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Glycoprotein Ⅱb/Ⅲa mAb Decreases Atherosclerosis by Inhibiting High-Mobility Group Box-1/Toll-like Receptor 4 Signaling in Apolipoprotein-E-deficient Mice*

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Abstract The effects of glycoprotein (GP) Ⅱb/Ⅲa inhibitors on the development of the atherosclerotic process has received scant attention. To investigate whether GP Ⅱb/Ⅲa blockade influences atherosclerosis lesion and HMGB-1/TLR4 signaling, we compared plaque formation in ApoE$^{-/-}$ mice: control group (n=10); IgG group (n=10, 50 μg) and GP Ⅱb/Ⅲa mAb group (n=10, 50 μg). All mice were fed on a Western diet (10% fat and 1.25% cholesterol) for 10 weeks. GP Ⅱb/Ⅲa blockade significantly decreased the atherosclerotic lesion and platelet adhesion to the vessel wall. Immunohistochemistry analysis showed that blocking GP Ⅱb/Ⅲa diminished MOMA-2 and VCAM-1 expression in aortic plaque in ApoE$^{-/-}$ mice. Western blot results indicated that HMGB-1, TLR4, and NF-κB levels were markedly reduced in arteries of ApoE$^{-/-}$ mice treated with GP Ⅱb/Ⅲa mAb (P < 0.05). Moreover, GP Ⅱb/Ⅲa mAb decreased plasma HMGB-1, IL-1β, TNF-α and MCP-1 concentrations. Our findings demonstrated that GP Ⅱb/Ⅲa mAb significantly decreased atherosclerotic lesions and HMGB-1, TLR4 and NF-κB expression in ApoE$^{-/-}$ mice (P < 0.05). The present study has suggested a possibility that GP Ⅱb/Ⅲa blockade attenuates atherosclerosis by inhibiting the HMGB-1/TLR4 pathway.

Key words GP Ⅱb/Ⅲa, atherosclerosis, HMGB-1, TLR4, NF-κB
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Atherosclerosis is a chronic inflammatory disease characterized by the accumulation of macrophages in the intima of arteries[1]. Increasing evidence has shown that platelet is involved in the development of atherosclerosis. However, the mechanisms by which platelet contributes to the pathogenesis of atherosclerosis are not fully understood. Recently, several studies have shown that the platelet-vessel wall interactions may play an important role in the development of atherosclerosis[2-3]. After vessel injury, activated platelets can adhere directly to the sub-endothelium of the damaged blood vessels through the collagen-von Willebrand factor-platelet glycoproteins axis. Among these platelet glycoproteins, the platelet glycoprotein Ⅱb/Ⅲa(GP Ⅱb/Ⅲa) integrin is thought to be the major class of receptor that mediates firm platelet adhesion. The platelet-vessel wall interaction *via* GP Ⅱb/Ⅲa can induce expression of inflammatory cytokines and adhesion molecules by activated cells, such as platelets and endothelial cells[4]. These processes promote monocytes recruitment to the vascular wall——a key mechanism in atherosclerosis. Correspondingly, GP Ⅱb/Ⅲa inhibitors improve the

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treatment of atherosclerosis diseases not only by inhibition of platelet adhesion but also through their anti-inflammatory effect [5]. However, less is known about the signaling cascades by which GP Ⅱb/Ⅲa inhibitors exert anti-inflammatory effect.

High mobility group box-1 (HMGB-1) is a highly conserved nuclear DNA-binding protein that stabilizes nucleosomes and facilitates transcription[6]. It is either passively released by injured or necrotic cells or actively secreted by monocytes/macrophages. Interestingly, HMGB-1 can be released by activated platelets and endothelial cells [7]. Recent studies indicate that HMGB1 as a potent extracellular cytokine is involved in cellular activation and proinflammatory response through interactions with its receptors. So far, HMGB-1 has been reported to signal mainly through 3 putative receptors, including the receptor for advanced glycation end products (RAGE), Toll-like receptor (TLR) 2, and TLR4. More recently, anti-HMGB-1 neutralizing monoclonal antibody has been shown to markedly attenuate atherosclerosis lesion in ApoE$^{-/-}$ mice[8]. Another study suggested that mice deficient in TLR4 but not TLR2 had a drastically reduced production of inflammatory cytokines in response to HMGB1 administration [9]. These observations demonstrate that HMGB1 plays important roles in the inflammatory cascade.

TLR4 is the major signal-generating pattern-recognition receptors, coordinating innate immunity and shaping inflammation response. It is mainly found on immune cells. Recently, TLR4 has been found to be expressed in platelet and play critical roles in LPS-induced thrombocytopenia [10]. Importantly, all of these cells are present in the atherosclerotic lesion and contribute to the inflammatory response. TLR4 can be activated both by extrinsic and endogenous ligands, such as heat shock proteins, matrix proteins and HMGB-1. After binding to its ligand, TLR4 engages a downstream cascade of signaling molecules, leading to the activation of two distinct signaling pathways. The activation of NF-κB results in the synthesis of a number of pro-inflammatory mediators and adhesion molecules. Our previous study indicated that platelet factors 4 play a key role in the progression of murine atherosclerotic lesions through TLR4 signaling [11]. Thus, we presume that GP Ⅱb/Ⅲa-mediated platelet-vessel interaction might induce the inflammation *via* activation of HMGB-1/TLR4 signaling, contributing to the pathogenesis of atherosclerosis.

In the present study, we showed evidence that GP Ⅱb/Ⅲa blockade protected against the progression of atherosclerosis lesions in ApoE$^{-/-}$ mice through anti-inflammatory biological properties. It inhibited the expression of HMGB-1/TLR4 pathways, leading to reduced expression of pro-inflammatory cytokines including VCAM-1, MCP-1, IL-1β, and TNF-α in ApoE$^{-/-}$ mice.

