Original Paper

High Mobility Group B-1 (HMGB-1) Promotes Apoptosis of Macrophage-Derived Foam Cells by Inducing Endoplasmic Reticulum Stress

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Key Words

High mobility group B-1 • Foam cell formation • Apoptosis • Endoplasmic reticulum stress

Abstract

Background/Aims: High mobility group B-1 (HMGB-1)-induced endoplasmic reticulum stress (ERS) has been implicated in inflammation and dendritic cell maturation. C/EBP-homologous protein (CHOP) is a vital component of ERS and apoptosis and plays a critical role in atherosclerosis. However, only a little information is available about the role of HMGB-1 in foam cell formation. Thus, the role of HMGB-1-induced ERS/CHOP pathway in apoptosis and formation of macrophage-derived foam cells is investigated. **Methods:** RAW264.7 cells were treated with oxidized low-density lipoprotein (oxLDL) in the absence and/or presence of HMGB-1, N-acetylcysteine (NAC, an antioxidant), glycyrrhizin (Gly, an HMGB-1 inhibitor), tunicamycin (TM, an ERS inducer), and 4-phenylbutyrate (4-PBA, an ERS inhibitor). Reactive oxygen species (ROS) production was examined by dihydroethidium (DHE) staining. Oil Red O staining, intracellular total cholesterol assay, and Dil-oxLDL uptake assay evaluated the accumulation of lipids in macrophages. Cell apoptosis was measured by flow cytometry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Western blot detected the expression of HMGB-1/ERS/CHOP pathway. **Results:** oxLDL induced HMGB-1 translocation and secretion in a dose- and time-dependent manner, which was inhibited by NAC. oxLDL-induced lipid accumulation in macrophages was promoted synergistically by HMGB-1 that was attenuated by Gly. Moreover, TM synergized with oxLDL induced lipid accumulation and apoptosis of macrophages; however, 4-PBA alleviated the oxLDL-induced apoptotic foam cells. Additionally, the inhibition of ERS with 4-PBA suppressed the expression of HMGB-1-induced CHOP. **Conclusions:** OxLDL triggered HMGB-1 secretion in macrophages via oxidative stress. Furthermore, HMGB-1 promoted the formation and apoptosis of macrophage-derived foam cells via activation of ERS/CHOP pathway.

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Introduction

Atherosclerosis is the leading cause of deaths worldwide, with deteriorated clinical manifestations such as myocardial infarction, peripheral vascular diseases, and stroke [1]. Macrophages play a crucial role in all the stages of atherosclerosis development. Oxidized low-density lipoprotein (OxLDL), the strongest atherogenic factor, has been considered to promote the transformation of macrophages into foam cells, contributing to fatty streaks in atheroma. In addition, the apoptosis of foam cells has been recognized as a critical component in advanced plaques, which is the essential hallmark of vulnerable plaques [2-4] and leads to thrombosis-related acute ischemic cardiovascular events. However, the molecular mechanisms underlying apoptosis of macrophage-derived foam cells remain poorly understood.

High mobility group B-1 (HMGB-1), a conserved DNA-binding protein, is primarily expressed by macrophages. It can translocate from nucleus to cytosol and release into extracellular milieu under stress conditions. Extracellular HMGB-1 functions as a pro-inflammatory cytokine and plays a critical role in various pathological processes [5-7]. Early reports showed increased levels of serum HMGB-1 in patients with coronary artery disease [8, 9]. In apolipoprotein E-deficient (apoE^{-/-}) mice fed high-fat diet, treatment with neutralizing antibodies against HMGB-1 significantly prevented the development of atherosclerosis [10]. Further evidence revealed the critical role of HMGB-1 in human atherosclerosis lesions from the aorta, indicating high expression of HMGB-1 in carotid arteries [11], coronary arteries [12], and peripheral vessels [13]. In addition, macrophages have been considered to be primarily responsible for HMGB-1 expression in atherosclerosis [14], suggesting that the protein might promote macrophage-derived foam cells in plaques. However, the exact role of HMGB-1 in macrophage-derived foam cells remains unclear.

