 min reperfusion. An intravenous injection of anti-HMGB1 mAb or control IgG was administered just before reperfusion. The infarct size was enlarged in the anti-HMGB1 mAb group in comparison with the control group (p<0.05). The treatment of anti-HMGB1 mAb significantly increased the plasma troponin-T and norepinephrine (NE) content in the heart in comparison with the control (p<0.05). Moreover, the production of dihydroxyphenylglycol was reduced in the anti-HMGB1-treated group (p<0.05).

Conclusion: This study shows for the first time the effects of treatment with neutralizing anti-HMGB1 mAb on I/R injury in the rat heart. The findings support the novel view that I/R-induced HMGB1 may be an important factor in the modulation of interstitial NE. (Circ J 2008; 72: 1178–1184)

Key Words: Coronary ischemia-reperfusion injury; HMGB1; Norepinephrine

Myocardial infarction is a disease that remains highly lethal despite recent advanced medical treatment. It is caused by the sudden interruption of coronary flow by occlusion of the coronary artery, which sequentially causes irreversible cardiomyopathy, tissue loss, and scar formation. Various pharmacological or surgical therapies have been reported for ischemia-reperfusion (I/R) injury and recent investigations have revealed the involvement of nitric oxide in myocardial injury during coronary reperfusion. Moreover, I/R induces an inflammatory response and cytokine release from inflammatory cells, such as monocytes, macrophages and leukocytes but there is still no satisfactory protective therapy for I/R injury by regulating inflammation.

High-mobility group box 1 protein (HMGB1) is a non-histone DNA-binding, multifunctional protein that has both nuclear and extracellular functions. HMGB1 was originally identified as a DNA-binding protein, and was described as a multifunctional transcriptional factor (amphoterin) that interacted with DNA. In addition, HMGB1 has a binding site for the receptor for advanced glycation end-products (RAGE), which was originally identified as a receptor for the AGEs that accumulate during hyperglycemia. Consequently, HMGB1-deficient mice die of hypoglycemia within 24 h of birth, suggesting that HMGB1 is associated with glycemic homeostasis. Nuclear HMGB1 is released into the extracellular space during inflammatory responses that markedly and extensively activate macrophages and monocytes, as well as from necrotic cells, being released in sepsis.

Recent animal model studies have shown that HMGB1 is associated with I/R injury in several organs. In liver I/R injury in the rat, plasma HMGB1 levels were increased at the early time point of 1 h after reperfusion and thereafter further increased in a time-dependent manner for 24 h. Treatment with a neutralizing antibody to HMGB1 significantly decreased liver damage after reperfusion injury. In cerebral I/R injury in the rat, extracellular HMGB1 prevented expansion of the lesion during acute neuronal damage processes, and treatment with anti-HMGB1 antibody improved brain infarction post-treatment. In I/R injury of the heart, cardiomyocyte inflammatory receptors are stimulated by pro-inflammatory mediators. Recent studies have suggested that I/R injury may be attenuated through therapeutic targeting of these inflammatory receptors, such as RAGE, toll-like receptor (TLR) 2, and TLR4. These observations predict that blocking the HMGB1 signal may protect the heart from I/R injury. However, another study of HMGB1 showed that exogenous HMGB1 induced myocardial cell proliferation and differentiation in the infarcted heart tissue. In the present study, neutralizing anti-HMGB1 monoclonal antibody (mAb) was used to elucidate the role of HMGB1 in cardiac I/R injury.
Methods

I/R Model

Male Wister rats, weighing 270–330 g, were obtained from Japan SLC, Inc (Hamamatsu, Japan) and were housed in a room at a temperature of 22±2°C and an alternating 12-h light and dark cycle. Food and water were given ad libitum. All studies conformed to the “Guide for the Care and Use of Laboratory Animals” published by the US NIH (National Institutes of Health Publication No. 85–23, revised 1996) and were approved by the Committee on Animal Experiments of Okayama University.