1 Materials and methods

1.1 Animals

Male, 5 weeks old ApoE$^{-/-}$ mice (C57BL/6J background) were obtained from Peking University Health Science Center (purchased from Jackson Laboratory). They were kept in the experimental room a week before the onset of the experiment in order to familiarize them with the testing environment. All mice were housed five per small polycarbonate cage (8 cm×13.5 cm×8.1 cm) and maintained under equivalent conditions of temperature (23 ± 1)℃, a 12 h light-dark cycle (lights on at 7∶00 AM and off at 7∶00 PM), and relative humidity (55% to 60%). Food and water were available *ad libitum*. At 6-weeks age, the 30 ApoE$^{-/-}$ mice fed a high-fat, cholesterol-rich diet containing 10% fat and 1.25% cholesterol were randomly divided into 3 groups: control group (vehicle treated group, n=10); immunoglobulin G (IgG) group (50 μg, irrelevant rat IgG, n=10) and GP Ⅱb/Ⅲa monoclonal antibody (mAb) group (50 μg, n=10). Vehicle(saline), IgG or mAb against mouse GP Ⅱb/Ⅲa was intraperitoneally injected twice a week for 10 weeks. All experiments were approved by the laboratory animals' ethical committee of University of South China and followed national guidelines for the care and use of animals (Permit Number: 20120035).

1.2 Main reagents

Rabbit anti-mouse TLR4(ab47093), anti-HMGB1 (ab18256), anti-NF-κB (ab52175), anti-MOMA-2 (ab33451), anti-VCAM-1(ab134047) and anti-GAPDH antibody (ab9485) were purchased from Abcam (Cambridge, UK). Against mouse GP Ⅱb/Ⅲa (JON/A) mAb was prepared as described [12]. Enzyme-linked immunosorbent assay (ELISA) kits for MCP-1 (SMJE00), IL-1β (MLB00C), and TNF-α (SMTA00B) were purchased from R&D Systems (Minneapolis, MN, USA). HMGB-1 ELISA kit (ABIN867711) was purchased from antibodies-online Inc (USA). Cholesterol (TC), triglycerides (TG), low-density lipoprotein-cholesterol (LDL-C), and high density

lipoprotein-cholesterol (HDL-C) kits were purchased from Sigma Diagnostics (USA).

1.3 Measurement of plasma lipid profile in ApoE$^{-/-}$ mice

Blood samples were taken by cardiac aspiration after the mice were anesthetized with 3% pentobarbital sodium. Blood was collected in ethylenediaminetetra-acetic acid-coated tubes and centrifuged at 2 000 *g* for 10 min at 4℃. Plasma samples were stored at −20℃. TC, TG, LDL-C, and HDL-C plasma concentrations were measured by ELISA kits. The assays were performed in accordance with the manufacturer's instructions.

1.4 Preparation of platelets for intravital microscopy

Blood from 5 ApoE$^{-/-}$ mice was drawn from the retro-orbital plexus and collected in 1.5 ml polypropylene tubes containing 0.1 ml volume of 38 mmol/L citric acid/75 mmol/L trisodium citrate/ 100 mmol/L dextrose. The blood was centrifuged at 300 *g* for 10 min and platelet-rich plasma was gently transferred to a fresh tube and the centrifugation was repeated at 2 000 *g* for 10 min. The pellet was resuspended in modified Tyrode-HEPES buffer containing 0.35% BSA and 5 mmol/L glucose. Isolated platelets were labeled with calcein-AM 0.25 mg/L for 3 min and adjusted to a final concentration of 3×10^8 /ml.

1.5 Intravital microscopy of platelet adhesion to the endothelium of ApoE$^{-/-}$ mouse carotid arteries

ApoE$^{-/-}$ mice were anesthetized, followed by cannulation of the trachea and right jugular vein. The periadventitial tissues around the left carotid arteries were carefully separated from the vessel. Most of the common carotid artery, external bifurcation and external branch were exposed and left intact. The carotid artery was viewed at 10 fold magnification using a SZX7-1063 microscope (Olympus, Japan). The calcein-AM labeled platelets(3×10^7 in 100 μl Tyrode-HEPES) were injected into 16-week-old ApoE$^{-/-}$ mice, and platelet adhesion was continuously monitored with a video system over an area of 100 μm × 100 μm. In each mouse, 3 non-overlapping fields were analyzed for 30 s in a slow-motion modus. Clusters of 2 or more platelets were defined as micro-aggregates. The total number of firm adhesion platelets at time = 3 min was calculated by the following formula that reflects concave shape of the vessel wall: Firm adhesion of platelets/mm^{-2} = vessel diameter/μm × π × 2× sin^{-1} (amplitude of measured area/μm)×length of measured area/μm. For image acquisition and analysis, Olympus cell^R software was used.

1.6 Assessment of atherosclerotic lesions in aortic sinus of ApoE$^{-/-}$ mice

The mice were fed a high-fat, high-cholesterol diet for 10 weeks. After anesthesia with 3% pentobarbital sodium, the mice were perfusion-fixed with 4% paraformaldehyde, and the proximal aortas attached to the heart were removed. To determine cross-sectional lesion area, the proximal aortas were embedded in OCT compound (Tissue Tek, Sakura, Torrance, CA), frozen on dry ice, and then stored at −70℃ until sectioning. Serial sections were collected on slides for immunohistochemistry or staining with Oil red O as described earlier. Cross sections of the aortic sinus and aortic valve were stained with Oil red O and counterstained with Gill Ⅲ hematoxylin (Sigma). Lesion areas were quantified with IMAGEPRO PLUS (Media Cybnetics, Silver Spring, MD). Results are expressed as the average lesion size per section or as the percent of the total cross sectional vessel wall area (normal plus diseased area/section, excluding the lumen) stained with Oil red O. For each animal, the average of 10 sections was determined, and data are expressed as lesion size or mean percent lesion area ± SEM.