Endoplasmic reticulum stress (ERS), characterized by the accumulation of misfolded proteins in the ER lumen, exhibits homeostatic signaling pathways such as unfolded protein response (UPR). Accumulating evidence illustrated that severe ERS plays a critical role in foam cell formation and the development of atherosclerosis [15, 16]. Furthermore, ERS induced C/EBP-homologous protein (CHOP)-related apoptotic signaling pathway in macrophages of advanced atherosclerotic plaques and promoted the rupture formation of plaques [2]. Thus, the present study aimed to investigate whether HMGB-1 induced the apoptosis of macrophage-derived foam cells via ERS/CHOP pathway.

Materials and Methods

Cell culture and reagents

RAW264.7 cells were obtained from Keygen Biotechnology (Nanjing, China). The cells were cultured in DMEM medium containing 10% fetal bovine serum at 37 °C in a humidified incubator with 5% CO₂ until subconfluent. Then, the medium was replaced with serum-free media, and the cells treated for different experiments. OxLDL was obtained from Yiyuan Biotech (Guangzhou, China). HMGB-1 was from R&D Systems (Minneapolis, MN, USA). N-acetylcysteine (NAC) was obtained from Beyotime Institute of Biotechnology (Haimen, China), glycylrhizin (Gly) and 4-phenylbutyrate (4-PBA) from Sigma-Aldrich (St. Louis, MO, USA), and tunicamycin (TM) from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Oil red O staining

After the designated treatment, the cells were washed with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 30 min at room temperature. Then, the cells were stained with 0.5% Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) solution for 20 min at room temperature and counterstained with hematoxylin (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) for 2 min, followed by PBS washes and microscopic enumeration.

Uptake assay

The cells were incubated with Dil-oxLDL for 24 h, following which, the medium was removed. After two PBS washes, the cells were observed under fluorescence microscope (Olympus, Tokyo, Japan).

Assessment of oxidative stress

The macrophages were seeded in 6-well plates and subjected to serum starvation, followed by treatment as indicated in the Fig. 2. Then, intracellular oxidative stress was determined using dihydroethidium (DHE) according to the manufacturer's instructions. Briefly, the cells were incubated with 10 $\mu\text{mol/L}$ DHE (Beyotime Institute of Biotechnology, Haimen, China) for 30 min at 37 °C in the dark. Then, the cells were washed three times with PBS and observed under fluorescence microscope. Three fields were selected randomly in each well, and the DHE intensities quantified using the Image J software.

Cell apoptosis assessment

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining assay and annexin V/propidium iodide (PI) double staining assays were used to quantify cell apoptosis. For TUNEL staining, the cells were fixed with 4% paraformaldehyde for 30 min at room temperature, washed two times with PBS, and permeabilized with 0.1% Triton X-100 for 5 min. Subsequently, the cells were subjected to TUNEL staining (Roche, Basel, Switzerland) for 1 h at room temperature in the dark, followed by 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO, USA) staining for the detection of cell nuclei. Images were acquired using a fluorescence microscope from three fields selected randomly in each well. The number of apoptotic cells was quantified using Image J software.

Annexin V/PI apoptosis detection kit was also used for examining cell apoptosis. Briefly, the cells were washed two times with cold PBS and resuspended in 500 μL binding buffer. Subsequently, 5 μL Annexin V and PI, each was added to the cells at room temperature in the dark. Finally, the cells were collected, resuspended in cold binding buffer for flow cytometry (Thermo Fisher Scientific, Waltham, MA, USA).

