Increased Expression of the DNA-Binding Cytokine HMGB1 in Human Atherosclerotic Lesions
Role of Activated Macrophages and Cytokines

N. Kalinina, A. Agrotis, Y. Antropova, G. DiVitto, P. Kanellakis, G. Kostolias, O. Ilyinskaya, E. Tararak, A. Bobik

Objective—Atherosclerosis is a chronic inflammatory response of the arterial wall to injury. High-mobility group box 1 (HMGB1) is a DNA-binding protein, which on release from cells exhibits potent inflammatory actions. We examined its expression in atherosclerotic lesions and regulation by cytokines.

Methods and Results—In atherosclerotic lesions, HMGB1 protein is expressed by endothelial cells, some intimal smooth muscle cells, and macrophages. As atherosclerosis develops and progresses from fatty streaks to fibrofatty lesion, the number of HMGB1-producing macrophages increases markedly. Studies using the THP-1 cell line indicated that HMGB1 mRNA expression could be markedly upregulated by inflammatory cytokines, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and also transforming growth factor (TGF)-β. IFN-γ, TNF-α, TWEAK, and TGF-β induced an intracellular redistribution of HMGB1 and stimulated secretion by THP-1 cells and human blood monocytes. Inhibitors of MEK1/MEK2, protein kinase C, and PI-3/Akt, which inhibit lysosomal degranulation and mRNA translation, attenuated cytokine-induced HMGB1 secretion.

Conclusions—Macrophage is the major cell type responsible for HMGB1 production in human atherosclerotic lesions. Inflammatory cytokines and TGF-β increase HMGB1 expression and secretion by monocyte/macrophages. HMGB1 appears to be a common mediator of inflammation induced by inflammatory cytokines and is likely to contribute to lesion progression and chronic inflammation. (Arterioscler Thromb Vasc Biol. 2004;24:2320-2325.)

Key Words: high-mobility group box 1 ■ macrophages ■ cytokines ■ inflammation ■ atherosclerosis

High-mobility group box chromosomal protein 1 (HMGB1; previously called HMG1 or amphoterin) is a ubiquitous nuclear protein present in many eukaryotic cells. As a nuclear protein it stabilizes nucleosomes and enables bending of DNA, which facilitates gene transcription. HMGB1 possesses 2 separate and characteristic DNA-binding domains, a 3-hydroxy-3-methylglutaryl (HMG) A box and B box. Recent studies unexpectedly revealed that extracellular released HMGB1 exerts distinctly different cellular actions from its intranuclear effects. When released from cells, HMGB1 is a potent stimulator of macrophages and a proinflammatory mediator of inflammation. Its effects on macrophages include elevations in the secretion of tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-1β, IL-6, and macrophage inflammatory proteins (MIP-1α and MIP-1β). It also elicits proinflammatory responses in endothelial cells (ECs) and induces chemotaxis in vascular smooth muscle cells (SMCs). HMGB1 plays a critical role in several inflammatory diseases such as septic shock, acute lung inflammation, and rheumatoid arthritis.

The mechanisms that govern HMGB1 production, secretion, and action are only partially understood. Inflammatory stimuli, such as lysophosphatidylcholine or interferon (IFN)-γ lead to the release of preformed HMGB1. HMGB1 can also be released from necrotic cells to trigger inflammation. Its actions appear dependent on interactions with several membrane receptors, including RAGE. Ligation of HMGB1 to receptors results in the activation of multiple kinases, including ERK1/ERK2, p38MAP kinase, and JNK kinase, the rapid phosphorylation, and nuclear localization of the cAMP response element-binding protein. In ECs this leads to increased expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, RAGE, and secretion of proinflammatory cytokines, TNF-α, IL-8, and monocyte chemotactic protein-1.

The pathogenesis of atherosclerosis is characterized by a chronic inflammatory fibroproliferative response of the arterial wall to injury. Because HMGB1 is a mediator of inflammatory processes and human diseases, and because its signaling...
receptor RAGE is expressed in human atherosclerotic lesions,18,19 we studied the expression and distribution of HMGB1 immunoreactivity in different stages of human atherosclerosis. We also sought to examine the contribution of cytokines commonly present in human atherosclerotic lesions to its expression and release by macrophages and potential signaling mechanisms regulating these processes.