1.7 Western blot

The descending arteries were dissected and used to analyze the protein levels by Western blot. Equal amounts of extracted proteins were separated by 10% SDS-PAGE gels and transferred to nitrocellulose membranes (BioRad, Hercules, PA, USA). The membranes were blocked with 1% bovine serum albumin and then subjected to Western blot analyses using anti-TLR4, anti-HMGB1, and anti-NF-κB. After three washes in Tris phosphate-buffered saline (TPBS) that contained 0.5% Tween 20 in PBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in TPBS. The bands were detected by chemiluminescent detection reagents. Blot densitometry was then performed, and the bands were analyzed using a Gene Genius Bio Imaging. Immunodetection was accomplished using appropriate horseradish peroxidase-linked secondary anti-bodies (KPL, 074-1516) and enhanced chemiluminescence system (KPL). The blots were exposed to films (Fuji RX FUJIFILM, Tokyo, Japan). Protein levels were

quantified by scanning densitometry using image-analysis systems.

1.8 Immunohistochemistry

Frozen sections of ApoE$^{-/-}$ mice aortic root were fixed with acetone for 5 min at room temperature and then immunostained with Rabbit anti-mouse MOMA-2 antibody (1 ∶ 50) or VCAM-1 (1 ∶ 100) antibody according to the instructions on DAB immunostaining kit. Rabbit IgG was used as a negative control.

1.9 Detection of plasma HMGB-1, TNF-α, IL-1β and MCP-1 levels by ELISA

The plasma concentrations of HMGB-1, TNF-α, IL-1β and MCP-1 were measured by ELISA kits according to the manufacturer's instructions. The minimum detectable concentrations were < 0.3 μg/L for HMGB-1, < 4.8 ng/L for IL-1 β, < 1 ng/L for TNF-α, and < 2 ng/L for MCP-1 respectively.

1.10 Statistical analysis

The results were presented as $\bar{x} \pm s$. The data were analyzed by Student's *t*-test and analysis of variance (ANOVA). Statistical significance was accepted when $P < 0.05$.

2 Results

2.1 Effects of GP Ⅱb/Ⅲa mAb on plasma lipid levels in ApoE$^{-/-}$ mice

The mice were fed a high-fat, cholesterol-rich diet for 10 weeks, and plasma lipid levels were measured by ELISA. As shown in Table 1, no significant differences in TC, TG, LDL-C, and HDL-C levels were found among three groups, respectively.

Table 1 Effects of GP Ⅱb/Ⅲa mAb on plasma lipid levels in ApoE$^{-/-}$ mice

mmol/L

Group	TC	TG	LDL-C	HDL-C
Control	18.63 ± 3.08	0.98 ± 0.27	12.71 ± 2.06	4.48 ± 1.17
IgG	19.52 ± 2.87	1.03 ± 0.22	13.08 ± 2.38	4.83 ± 0.94
GPⅡb/Ⅲa mAb	18.07 ± 2.53	0.92 ± 0.36	12.47 ± 1.82	4.16 ± 0.68

Plasma lipid profiles in each group mice were detected by ELISA kits in accordance with the manufacturer's instructions (n = 10 in each group). Data are $\bar{x} \pm s$. No significant differences in TC, TG, LDL-C, and HDL-C levels were found among three groups, respectively.

2.2 Effects of GP Ⅱb/Ⅲa mAb on aortic sinus atherosclerotic lesions in ApoE$^{-/-}$ mice

To investigate the effects of GP Ⅱb/Ⅲa blockade on atherosclerosis, we evaluated aortic sinus atherosclerotic lesions in ApoE$^{-/-}$ mice. The percentage of lesion area was determined by quantitative histomorphology of Oil Red O staining. As demonstrated in Figure 1, after 10 weeks fed on the atherogenic diet, a large number of atherosclerois plaques appeared on the surface of the aortic sinus both in control group and IgG group mice. In contrast, compared with the control group, there was a markedly decreased tendency of the plaque area in GP Ⅱb/Ⅲa mAb group ((0.21 ± 0.05) mm^2 *vs* (0.09 ± 0.02) mm^2, $P < 0.05$). Similarly, there was a significant decrease in the percentage of lesion area in GP Ⅱb/Ⅲa mAb group compared with the control ones ((14.82 ± 3.41)% *vs* (5.83 ± 1.72)%, $P < 0.05$). However, no significant difference in the aortic sinus atherosclerotic lesions was found between the control group and IgG group. These results indicate that GP Ⅱb/Ⅲa mAb has protective effects against atherosclerosis in ApoE$^{-/-}$ mice.

2.3 Effects of GP Ⅱb/Ⅲa mAb on platelet adhesion to the common carotid artery wall of ApoE$^{-/-}$ mice

Platelet adhesion to vascular surface plays an important role in the process of atherosclerosis. Here, we addressed whether GP Ⅱb/Ⅲa mAb reduced atherosclerosis lesions related to inhibition of platelet adhesion in ApoE$^{-/-}$ mice. Platelet adhesion to artery wall was observed by intravital microscopy. As indicated in Figure 2, there was a significant increase in the number of platelets firmly adhering to the carotid artery both in control (46 ± 8)/mm^2 and IgG group (43±6)/mm^2 ApoE$^{-/-}$ mice. In contrast, a markedly reduced number of platelets firm adhesion to the endothelium of the carotid artery was seen in ApoE$^{-/-}$ mice injected with GP Ⅱb/Ⅲa mAb (8 ± 2)/mm^2. The image provides a visual representation of GP Ⅱb/Ⅲa involved in atherogenesis.