Western blot

The nuclear and cytosolic proteins were extracted from the cells using the nuclear and cytoplasmic protein extraction kit (Beyotime Biotechnology, Haimen, China) according to the manufacturer's instructions. Total proteins were harvested in RIPA lysis buffer (Beyotime Biotechnology, Haimen, China) containing protease and phosphatase inhibitor tablets (Roche, Basel, Switzerland). The concentrations of the collected proteins were determined by bicinchoninic acid protein assay (BCA; Pierce, Rockford, IL, USA). Equivalent amounts of protein samples or cell supernatant were separated on 10–15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). After blocking with 5% non-fat milk in tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, the membranes were probed with the following primary antibodies overnight at 4 °C: GAPDH (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Lamin B (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), HMGB-1 (1:2000, Bioworld Technology Inc., St. Louis, MN, USA), Glucose regulated protein 78 (GRP78) (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), phosphorylated inositol-requiring protein 1 (pIRE1) (1:1000, Cell Signaling Technology, Danvers, MA, USA), phosphorylated protein kinase RNA-like ER kinase (pPERK) (1: 1000, Cell Signaling Technology, Danvers, MA, USA), and CHOP (1: 2000, Cell Signaling Technology, Danvers, MA, USA). Subsequently, after three washes with TBST buffer, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000, Santa Cruz Biotechnology) at room temperature for 2 h. The immunoreactive bands were assessed using an enhanced chemiluminescence reagent (Pierce, Rockford, IL, USA). GAPDH and Lamin B were used as internal controls, and protein levels were quantified using Quantity One analysis software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Immunofluorescence

After the designated treatment, the cells were washed three times with cold PBS, fixed in 4% paraformaldehyde for 20 min, and permeabilized with 0.1% Triton X-100 for 10 min to increase antigen accessibility. After blocking with 1% BSA for 30 min, the cells were reacted with a primary antibody against HMGB-1 (1:200, Bioworld Technology, Inc., St. Louis, MN, USA) overnight at 4 °C. Subsequently, the cells were incubated with Alexa Fluor[®] 594-conjugated goat anti-rabbit secondary antibody (1:400,

Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 1 h at 37 °C in the dark. Then, the cells were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA) and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Statistical analysis

All experiments were conducted at least three times independently. The data were normalized to the respective control mean values and represented as means $\pm$ SEM. Statistical analyses were performed using SPSS software (version 21.0; IBM SPSS, Armonk, NY, USA). ANOVA was used for multiple group comparison. Student–Neuman–Keuls was used for post-hoc multiple comparisons. The statistical significance was set at 0.05.

Results

OxLDL induced HMGB-1 translocation and secretion in macrophages in a dose- and time-dependent manner

To determine the role of oxLDL on the translocation and secretion of HMGB-1 in macrophages, RAW264.7 cells were incubated with oxLDL at different concentrations ranging from 0–100 μ g/mL for 24 h or 0, 3, 6, 12, and 24 h. Western blot analysis revealed upregulation of HMGB-1 level in cell supernatant in a dose-dependent manner that reached a significant difference at 100 μ g/mL (Fig. 1A, $p < 0.01$). Also, we found that 100 μ g/mL oxLDL induced the temporal profiles of HMGB-1 expression in cell supernatant (Fig. 1B). Thus, cells treated with 100 μ g/mL oxLDL for 24 h were selected for the subsequent studies. The results shown in Fig. 1C indicated that oxLDL promoted the translocation of HMGB-1 from the nucleus to the cytoplasm, as evaluated by immunofluorescence.

Oxidative stress contributed to oxLDL-induced HMGB-1 translocation in macrophages

Oxidative stress was involved in HMGB-1 release in various cells. NAC, an antioxidant, was used to determine the role of oxidative stress in oxLDL-induced HMGB-1 translocation. As illustrated in Fig. 2A, oxLDL elevated the reduced nuclear HMGB-1 expression ($p < 0.01$), which was significantly suppressed by NAC ($p < 0.05$). Conversely, NAC partially but significantly attenuated the oxLDL-induced increased HMGB-1 expression in the cytoplasm ($p < 0.05$) (Fig. 2B). Furthermore, pretreatment with NAC normalized the elevated production of reactive oxygen species (ROS) induced by oxLDL in macrophages (Fig. 2C).

