EXPERIMENTAL STUDY

Porphyromonas Gingivalis Elevated High-Mobility Group Box 1 Levels After Myocardial Infarction in Mice

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Summary

High mobility group box 1 (HMGB1) is a nuclear protein released from necrotic cells, inducing inflammatory responses. Epidemiological studies suggested a possible association between periodontitis and cardiovascular diseases (CVDs). Due to tissue damage and necrosis of cardiac cells following myocardial infarction (MI), HMGB1 is released, activating an inflammatory reaction. However, it remains unclear whether periodontitis is also involved in myocardial damage. The purpose of this study was to determine the effect of the periodontal pathogen Porphyromonas gingivalis (P.g.) after MI in mice.

C57BL/6J wild type mice in post-MI were inoculated with P.g. in the infected group (P.g.-inoculated MI group) and with phosphate buffer saline (PBS) in the control group (PBS-injected MI group). Plasma samples and twelve tissue samples from mice hearts after MI were obtained. We determined the expression of HMGB1 by ELISA and immunohistochemistry.

The level of HMGB1 protein in the P.g.-inoculated MI group was significantly higher than in the PBS-injected MI group on day 5, but not on day 14. Immunohistochemistry analysis revealed that HMGB1 was mainly expressed in cardiomyocytes, immune cells, and vascular endothelial cells in the PBS-injected MI group, while HMGB1 was seen broadly in degenerated cardiomyocytes, extracellular fields, immune cells, and vascular endothelial cells in the P.g.-inoculated MI group. A significant increase in the number of HMGB1 positive cells was observed in the P.g.-inoculated MI group compared to the PBS-injected MI group.

Infection with P.g. after MI enhanced myocardial HMGB1 expression. There is a possible relationship between periodontitis and post-infarction myocardial inflammation through HMGB-1.

Key words: Cardiovascular disease, Periodontopathic bacteria, Post-infarction myocardial inflammation

High mobility group box protein 1 (HMGB1) was initially identified as a nuclear protein implicated in maintaining the nucleosome structure and the regulation of gene transcription. Furthermore, HMGB1 acts as a cytokine released actively by immune cells and passively by necrotic cells under inflammatory or injurious conditions. It is widely accepted that HMGB1 is a danger signal, acting as a link between cellular damage and inflammation, activating the immune system. Most cells, including macrophages and monocytes, express HMGB1 mRNA and protein in un-stimulated conditions. Macrophages and monocytes actively release HMGB1 in response to pathogenic products (LPS) or endogenous pro-inflammatory cytokines (TNF, IL-1β, IFN-γ) in a time- and dose-dependent manner. HMGB1 can also be passively released by necrotic or damaged cells. HMGB1 is capable of activating an inflammatory response, then transferring the injury signal to nearby immune cells. Cells undergoing apoptosis also release HMGB1, but they are immunologically inactive. Thus, cells undergoing apoptosis fail to significantly stimulate inflammatory responses. HMGB1 interacts with several receptors that can be activated by exogenous (TLR-2, TLR-4, TLR-9) and endogenous (RAGE) ligands. Moreover, HMGB1 lost their pro-inflammatory activity, following administration of HMGB1 antagonists and knockout their receptor (TLR-
4) techniques.

Myocardial infarction (MI) is an irreversible necrosis of heart muscle caused by the occlusion of a coronary artery secondary to prolonged lack of oxygen supply, leading to tissue necrosis and defect formation. Increasing evidence exists for the importance of extracellular HMGB1 in the pathophysiology of MI. In this context, recent studies have shown that HMGB1 was upregulated and released by ischemic tissue necrosis in vivo, including the brain, liver, and heart. Elevated plasma levels of HMGB1 have been detected in rat and human patients after MI. HMGB1 displays specific danger signal functions because it is released by necrotic cells. The prolonged presence of active inflammation can be harmful for the injured heart and eventually results in heart failure.

Periodontitis is a chronic inflammatory disease caused by gram-negative anaerobic bacteria that results in bone resorption, destruction of the connective tissue, and loss of teeth. Porphyromonas gingivalis (P. g.) is a major pathogen in human periodontitis. Recently, researchers have indicated that there might be a connection between periodontitis and cardiovascular disease (CVD), including MI. It has been reported that 10% to 15% of the periodontal patients had been linked to CVD. We previously showed that periodontopathic pathogens deteriorated ventricular remodeling after MI and other cardiovascular diseases. However, a detailed mechanism is still to be elucidated. We hypothesized that infection with a periodontal pathogen, P. g., could cause an adverse outcome after MI via HMGB1. Thus, the purpose of this study was to investigate the effect of P. g. on HMGB1 expression after MI in mice.

