plc, Cambridge, UK) was used as the primary Ab, followed by incubation with HRP-conjugated goat anti-mouse IgG (MBL international, Inc., Woburn, MA, USA). Diaminobenzidine (Sigma-Aldrich Co., St. Louis, MO, USA) and H_2O_2 were used as the substrates for the reaction.

Western blotting for HMGB1 in cerebrospinal fluid

Cerebrospinal fluid samples were collected from the cistern magna according to the previously reported method[1]. The samples were mixed with SDS-PAGE sample loading buffer under reducing condition. We loaded 15 μL of the samples per lane on SDS-PAGE. After transferring the proteins to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the membranes were blocked with TBS containing 0.1% Tween 20 (T-TBS) and 10% skimmed milk, and then probed with the anti-HMGB1 mAb (#10-22) labeled by horseradish peroxidase using a Peroxidase Labeling Kit-NH2 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After washing in T-TBS, an ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to visualize the HMGB1 band.

Western blotting using brain homogenate samples

Rat brain samples were collected at the time point of 12 h after reperfusion. Brain slices were cut from 0.5 mm anterior from the bregma with the thickness of 2 mm. The ischemic core area in the striatum and the cortex indicated as Supplemental Figure 1B were cut and homogenized in cold Radio Immuno Precipitation Assay buffer (RIPA) (150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1 % SDS, and 50 mM Tris-HCl, pH 8.0) with cocktail protease inhibitors (Sigma-Aldrich Co., St. Louis, MO, USA). The brain homogenate were then centrifuged at 10,000 g for 20 min. The protein concentration in the supernatant were detected by Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and adjusted to 250 μg/mL as the final concentration by Western sample buffer, 10 μL of each sample was loaded to the SDS-PAGE. After transferring the proteins to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the membranes were blocked with T-TBS and 10% skimmed milk, and then probed with the anti-HMGB1 mAb (#10-22) labeled by horseradish peroxidase using a Peroxidase Labeling Kit-NH2 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). β-actin was probed with a mouse anti-β-actin mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) followed by a HRP conjugated goat anti-mouse Ab. After washing in T-TBS, an ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to visualize the bands of HMGB1 and β-actin.

***In vitro* BBB permeability assay**

An *in vitro* BBB kit™ (RBE-12, PharmaCo-Cell Co. Ltd., Sakamoto, Japan) was used to assess the effects of rHMGB1 and the mAb to the BBB unit according to the instruction of the manufacturer[2]. The BBB system was composed of rat brain vascular endothelial cells, pericytes and astrocytes. The endothelial cells were cultured on the bottom of polyester

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membrane (radius, 6 mm; thickness, 10 μm; pore size, 0.4 μm) of the insert well. The pericytes were present below the membrane of the insert well. The astrocytes were cultured on the bottom of the lower chamber. RHMGB1 expressed and purified from Sf9 insect cells. RHMGB1 alone or together with the anti-HMGB1 mAb after pre-incubation for 30 min was added into the lower chamber which is supposed to be the brain side. Trans Endothelial Electrical Resistance (TEER) was measured before and 30 min after stimulation with rHMGB1 using an EVOM resistance meter (World Precision Instruments, Sarasota, FL) as previously reported (Hiu *et al.*, 2008). The values are shown as $\Omega \times cm^2$. Thereafter, Evans' blue (165 μg/mL) bound to 0.1% BSA[3] was added to the insert well which is supposed to be the blood vessel side. The plate was further incubated for 30 min at 37°C. The media in the lower compartment was then collected, and the absorbance of Evans blue at 595 nm was detected using a spectrophotometer (U-1500, Hitachi Ltd., Tokyo, Japan) to study the leakage of Evans blue-albumin complex.

The insert wells were washed by PBS, and the cells in the insert wells were fixed by 4% paraformaldehyde for 30 min at room temperature. The fixed cells were then washed by PBS and incubated with 0.1% Triton-X100 for 5 min at room temperature. An alexa-488 labeled phalloidin (Invitrogen Co., Branford, CT, USA) was added to the insert wells for F-actin staining. Cell nuclei were visualized by staining with DAPI (0.3 μM). The endothelial cells and pericytes in the insert wells were examined using a LSM 510 confocal imaging system (Carl Zeiss, Inc., Jena, Germany). The autofluoresence of Evans blue uptaken by astrocytes was observed in a fluorescence microscope (BZ-8000, Keyence Co., Osaka, Japan).

In vivo brain microdialysis of glutamate

Rats were anesthetized using the same condition of MCAO procedure. Rats were placed in a stereotaxic apparatus. Guide cannulae were implanted into the striatum (posterior: 0.25 mm, lateral: 4.0 mm from bregma; below the skull surface: 7.0 mm) through a hole drilled in the skull and fixed with two anchor screws and dental cement. One week after surgery, microdialysis probes (Eicom Co., Kyoto, Japan) with a membrane length of 3 mm were carefully inserted through the guide cannulae. The probe was perfused at a flow rate of 4 μL/min with Ringer's solution (Na^+: 147 mM, K^+: 4 mM, Ca^{2+}: 4.5 mM, Cl^-: 155.5 mM). After a stabilization period (approximately 2–3 hours), the microdialysis samples were collected every 15 min before, during and after MCAO. Samples were precisely reacted with OPA working solution (OPA, β-ME, sodium tetraborate) for 4 min at 32°C according to the previously reported method[4], and detected using a Shimadzu HPLC system (Shimadzu Co., Kyoto, Japan) equipped with a C18 column (TSKgel ODS-100V, Tosoh Bioscience, Tokyo, Japan) and a fluorescence detector (RF-530, Shimadzu Co., Japan). The mobile phase was 0.1 M Na_2HPO_4 containing 25% methanol (pH 6.75 adjusted by H_3PO_4). Before experiments, the *in vitro* recovery of each microdialysis probe was determined and all probes had 14% recovery at a flow rate of 4 μL/min.

Determination of 4-hydroxynonenal (4-HNE) adducts

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Plasma samples were prepared from 1 mL of blood collected via the inferior vena cava of sham and MCAO operated rats treated with control Ab or anti-HMGB1 mAb. The levels of 4-HNE adducts in each sample were determined using an OxiSelect™ HNE-His Adduct ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) with HNE-BSA as the standard. The determination was performed on triplicate samples.

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Supplemental Table

Table 1. Basic physiological parameters for study groups.