Fig. 1. Effect of oxLDL on HMGB-1 translocation and secretion in RAW264.7 cells. A, Effect of different concentrations of oxLDL on the HMGB-1 levels in cell supernatant. RAW264.7 cells were incubated with oxLDL for 24 h at different concentrations (0, 10, 50, 100 μ g/mL). B, Effect of 100 μ g/mL oxLDL on HMGB-1 secretion for different time points in RAW264.7 cells. RAW264.7 cells were stimulated with 100 μ g/mL oxLDL for 0, 3, 6, 12, 24 h. C, Translocation of HMGB-1 in RAW264.7 cells induced by 100 μ g/mL oxLDL for 24 h (arrow indicates HMGB-1 localization). N (number of times the experiment was repeated) = 3 in each group. NS, no difference between the two groups. ** $p < 0.01$ between the two groups. Scale bar=100 μ m.

Fig. 2. Role of oxidative stress in oxLDL-induced HMGB-1 translocation in RAW264.7 cells. Cells were pretreated with 10 mmol/L NAC for 2 h, followed by incubation with 100 μ g/mL oxLDL for 24 h. A, Expression of nuclear HMGB-1 in oxLDL-induced RAW264.7 cells with or without NAC treatment. B, Expression of cytoplasmic HMGB-1 in oxLDL-induced RAW264.7 cells with or without NAC treatment. C, Representative images of DHE staining in oxLDL-induced RAW264.7 cells with or without NAC treatment. NS, no difference between the two groups. * p <0.05 and ** p <0.01 between the two groups. The experiment was repeated three times in each group. Scale bar=50 μ m.

Fig. 3. Effect of HMGB-1 on foam cell formation in macrophages. After incubation with HMGB-1 (100 μ g/mL) or Gly (100 μ mol/L) for 2 h, RAW264.7 cells were treated with oxLDL (100 μ g/mL) or Dil-oxLDL (50 μ g/mL) for 24 h. A, Oil Red O staining in oxLDL-induced macrophages with HMGB-1 or Gly. Scale bar=20 μ m. B, The average IOD of lipid droplets stained using Oil Red O in oxLDL-induced macrophages with HMGB-1 or Gly. C, Quantitative analysis of intracellular total cholesterol content in oxLDL-induced macrophages with HMGB-1 or Gly. D, Fluorescence microscopy showed Dil-oxLDL uptake by RAW264.7 cells with HMGB-1 or Gly. Scale bar=50 μ m. The experiments were repeated three times in each group. * p <0.05 and ** p <0.01 between the two groups.

HMGB-1 promoted oxLDL-induced foam cell formation in macrophages

The present study examined the effect of HMGB-1 on macrophage-derived foam cells. As depicted in Fig. 3A, HMGB-1 facilitated the formation of foam cells induced by oxLDL in macrophages, as reflected by intense Oil Red O staining. To further substantiate the effect of HMGB-1 in oxLDL-induced foam cell formation, an inhibitor of HMGB-1 (Gly) was used in the subsequent experiments. Gly treatment significantly prevented the oxLDL-induced foam cells formation (Fig. 3A and B, p <0.05). Similar results were obtained by intracellular total cholesterol assay, indicating that HMGB-1 promoted oxLDL-induced lipid content in RAW264.7 cells (Fig. 3C, p <0.05); however, Gly attenuated the level of lipid content in RAW264.7 cells induced by oxLDL (Fig. 3C, p <0.05). Furthermore, oxLDL-induced uptake and binding of Dil-oxLDL in RAW264.7 cells were enhanced by HMGB-1 but inhibited by Gly (Fig. 3D).

HMGB-1 promoted oxLDL-induced apoptosis in macrophages

To explain the effect of HMGB-1 on the apoptosis of oxLDL-treated macrophages, TUNEL staining (Fig. 4A and B) and Annexin/PI staining (Fig. 4C and D) were performed in this section. Both assays indicated that HMGB-1 boosted the apoptosis of macrophages induced by oxLDL (p <0.01 and p <0.05, respectively); however, pretreatment with Gly suppressed the oxLDL-induced apoptosis in macrophages (both p <0.05).