Materials and Methods

Aortic Tissues
Thoracic and abdominal aortas were collected during autopsy, not later than 6 hours after death, from 20 males and females, at the Russian Cardiology Research Complex, Moscow (please see http://atvb.ahajournals.org for specific details and Table I). Specimen collection was approved by the Ethics Committee of the Cardiology Research Industrial Complex, Moscow. Aortic segments were frozen in OCT (Miles Inc, Elkhart, Ind) and stored at −80°C. Atherosclerotic lesions were classified according to American Heart Association guidelines after staining with Oil Red O, hematoxylin and eosin, and/or trichrome.20,21

Antibodies
Please see online Methods, available at http://atvb.ahajournals.org, for specific details. Anti-RAGE antibodies are described elsewhere.22

Immunohistochemical Procedures
The expression of HMGB1 was examined in aortic cross-sections, as previously described.23 Please see http://atvb.ahajournals.org for specific details.

Immunofluorescence and Confocal Imaging
Immunofluorescent staining of cultured monocytes/macrophages was performed as previously described11 and HMGB1 distribution was examined using a Zeiss META Channel confocal microscope. Please see http://atvb.ahajournals.org for specific details.

Monocytes and HMGB1 Secretion
Human blood monocytes were isolated from venous blood of healthy donors using Histopaque (Sigma) as described previously.11 Monocytes were cultured in RPMI-1640 medium (Gibco BRL) containing heat-inactivated 15% fetal calf serum (JRH BioSciences). The promonocyte (THP-1) human cell line (American Cell Type Collection) was cultured in 10% fetal serum/RPMI-1640 medium and 1 μmol/L β-mercaptoethanol (Sigma). Please see http://atvb.ahajournals.org for specific details of stimulation of HMGB1 secretion by monocytes and THP-1 cells. To evaluate cytotoxicity in THP-1 cultures, lactate dehydrogenase (LDH) activity was assessed using a CytoTox96 colorimetric kit (Promega).

Western Blotting
Cells were lysed in lysis buffer (1% Nonidet P-40, 2.5 mmol/L Na2VO4, 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L EDTA, 100 mmol/L NaF, 50 mg/mL aprotinin, 50 mg/mL leupeptin, and 1 μmol/L of PMSF). Proteins for Western blotting were quantitated using the “Coomassie plus” protein assay kit (Pierce) with bovine serum albumin as standard. Volumes of culture media taken for analysis were normalized to cell counts. Western blots using 40 μg of protein or normalized volumes of culture media were performed using the ECL Western Blotting System (Amersham) as previously described.24 To ensure equal protein loading, polyvinylidene fluoride were stained by Ponceau S (Sigma) before probing with antibodies.

Reverse-Transcription Polymerase Chain Reaction
Messenger RNA encoding HMGB1 was assessed using RT-PCR25 and DNase-treated RNA isolated from THP-1 cells using RNAeasy kit (Quagen). Please see http://atvb.ahajournals.org.

SMCs Migration Assay
Migration of SMCs in response to HMGB1-containing medium was examined using Boyden chambers as described previously.24 Please see http://atvb.ahajournals.org.

Statistical Analysis
Ratios of HMGB1-expressing cells in human atherosclerotic lesions were analyzed using Kruskal-Wallis 1-way test on ranks (please see online Methods, available at http://atvb.ahajournals.org, for cell count criteria). Numbers of migrated SMCs were analyzed using Student t test. Differences with P<0.05 were considered as statistically significant. Data are expressed as mean±SEM.

Results

HMGB1 Expression in Normal Intima and Atherosclerotic Lesions
In normal intima, SMCs expressing HMGB1 were mostly localized close to the endothelium (Figure 1B). HMGB1 was also present in endothelium of normal intima, as well as in microvessels within adventitia (Figure 1B, 1H, and 1J). Most of solitary CD68-positive macrophages observed within normal intima expressed HMGB1. Approximately 50% of those cells contained HMGB1 in their cytoplasm. Medial SMC did not express HMGB1 (Figure 1C).