	Pre	Control IgG		Anti-HMGB1	
		10 min	8 h	10 min	8 h
pH	7.4 ± 0.08	7.4 ± 0.01	7.4 ± 0.02	7.4 ± 0.01	7.3 ± 0.03
PCO_2 (mmHg)	43 ± 2.45	43.2 ± 1.35	46.4 ± 2.67	39.9 ± 1.99	42.9 ± 2.48
PO_2 (mmHg)	162.6 ± 17.6	170.6 ± 6.05	153.4 ± 6.17	148.5 ± 3.7#	156.1 ± 2.8
Base excess (mmol/L)	-0.5 ± 1.6	-1.1 ± 0.8	-0.7 ± 0.8	-0.7 ± 0.2	-2.5 ± 1.1
HCO_3^- (mmol/L)	22.4 ± 1.7	23.9 ± 0.7	24.7 ± 0.7	27.2 ± 3.8	22.7 ± 0.8
Hemoglobin (g/dl)	11.2 ± 0.6	12 ± 0.3	12.3 ± 0.9	11.1 ± 0.4	12.8 ± 0.3
Na^+ (mmol/L)	127 ± 2.4	130.8 ± 1.3	135.6 ± 0.7	130 ± 1.1	136 ± 1.6
K^+ (mmol/L)	3.8 ± 0.1	6.5 ± 0.7*	4.0 ± 0.1	3.4 ± 0.1#	3.6 ± 0.1
Ca^{2+} (mmol/L)	0.8 ± 0.07	1.0 ± 0.06	1.1 ± 0.02	0.8 ± 0.07	1.1 ± 0.05
Cl^- (mmol/L)	95 ± 1.2	102.8 ± 0.5	107.4 ± 0.8	99 ± 1.9	110 ± 2.4
Glucose (mg/dl)	158.6 ± 8.7	202.6 ± 13.9*	165.2 ± 9.7	176.8 ± 10.4	176.2 ± 7.4
Lactate (mg/L)	19.4 ± 1.5	18.2 ± 0.5	16 ± 1.2	16 ± 2.2	12.2 ± 1.1#

Control IgG or anti-HMGB1 mAb was administered intravenous under halothane anesthesia, and physiologic variables were determined 10 min and 8 h after mAb administration. Each value represents the mean ± s.e.m of 5 rats. *P<0.05 compared with the Pre value, #P<0.05 compared with the value at the same time point in control IgG group. Pre, pre-ischemia.

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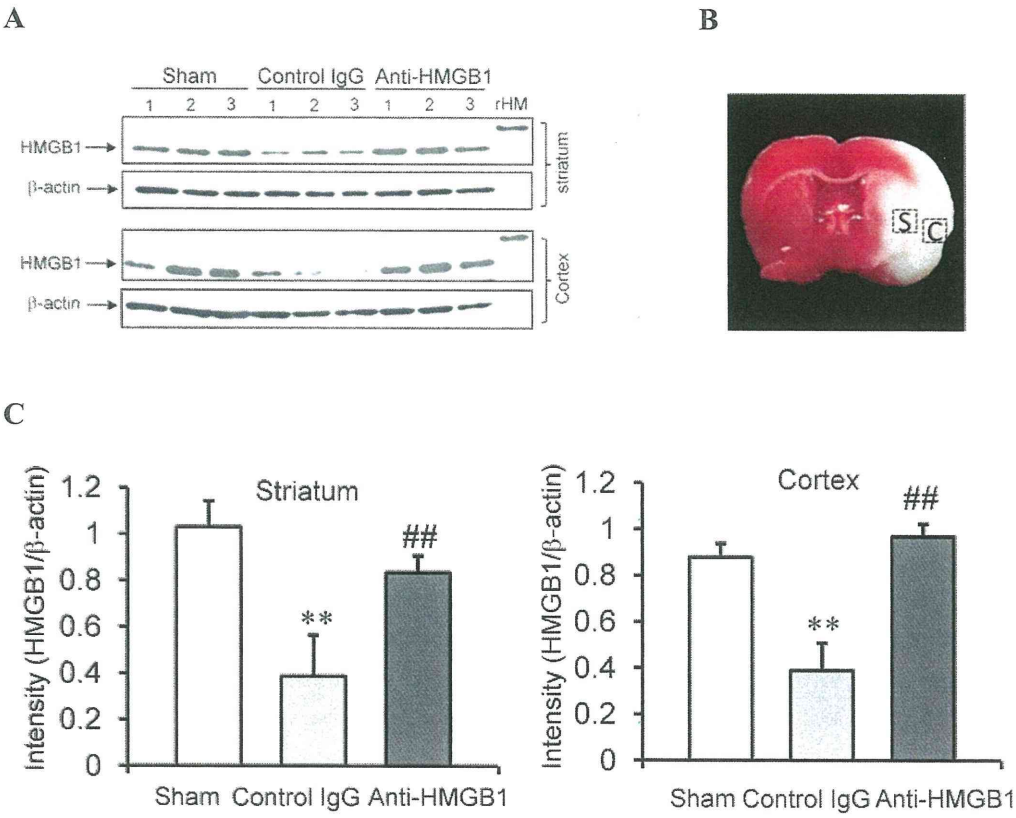
Supplemental Figures



Supplemental Figure 1

Detection of HMGB1 in the ischemia core area using Western blotting. The rat brain in control IgG-treated group and anti-HMGB1 treated group was perfused by cold saline, collected and homogenized in RIPA solution with protease inhibitors as indicated in supplemental methods. HMGB1 was blotted by a peroxides-labeled anti-HMGB1 mAb (#10-22) and visualized by ECL system. β-actin was used as the internal control. (**A**) The bands of HMGB1 and β-actin detected by Western blotting from 3 representative brain samples in sham (n=5), control IgG (n=8), and anti-HMGB1 (n=8) groups. (**B**) A representative image of TTC staining of a brain slice from a MCAO rat 12 h after reperfusion. The squares circled by dash line indicate the positions where the brain samples were collected. S: striatum, C: cortex. (**C**) Quantitative results of the bands detected by Western blotting using NIH image J 1.42q software. The results are the means ± s.e.m. **P<0.01 compared with sham control, ##P<0.01 compared with control IgG group.