Fig. 4. Effect of HMGB-1 on oxLDL-induced apoptosis of RAW264.7 cells. Cells were treated as described in Fig. 3. A, Representative microphotographs of TUNEL staining in oxLDL-induced RAW264.7 cells with HMGB-1 or Gly. Nuclei were stained blue (DAPI) and green punctate staining represented TUNEL-positive cells; apoptotic cells were identified with DAPI and TUNEL double stained cells. B, Quantitative analysis of the percentage of TUNEL-positive cells relative to total oxLDL-induced RAW264.7 cells with HGMB-1 or Gly. C, The percentage of oxLDL-induced apoptotic cells with HGMB-1 or Gly by flow cytometry. D, Representative histograms of Annexin/PI staining. The experiments were repeated three times in each group. * $p < 0.05$ and ** $p < 0.01$ between the two groups. Scale bar=100 μm .

Fig. 5. Involvement of ERS in HMGB-1-induced apoptotic foam cells. RAW264.7 cells were pretreated with TM (4 $\mu\text{g/mL}$), HMGB-1 (100 $\mu\text{g/mL}$), or 4-PBA (5 mmol/L) for 2 h, followed by incubation with oxLDL (100 $\mu\text{g/mL}$) for 24 h. A, Oil Red O staining in oxLDL-induced macrophages with TM, HMGB-1, or 4-PBA. Scale bar=50 μm . B, Average IOD of lipid droplets with Oil Red O staining in oxLDL-induced macrophages with TM, HMGB-1, or 4-PBA. C, Quantitative analysis of intracellular total cholesterol content in oxLDL-induced foam cells with TM, HMGB-1, or 4-PBA. D, Representative images of oxLDL-induced apoptotic cells with TM, HMGB-1, or 4-PBA. Nuclei were stained blue (DAPI) and green punctate represented TUNEL-positive cells; apoptotic cells were identified with DAPI and TUNEL double stained cells. Scale bar=100 μm . E, Quantitative analysis of the percentage of TUNEL-positive cells relative to the total oxLDL-induced RAW264.7 cells with TM, HMGB-1, or 4-PBA. The experiments were repeated three times in each group. * $p < 0.05$ and ** $p < 0.01$ between the two groups.

HMGB-1 promoted foam cell formation and apoptosis in oxLDL-induced macrophages via ERS

Considering the ERS is a key modulator in the formation of foam cells, we determined whether HMGB-1 promoted apoptosis of foam cells in oxLDL-induced macrophages via ERS. Oil Red O staining (Fig. 5A and B) and intracellular total cholesterol quantitative assay (Fig. 5C) showed that the induction of ERS by tunicamycin (TM) exaggerated the oxLDL-induced foam cell formation as well as the level of HMGB-1; however, ERS inhibitor 4-phenylbutyrate (4-PBA) reversed the effect of HMGB-1 in macrophage-derived foam cells induced by oxLDL. As depicted in Fig. 5D, TM significantly promoted the apoptosis of oxLDL-treated macrophages ($p < 0.05$), which was mimicked by the pretreatment with HMGB-1 ($p < 0.01$). However, the stimulatory effect of HMGB-1 on apoptosis of macrophages was partially abolished by 4-PBA ($p < 0.05$).

Fig. 6. HMGB-1 induced ERS/CHOP signaling pathway in oxLDL-treated macrophages. RAW264.7 cells were stimulated with HMGB-1 (100 μ g/mL) for 24 h after pre-incubation with 4-PBA (5 mmol/L) for 2 h. A, 4-PBA attenuated the expression of HMGB-1-induced GRP78 induced in RAW264.7 cells. B, 4-PBA attenuated the expression of HMGB-1-induced pPERK in RAW264.7 cells. C, 4-PBA did not exhibit any effect on HMGB-1-induced pIRE1 expression in RAW264.7 cells. D, 4-PBA attenuated the expression of HMGB-1-induced CHOP in RAW264.7 cells. The experiments were repeated three times in each group. * $p < 0.05$ and ** $p < 0.01$ between two groups.