In fatty streaks and fibrofatty lesions, 13.6±1.9% of the SMC population expressed HMGB1, a frequency similar to that observed in normal intima (12.8±1.9%; for difference P>0.05), suggesting that only a limited number of intimal SMCs are capable of expressing this peptide in amounts detected by immunohistochemistry (Figure 1D, 1E, 1F, and 1G). Although the number of macrophages increased markedly in fatty streaks and fibrofatty lesions, the proportion that expressed HMGB1 did not alter significantly (95.3±3.1% in fatty streaks and 95.1±2.9% in fibrofatty lesions, P>0.05). However, the proportion of macrophages containing HMGB1 in both cytoplasm and nuclei increased markedly (93.8±8.8% in normal intima; P<0.01), suggesting secretion of HMGB1 in atherosclerotic lesions. Intense HMGB1 immunostaining was also observed in regions adjacent to necrotic core of lesions.

Most of the HMGB1-positive cells within fatty streaks and fibrofatty lesions also expressed RAGE (please see Figure I, available online at http://atvb.ahajournals.org).

Cytokines Elevate HMGB1 mRNA Expression
Because HMGB1 is upregulated in macrophages of atherosclerotic lesions, we examined the dependency of upregulation of its mRNA on cytokines. IFN-γ induced a time-dependent increase in HMGB1 mRNA, which was maximal after 24 hours (Figure 2A). TNF-α induced a similar time-dependent increase in mRNA. TGF-β also elevated HMGB1 mRNA levels (Figure 2B). HMGB1 mRNA did not increase in unstimulated monocytes after 24 hours in culture (control in Figure 2A) or after 48, 72, and 96 hours (not shown). Because IFN-γ, TNF-α, and TGF-β can signal via extracellular signal kinase (ERK1/ERK2),25-27 we examined the extent to which ERK1/ERK2 might contribute to the elevations in HMGB1 mRNA. Pre-exposure to PD98059 did not affect the ability of IFN-γ, TNF-α, or TGF-β to elevate HMGB1 mRNA (Figure 2B). Similarly, protein kinase C, which is also implicated in signaling by these
Thus, PI-3 kinase appears essential for PI-3 kinase has previously been implicated in cell signaling by elevated the elevations in HMGB1 by all 3 cytokines (Figure 2); mRNA elevations (not shown). In contrast, the mRNA, because bisindolylmaleimide did not attenuate the secretion also affect its cellular distribution, THP-1 monocytes (please see Figure II, available online at http://atvb.ahajournals.org).

Control unstimulated THP-1 monocytes secreted very low amounts of HMGB1 into the medium (Figure 3). Proinflammatory members of the TNF superfamily, TNF-α, TWEAK, CD40L (not shown), and IFN-γ markedly increased the secretion of HMGB1 into the medium (Figure 3A). Secretion increased in a time-dependent manner over 96 hours. TGF-β also stimulated HMGB1 secretion, which was apparent after 72 hours and continued through to at least 96 hours. In all instances, intracellular levels of HMGB1 remained relatively constant, suggesting that the increased release of HMGB1 was also accompanied by significant increases in HMGB1 protein synthesis. HMGB1-containing media concentrated from THP-1 cells markedly increased ERK1/2 phosphorylation in rat aortic SMCs and induced their migration (44.2±1.6 cells/field in HMGB1-containing medium versus 10.2±0.5 cells/field in control medium; \( P<0.05 \)) (please see Figure III, available online at http://atvb.ahajournals.org). Inhibition of SAPK/p38 MAPK and NF-κB by pre-incubating the monocytes with SB203580 and isohelenin did not affect the IFN-γ, TNF-α, and TGF-β-stimulated increases in HMGB1 secretion (Figure 3B). In contrast inhibitors of ERK1/ERK2 (PD098059), protein kinase C (bisindoylmaleimide) and PI3-kinase (wortmannin) all attenuated secretion stimulated by these cytokines (Figure 3B), suggesting that ERK1/ERK2, protein kinase C, and PI3-kinase activities are necessary for cytokine-stimulated HMGB1 secretion.

HMGB1 lacks a secretory peptide and is thought to be secreted via endolysosomes in a manner analogous to IL-1β. Because endolysosomes contain cathepsin D, we sought to determine whether IFN-γ, TNF-α, and TGF-β affect cathepsin D secretion in a manner similar to HMGB1. In contrast to HMGB1, only TNF-α and TGF-β elevated the secretion of cathepsin D into the medium (Figure 3B); inhibitors of ERK1/ERK2, protein kinase C, and PI3-kinase did not uniformly attenuate its secretion. It would appear that HMGB1 secretion occurs independently of endolysosomes containing cathepsin D. Because cell necrosis can also be responsible for HMGB1 release, we examined cytotoxicity in cultures by measuring LDH activity. LDH activity in monocytes treated with cytokines averaged 103.4±7.9% compared with control (100%) over 3 cytokines (IFN-γ, TNF-α, and TGF-β), whereas HMGB1 release was ≈5-times greater compared to control (Figure 3). Therefore, the increase in HMGB1 secretion is unlikely to be increased because of spontaneous release from necrotic cells.