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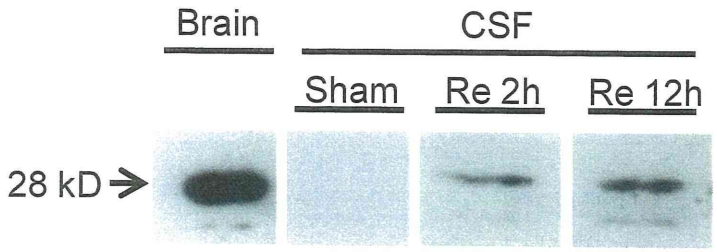

Supplemental Figure 2

Detection of HMGB1 in the cerebrospinal fluid after brain ischemia using Western blotting. Cerebrospinal fluid was collected from the cisterna magna at different time points. Homogenate sample of normal rat brain was used as the positive control. HMGB1 was blotted by a peroxides-labeled anti-HMGB1 mAb (#10-22) and visualized by ECL system as described in supplementary methods. (Re: reperfusion)

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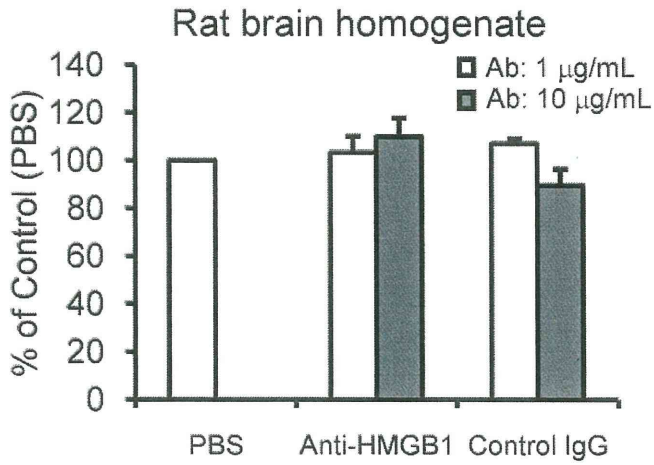
A



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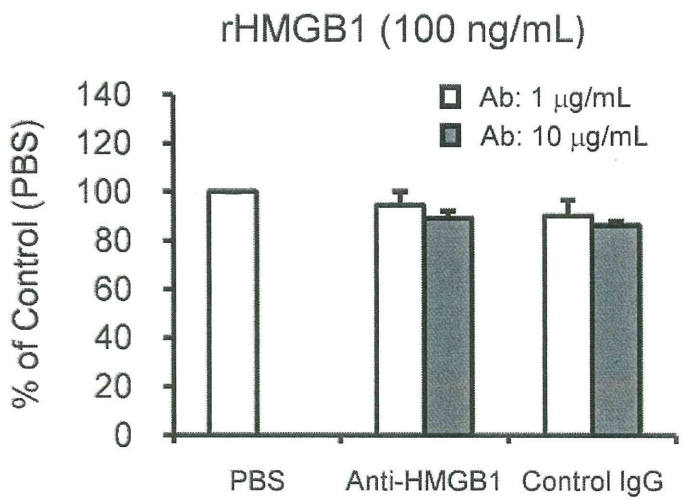

Supplemental Figure 3

Effects of anti-HMGB1 mAb (#10-22) on the ELISA. Different concentrations of anti-HMGB1 mAb were added to the incubation mixture with the brain homogenate samples containing HMGB1 (**A**) and rHMGB1 (**B**). The brain homogenizing samples were parepared by the rat brain using 50 mM Tris-HCL (pH 8.0), and diluted to 80~100 ng HMGB1 equivalent/mL by the sample buffer provided in the ELISA kit. The results are the means ± s.e.m. of three determinations.

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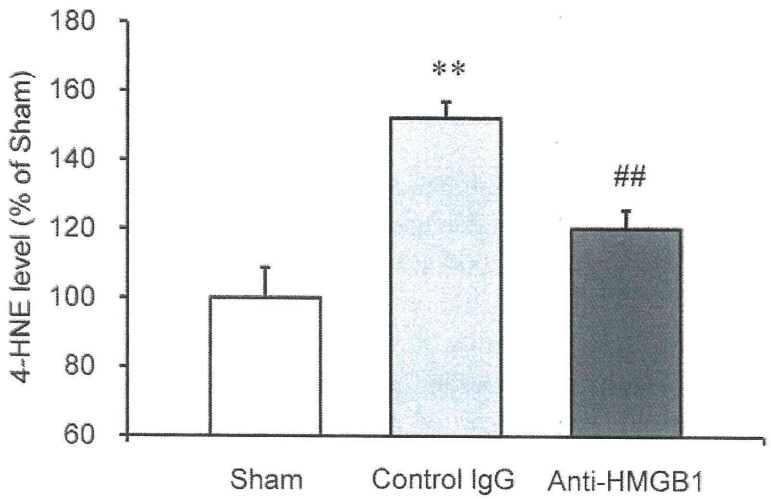

Supplemental Figure 4

Plasma 4-HNE adducts levels in control IgG-treated and anti-HMGB1 mAb-treated rats. Plasma samples were collected from ischemic rats at 6 h after reperfusion. 4-HNE adducts in the samples were detected by ELISA using 4-HNE-BSA as the standard. The results are the means ± SEM of 4 (sham), 5 (control IgG- and anti-HMGB1 mAb-treated group) rats. **P<0.01 compared with sham control, ##P<0.01 compared with control IgG group.

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High-Mobility Group Box Protein 1 Neutralization Reduces Development of Diet-Induced Atherosclerosis in Apolipoprotein E–Deficient Mice

Peter Kanellakis, Alex Agrotis, Tin Soe Kyaw, Christine Koulis, Ingo Ahrens, Shuji Mori, Hideo K. Takahashi, Keyue Liu, Karlheinz Peter, Masahiro Nishibori, Alex Bobik

Objective—High-mobility group box protein 1 (HMGB1) is a DNA-binding protein and cytokine highly expressed in atherosclerotic lesions, but its pathophysiological role in atherosclerosis is unknown. We investigated its role in the development of atherosclerosis in ApoE−/− mice.

Methods and Results—Apolipoprotein E–deficient (ApoE−/−) mice fed a high-fat diet were administered a monoclonal anti-HMGB1 neutralizing antibody, and the effects on lesion size, immune cell accumulation, and proinflammatory mediators were assessed using Oil Red O, immunohistochemistry, and real-time polymerase chain reaction. As with human atherosclerotic lesions, lesions in ApoE−/− mice expressed HMGB1. Treatment with the neutralizing antibody attenuated atherosclerosis by 55%. Macrophage accumulation was reduced by 43%, and vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1 expression was attenuated by 48% and 72%, respectively. CD11c+ dendritic cells were reduced by 65%, and the mature (CD83+) population was reduced by 60%. Treatment also reduced CD4+ cells by nearly 50%. mRNAs in lesions encoding tumor necrosis factor-α and interleukin-1β tended to be reduced. Mechanistically, HMGB1 stimulated macrophage migration in vitro and in vivo; in vivo, it markedly augmented the accumulation of F4/80+Gr-1(Ly-6C)+ macrophages and also increased F4/80+CD11b+ macrophage numbers.