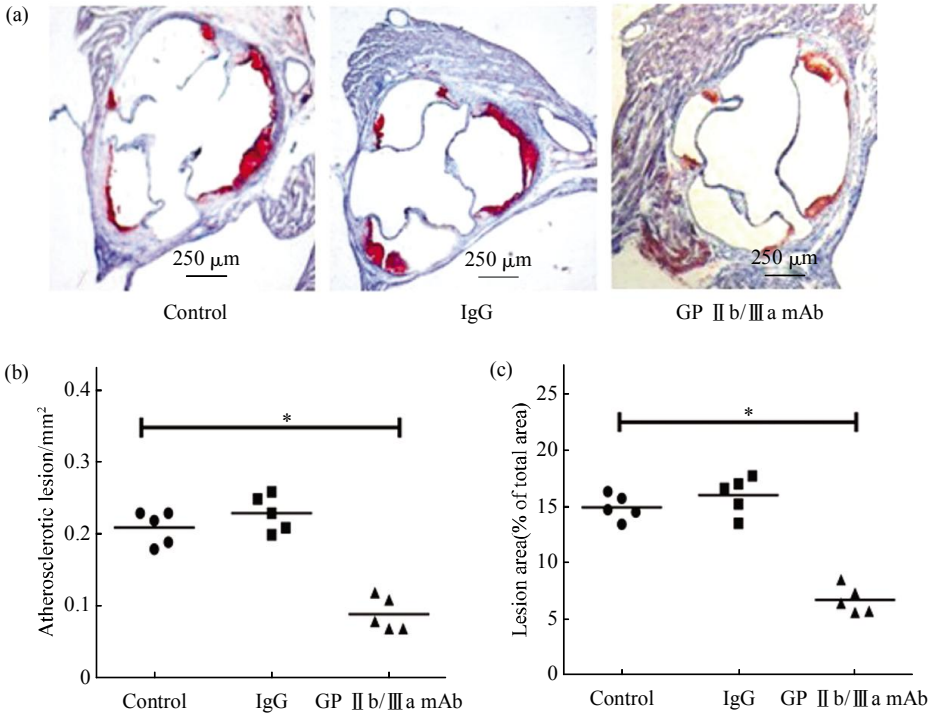

Fig. 1　GP Ⅱb/Ⅲa mAb attenuates atherosclerotic lesion formation in ApoE$^{-/-}$ mice

(a) Representative photographs of Oil Red O-stained cross-sections from the aortic sinus in each group. 6-week-old ApoE$^{-/-}$ mice were treated with vehicle (Control), irrelevant rat IgG, or GP Ⅱb/Ⅲa mAb for 10 weeks. Atherosclerotic lesion formation was assessed in the aortic sinus by Oil Red O-stained cross-sections (8 μm frozen sections). (b) Quantification of plaque areas in aortic sinuses in ApoE$^{-/-}$ mice stained for lipid deposition with oil red O. Total plaque area in mAb treated mice was significantly decreased compared with the control ones; atherosclerotic lesion area in the aortic sinus was presented by mm^2. (c) The percentage of aortic lesion area (lesion area compared to total aortic sinus area) was measured by quantitative histomorphology of Oil Red O staining. Data represent the $\bar{x} \pm s$ (n = 5 in each group). * $P < 0.05$ versus control group.

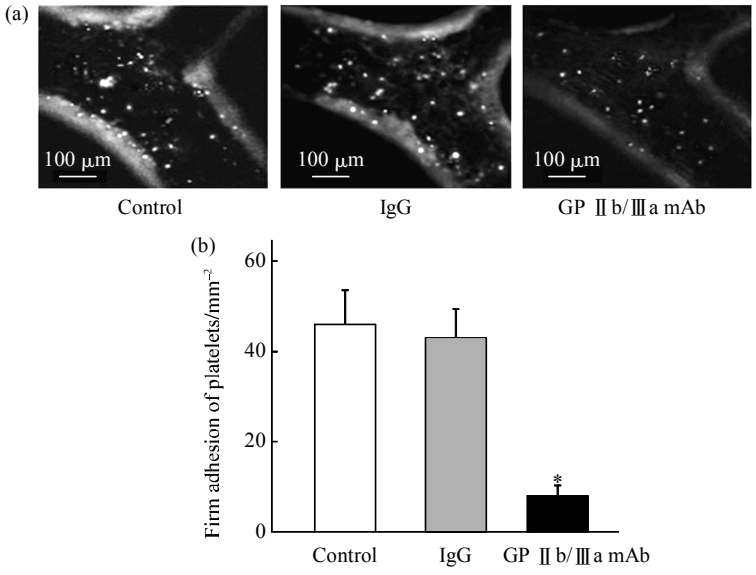

Fig. 2　GP Ⅱb/Ⅲa mAb suppresses platelet adhesion to the vessel wall of ApoE$^{-/-}$ mice

(a) The microphotographs show representative *in vivo* fluorescence microscopy images in each group. The calcein-AM labeled platelet adhesion was quantified in the proximal (nonlesion-prone) carotid artery and adjacent to the carotid bifurcation (lesion-prone) in 16-week-old ApoE$^{-/-}$ mice. (b) Quantification of firm platelet adhesion in ApoE$^{-/-}$ mice. Data are expressed as interactions per mm^2 (n = 5 in each group). *$P < 0.05$ versus the control value.

2.4 The effects of GP Ⅱb/Ⅲa mAb on the expressions of TLR4, HMGB-1, and NF-κB (p65) in arteries of ApoE$^{-/-}$ mice

To determine whether GP Ⅱb/Ⅲa mAb inhibits the development of atherosclerosis in ApoE$^{-/-}$ mice through HMGB-1/TLR4 signaling pathway, the expressions of TLR4, MGB-1, and NF-κB (p65) in descending arteries were detected by Western blot. As shown in Figure 3, GP Ⅱb/Ⅲa mAb administration led to a significant decrease in the expression of TLR4, HMGB-1, and NF-κB compared with the control group mice, respectively.