**Regulation of HMGB1 Cellular Localization by Cytokines**

To determine whether the cytokines that induce HMGB1 secretion also affect its cellular distribution, THP-1 monocyte/macrophage cultures were immunostained with anti-HMGB1 antibodies and nuclei identified using propidium iodide (PI). Control monocyte/macrophages constitutively expressed HMGB1 and maintained an intracellular pool mostly localized in the nucleus (Figure 4). Exposure to TGF-β1 induced a redistribution of HMGB1 from the nucleus and into cytoplasm (Figure 4). IFN-γ also induced a similar localization of HMGB1 in monocytes/macrophages treated with cytokines.

**Regulation of HMGB1 Protein Expression and Secretion by Cytokines**

Lysophosphatidylcholine and IFN-γ increase HMGB1 secretion by monocytes. Consequently, we examined whether other cytokines that are expressed in human atherosclerotic lesions might also elevate HMGB1 secretion by monocyte/macrophages. TNF-α, IFN-γ, and TGF-β induced secretion of HMGB1 in freshly isolated blood monocytes (please see Figure II, available online at http://atvb.ahajournals.org).

Figure 1. Immunohistochemical identification of HMGB1 expression in human aorta. A to C, In normal aorta, HMGB1 (black stain) is located in ECs, some intimal SMCs (double yellow arrows), and CD68-positive monocytes (red–brown stained cells), but not in the medial SMCs. In fatty streak (D and E) and fibrofatty lesion (F and G), HMGB1 (black) is present within the cytoplasm and nuclei of almost all CD68-positive macrophages (cells stained red–brown, yellow arrows). H to J, In adventitia, HMGB1 is present in ECs of microvessels (mv) (double yellow arrows) and macrophages (yellow arrows). Green arrowhead indicates CD68-positive macrophage not expressing HMGB1. I, Region parallel to (E) incubated with nonspecific rabbit IgGs instead of the primary antibody. Original magnification in A, D, E, and H was ×80; B, C, E, G, J, and I magnification ×400.
redistribution, which was most apparent after 2 days when HMGB1 was being actively secreted into the medium. In contrast, TNF-α did not induce substantial redistribution of HMGB1 during this time despite inducing active secretion of HMGB1 (Figure 4). Thus, it would appear that although a number of cytokines induce translocation of HMGB1 from the nucleus to the cytoplasm, when substantial amounts of HMGB1 are present in the cytoplasm, this redistribution is apparently not necessary for active secretion of HMGB1. Pretreatment of cells with inhibitors of SAPK/p38 MAP kinase, NF-κB, MEK1/2, protein kinase C, or PI-3 kinase did not affect cytokine-induced redistribution of HMGB1 in the cells (not shown).

Discussion
This study demonstrates that HMGB1 is highly expressed in human atherosclerotic lesions and may contribute to the sustained inflammation of fatty rich lesions. HMGB1 expression is
highest in fibrofatty lesions and mostly associated with macrophages. Cytokines TNF-α and other members of the TNF superfamily, as well as TGF-β and IFN-γ, appear responsible for its upregulation in monocyte/macrophages and its secretion. Upregulation of its mRNA was dependent on PI-3 kinase, whereas secretion stimulated by the cytokines appears dependent on MEK1/2, protein kinase C, and PI-3 kinase.

HMGB1, a 30-kDa member of the high-mobility group nonhistone chromosomal protein family, is also a mediator of delayed endotoxin lethality and systemic inflammation. Recent studies have associated local expression of HMGB1 with local sites of tissue inflammation. For example, in rheumatoid arthritis, HMGB1 was localized to the cytoplasm of CD68-positive cells infiltrating the sublining layer, suggesting that it is secreted by synovial macrophages. In normal aortic intima, arthritis, HMGB1 was localized to the cytoplasm of CD68-positive cells, suggesting that it is secreted by synovial macrophages. In normal aortic intima, arthritis, HMGB1 was localized to the cytoplasm of CD68-positive cells, suggesting that it is secreted by synovial macrophages.