The myocardial I/R model were established according to a method reported by Yorozuya et al. Each rat was anesthetized by an intraperitoneal injection of sodium pentobarbital (50mg/kg), placed in the supine position and underwent a tracheotomy. Respiration was maintained by ventilating the animal with positive pressure respiration of oxygen (1.0 L/min) using a Shinano respirator (SN-480–4). A catheter for intravenous (iv) injection was inserted into the right internal jugular vein. An interarterial catheter was inserted into the femoral artery for measuring arterial blood pressure and heart rate (HR). Following a thoracotomy in the left 4 intercostal spaces, the pericardium was opened. The anterior descending left coronary artery and apex were occluded temporarily with 4-0 silk thread for 30 min. Cardiac ischemia was confirmed by a change in ventricular color, from fresh-red to dark red, and by the detection of arrhythmias. After 30-min ischemia and before reperfusion, either anti-HMGB1 mAb or class-matched mAb against keyhole limpet (KHL) hemocyanin was injected iv in a volume of 0.5 ml through the right internal jugular vein followed by a 1-h reperfusion. Sham rats underwent a similar operation without I/R or antibody infusion. The non-ischemia group was treated with anti-HMGB1 mAb without I/R.

Measurement of Cardiac Function

The indexes of cardiac function, including HR and mean arterial blood pressure (MBP), were monitored using model AP-601G and AP-641G blood pressure amplifiers (Nihon Kohden, Tokyo, Japan) and analyzed using a cardiograph (Power lab; AD Instruments, Otago, New Zealand).

Measurement of Infarct Size and the Salvage Rate

The heart was perfused with saline from the ascending aorta following I/R. The left anterior descending coronary artery was occluded at the same position as before and 4 ml of fluorescent particles (Fluorescent polymer microspheres, aorta following I/R. The left anterior descending coronary artery was occluded for 30 min, followed by a 1-h reperfusion. Sham rats underwent a similar operation without I/R or antibody infusion. The non-ischemia group was treated with anti-HMGB1 mAb without I/R.

Estimation of Plasma Troponin-T (TpT), Lactate Dehydrogenase (LDH) and Aspartate Aminotransferase (AST)

Blood samples (0.5 ml) were collected via the arterial catheter at 4 time points (pre-ischemia (~30 min), pre-reperfusion (0 min), and post-reperfusion (30 min/60 min)), and centrifuged to obtain plasma samples. TpT, LDH, AST and norepinephrine (NE) levels were analyzed using standard methods established by the SRL Corp (Tokyo, Japan).

Immunohistochemistry

Following I/R, the rat heart was immediately harvested and perfused through the left ventricle with 50% formalin. The fixed heart specimens were embedded in paraffin and used for histological examination. A mouse anti-HMGB1 antibody (1:1,000; Santa Cruz, Santa Cruz, CA, USA) was used for immunohistochemical staining.

Catecholamine Analysis

Cardiac samples were prepared by homogenization of heart tissue in 0.2 mol/L HClO4 and the levels of NE and dihydroxyphenylglycol (DHPG) were determined by high-performance liquid chromatography (ODS-100V; Tos, Tokyo, Japan) combined with electrochemical detection according to method described by Ogawa et al. NE and DHPG data were expressed as nanograms per gram tissue (ng/g).

ELISA Analysis

Blood samples (0.5 ml) were collected via the arterial catheter, and centrifuged (1,000 rpm, 30 min) to obtain plasma samples and levels of HMGB1 were determined according to the manufacturer’s protocol (Shino-Test, Sagamihara, Japan) and expressed as nanograms per milliliter (ng/ml).

Monoclonal Antibodies

The preparation and characterization of the neutralizing anti-HMGB1 mAb (#10–22) have been described previously. Class-matched (IgG2a) anti-KHL hemocyanin mAb was used as a control.

Real-Time Polymerase Chain Reaction (PCR)

The myocardium corresponding to the core infarct area was dissected on ice, and preserved at ~8°C until homogenization. Total RNA was isolated with Bio-Robot EZ1 (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA (1 µg) was reverse-transcribed with avian myeloblastosis virus reverse transcriptase XL and Oligo dT-primers (Takara RNA Kit; Takara Biomedicals, Shiga, Japan). PCR was performed using Takara Ex taq HS DNA polymerase (Takara). The sense and antisense primers used for the analysis of the expression of tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS) and GAPDH were as follows: TNF-α: 5'-AATCTGAGACAA-GCCCGTAG-3' and 5'-GAACAGTCTGTAGTGATTTAT-3'; iNOS: 5'-GATTCAGATGCAGGATAGTCTTTG-3' and 5'-GAAGCTTCCGCGGAGCTC-3'; GAPDH: 5'-AACTCGAGACAAG- GCCCGTAG-3' and 5'-GATTCCACGATTTGTCTTTGA-3'. Real-time PCR was performed with SYBR Premix EX Taq (Takara) in a light Cycler instrument (Roche, Basel, Swiss) according to the manufacturer’s instructions. The expression of GAPDH was used to normalize the cDNA levels. PCR products were analyzed by a melting curve to ascertain the specificity of amplification.