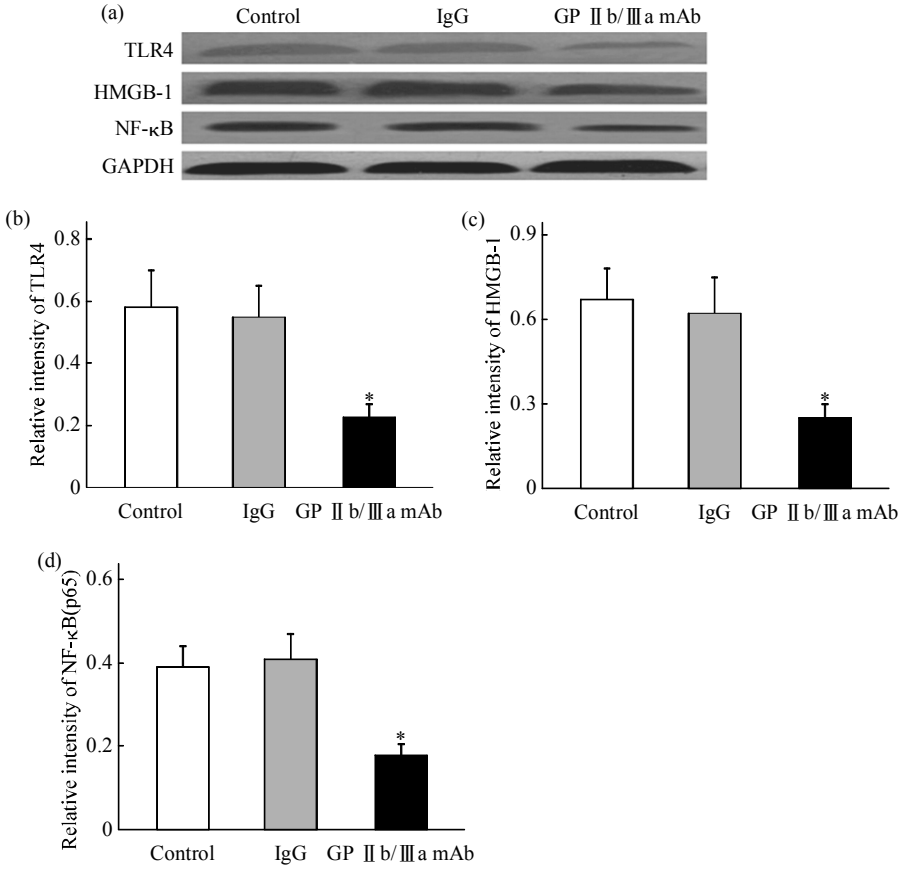

Fig. 3 GP Ⅱb/Ⅲa mAb decreases HMGB-1, TLR4 and NF-κB (p65) expression in ApoE$^{-/-}$ mice

(a) The protein levels of HMGB-1 (29 ku), TLR4 (100 ku), NF-κB p65 (70 ku) and GAPDH (40 ku) in arteries were detected by Western blot. ApoE$^{-/-}$ mice fed a high fat diet were treated with vehicle (Control), irrelevant rat IgG, or the mAb for 10 weeks, and proteins were prepared from arteries of each group ApoE$^{-/-}$ mice. Each lane shows representative Western blots using anti-HMGB-1, TLR4, NF-κB p65, or anti-GAPDH bodies, respectively. Each panel summarizes densitometric readings of band intensities normalized to GAPDH, which was measured by densitometry with Image J image analysis software. (b), (c) and (d) Represent densitometric measurements of TLR4, HMGB-1 and NF-κB p65 from Western blots, respectively. Data are $\bar{x} \pm s$. (n = 5 in each group). * P < 0.05 compared with the control. The data are representative of three experiments.

2.5 Effects of GP Ⅱb/Ⅲa mAb on the expressions of MOMA-2 and VCAM-1 in atherosclerotic lesions of ApoE$^{-/-}$ mice

Inflammatory processes within the vessel wall mediate the initiation and progression of atherosclerosis. In this context, monocytes invasion into the arterial wall and differentiation into resident macrophages contributes to atherosclerotic plaque development. Therefore, the effects of GP Ⅱb/Ⅲa mAb on monocytes/macrophages(MOMA) accumulation in aortic root and the expression of VCAM-1 in the aortic wall were determined by quantitative immunohistochemistry imaging analysis. As shown in Figure 4, compared with the control group, the positive area of MOMA-2 within the lesion area was decreased by 65.1% in GP Ⅱb/Ⅲa mAb group mice. Similarly, the immunoreactivity in the mAb treated mice was reduced by 54.7% compared with the control group. These results suggest blocking GP Ⅱb/Ⅲa can reduce MOMA-2 and VCAM-1 expression in plaque in ApoE$^{-/-}$ mice.