HMGB1 can profoundly affect the function of cells associated with atherosclerotic lesions, particularly macrophages, ECs, and vascular SMCs, promoting local inflammation, the accumulation of monocyte/macrophages, and even remodeling of the lesion. HMGB1 induces human monocytes to release a large array of inflammatory cytokines, including TNF-α, IL-1α, IL-1β, IL-1RA, IL-6, IL-8, and MIP-1α and MIP-1β. Its actions on ECs include increasing the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and RAGE, as well as increasing the secretion of TNF-α, IL-8, monocyte chemotactic protein-1, and tissue plasminogen activator. HMGB1 also induces cytoskeleton reorganization and chemotaxis in vascular SMCs.

Our findings that the proinflammatory cytokines IFN-γ and TNF-α, as well as TGF-β, elevate HMGB1 mRNA in monocyte/macrophages suggest that HMGB1 exerts its inflammatory action downstream of proinflammatory cytokines. The finding that TNF-α induces HMGB1 secretion is particularly important because the secreted HMGB1 may in turn induce further secretion of TNF-α, raising the possibility that a proinflammatory loop exists between TNF-α and HMGB1, which increases the severity of inflammation and prolongs its duration. Whether such a proinflammatory loop also exists for other inflammatory cytokines of the TNF superfamily and HMGB1 remains to be determined. Our findings also suggest that PI-3 kinase is essential for the upregulation of HMGB1 mRNA by these cytokines. PI-3 kinase is known to regulate the activity of S1p-responsive promoters, which are abundant in the 5′-region of the human HMGB1 gene. TGF-β, TNF-α, and IFN-γ are all known to potentiate translation of Akt. HMGB1 is released from necrotic cells, inducing local inflammation. In macrophages, release of HMGB1 can also be induced by lysophosphatidylcholine and IFN-γ. IFN-γ-dependent release of HMGB1 could be inhibited by a specific inhibitor of Janus kinase 2. In both instances, release appeared to be associated with its translocation from the nucleus to the cytoplasm. We have demonstrated that a number of proinflammatory cytokines of the TNF superfamily, TNF-α, TWEAK, and CD40L (not shown), as well as TGF-β, and IFN-γ, are also capable of stimulating HMGB1 secretion from human monocyte/macrophages. These cytokines stimulated secretion in a somewhat delayed manner, being most apparent after 48 hours. Secretion was accompanied by increased HMGB1 protein synthesis, because intracellular HMGB1 levels were unaltered during this time. Our findings indicate that HMGB1 secretion stimulated by cytokines is in part dependent on transient elevations in its mRNA and elevations in its biosynthesis. Secretion can be also accompanied by its translocation from the nucleus to the cytoplasm. This was most apparent with TGF-β, IFN-γ, and CD40L. TNF-α was least effective in inducing such translocation, although it is a potent stimulant of HMGB1 secretion. Because HMGB1 lacks a hydrophobic signal sequence, it appears to be secreted via a yet to be fully defined nonclassical, vesicle-mediated secretory pathway analogous to that used by IL-1β. However, in contrast to IL-1β-containing secretory lysosomes, those responsible for HMGB1 secretion do not apparently contain cathepsin D. We found no correlation between HMGB1 and cathepsin D secretion under a variety of different conditions. Cytokine-induced HMGB1 secretion could be attenuated by inhibitors of MEK1/MEK2, protein kinase C, and PI-3 kinase/Akt, suggesting that such agents might be useful in attenuating HMGB1-mediated inflammation. Although the mechanisms by which these agents attenuate HMGB1 secretion needs to be clarified, it is possible that they inhibit lysosomal degranulation and/or HMGB1 protein synthesis via effects on mRNA translation. Protein kinase C, MEK1/MEK2, and PI-3 kinase appear essential for lysosomal degranulation and also regulate mRNA translational processes.

In summary, this is the first study to our knowledge to identify that macrophages are a major source of HMGB1 in human atherosclerotic lesions. Its expression and secretion by macrophages is highly regulated by cytokines. Upregulation and secretion of HMGB1 has the potential to amplify inflammatory responses and may also contribute to macrophage accumulation, thereby promoting atherogenesis. Definition of its precise roles in the development and progression of atherosclerosis will require additional in vivo investigations.