HMGB-1 upregulated CHOP expression via ERS in macrophages

Since ERS/CHOP pathway contributed to the apoptosis macrophages, we hypothesized that HMGB-1 regulated the CHOP expression via ERS in macrophages. To address this issue, we analyzed the expressions of ERS-related molecules, GRP78, pIRE1, pPERK, and CHOP. Western blot results (Fig. 6A-C) indicated that HMGB-1 promoted ERS in macrophages, as evident by the upregulated expression of GRP78 ($p < 0.05$), pIRE1 ($p < 0.05$), and pPERK ($p < 0.01$), and pretreatment with 4-PBA reversed the effect of HMGB-1 on GRP78 and pPERK protein levels. However, 4-PBA did not affect HMGB-1-induced pIRE1 expression in RAW264.7 cells. Additionally, in response to the HMGB-1 challenge, RAW264.7 cells showed an increased expression of CHOP (Fig. 6D, $p < 0.01$), which was normalized by 4-PBA (Fig. 6D, $p < 0.05$).

Discussion

The findings of the current study suggested that oxLDL upregulated the HMGB-1 translocation and secretion in macrophages. Additionally, HMGB-1 promoted the oxLDL-induced foam cell formation in macrophages via ERS. Also, HMGB-1-induced the apoptosis of macrophages via ERS/CHOP pathway. Therefore, these findings further demonstrated that ERS contributed to HMGB-1-induced apoptosis of macrophage-derived foam cells (Fig. 7).

HMGB-1, a ubiquitously expressed protein, is localized in the nucleus. It can be passively released by necrotic cells or actively secreted from active immune cells under specific conditions. Emerging evidence indicated that HMGB-1 could be released from macrophages by stimulators such as lipopolysaccharide [17] and inflammatory cytokines [14]. Interestingly, oxLDL induced HMGB-1 secretion in macrophages in a dose- and time-dependent manner. These findings were consistent with those reported previously that cholesterol loading upregulated the HMGB-1 expression in vascular smooth muscle cells [18]; however, the underlying mechanisms are yet to be elucidated.

Accumulating evidence has indicated that oxidative stress is a primary modulator of HMGB-1 expression under pathological conditions. Our recent study showed that ROS genesis contributed towards the secretion of HMGB-1 from endothelial progenitor cells exposed to advanced glycation end production [19]. Concurrently, we also demonstrated that the inhibition of oxidative stress attenuated oxLDL-induced HMGB-1 translocation to cytoplasm and secretion, postulating that oxLDL promoted HMGB-1 secretion from macrophages via oxidative stress.

During the last decade, several studies revealed a correlation between HMGB-1 and atherosclerosis. Kalinina et al. demonstrated an increased expression of HMGB-1 in human atherosclerotic lesions [14], which was supported by subsequent studies. Another recent finding showed a local extension of HMGB-1 in atherosclerotic lesions of main cerebral and

carotid arteries in humans [20]. In atherosclerosis animal models, HMGB-1 was increased in plaques from diabetic apoE deficiency mice [21]. Notably, the administration of anti-HMGB-1 neutralizing antibody suppressed the atherosclerosis by 55% in apoE-deficient mice [10]. Thus, HMGB-1 may promote the *in vivo* development of atherosclerosis. Furthermore, macrophage-derived foam cells and cell apoptosis were critical hallmarks in atherosclerotic lesions. Thus, the role of HMGB-1 in oxLDL-induced foam cell formation was investigated. Consequently, we found that the treatment of HMGB-1 significantly promoted oxLDL-induced foam cell formation, as assessed by intense Oil Red O staining and intracellular cholesterol levels in HMGB-1-treated RAW264.7 cells. Furthermore, pretreatment with Gly, an inhibitor of HMGB-1, reversed the effect of oxLDL on cholesterol uptake in macrophages, which confirmed the critical role of HMGB-1 in oxLDL-induced foam cell formation. Interestingly, macrophage apoptosis played a major role in vulnerable atherosclerotic plaques, a key characteristic of acute myocardial infarction [3, 22]. Thus, in addition to its ability to promote foam cell formation, HMGB-1 has also been shown to facilitate apoptosis of oxLDL-treated RAW264.7 cells in the current study. These results were in agreement with those stated previously, wherein HMGB-1 induced the apoptosis of macrophages [23] and lymphocytes [24].