Conclusion—HMGB1 exerts proatherogenic effects augmenting lesion development by stimulating macrophage migration, modulating proinflammatory mediators, and encouraging the accumulation of immune and smooth muscle cells. (***Arterioscler Thromb Vasc Biol.* 2011;31:313-319.)**

Key Words: atherosclerosis ■ macrophages ■ HMGB1

Atherosclerosis is a chronic inflammatory disease characterized by intimal accumulation of atherogenic lipoproteins, extracellular matrix, smooth muscle cells, and inflammatory cells. Cytokines within atherosclerotic lesions play a key role in both the development and progression of atherosclerosis.[1,2] Recently, we and others have identified a novel cytokine in human atherosclerotic lesions, high-mobility group box 1 (HMGB1), that could be important for regulating development of atherosclerosis.[3–5] HMGB1 has 2 main functions. As a nuclear protein, it stabilizes nucleosomes and bending of DNA, which facilitates gene transcription. It can also be released from necrotic cells[6] or secreted by inflammatory cells, such as macrophages,[3] and natural killer cells,[7] triggering inflammation.[8] HMGB1-nucleosome complexes released by necrotic cells activate macrophages and dendritic cells to produce cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-10[9]; also, native secreted HMGB1 stimulates monocytes to secrete proinflammatory cytokines.[10] With respect to the vasculature, HMGB1 stimulates the migration and proliferation of vascular smooth muscle cells[4,11] and activates endothelial cells.[12] It induces the migration of macrophages, activates dendritic cells,[13,14] and is required for dendritic cells maturation.[14,15] Notably, dendritic cells control T-cell activation by secreting HMGB1.[16]

Structurally, HMGB1 has a tripartite domain organization. It contains 2 similar DNA-binding domains, HMG Box A and Box B, and a unique C-terminal domain consisting of an acidic tail of 30 amino acids all connected by short amino acid sequences.[17] The proinflammatory activity of HMGB1 has been localized to Box B, and antibodies raised against Box B prevent HMGB1 actions[17,18]; a receptor for advanced glycation end products (RAGE)-binding domain is localized within the C-terminal component of Box B and the segment connecting to the acidic tail.[19] In contrast, Box A attenuates HMGB1-induced release of proinflammatory cytokines.[20,21] The acidic tail is thought to be unstructured and interacts with specific basic residues in both boxes, possibly regulating their conformation.[22]

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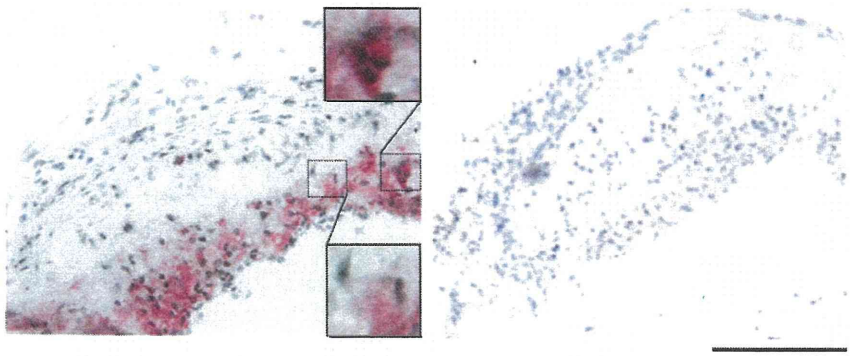

Figure 1. Immunohistochemical identification of HMGB1 expression in aortic sinus atherosclerotic lesions in ApoE−/− mice. Left, HMGB1 (red staining) was localized within intimal cells (top insert) and also diffusely distributed within lesions (bottom inset). Cells within the media did not express HMGB1. Right, Region similar to that shown in A incubated with nonspecific rabbit IgG instead of the primary rabbit HMGB1 IgG. Section was counterstained with hematoxylin. Scale bar represents 100 μm.

The proinflammatory effects of HMGB1 have been attributed to its interaction with a number of receptor types, which appears dependent on factors bound to HMGB1. RAGE, toll-like receptor (TLR)2, and TLR4 appear to mediate proinflammatory effects in HMGB1-stimulated macrophages.[23,24] HMGB1 in DNA immune complexes and nucleosome complexes appears to exert proinflammatory effects via TLR9 and TLR2, respectively.[12,25] More recently, HMGB1 has also been shown to interact with CD24 and Siglec-10 to attenuate tissue damage induced immune responses[26]; a balance between these and the detrimental effects of HMGB1 has been suggested to determine the overall magnitude of its detrimental effects.[26]

HMGB1 has been implicated in a number of immune-driven diseases, including systemic lupus erythematosus, autoimmune diabetes, and arthritis.[9,27,28] In arthritis, it triggers inflammation by activating macrophages and inducing IL-1.[28] Because HMGB1 is overexpressed in atherosclerotic lesions[3–5] and activates immune processes that can augment atherosclerosis,[4,11,13–15] we examined its role in the development of atherosclerosis in apolipoprotein E–deficient mice (ApoE−/− mice). We used an anti-HMGB1neutralizing monoclonal antibody, which interacts specifically with the C-terminal sequence of HMGB1 within the acidic tail, to determine its role in atherosclerosis. This antibody reacts with HMGB1 and not HMGB2 and has been shown to inhibit HMGB1 responses in macrophages and ameliorates brain infarction induced by transient ischemia.[29]

Materials and Methods

Six-week-old ApoE−/− mice were fed a high-fat diet for 8 weeks and administered either an anti-HMGB1 monoclonal antibody or IgG2a control (400 μg IV twice weekly). At the end of the study, mice were killed with an overdose of pentobarbitone, and blood, aortic sinus and arch, spleen, and lymph nodes were collected for histological and molecular studies. Monocyte proliferation in vivo was assessed after administering bromodeoxyuridine (1 mg IP) for 3 days before the mice were killed. The chemotactic effects of HMGB1 on monocytes was assessed in mice 5 hours after administering HMGB1 (20 μg) into the peritoneal cavity.

A detailed Supplemental Methods section is available online at http://atvb.ahajournals.org.

Results

HMGB1 Expression in Atherosclerotic Lesions

HMGB1 is highly expressed in human atherosclerotic lesions, mostly by macrophages in which nearly 50% of the cells contained HMGB1 in their cytoplasm.[3] Therefore, we investigated whether atherosclerotic lesions in ApoE−/− mice also expressed HMGB1. HMGB1 was expressed within the aortic sinus of ApoE−/− mice and restricted to cells within the atherosclerotic intima (Figure 1). HMGB1 appeared to be cell associated, frequently within the cytoplasm and also diffusely distributed within lesions; the latter most probably reflected secreted HMGB1 (Figure 1).