References


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Supplementary Data - MS ID#: ATVB/2004/072876 – R1: Kalinina et al. HMGB1 in Human Atherosclerosis

**Methods**

**Antibodies**

The antibody to HMGB1 (BD Pharmingen, #556528) and to total Erk1/2 from Santa Cruz, # sc-93 ) were rabbit polyclonal. Antibodies to Cathepsin D (Oncogene, #IM-03), phospho-Erk1/2 (Cell Signalling, # 9106S) and CD-68 (DAKO Corp.), were mouse monoclonal. A rabbit anti-RAGE antibody was a kind gift of Dr. J. Forbes\(^1\). Non-specific rabbit IgGs were from Zymed. Secondary biotinylated horse anti-mouse, rabbit-anti-goat and goat-anti-rabbit antibodies, streptavidin-peroxidase, conjugates and the visualization kit for red streptavidin peroxidase were from Vector Laboratories. A secondary goat anti-rabbit antibody-Alexa488 conjugate was from Molecular Probes. 3,3’-Diaminobenzidine tetrachloride (DAB) was from Sigma Chemical Company.

**Immunohistochemistry**

The expression of HMGB1 was examined in aortic cross-sections, essentially as previously described.\(^2\) Briefly, 7\(\mu m\) frozen sections were fixed in cold (-20\(^\circ\)C) acetone for 20 min. The sections were then sequentially incubated in 3% H\(_2\)O\(_2\) in PBS, 10% normal goat serum (NGS)/PBS and biotin/avidin blocking reagents (Vector Laboratories). Thereafter, the sections were incubated (1 hr) with the anti-HMGB1 antibody in NGS/PBS (0.125\(\mu g/ml\)). Incubation (40 min) with the secondary antibody (0.3\(\mu g/ml\) in NGS/PBS) was followed by incubation with streptavidin horseradish peroxidase complex. Antigens were visualized using DAB solution containing 0.5% NiCl\(_2\). To simultaneously detect macrophages or expression of RAGE, unreacted DAB was removed by washing and incubating the sections with 10% normal horse serum (NHS)/PBS and the biotin/avidin blocking kit was performed, before incubation with anti-CD-68 (0.150\(\mu g/ml\) NHS/PBS) or anti-RAGE (1:500) antibodies for 1 hour. Then the appropriate biotinylated secondary antibody and the streptavidin alkaline phosphatase complex were used to visualize
immunoreactive peptides as a red colour, using the ABC red visualization kit. For negative controls the primary antibodies were replaced with rabbit non-specific IgGs (0.125µg/ml in NGS/PBS). Nuclei were counterstained with haematoxylin.

**Immunofluorescence and Confocal Imaging**

Immunofluorescent staining of cultured monocytes/macrophages was performed with cells either attached to glass slides (8-well cell chambers, LabTek) or in suspension. Suspended cells were examined as smears. The cells were fixed in 4% paraformaldehyde followed by permeabilization with Triton-X100, and blocking was performed in 10% NGS followed by incubation with the anti-HMGB1 antibody (0.125µg/ml in NGS/PBS), overnight at +4°C. Then cells were incubated with Alexa488-conjugated anti-rabbit antibodies (10µg/ml in PBS), nuclei were counterstained by propidium iodide (PI) (Sigma) and cells were mounted in Vectashield mounting media (Vector Inc). HMGB1 distribution was then examined using a Zeiss META Channel confocal microscope and the multitrack method for simultaneous detection of Alexa488 and PI with less then 3% overlapping in detection channels. For negative controls the primary antibodies were replaced with rabbit non-specific IgGs (0.125µg/ml in NGS/PBS).

**RT-PCR**

Messenger RNA encoding HMGB1 was assessed using RT-PCR and DNAase treated RNA isolated from THP-1 cells using RNAeasy kit (Qiagen). Amplification was optimized so that the amount of PCR product reflected mRNA levels. RT-PCR was performed using a one-step procedure (SuperScript RT-PCR System, Life Technologies), in a mixture containing reaction buffer, RT/Taq polymerase, RNA (100ng) and oligonucleotide primer pairs (10µM). Reverse transcription was at 50°C for 30 minutes, initial denaturation at 94°C for 2 minutes, followed by 21 cycles of PCR: 94°C for 30 seconds, 60°C for 30 seconds,
Supplementary Data - MS ID#: ATVB/2004/072876 – R1: Kalinina et al. HMGB1 in Human Atherosclerosis

70°C for 1 minute and after the final cycle the mixture was maintained at 70°C for 8 minutes. PCR products were electrophoresed on 2% agarose gels at 120 mV (BioRad), together with Hae III-digested φX174 DNA size markers (Promega).