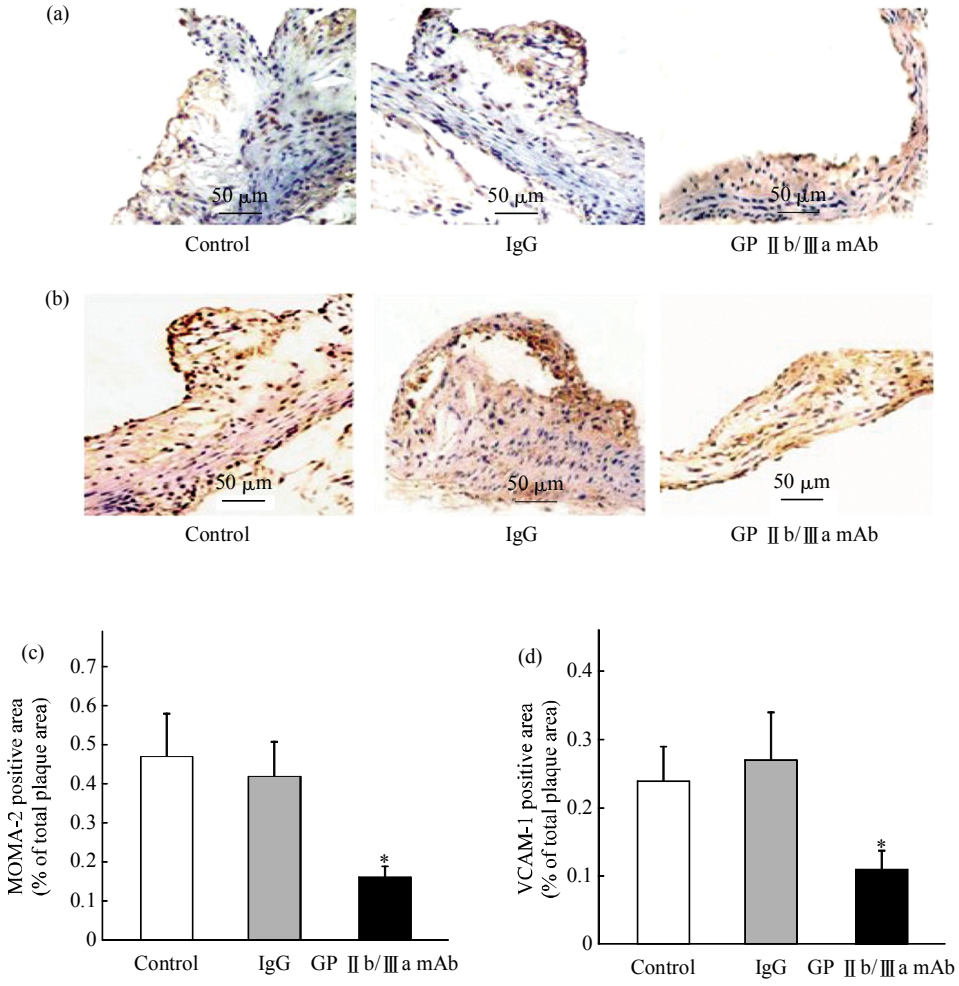

Fig. 4　GP Ⅱb/Ⅲa mAb reduces MOMA-2 and VCAM-1 expression in atherosclerotic lesions of ApoE$^{-/-}$ mice

5 μm frozen sections of the ApoE$^{-/-}$ mouse aortic sinus were fixed with acetone for 5 min at room temperature and then immunostained with Rabbit anti-mouse MOMA-2 antibody (1∶50) or VCAM-1 antibody (1∶100). (a), (b) Representative of immunostained aortic sinus sections using specific antibodies against MOMA-2 and VCAM-1, respectively (blue: nuclei, brown: target protein). (c) Quantitative analysis of MOMA-2 immunoreactivity in aortic sinus plaques of ApoE$^{-/-}$ mice, expressed as a proportion of the total plaque areas (n = 5 in each group). (d) Quantitative analysis of VCAM-1 immunoreactivity in aortic sinus plaques of ApoE$^{-/-}$ mice (n = 5 in each group). Data represent the $\bar{x} \pm s$. * $P < 0.05$ versus control group.

2.6　Effects of GP Ⅱb/Ⅲa mAb on plasma HMGB-1, TNF-α, IL-1β and MCP-1 levels in ApoE$^{-/-}$ mice

Inflammation and immune responses might potentially contribute to the progression process of atherosclerosis, we further asked whether GP Ⅱb/Ⅲa mAb attenuated atherosclerotic lesion formation might be paralleled by reduced inflammation cytokine levels. To test this, plasma HMGB-1, TNF-α, IL-1β and MCP-1 levels were detected by ELISA in the present study. As revealed in Figure 5, compared with the control group, GP Ⅱb/Ⅲa mAb potently decreased the levels of HMGB-1, TNF-α, IL-1β and MCP-1 by 69.4%, 64.1%, 57.2%, and 52.8%, respectively. These results further indicate that GP Ⅱb/Ⅲa mAb significantly suppressed vascular inflammation in ApoE$^{-/-}$ mice.