Effect of HMGB1 Neutralization on Atherosclerotic Lesion Size

At 14 weeks of age (8 of weeks antibody treatment) total plasma cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were not different between mice treated with control or anti-HMGB1 neutralizing antibody (Supplemental Table I). However, analysis of Oil Red O–stained aortic sinus sections revealed significant differences in lipid deposition and atherosclerotic plaque size (Figure 2). Morphometric analysis showed that the anti-HMGB1 neutralizing antibody reduced lesion size compared with treatment with control antibody, 89 253±13 098 μm^2 versus 183 903±11 784 μm ($P<0.05$; Figure 2). Similarly, macrophage accumulation was reduced in lesions of mice treated with the neutralizing antibody. Cross-sectional area of the aortic sinus that stained with anti-CD68 antibody averaged 83 400±15 451 μm^2 in mice treated with the anti-HMGB1 neutralizing antibody compared with 141 364±12 608 μm^2 with control antibody (Figure 2). To determine whether the effects of the HMGB1 antibody were due to neutralizing circulating HMGB1, we measured plasma HMGB1 levels in control and anti-HMGB1 antibody treated mice using a specific HMGB1 ELISA. Plasma HMGB1 levels were undetectable (<1 ng/mL) in both instances, similar to earlier reports in nonatherosclerotic mice,[30] suggesting that effects of the neutralizing antibody were local at the site of developing lesions.

Effect of Neutralizing HMGB1 on Atherosclerotic Lesion Composition

Analysis of atherosclerotic lesion composition also revealed differences between the 2 groups in terms of cellular composition. Because HMGB1 can stimulate the migration of dendritic cells,[14,15] we investigated whether neutralizing HMGB1 activity attenuated their accumulation in developing lesions. Immunohistochemical studies and quantitative analysis indicated a marked reduction in dendritic cell accumu-

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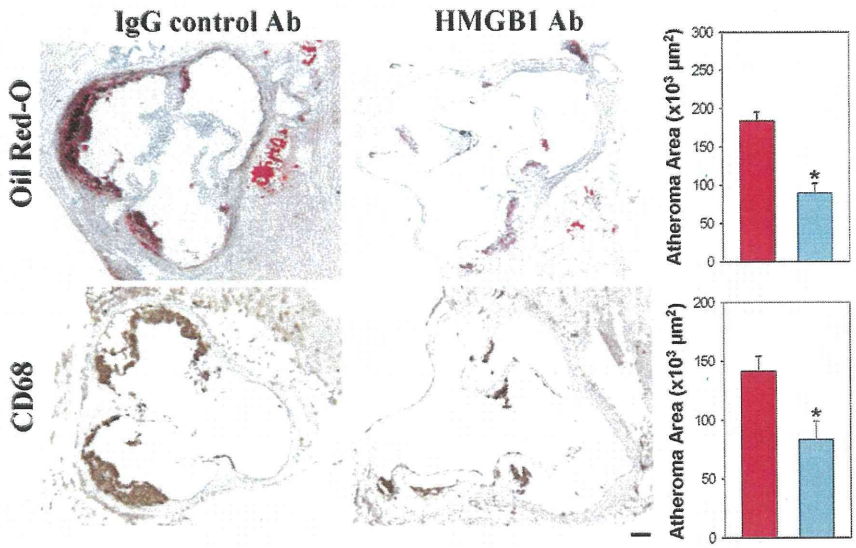

Figure 2. Photomicrographs of aortic sinus atherosclerotic lesions from ApoE−/− mice fed a high-fat diet and treated with control antibody (Ab; left) and anti-HMGB1 neutralizing antibody (right). Top sections show sections stained with Oil Red O and mean areas of staining (bar graph) in the 2 groups of mice. Bottom sections, Immunohistochemical staining using anti-CD68 (macrophage) antibody and mean areas of staining (bar graphs) in the 2 groups of mice. Red indicates control antibody; blue, anti-HMGB1 neutralizing antibody. *$P<0.05$ compared with control. Scale bar represents 100 μm.

lation, with CD11c staining averaging 8±1% of lesion area in aortic sinus of mice treated with anti-HMGB1 neutralizing antibody compared with 24±3% with control antibody ($P<0.05$; Figure 3). Because HMGB1 can also promote maturation of dendritic cells,[15] we investigated whether expression of CD83, a marker of dendritic cell maturation, was affected. CD83 expression in the lesions represented only a small fraction of the dendritic cell population, indicating that the dendritic cell population was largely immature (Figure 3). Treatment with the anti-HMGB1 antibody reduced the CD83 cell population by nearly 60% ($P<0.05$; Figure 3). Mature dendritic cells in atherosclerotic lesions are known to produce T-cell–attracting chemokines CCL19 and CCL21.[31] Consequently, we examined whether CD4+ T-cell accumulation was affected in mice treated with the anti-HMGB1 neutralizing antibody. Treatment with anti-HMGB1 neutralizing antibody reduced CD4+ T-cell accumulation in lesions by ≈50% ($P<0.05$; Figure 4). To determine the different T-cell subtypes that might be affected, we analyzed subset-specific mRNA expression. In lesions of anti-HMGB1 antibody–treated mice, we observed no change in expression of Tim-3, a known marker and negative regulator of Th1 lymphocytes ($P>0.05$; Figure 4)[32]; Tim-1, a marker of Th2 lymphocytes, was undetectable (Figure 4).[33]

Because HMGB1 stimulates the in vitro migration and proliferation of vascular smooth muscle cells,[4,11] and endothelial cells,[34] we also investigated whether it affected smooth muscle cell and endothelial cell numbers in developing lesions. Treatment with the anti-HMGB1 neutralizing antibody reduced smooth muscle cell accumulation within developing lesions by nearly 50% ($P<0.05$; Supplemental Figure I) but did not affect lesion-associated endothelial cells (Supplemental Figure I). Treatment with the neutralizing antibody was also associated with a 35% reduction in the number of proliferating cells within the lesions ($P<0.05$; Supplemental Figure I).

To further confirm that these cellular effects were largely restricted to developing atherosclerotic lesions, we also assessed lymphocyte populations in blood, spleen, and paraaortic lymph nodes. In these tissues anti-HMGB1 antibody treatment