Oligonucleotide primer pairs for RT-PCR were designed using previously defined criteria, and purchased from Geneworks (Adelaide, Australia). HMGB1, sense: 5’-gcatttttttgcaactgtcgggagg-3’, antisense: 5’-atcaggctttcttagctatgcagc-3’ (product length 459bp); GAPDH, sense: 5’-aaggtgaaggtcggagtcaacg-3’, antisense: 5’-cttgaggctgttgcatctctcag-3’ (product length 429bp).

Monocytes and HMGB1 Secretion

To induce HMGB1 secretion by monocytes RPMI-1640 containing heat-inactivated 15% FCS was replaced with macrophage serum-free media (Gibco) and 50ng/ml of TNF-α (R&D), 300 U/ml IFN-γ (Roche) or 10 ng/ml TGF-β (R&D) were added to cells for following 24 hrs.

To stimulate HMGB1 expression in THP-1 monocytes cells were plated at 2-3x10⁶ cells/ml in RPMI-1640 supplemented with 0.5% FCS, non-essential amino acid mix & insulin/transferring/selenium supplement (both from Gibco) to maintain cell surviving. Cells were treated with 50ng/ml of TNF-α (R&D), 300 U/ml IFN-γ (Roche), 10 ng/ml TGF-β (R&D), 1µg CD40L (R&D) or 500ng/ml TWEAK (Peprotech) for 24, 48, 72 and 96 hrs. To investigate the involvement of various signaling pathways in regulating HMGB1 expression, cells were pre-treated for 1 to 2 hrs with various protein kinase inhibitors (please see Table 2 for details) and then treated by cytokines for 48 hrs. Cell numbers were determined prior to harvesting using Z2 Coulter Counter (Beckman Coulter).
SMCs Migration Assay

Rat aortic SMCs migration assay was performed using Boyden chambers. Briefly, cell culture inserts with 8µm pores were coated on both sides by 100 µg/ml of human fibronectin (Roche). Macrophage-SFM media (Gibco) was collected from THP-1 cells cultured for 4 days and concentrated (x 25 times) using Amicon Microcon YM-10 filters. Cultured aortic smooth muscle cells (2 x 10⁵ cells) in 250µl of serum free LoPro medium were placed in the upper chamber and a 1 in 2.5 dilution of HMGB1 concentrate, recombinant PDGF (10ng/ml), 10% serum or serum free-control media placed in the lower chamber. Migration was allowed to proceed at 37°C for 24 hours. Then cells were washed (x 2) with PBS and cells from the top of the membrane were mechanically removed. Cells were fixed by methanol : glacial acetic acid (3:1) for 5 min and stained with 0.1% crystal violet solution (Merk) in the presence of 2% ethanol for 2 hrs. After several washes by PBS cells were counted using x25 objective in 5 arbitrary fields per chamber.

Cell count criteria

Cell counts were performed on 7µm cross-sections within the entire intima area (from lumen to internal elastic lamina) by two independent observers using following criteria. Cells possessing brown to black staining only were assessed as HMGB1 - positive smooth muscle cells, nuclei without apparent surrounding black staining were counted as HMGB1- negative smooth muscle cells, cells showing double – red and black staining were counted as HMGB1- positive macrophages and very few red-stained cells without apparent black staining in them were counted as HMGB1- negative macrophages. 567 cells on average (350 minimum and 1100 maximum) were counted from each section.
Figure I.
Figure II.
Figure III.