There is accumulating experimental evidence of the critical role of ERS in atherosclerosis development [25, 26] and foam cell formation [27-29]. In accordance with previous studies, our experiments showed that TM, an ERS inducer, promoted oxLDL-induced lipid accumulation and apoptosis of macrophages. Interestingly, it appears clearly from our experiments that the effects of ERS on foam cell formation are mimicked by the addition of HMGB-1. More recently, HMGB-1 has been shown to promote osteoblastic differentiation via ERS in aortic valve interstitial cells [30]. Therefore, it is not completely understood but substantial data suggest that the proatherosclerotic effect of HMGB-1 on macrophages may be mediated via ERS. UPR is characterized by accumulation of unfolded proteins, mis-folded proteins and defective calcium homeostasis [31]. As an evolutionarily adaptive action, UPR is triggered to alleviate the disturbance of ER homeostasis. GRP78, a molecular chaperones, regulates protein folding and protein trafficking in ER lumen by binding to three stress sensors including PERK, IRE1 and activating transcription factor 6 (ATF6) [32]. However, GRP78 dissociates from the three proteins under stress conditions, leading to activation of the stress sensors, and eventually promotes initiation of ERS [33]. As shown previously, HMGB-1 induced the activation of ERS in endothelial cells in a dose-dependent manner, as reflected by the increased expression of PERK and IRE1 [34]. Thus, in the present study, exogenous HMGB-1 induced the expression of ERS markers (GRP78, pPERK, pIRE1) in macrophages. Additionally, HMGB-1-induced expression of GRP78 and pPERK was reversed by 4-PBA (an inhibitor of ERS). Therefore, HMGB-1 might facilitate the oxLDL-induced lipid accumulation in macrophages via ERS.

Another interesting finding in the present study was that HMGB-1 promoted oxLDL-induced macrophage apoptosis that was significantly suppressed by 4-PBA. Accumulating evidence illustrated that sustained ERS could lead to the activation of apoptosis signaling pathway [27, 35]. These data provided additional evidence to the hypothesis that HMGB-1 may promote oxLDL-induced foam cell apoptosis via ERS. CHOP is a well-known apoptosis-related signaling protein in macrophage-derived foam cells, contributing to the instability of atherosclerosis plaques [2, 36]. In the current study, Western blot analysis showed that pretreatment with 4-PBA attenuated the HMGB-1-induced activation of ERS/CHOP signaling

Fig. 7. Proposed mechanism, wherein HMGB-1 promoted oxLDL-induced apoptotic foam cells via ERS in macrophages. ROS, reactive oxygen species; ERS, endoplasmic reticulum stress.

pathway, which was accompanied by a similar trend with the incidence of macrophage apoptosis. Taken together, these findings suggested that HMGB-1 facilitated oxLDL-induced apoptosis of macrophages via the ERS/CHOP pathway.

In conclusion, our results implied that HMGB-1 promoted oxLDL-induced macrophage-derived foam cell apoptosis via ERS/CHOP pathway. Thus, drugs targeting HMGB-1 might be therapeutic strategies for preventing the development of atherosclerosis. However, the study presents some limitations. Herein, we investigated the effects of HMGB-1 on macrophages cell line; the data strength can be significantly improved by extrapolation into primary macrophages in future experiments. The HMGB-1 inhibitor was used in this study; nonetheless, additional genetic inhibition of HMGB-1 might improve the findings. Furthermore, additional investigations would provide the mechanism underlying the HMGB-1-induced ERS in macrophages.

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Disclosure Statement

The authors declare that they have no competing interests.

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