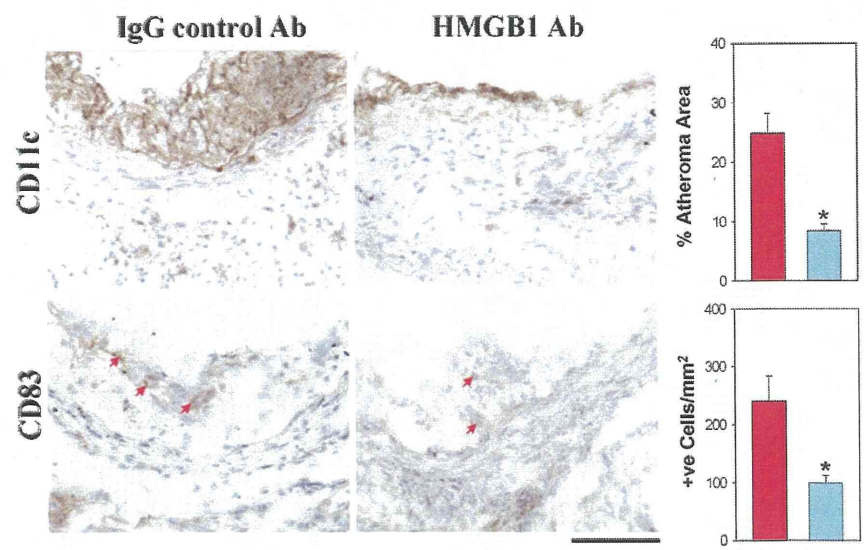

Figure 3. Immunohistochemistry of aortic sinus atherosclerotic lesions from control antibody (Ab; left) and anti-HMGB1 antibody (right)–treated ApoE−/− mice fed a high-fat diet. Cross-sections were stained with anti-CD11c antibody to detect dendritic cells (top) and anti-CD83, a marker of mature dendritic cells (bottom). Bar graphs represent the extent of immunostaining in the 2 groups. Red indicates control antibody; blue, anti-HMGB1 neutralizing antibody. *$P<0.05$ compared with control. Scale bar represents 100 μm.

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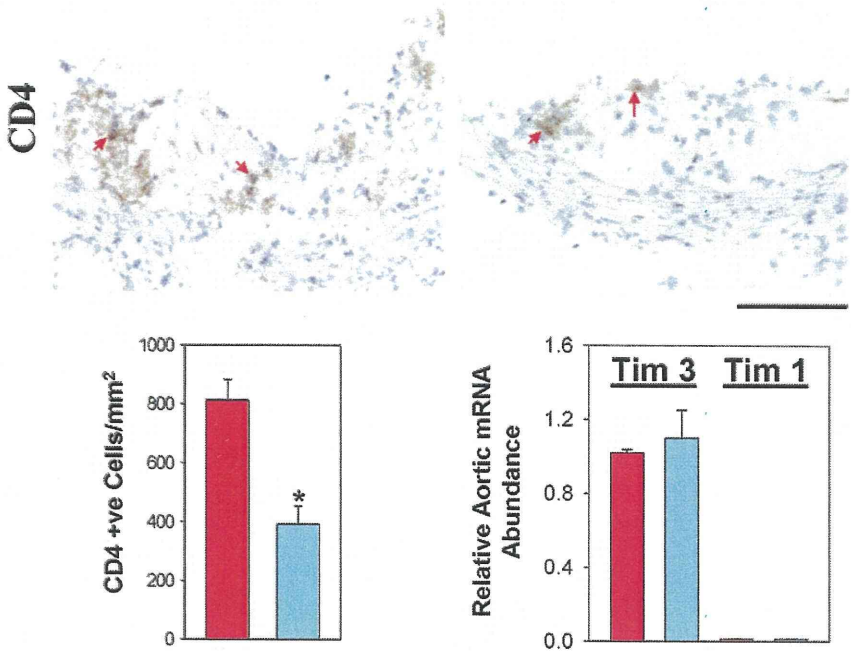

Figure 4. Distribution of CD4+ T cells in aortic sinus atherosclerotic lesions and their polarization following treatment of ApoE−/− mice with either control antibody or anti-HMGB1 neutralizing antibody. Top, cross-sections were stained with anti-CD4 antibodies to detect CD4+ lymphocytes in lesions of control (left) and anti-HMGB1 antibody (right)–treated mice. Bottom left, Bar graph indicating CD4+ lymphocyte density in the lesions. Bottom right, Bar graph indicating the relative abundance of mRNAs encoding Tim3 and Tim1 in lesions. Red indicates control; blue, anti-HMGB1 treatment. *$P<0.05$ compared with control. Scale bar represents 100 μm.

did not affect CD4+ T cells, B cells, natural killer (NK) or natural killer T cell or CD4+Foxp3+ regulatory T-cell numbers (all $P>0.05$; Supplemental Figure II). In addition, we assessed monoblast/promonocyte proliferation, as well as monocyte and dendritic cell numbers in blood of control and anti-HMGB1–treated mice and dendritic cell numbers in lymph nodes. BrdU+CD11b^{hi}Ly-6C^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo} Ly-6G^{lo}CD22^{lo}, CD11b^{hi}Ly-6C^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, and CD11b^{hi}CD90^{lo}Cd49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocytes in blood were unaffected by anti-HMGB1 antibody treatment ($P>0.05$; Supplemental Table II). Similarly, BrdU+ CD11c^{hi}CD11b^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, CD11c^{hi}CD11b^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo}, and CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocyte-derived dendritic cells in blood were unaffected ($P>0.05$; Supplemental Table II). Also, CD11c^{hi}CD11b^{hi}I-A$^{b(hi)}$CD115^{hi}CD90^{lo}CD49b^{lo}NK1.1^{lo}Ly-6G^{lo}CD22^{lo} monocyte-derived dendritic cells in inguinal and mediastinal lymph nodes were unaltered by anti-HMGB1 antibody treatment; CD11c+I-A^{b}+CD115+ are thought to emigrate from lesions to lymph nodes during regression of lesions.[35] Together, these results indicate that monocytes/dendritic cell numbers in blood do not account for the reduction in macrophages and dendritic cells in lesions of anti-HMGB1 treated mice. Similarly, emigration of dendritic cells from lesions does not appear to be affected by anti-HMGB1 antibody treatment.

HMGB1 Neutralization and Proinflammatory Mediators in Lesions

HMGB1 can increase the expression of a number of proinflammatory mediators including MCP-1, vascular cell adhesion molecule-1 (VCAM-1) and a variety of cytokines including IL-1β, IL-6, and tumor necrosis-α (TNF-α).[12,36,37] We investigated whether treatment with anti-HMGB1 neutralizing antibodies might affect expression of such proinflammatory mediators in developing lesions. Treatment with anti-HMGB1 neutralizing antibodies reduced expression of VCAM-1 in lesions by 50% and MCP-1 expression by nearly 70% (both $P<0.05$; Supplemental Figure III). There also tended to be reductions in the expression of TNF-α, IL-1β, and interferon-α, whereas IL-6 was unaffected (Supplemental Figure III).

HMGB1 and Cell Migration

Because anti-HMGB1 antibody treatment affected immune cell accumulation in lesions, in particular macrophages, to a much greater extent than proinflammatory cytokines, we examined the possibility that HMGB1 might be influencing macrophage migration to developing lesions. In vitro, HMGB1 stimulates macrophage migration.[13] To confirm the chemoattractant actions of HMGB1 on mouse macrophages, we first assessed its ability to stimulate mouse RAW264.7 macrophage migration using 24-well chemotaxis chambers. HMGB1 stimulated their migration, more than doubling the number of migrated macrophages ($P<0.05$; Figure 5). To determine whether HMGB1 also stimulated macrophage cell migration in vivo, we injected HMGB1 into the peritoneal cavity of mice and 5 hours later assessed its effects on peritoneal macrophage. HMGB1 markedly increased the number of F4/80+Gr1(Ly-6C)+ macrophages ($P<0.05$; Figure 5) and also increased F4/80+CD11b+ macrophages ($P<0.05$; Figure 5).