Methods

Mice and subcutaneous chamber model: A subcutaneous chamber model was used as previously described. Male C57BL/6J wild type mice (7 weeks, 20-25 g) were obtained from Japan Clea, Co. (Tokyo, Japan). All animal care and experimental procedures were approved by the Tokyo Medical and Dental University for the Care and Use of Laboratory Animals (permit number: 0150166 A) and by the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Euthanasia was properly performed by overdose application of 3.6% chloral hydrate (intraperitoneal administration).

Coil-shaped chambers (length 10 mm, diameter 5.0 mm), prepared from 0.5 mm stainless-steel wire were surgically implanted subcutaneously in the dorsal region of each mouse. After a 2-week of healing period, the chamber was used as a biological compartment to inoculate bacteria by injection. P. g. (0.1 mL of 10^6 CFUs/mL) or phosphate-buffered saline (PBS) (0.1 mL) was injected into the lumen of the chambers to induce inflammation once a week.

Bacterial growth and infection of periodontal pathogens: P. g., strain A7A1-28, was grown on blood agar plates in an anaerobic chamber with 85% N₂, 5% H₂, 10% CO₂, and incubated at 37 °C for two to three days. Bacterial cells were inoculated in a peptone yeast extract for one-week incubation under the same conditions. The bacterial concentrations were standardized to 10^6 colony forming units (CFUs/mL). Two weeks after chamber implantation, mice were injected with live P. g. (0.1 mL of 10^6 CFUs/mL), or PBS once a week for two weeks and subjected to MI induction.

Experimental myocardial infarction model: MI was induced as previously described. Briefly, the mice were anesthetized with 3.6% chloral hydrate (intraperitoneal administration, 0.1 mL/10 g body weight) and intubated with a small rodent respirator (MiniVent 845, Harvard Apparatus, Holliston, MA). Left lateral thoracotomy was performed, and the left anterior descending (LAD) coronary artery was ligated after removal of the pericardium using an 8/0 nylon suture. The chest was closed, and the mice were removed from the respirator. The mice were allowed to recover on a warmed surface. The mice were evaluated for up to five days and fourteen days after MI and then sacrificed to obtain samples. An autopsy was performed on all mice as described previously.

Measurement of plasma levels of HMGB1: Plasma samples were obtained when the mice were sacrificed on days 5 and 14, and the level of HMGB1 was determined by an enzyme-linked immunosorbent assay (ELISA) with an immunoassay kit (Shino-Test, Tokyo, Japan). We also measured the control levels of HMGB1 on day 0 prior to the MI induction (P. g. inoculation only), and mice with sham operation. The ELISA was performed according to the manufacturer’s instructions.

Histopathological examinations: The sections were prepared through the heart on day 5 for immunohistochemistry. The tissue specimens were routinely fixed in formalin and embedded in paraffin. The embedded tissues were sectioned into 5 micrometers thick tissue sections using microtome. The sections were dewaxed with xylene and rehydrated through a series of ethanol gradients, and then, antigen retrieval and blocking was performed. The sections were then incubated with rabbit polyclonal anti-HMGB1 as the primary reagent (1:1000, Abcam, Cambridge, UK) at 4 °C for overnight. After a thorough washing with PBS on shaker, the sections were incubated with biotinylated donkey anti-rabbit antibodies (1:200, Dianova, Hamburg, Germany) as the secondary reagent in room temperature for 1 hour. After washing with PBS, the sections were developed with an avidin-biotin alkaline phosphatase (ABC-AP, Dako, Hamburg, Germany) and observed under a microscope. In all cases, parallel incubations with nonimmune IgGs of the relevant species served as negative control. The immunohistochemical results were determined by counting the positively stained cells in each section under a light microscope (X 400 microscopic fields). We counted the positive cells in each whole heart sample and compared them among the following myocardial areas: 1) infarcted area (anterior wall), 2) peri-infarcted area (lateral and septal wall), and 3) remote viable area (inferior wall) in ×100 microscopic fields.