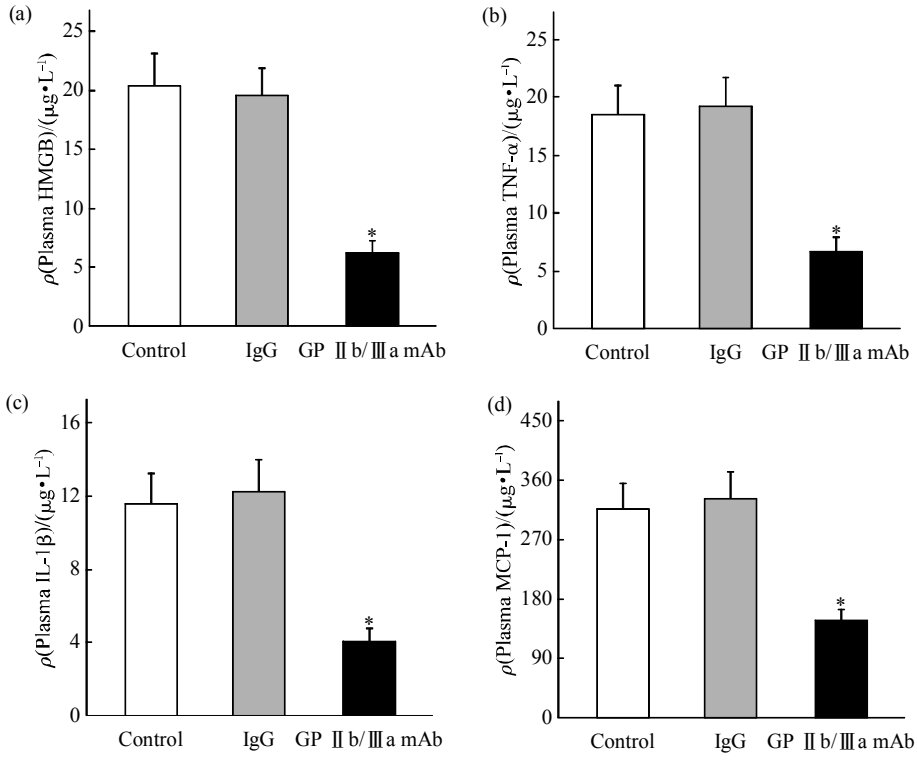

Fig. 5 GP Ⅱb/Ⅲa mAb reduces plasma HMGB-1, IL-1β, TNF-α and MCP-1 levels in ApoE$^{-/-}$ mice

Plasma HMGB-1 (a), TNF-α (b), IL-1β (c), and MCP-1 (d) concentrations were assayed by ELISA kits according to the manufacturer's instructions. Data are $\bar{x} \pm s$ (n = 5 in each group). *P < 0.05 versus the control value.

3 Discussion

Platelet integrin GP Ⅱb/Ⅲa plays an important role in pathogenesis of various cardiovascular diseases, such as atherosclerosis and ischemia/reperfusion injury[12]. Understanding the mechanisms for GP Ⅱb/Ⅲa in atherosclerosis might be beneficial to prevent and treat platelet-mediated cardiovascular disease. The present study investigated whether GP Ⅱb/Ⅲa blockade can decrease the development of atherosclerosis and the expression of HMGB-1/TLR4 signaling in ApoE$^{-/-}$ mice. Our major findings are as follows: (1) GP Ⅱb/Ⅲa mAb not only profoundly reduced atherosclerotic lesions in ApoE$^{-/-}$ mice, but also substantially decreased platelet adhesion to atherosclerosis lesions; (2) Macrophages and the inflammatory proteins (such as TNF-α and IL-1β) were also proportionally decreased in atherosclerosis lesions; (3) Blocking GP Ⅱb/Ⅲa appeared to have providently inhibitory effects on TLR4 and HMGB-1 expression in plaque; (4) Blocking GP Ⅱb/Ⅲa had no effects on plasma lipid profile, but it markedly decreased inflammatory cytokines (such as IL-1β and MCP-1).

It has been well known that long-term dyslipidemia is an established predictor of cardiovascular disease risk[13]. In present study, the effects of GP Ⅱb/Ⅲa mAb on plasma lipid profile were investigated. The results demonstrated that GP Ⅱb/Ⅲa mAb had no effects on lipid levels. Current evidence suggested that the mechanism of GP Ⅱb/Ⅲa blockade decreased the development of atherosclerotic lesion in ApoE$^{-/-}$ mice may be distinct from lipid lowering drugs.

Platelet GP Ⅱb/Ⅲa has been demonstrated to largely contribute to endothelial platelet adhesion [14], making it a good candidates for inhibition. In this study, we also evaluate the effect of GP Ⅱb/Ⅲa mAb on platelet adhesion to vascular wall during atherogenesis. Our findings indicated that inhibition of GP Ⅱb/Ⅲa significantly reduced platelet adhesion

to the atherosclerotic area. Since engagement of GP Ⅱb/Ⅲa during platelet adhesion and aggregation at sites of endothelial dysfunction may contribute to the inflammatory response triggered by activated platelets and endothelial cells. These findings provide strong evidence for a critical role of GP Ⅱb/Ⅲa-mediated platelet adhesion in the development of atherosclerosis in response to elevated cholesterol.

It is now widely accepted that inflammatory processes play a critical role in all phases of atherosclerosis. Accumulating evidence suggests that GP Ⅱb/Ⅲa might also contribute significantly to the inflammatory processes that initiate atherosclerotic lesion formation[4]. To address the pathophysiological role of GP Ⅱb/Ⅲa in the inflammatory process of atherosclerosis, we assessed monocytes/macrophages recruitment to vessel wall in ApoE$^{-/-}$ mice. In this study, Western blot and quantitative immunohistochemistry imaging revealed significant decreases in VCAM-1 and MOMA-2 within the lesion area of ApoE$^{-/-}$ mice treated with GP Ⅱb/Ⅲa mAb. As we know, monocytes adhesion to the vessel is mediated primarily by VCAM-1, and the invasion of neutrophils into the arterial wall is facilitated by VCAM-assisted trafficking[15], which also contributes to atherosclerotic lesion initiation and progression. Since monocytes invasion into lesion-prone areas in the arterial wall and differentiation of monocytes into resident macrophages contributes to atherosclerotic plaque development, these data suggested that GP Ⅱb/Ⅲa blockade exerts anti-inflammatory effects in the aorta may be through reduction monocytes invasion and differentiation. This study indicated that inhibition of GP Ⅱb/Ⅲa was paralleled by reduced monocytes recruitment into the lesion area and protected ApoE$^{-/-}$ mice from atherosclerotic plaque formation, supporting a critical role of platelet GP Ⅱb/Ⅲa integrin in atherosclerosis.