Discussion

We have previously reported that HMGB1 is expressed by macrophages in human aortic fatty streaks and fibrofatty lesions.[3] In advanced atherosclerotic plaques, HMGB1 is also expressed by vascular smooth muscle cells.[5] Also, serum HMGB1 levels have been associated with coronary artery

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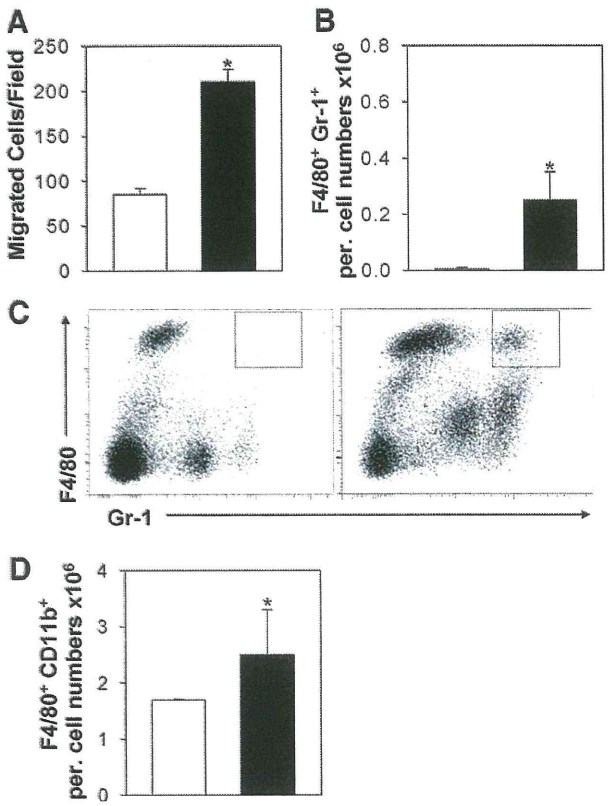

Figure 5. HMGB1-stimulated macrophage chemotaxis in vitro and intraperitoneal macrophage recruitment in vivo. A, Migration of mouse RAW264.7 macrophages in vitro in response to vehicle or HMGB1 (4 μg/mL). B, Number of F4/80+Gr-1(Ly-6C)+ macrophages recruited to the peritoneal (per.) cavity 5 hours after IP injection of vehicle or HMGB1 (20 μg/mL). C, Flow cytometric analysis of peritoneal lavage effluent demonstrating F4/80 and Gr-1 populations 5 hours after injection of vehicle or HMGB1. Boxed areas show the F4/80+Gr-1(Ly-6C)+ population. D, Number of F4/80+CD11b+ macrophages recruited to the peritoneal cavity 5 hours after IP injection of vehicle or HMGB1. *$P<0.05$ compared with vehicle control.

disease.[38,39] In this study, we demonstrated that atherosclerotic lesions within the aortic sinus of ApoE−/− mice also express HMGB1, which contributes to lesion development. Neutralizing HMGB1 using a monoclonal antibody targeting amino acids within the acidic tail,[29] attenuated development of atherosclerosis. Our study suggests that HMGB1 in lesions rather than circulating HMGB1 contributes to atherosclerosis, stimulating macrophage migration and increasing the accumulation of other immune cell types, as well as proinflammatory mediators.

HMGB1 exerts multiple effects on monocyte/macrophages in vitro. It stimulates macrophage migration,[13] monocyte adherence and spreading, monocyte-matrix interactions,[40] and dendritic cell migration.[14] Our observations extend these findings and indicate that HMGB1 is also chemoattractive in vivo for macrophages; HMGB1 stimulated the migration of F4/80+Gr-1(Ly-6C)+ macrophages, a subtype known to accumulate in atherosclerotic lesions.[41] It does not appear to influence monoblast/promonocyte proliferation, monocyte numbers in blood, or efflux of dendritic cells from lesions.

Our studies suggest that CD4+ T cells in lesions are also affected by HMGB1; CD4+ T-cell numbers were reduced in lesions of mice treated with the HMGB1 neutralizing antibody but unaffected in blood, spleen, or lymph nodes. In vitro HMGB1 acts as a costimulatory factor together with T-cell receptor stimulation to augment proliferation.[42] Also, dendritic cells produce and respond to HMGB1 elevating IL-12 secretion, which in turn polarizes CD4+ T cells to the Th1 phenotype.[37] Our finding that Tim-3 expression is unaffected by anti-HMGB1 antibody treatment, despite reductions in CD4+ T cells, suggests that the function of these cells may be impaired; Tim-3 is a negative regulator of Th1 cell function.[32,43] Because HMGB1 appears to regulate immune cell numbers in lesions we also investigated whether it affected cytokine levels. In vitro, HMGB1 potently stimulates macrophages and endothelial cells to secrete proinflammatory cytokines, TNF-α, IL-1β, and IL-6.[12,37] Surprisingly, we found that neutralizing HMGB1 only had small effects on the expression of proinflammatory cytokines, suggesting that HMGB1 in lesions was affecting immune cell function rather than expression of proinflammatory cytokines in lesions. However, HMGB1 augmented the expression of proinflammatory mediators VCAM-1 and MCP-1.

In addition to affecting immune cells, HMGB1 can affect vascular smooth muscle cells, stimulating their migration[11] and proliferation.[4] Treatment with anti-HMGB1 neutralizing antibodies reduced smooth muscle cell accumulation and the number of proliferating cells within developing lesions. The intima is considered to be the "soil" in which atherosclerosis develops,[44] and attenuating its development/growth by neutralizing HMGB1 activity and reducing the intimal smooth muscle population could contribute to the reductions in lesion size. Although HMGB1 has also been shown to stimulate endothelial cell proliferation and angiogenesis, endothelial cells did not appear to be affected by treatment with the anti-HMGB1 neutralizing antibody.

Although our studies indicate that HMGB1 contributes to lesion development by stimulating macrophage migration and activating dendritic cells, the receptors through which these effects are mediated remain to be identified. It is interesting to note that the effects we observed on macrophages, dendritic cells, and T cells, as well as vascular smooth muscle cells, have been largely attributed to interactions with RAGE.[11,13,15,16] Inhibition of RAGE attenuates lesion development and is associated with reductions in macrophage and smooth muscle cell accumulation, as well as reductions in VCAM-1 expression,[45] effects that we observed following neutralization of HMGB1. However, we cannot exclude the possibility that HMGB1 also interacts with other receptors during development of atherosclerosis, including TLR2, TLR4, or the CD24/Siglec-10 system. Our findings provide an encouraging basis for the development of a novel therapeutic approach for atherosclerosis, possibly using newer formats of recombinant antibodies.