Statistical analysis: All data are expressed as the mean ± SEM. All statistical analyses were performed using an unpaired Student’s t-test. A value of P < 0.05 was considered to be significant.
Results
Changes in plasma levels of HMGB1 after MI for \textit{P.g.}-inoculated MI mice: As we previously described,21) \textit{P.g.} infection deteriorated myocardial remodeling after MI compared to PBS injected MI mice. To evaluate the effect of \textit{P.g.} infection on the level of plasma HMGB1 in MI mice, we performed ELISA. The level of HMGB1 protein significantly increased in the \textit{P.g.}-inoculated MI mice group compared to the PBS-injected MI mice on day 5 (27.75 ± 0.89 versus 12.67 ± 0.20, \(P < 0.05\)), but not on day 14 (24.31 ± 2.23 versus 21.05 ± 1.21). The control serum samples on day 0 prior to the MI induction and mice with sham operation showed low HMGB1 levels (Figure 1).

Expression of HMGB1 in the \textit{P.g.}-inoculated MI mice heart: In order to confirm the effect of \textit{P.g.} infection on HMGB1 expression in MI hearts, we performed immunohistochemistry on day 5. In the PBS-injected MI group, HMGB1 was mainly expressed in cardiomyocytes, immune cells, and vascular endothelial cells. However, HMGB1 was seen broadly in degenerated cardiomyocytes, extracellular fields, immune cells, and vascular endothelial cells in the \textit{P.g.}-inoculated MI group. A significant increase in the number of HMGB1 positive cells was observed in the \textit{P.g.}-inoculated MI group compared to the PBS-injected MI group. (6023.33 ± 1406.74 versus 3139.33 ± 940.44, \(P < 0.05\)) (Figures 2, 3A). We compared the HMGB1 positive cell numbers among the following myocardial areas: 1) infarcted area (anterior wall), 2) peri-infarcted area (lateral and septal wall), and 3) remote viable area (inferior wall). The HMGB1 positive cells in all areas in the \textit{P.g.}-inoculated MI group showed significant increase compared to those in the PBS-injected MI group (Figure 3B-3E).

Discussion
The present study revealed that periodontopathic bacteria, \textit{P.g.} affect MI in mice. The major finding of this study was that \textit{P.g.} increased the release of plasma HMGB1 in post-MI mice. It also resulted in a significant rise in the number of HMGB1 positive cells in MI hearts compared with PBS-injected MI mice. These data suggest that there is a possible relationship between periodontitis and post-infarction myocardial inflammation.

HMGB1 acts as a cytokine passively released from a variety of cells undergoing necrotic cell death or damaged cells and serves as a signal for inflammation. In agreement with our present study, Kitahara, \textit{et al.} reported that HMGB1 was released from the necrotic cardiomyocytes into the circulation in HMGB1-transgenic mice and wild-type littermate mice after MI.14) Wang, \textit{et al.} also demonstrated that HMGB1 mRNA and protein expression increased in early stage (1 week) after MI, and significantly down-regulated in later stage (4-8 weeks).31) After released, HMGB1 acted as a mediator of inflammation and organ damage in ischemic-reperfusion injury of the heart, activating of pro-inflammatory pathways and enhanced myocardial injury.12,32) Previous studies have shown that HMGB1 functions as a pro-inflammatory cytokine in certain cardiovascular diseases, and it was related to the severity of coronary artery disease.33,34) Yao, \textit{et al.} demonstrated that myocardial expression of HMGB1 significantly increased in rats with acute MI.35) High serum levels of HMGB1 may cause cardiac inflammation and left ventricular dysfunction after MI.

Periodontopathic bacteria, especially \textit{P.g.} which is strongly associated with the most common type of periodontitis, appear in specimens of atherosclerotic plaques36) and were also found in thrombus obtained from the oc-
Numerous studies have shown that patients with periodontitis were at risk for bacteremia from brushing or dental treatment. The periodontal bacteria may invade the peripheral vessels, and some bacteria may infiltrate in the heart. Akamatsu, et al. reported that experimental transient P. g. bacteremia could induce MI in mice and it might possibly induce severe MI by repeat P. g. infection. It is well recognized that periodontal pathogens increase the expression of pro-inflammatory cytokine including HMGB1. Significantly elevated levels of HMGB1 and a large number of positive cells were found in gingival crevicular fluid and inflamed gingival epithelial cells in periodontal patients as compared to healthy patients. Even now, the direct relationship between P. g. infection and HMGB1 expression is unknown. However, there are many papers showing the deep relationship between HMGB1 and Toll-like receptors (TLRs). Pahwa, et al. demonstrated that TLRs play a significant role in the enhancement of HMGB1 expression in diabetic vascular disease. Böhm, et al. also revealed that TLR-2, TLR-4, and HMGB1 were enhanced synergistically in 661W cells exposed to elevated pressure. Deng, et al. demonstrated that TLR-4 mediated acute lung injury induced by HMGB1. Because P. g. is recognized via these TLRs, its infection can aggravate HMGB1-induced MI.