We and others have previously reported that HMGB-1 and TLR4 are expressed in atherosclerotic lesions [16–17]. Recently, the HMGB-1/TLR4-NF-κB pathway has been suggested as a link between inflammation and cardiovascular disease[18]. To further reveal the mechanisms for GP Ⅱb/Ⅲa blockade reducing atherosclerosis lesions, the effects of GP Ⅱb/Ⅲa mAb on HMGB-1/TLR4 pathway were investigated in the present study. The results showed that GP Ⅱb-Ⅲa mAb significantly reduced the expressions of HMGB-1, TLR4, and NF-κB (p65) in arteries of ApoE$^{-/-}$ mice. HMGB-1 is an evolutionarily conserved protein present in the nucleus of almost all eukaryotic cells, where it functions to stabilize nucleosomes and acts as a transcription factor. In addition to its nuclear roles, HMGB-1 was identified as a potent proinflammatory cytokine in experiments showing that HMGB-1 is actively secreted by activated macrophages and functions as a late mediator of lethal endotoxemia in sepsis models. Furthermore, HMGB-1 is an endogenous protein in platelets, which is secreted to outside the cell during platelet activation, which may be mediated by platelet GP Ⅱb-Ⅲa integrin through inside-out signaling[9]. In this study, we confirmed up-regulation of HMGB-1 within atherosclerotic arteries of ApoE$^{-/-}$ mice fed by atherogenic diet. In contrast, GP Ⅱb/Ⅲa blockade decreased HMGB-1 expression both in plasma and arteries. The mechanisms for this reduction might be related to its roles of inhibition platelet adhesion, aggregation, and activation, reduction monocytes invasion, and differentiation within arteries.

Growing evidence has showed that TLR4 signaling-mediated innate immune response plays an important role in the pathogenesis of atherosclerosis. TLR4 not only recognize specific molecular patterns that are present on invading microorganisms, but also recognize endogenous ligands released by damaged cells such as HMGB-1. Platelet adhesion to endothelium causes injured tissues to express or release a variety of endogenous TLR4 ligands, including heat-shock proteins, HMGB-1, and fibronectin. HMGB-1 has been reported to trigger cellular signaling by interacting with three receptors: RAGE, TLR2, and TLR4[6]. HMGB-1 binding to TLR4 leads to NF-κB activation through a MyD88-dependent pathway to promote inflammatory responses including the release of proinflammatory cytokines and chemokines. In present study, we further explored the possible relevant molecular mechanisms for blocking GP Ⅱb/Ⅲa anti-inflammation. Our data demonstrated that the expressions of HMGB-1, TLR4, and NF-κB significantly reduced in arteries of ApoE$^{-/-}$ mice administered with GP Ⅱb/Ⅲa mAb. Accordingly, the activation of NF-κB downstream signaling molecules such as IL-1β, TNF-α, and MCP-1 levels were decreased in plasma. Moreover, studies have identified that NF-κB is mainly responsible for the expression of these pro-inflammatory cytokines when TLR4 signal pathway is activated[18–19]. Although

the present results cannot exclude the possibility that GP Ⅱb/Ⅲa mAb directly suppress the activation of TLR2 or RAGE by HMGB-1 to achieve anti-inflammation effects. Taken together, GP Ⅱb/Ⅲa blockade inhibited the progression of atherosclerosis in ApoE$^{-/-}$ mice, at least partly, by suppressing the activation HMGB-1/TLR4 pathway and subsequently decreasing adhesion molecule-induced monocytes/ macrophage infiltration in the damaged endothelial cell.

In conclusion, our data indicated that GP Ⅱb/Ⅲa integrin is involved in atherosclerosis in ApoE$^{-/-}$ mice, and blocking GP Ⅱb/Ⅲa protected against atherosclerosis by attenuating inflammation. The precise mechanism of action and possible clinical applications will need to be addressed in future studies.

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膜糖蛋白Ⅱb/Ⅲa单抗通过抑制HMGB1/TLR4途径减轻ApoE$^{-/-}$小鼠动脉粥样硬化*

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摘要　观察膜糖蛋白(GP) Ⅱb/Ⅲa单抗对小鼠动脉粥样硬化(atherosclerosis，As)病变和HMGB1/TLR4途径基因表达变化的影响，以探讨膜糖蛋白Ⅱb/Ⅲa受体拮抗剂对As进程的影响及其机制．30只5周龄雄性ApoE$^{-/-}$小鼠随机均分为3组：溶剂对照组(生理盐水50 μl，腹腔注射)，IgG对照组(50 μg，腹腔注射)，GP Ⅱb/Ⅲa单抗组(50 μg，腹腔注射)．实验ApoE$^{-/-}$小鼠均已高脂、高胆固醇饲料喂养，10周后处死动物．油红O染色观察主动脉窦As病变；活体荧光显微镜观察颈总动脉As病变处血小板黏附；Western blot检测HMGB-1、TLR4与NF-κB蛋白的表达；免疫组化观察主动脉窦As病变部位MOMA-2和VCAM-1的表达；ELISA法检测血浆中HMGB-1、IL-1β、TNF-α与MCP-1的含量．研究结果表明：与对照组相比，GPⅡb/Ⅲa单抗组ApoE$^{-/-}$小鼠As病变和血小板黏附显著减少($P < 0.05$)；且该组小鼠主动脉TLR4与NF-κB蛋白的表达明显降低；其血清中的HMGB-1、IL-1β、TNF-α与MCP-1的水平也明显下降($P < 0.05$)．此外，GP Ⅱb/Ⅲa单抗治疗显著减少As病变处MOMA-2和VCAM-1的表达($P < 0.05$)．GP Ⅱb/Ⅲa单抗减轻ApoE$^{-/-}$小鼠As病变可能与抑制HMGB1/TLR4途径介导的炎症有关．

关键词　膜糖蛋白Ⅱb/Ⅲa，动脉粥样硬化，高迁移族蛋白-1，Toll样受体4，核转录因子-NF-κB
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