In conclusion, our data extend earlier findings on the expression of HMGB1 in human atherosclerotic lesions and demonstrate that locally, I expressed HMGB contributes to lesion development by stimulating macrophage migration and modulating proinflammatory mediators such as MCP-1 and VCAM-1. HMGB1 also indirectly contributes to accumula-

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tion of dendritic cells and CD4+ T cells. The findings define a new potential therapeutic target for atherosclerosis.

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Disclosures

None.

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Supplemental Material

Peter Kanellakis et al. HMGB1 Neutralization Reduces Development of Diet-Induced Atherosclerosis in Apolipoprotein E-Deficient Mice

Supplemental Materials and Methods

Animal Experiments

Twenty four male ApoE-/- mice on the C57BL/6 background were obtained from the Precinct Animal Facility, AMREP, Melbourne, Australia and fed a high-fat diet containing 0.15% cholesterol and 21% fat (Speciality Feeds) from 6 weeks of age for 8 weeks. Mice (12 per group) also received either a control IgG2a against *Keyhole Limpet* hemocyanin (400μg iv twice weekly) or anti-HMGB1 monoclonal antibody (400μg iv twice weekly) for the duration of the dietary feeding. This dose regime was based on our earlier findings that 400μg anti-HMGB1 i.v. is highly effective in reducing cerebral infarct volumes,[1] and the long plasma half-life of IgG2a, approximately 5 days.[2,3] In vitro, 1μg of the anti-HMGB1 neutralizing antibody attenuates the stimulatory effect of 10μg HMGB1 on macrophages, assessed as an increase in ICAM-1 expression, by 67% whilst 100μg of antibody attenuates this response by 93%.[1] At the end of the study mice were killed with an overdose of pentobarbitone (120mg/kg i.p.), blood collected by cardiac puncture and aortic sinus and arch collected for histology and molecular studies. To assess the effects of the anti-HMGB1 antibody treatment on monocytes and dendritic cells and proliferation of their precursors an additional 4 mice in each group were treated with bromodeoxyuridine (1mg, i.p. daily) for three consecutive days prior to culling. All experiments were approved by the Alfred Medical Research Education Precinct (AMREP) Animal Ethics Committee.

Monoclonal Antibodies

Monoclonal antibodies (anti-HMGB1 and control) were purified from culture media of growing hybridomas producing the anti-HMGB1 and anti-*Keyhole Limpet* hemocyanin

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(control) antibodies using Mep HyperCel (Pall Life Sciences, Cerg, FGrance) and DEAE cellulose chromatographies. HMGB1 neutralizing activity was confirmed by determining its ability to attenuate HMGB1 bioactivity on cultured macrophages as previously described.[1]

HMGB1

Full-length recombinant HMGB1 was purchased from HMGBiotech (Milano, Italy); it was purified as previously described and endotoxins were removed by passage through Detoxy-Gel columns (Pierce Chemical Co).[4,5] HMGB1 was rigorously tested to be LPS-free.[6]

Plasma Cholesterol and Triglycerides

Plasma LDL- and HDL-cholesterol were determined enzymatically using a Cobas Mira Plus Autoanalyzer and a HDL and LDL/VLDL cholesterol quantification kit (BioVision, Mountain View, CA).Plasma triglycerides were determined using a triglyceride quantification kit (BioVision, Mountain View, CA).

Quantification of Atherosclerotic Lesions

The heart and proximal aorta were dissected from mice, embedded in OCT compound (Tissue-teck) and frozen at -80°C. Frozen sections (6μm) were cut from the aortic sinus, from where the valves or valve cusps first become visible to where the left and right coronary arteries branch off, a distance of approximately 250μm.[7] A total of 4 sections taken from identical aortic sinus locations in each mouse at 60μm intervals were stained with Oil Red O to delineate lipid deposits and counter stained with haematoxylin.[8] The aortic sinus was evaluated because this region of the aorta is particularly susceptible to the development of atherosclerotic lesions in mice fed a high fat diet.[7] Sections were examined using light microscopy and the cross-sectional area of lipid depositions quantified using image analysis software (Optimus 6.2 VideoPro-32). For each mouse, the

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lesion size was measured in 4 cross-sections and lesion size per cross-section averaged to provide the mean lesion size per mouse.

Immunohistochemistry

Twenty seven 6μm cryo-sections from similar parts of the aortic sinus of ApoE-/- mice treated with control or anti-HMGB1 neutralizing antibody were used for immunohistochemistry to assess macrophage accumulation (CD68), CD4+ T-lymphocytes, dendritic cells (CD11c), vascular smooth muscle cells (alpha SM actin) and endothelial cells (CD31), VCAM-1, MCP-1, CD83, HMGB1 and PCNA. Briefly, sections were fixed in cold (-20°C) acetone for 20 min. The sections were then incubated in 3% hydrogen peroxide in PBS, 10% normal serum and biotin/avidin blocking reagents (Vector Laboratories). Then the sections were incubated (1hr) with primary antibodies in serum, rat anti-mouse CD68 (1-100; Serotec: cat#MCA1957), Armenian hamster anti-mouse CD11b (1-50; eBioscience: cat#14-0114), rat anti-mouse CD4 (1-20; BD Pharmingen: Cat#550280), rabbit anti-alpha smooth muscle actin (1-100; Abcam: #ab5694), rat anti-mouse CD31 (1-100, BD Pharmingen: cat# 550274), rat anti-mouse VCAM-1 (1-50; BD Pharmingen: cat#550547), rabbit anti rat MCP-1 (1-50; Abcam: cat#ab7202), rat anti-mouse CD83 (1-50; eBioscience: cat#14-0831),rabbit anti-HMGB1 antibody (0.125μg/ml; BD Pharmingen: cat#556528) and rabbit anti-human PCNA (1-50; Abcam: cat#ab2426). Subsequently the sections were washed and incubated with the appropriate secondary antibody [biotinylated mouse anti-rat (1-200; BD Pharmingen: cat#550325), biotinylated mouse anti-Armenian hamster (1-200; eBioscience: cat#13-4113-85) or biotinylated anti-rabbit (1-200; Vector Labs: cat#BA-1000)] for 40 minutes, followed by incubation with streptavidin horseradish peroxidase complex (Vector Laboratories). Antigens were visualized using 3,3-diaminobenzidine.[9] Sections were counterstained with hematoxylin. Expression of antigens was quantified either by cell counting or measuring stained areas using Optimus 6.2 VideoPro-32 and results expressed either as stained cross-sectional

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