Data Analysis

All data are expressed as the mean±SEM. Group com-
Comparisons were performed using 1-way ANOVA of repeated measurements, followed by Student's t-test. For post hoc analysis, the Bonferroni test was applied; p<0.05 was considered to be statistically significant.

Results

HMGB1 Expression After I/R Injury in Rat Hearts

Immunohistochemical studies were performed in normal rat hearts and hearts that underwent I/R to determine the cellular localization of HMGB1. Immunoreactivity of HMGB1 was observed in the nuclei of cardiac muscle cells in the normal heart (Fig 1B). The distribution of HMGB1 after I/R injury changed drastically from the nucleus to the cytoplasm, associated with a marked increase in the density of the immunoreactivity (Fig 1D). In hematoxylin-eosin staining, it appeared that the muscle fibers were disrupted, and an enlargement of the extracellular space was observed after I/R (Fig 1C), whereas no such damage was observed in the hearts from normal rats (Fig 1A). The plasma levels of HMGB1 at 60 min after reperfusion increased 2.6-fold in comparison with the values obtained immediately before (pre I/R) and 60 min after reperfusion (after I/R). Data are means±SEM; *p<0.05 vs pre I/R value.

Effects of Anti-HMGB1 mAb on Post-Ischemia Cardiac Function

There were no significant differences in the baseline hemodynamic variables (HR, MBP, and rate-pressure product) (Table 1). There were no significant differences in the hemodynamic variables at 15 min of occlusion, 5 min of reperfusion, 30 min of reperfusion, and 60 min of reperfusion compared with the baseline variables within each group.

I/R Injury in the Anti-HMGB1 mAb Treated Rats

There were no significant differences in the heart weight or the volume of the ischemia risk area between the control mAb treatment group and the anti-HMGB1 mAb treatment group. Representative transverse sections of control IgG-treated and anti-HMGB1 mAb-treated hearts after I/R are shown in Fig 2. In the anti-HMGB1 mAb-treated heart, the necrotic area was enlarged significantly in comparison with the control IgG-treated heart. The infarct size as a percentage of the size of the at-risk area was 40.9±4.2% and 53.7±3.9% in the control IgG and anti-HMGB1 mAb-treated rats, respectively, showing that the relative infarct size in the anti-HMGB1 mAb-treated rat was 31% greater than that in the control IgG-treated rats (p<0.05, Fig 2E). The plasma TpT levels were undetectable before the induction of ischemia, but after reperfusion, the levels of TpT, AST, and LDH were markedly elevated in both groups, though significantly greater in the anti-HMGB1 mAb-treated rats (Fig 3).
the levels of NE and its metabolite, DHPG, were analyzed. In the control rats, there was a marked increase in DHPG levels in comparison with the sham-operation group, whereas NE was maintained at the same level as in the sham-operation group. Anti-HMGB1 mAb treatment significantly increased the NE levels in comparison with both the sham and control IgG antibody (Ab)-treated groups (Figs 4,5). On the other hand, the DHPG levels of the anti-HMGB1 mAb group were significantly lower than those of the control Ab-treated group (Fig 5). NE and DHPG levels in the non-ischemia group were not different from those in the sham-operated group.
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Analysis of the Expression of TNF-α and iNOS in the Heart

The expressions of the mRNAs of TNF-α and iNOS were quantified by real-time PCR. Cardiac I/R significantly upregulated the expression of TNF-α and iNOS compared with sham-operated and non-ischemia hearts (Fig 6). Anti-HMGB1 mAb treatment slightly increased the expression of iNOS compared with the values in the control IgG treated rats, but the increase did not reach a significant level. Treatment with anti-HMGB1 mAb alone (non-ischemia group) also had no effect on TNF-α and iNOS expression.