In the present study, we demonstrated that the level of HMGB1 protein was significantly increased in the P. g.-inoculated MI mice group compared to the PBS-injected MI mice on day 5 but not on day 14. This result implied that P. g. elevated the early release of HMGB1 from post-MI damaged cells, necrotic cell and inflammatory cells that were likely to trigger and sustain the initial inflammation. We compared the HMGB1 positive cell numbers among infarcted, peri-infarcted and remote viable areas. Our results showed that the HMGB1 positive cells in all areas in the P. g.-inoculated MI group increased compared to those in the PBS-injected MI group. This means that the myocardial ischemia and chronic inflammation induced by a periodontal pathogen influenced all areas of the hearts.

A previous study showed that HMGB1 significantly enhances the production of TNF-α both in vitro and in vivo. Pro-inflammatory cytokines might further promote inflammatory cell adhesion and infiltration into the myocardium and enhance tissue injury. Immunohistochemical results in this study showed that P. g. accelerated the number of HMGB1 positive cells in post-MI mice as compared with PBS-injected post-MI mice. HMGB1 is released by many cell types including cardiomyocytes, immune cells, and vascular endothelial cells. In the previous study, HMGB1 was released by hypoxic cardiomyocytes in vitro and is increased in the infarcted myocardium in vitro.
Activated macrophages and monocytes secrete HMGB1 as a mediator of inflammation. The mechanism of inflammation is bound to TLRs. It also mediates HMGB1-dependent activation of macrophage cytokine release. Vascular endothelial cells also express HMGB1, thus, HMGB1 may be implicated in the induction of adhesion molecules in an infarcted myocardium and occupy a rule in mediating inflammation that activates cytokines and endothelial activation.

The present study demonstrated that *P. g.* significantly increased HMGB1 plasma levels and the number of HMGB1 positive cells in mice hearts after MI. These results indicate that *P.g.* might aggravate myocardial injury following MI by elevating the expression of HMGB1. Experimental research that mainly target *P.g.* infection which induce HMGB1 expression effectively prevent synergistic activation of inflammation and will attenuate cell injury or tissue damage in post-MI. It is also well known that there is a relationship between apoptosis and HMGB1 expression. We previously evaluated apoptotic cells in MI hearts with or without *P.g.* infection in our previous paper. We clearly showed that TUNEL positive apoptotic cells increased in *P.g.* infected MI hearts compared to non-infected MI hearts. Excessive inflammation post-MI has been shown to impair infarct healing. Andrassy, et al. have revealed HMGB1 as a precise mediator of inflammatory processes in the initiation of MI and subsequent myocardial remodeling. This is because its active release by mononuclear cells and passive release from necrotic or damaged cells.

It has been reported that there are several patents proposed for controlling the production, secretion and neutralization of HMGB1 and consequently the inflammatory process. Among them, anti-HMGB1 antibodies and HMGB-A box as a competitive antagonist of HMGB1 are the most reliable methods to regulate HMGB1 directly. Furthermore, anti-TLR-2 antibodies might be effective in the prevention of *P.g.* induced HMGB1 expression. Therapeutic target phase of HMGB1 on MI should also be discussed. Loukili, et al. showed that the expression of HMGB1 in hearts increased significantly after 1 hour of reperfusion, further increased after 2 hours of reperfusion, and then no further increase was observed thereafter. As we showed in this study, *P.g.* infection significantly elevated serum HMGB1 levels on day 5, but not on day 14. These results suggest that early HMGB1 increase might influence MI hearts, thus, the therapeutic target phase of HMGB1 on MI with *P.g.* infection might be early time points than late phases after MI. Future studies are needed to clarify its effects and safety before it is used in clinical settings.

Disclosures

Conflicts of interest: None.

References


8. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol 2007; 81: 1-5. (Review)