A

- 44kD
- 42kD

control
HMGB1

44kD
42kD

(P)-Erk1/2

Total Erk1/2

B

Cell count

0 20 40 60 80 100 120

control  PDGF  FCS  HMGB1
Table 1. Characteristics of Subjects and Thoracic Aortas

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<td>F</td>
<td>45</td>
<td>Acute Haemorrhage</td>
<td>30% FS, FFL</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>50</td>
<td>Myocardial Infarction</td>
<td>50% FS, FFL</td>
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<td>11</td>
<td>M</td>
<td>50</td>
<td>Skull-brain Trauma</td>
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<tr>
<td>12</td>
<td>M</td>
<td>52</td>
<td>Acute Haemorrhage</td>
<td>60% FS, FFL, FP, CL</td>
</tr>
<tr>
<td>13</td>
<td>M</td>
<td>53</td>
<td>Myocardial Infarction</td>
<td>50% FS, FFL</td>
</tr>
<tr>
<td>14</td>
<td>M</td>
<td>53</td>
<td>Heart Failure</td>
<td>50% FS, FFL</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>58</td>
<td>Traffic Accident</td>
<td>40% FS, FFL</td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>59</td>
<td>Heart Failure</td>
<td>99% FS, FFL, FP, CL</td>
</tr>
<tr>
<td>17</td>
<td>M</td>
<td>60</td>
<td>Traffic Accident</td>
<td>80% FS, FFL, FP, CL</td>
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<tr>
<td>18</td>
<td>M</td>
<td>61</td>
<td>Sudden Death</td>
<td>99% FS, FFL, FP, CL</td>
</tr>
<tr>
<td>19</td>
<td>M</td>
<td>67</td>
<td>Myocardial Infarction</td>
<td>80% FS, FFL, FP, CL</td>
</tr>
<tr>
<td>20</td>
<td>F</td>
<td>80</td>
<td>Sudden Death</td>
<td>99% FS, FFL, FP, CL</td>
</tr>
</tbody>
</table>

* FS = fatty streak, FFL = fibrofatty lesion, FP = fibrous plaque, CL = complex lesions
Table 2

Protein kinase inhibitors used in the study

<table>
<thead>
<tr>
<th>Name and manufacturer</th>
<th>Target</th>
<th>Conc*</th>
<th>Positive control</th>
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</thead>
<tbody>
<tr>
<td>Bisindoylmalieimide Sigma</td>
<td>PKC</td>
<td>1µM</td>
<td>Inhibited HMGB1 secretion</td>
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<tr>
<td>Isohelenin Calbiochem</td>
<td>NF-κB</td>
<td>20µM</td>
<td>30 min pre-treatment blocked elevation of MMP-1 mRNA expression induced by CD40L</td>
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<tr>
<td>PD 098059 Calbiochem</td>
<td>MEK1/2</td>
<td>50 µM</td>
<td>Inhibited HMGB1 secretion</td>
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<tr>
<td>SB 203580 Calbiochem</td>
<td>p38/SAPK</td>
<td>10 µM</td>
<td>30 min pre-treatment blocked phosphorylation of p38/SAPK induced by 0.5M sorbitol</td>
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<tr>
<td>Wortmannin Sigma</td>
<td>PI-3K/Akt</td>
<td>10nM</td>
<td>Inhibited HMGB1 secretion and mRNA expression</td>
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</tbody>
</table>

* Final concentration used in experiments
References


Figure I. Immunohistochemical identification of HMGB1 and RAGE in human fibrofatty lesion. HMGB1 protein (black stain) is co-localized with a large degree with RAGE (red stain) in cells comprising fibrofatty intima. Control staining non-immune rabbit IgG is shown in Figure 1 (I). Nuclei are counterstained by hematoxylin. Original magnification x 16.

Figure II. Analysis of HMGB1 secretion by human blood monocytes using Western Blotting. Incubation of monocytes with 50ng/ml of TNF-α (R&D), 300 U/ml IFN-γ (Roche) or 10 ng/ml TGF-β (R&D) for 24 hrs induced elevation in HMGB1 levels in culture media.

Figure III. Effects of HMGB1 containing media from THP-1 cells on Erk1/2 phosphorylation and rat aortic SMC migration. Serum free media was collected from THP-1 cells cultured for 4 days and secreted HMGB1 concentrated (x25) using Amicon Microcon YM-10 filters (10kD cut off filters). Cultured rat aortic SMCs were then exposed to a 1 in 2.5 dilution of this medium diluted in LoPro medium and effects on Erk1/2 phosphorylation and cell migration determined. (A). Twenty minute exposure of SMC to secreted HMGB1 induces phosphorylation of Erk1/2. (B). Twenty four hour treatment of SMCs with secreted HMGB1 induced their migration in Boyden chambers, as did recombinant PDGF-BB (10ng/ml) and 10% fetal calf serum (FCS). Average of two independent experiments performed in duplicate is shown.