Discussion

This study has demonstrated that anti-HMGB1 mAb treatment exacerbated myocardial infarction in the rat after I/R, associated with elevated plasma levels of markers of cardiomyocyte damage, such as TpT, AST, and LDH, in a comparison with control IgG treated rats. The results also show that anti-HMGB1 mAb increased the cardiac NE content and suppressed the level of its metabolite (DHPG), suggesting that anti-HMGB1 mAb treatment strongly inhibited the transport of NE accumulated during reperfusion, and the resulting metabolism of NE in sympathetic nerve terminals. It is probable that the prolonged elevation of interstitial NE induced by anti-HMGB1 treatment resulted in cardiac damage in the I/R heart (ie, NE toxicity). The mAb used in the present study was specific for HMGB1, so these results suggest that endogenous HMGB1 released from cardiomyocytes may modulate, in part, NE metabolism during I/R injury in the rat heart.

HMGB1 and NE Release

The current study demonstrated that myocardial NE levels significantly increased and DHPG levels were suppressed with anti-HMGB1 mAb treatment following ischemia. Other studies have indicated that high concentrations of interstitial NE are deleterious to the heart. The interstitial NE concentration is regulated by various factors such as exocytotic release from the cytoplasmic fraction of sympathetic nerve endings, from storage vesicles, and NE transport via uptake carriers. Interstitial NE levels, as determined by microdialysis, have been reported as gradually increasing during ischemia, and then rapidly decreasing after reperfusion because of a washout flow effect and recovery of NE uptake by cardiac sympathetic nerve endings. In addition, DHPG production has been widely used to evaluate intraneuronal...
NE metabolism after re-uptake into presynaptic terminals, as well as NE transport into sympathetic nerve endings. The myocardial DHPG levels significantly increase after reperfusion, because re-oxygenation following I/R causes functional recovery of the uptake carriers that transport NE from the interstitial space to the cardiac sympathetic nerve endings, thus facilitating oxidation of NE by monoamine oxidase (MAO), which is involved in the formation of DHPG. In fact, the interstitial concentration of NE during 30 min of ischemia has been reported to reach to 100-fold of normal plasma concentration, which may cause cardiomyocyte necrosis even in a non-ischemic heart. In the current study, anti-HMGB1 mAb treatment induced an approximately 1.5-fold increase in NE content in comparison with sham-operated heart tissue specimens and the control IgG-treated I/R heart. On the other hand, the DHPG content in the anti-HMGB1 treated heart was suppressed to 50% of that in the control IgG-treated rat heart. This pattern of change in NE dynamics raises the possibility of inhibition of uptake of interstitial NE and resultant reduced NE metabolism, reflecting a sustained increase in interstitial NE in the reperfused heart. It is probable that the increased levels of DHPG content in the IgG-treated-control heart reflect the facilitation of NE uptake and metabolism via recovered MAO activity by re-oxygenation during reperfusion. The dramatic decrease in the suppression of the DHPG production in the anti-HMGB1 mAb-treated heart was consistent with the idea that the anti-HMGB1 mAb markedly inhibited the re-uptake of NE into the metabolic pool. The in vivo observation of a high NE content and low DHPG induced by anti-HMGB1 mAb production suggests that HMGB1 may modulate the dynamics of interstitial NE in the I/R heart.

HMGB1 and Inflammation in the I/R Heart

The cardiomyocyte expresses several HMGB1 receptors, including TLR2, TLR4, and RAGE, and recent studies have suggested that these receptors perform very important roles in inflammation and I/R injury. RAGE signaling may affect myocardial energy metabolism while decreasing contractile function during I/R. TLR2 signaling may play an important role in left ventricle dysfunction following I/R and the innate immune responses mediated by TLR4 signaling may also exert a proinflammatory role during I/R in the heart. Therefore, blocking these receptors might induce a protective effect. Furthermore, we previously observed that anti-HMGB1 mAb suppressed I/R-induced brain injury in a transient middle cerebral artery occlusion model in rats. From these findings it is possible to predict that inhibition of HMGB1 activity with neutralizing mAb may thus significantly decrease the progression of cardiac I/R damage. However, in the present study rat hearts treated with the neutralizing anti-HMGB1 mAb-treated had remarkably increased cardiomyocyte damage following I/R. Moreover, markers of inflammation, such as TNF-α and iNOS, were not suppressed by anti-HMGB1 treatment. These results strongly suggest that endogenous HMGB1 in the I/R heart may not produce any deleterious inflammatory responses, in sharp contrast to the I/R injuries observed in other organs.

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References


