Original article

High-mobility group box 1 protein blockade suppresses development of abdominal aortic aneurysm

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Received 28 July 2011; received in revised form 9 December 2011; accepted 9 January 2012
Available online 24 February 2012

KEYWORDS
Aorta;
Aneurysms;
Cytokines

Summary
Background: Abdominal aortic aneurysm (AAA) expansion is characterized by chronic inflammatory cell infiltration and extracellular matrix degradation. High-mobility group box 1 protein (HMGB1) is one of the damage-associated molecular pattern molecules derived from injured/necrotic and activated inflammatory cells. We investigated the expression of HMGB1 in human AAA and mouse experimental AAA. Then, we evaluated the effect of HMGB1 blockade on AAA formation in the mouse model.

Methods and results: Human AAA samples showed increased HMGB1 expression compared with normal aortic wall. In a mouse CaCl\textsubscript{2}-induced AAA model, the expression of HMGB1 was increased compared with that in sham, and was positively correlated with matrix metalloproteinase
Introduction

Chronic inflammation of the aortic wall has been implicated in the development, expansion, and rupture of abdominal aortic aneurysm (AAA) [1—6]. Inflammatory cells in the media and adventitia are significant sources of cytokines that stimulate proteolytic enzymes such as matrix metalloproteinases (MMPs) [3,7,8]. MMPs mediate fragmentation of extracellular matrix (ECM) elastin fibers with a reduction of their concentration in ECM, which is the pathologic hallmark of AAA [7—9]. However, the molecular mechanism that maintains the inflammatory response and MMP activation in aneurysmal tissue is still not fully understood.

High-mobility group box 1 protein (HMGB1) was originally identified as a nonhistone DNA-binding nuclear protein produced by nearly all cell types [10]. It stabilizes nucleosomes and enables binding of DNA, which facilitates gene transcription. Recently, it has been clarified that HMGB1 is released passively from injured/necrotic cells, and is actively secreted by inflammatory cells in response to proinflammatory cytokines [10—12]. As extracellular HMGB1 itself can elicit proinflammatory responses by increasing the expression of proinflammatory cytokines [10,13], HMGB1 participates in perpetuation of inflammation and has been reported to play an essential role in the pathogenesis of inflammatory diseases such as sepsis, rheumatoid arthritis, and atherosclerosis [10,12,14—18]. Various stimuli have been linked to the inflammation observed in AAA, including oxidative stress and proinflammatory cytokines [2,3,19]. These stimuli also activate HMGB1 expression in macrophages, monocytes, and smooth muscle cells (SMCs) [10,12,16,17,20]. Increased HMGB1 expression is also reported to be linked to MMP activation. A previous study demonstrated that blockade of HMGB1 suppressed inflammatory cell infiltration and MMP-9 induction during ischemia/reperfusion injury [21]. Additionally, HMGB1 directly stimulated the production of MMPs in vitro [17,22,23]. The importance of HMGB1 in inflammation and MMP activation led us to hypothesize that HMGB1 might play a key role in the progression of AAA through the persistence of inflammation and ECM degradation.

To test this hypothesis, we first examined the expression level and distribution of HMGB1 in aortic tissue in both human and experimental AAA. We then evaluated whether blockade of HMGB1 could attenuate aneurysm formation in a mouse AAA model.

Materials and methods

Clinical study protocol

For determination of serum HMGB1 level, serum was collected from 40 patients with atherosclerosis-associated AAA who were admitted to Keio University Hospital. A diagnosis of AAA was made on the basis of abdominal computed tomography showing dilatation of the abdominal aorta, with a diameter ≥ 5 cm. The study population consisted of patients who had survived a ruptured aorta (n = 6) and patients in whom the diagnosis had been made through computed tomography performed for other reasons (n = 34). There was no patient with recent infection, active inflammatory disorder, malignancy, collagen disease, or chronic renal failure. The shoulders (between the edge and the maximal side) of the AAA were obtained from patients during elective surgical repair of AAA (n = 5). Control aortic samples were obtained from autopsy specimens of patients who had died of unrelated causes (n = 5). This clinical investigation was approved by the institutional medical ethical committee and conducted according to the ethical guidelines outlined in the Declaration of Helsinki.

Mouse model of experimental AAA

We induced AAA in 8-week old mice (C57BL/6J) by periaortic application of 0.5 M calcium chloride (CaCl2) as described previously, with minor modifications [24,25]. The sham group received saline instead of CaCl2. To determine HMGB1 expression, mice were killed 6 weeks after operation for histological (n = 6) and protein analyses (n = 6).

In the HMGB1 blockade study, AAA mice were randomly assigned to two groups: (1) neutralizing polyclonal chicken IgY anti-HMGB1 antibody (10 mg/kg/day, donated by Shino-Test Corporation, Sagamihara, Japan) administered subcutaneously 24 h after operation and subsequently every 3 days for 6 weeks (AAA/anti-H), and (2) control chicken IgY antibody (AAA/C). To prepare neutralizing anti-HMGB1 antibody, IgY class antibody from the egg yolk of HMGB1-immunized hens was isolated and purified [14,18]. The dosage of neutralizing anti-HMGB1 antibody was determined according to a previous study showing improved survival in a rodent sepsis model [14]. Mice were killed 6 weeks after operation for histological (n = 6), mRNA (n = 6), and protein
analyses (n = 6). All procedures were performed in accordance with the Keio University animal care guidelines.

**Histological study**

For histological analyses, mice were killed by an overdose of pentobarbital and perfusion-fixed with a mixture of 10% formaldehyde in phosphate buffered saline at physiological perfusion pressure. We excised the abdominal aortas, photographed them to determine the external diameter and used them for histological analyses. Paraﬁn-embedded cross-sections (6 μm thick) were stained with haematoxylin—eosin (HE) and elastica van Gieson (EVG). Immunohistochemical studies were performed using the antibodies against HMGB1 (Shino-Test Corporation) and Mac-3 (BD Pharmingen, San Jose, CA, USA) as described previously [18,25].

**Western blotting**

Frozen tissue was homogenized in cell lysis buffer (Cell Signaling, Danvers, MA, USA) containing 1% Triton X-100 and protease inhibitors. Western blot analysis with rabbit polyclonal HMGB1 antibody (Shino-Test Corporation) was carried out as previously described [18].

**Real-time quantitative reverse transcriptase polymerase chain reaction**

Total RNA was isolated by acid—phenol extraction in the presence of chaotropic salts (TRizol, Invitrogen, Carlsbad, CA, USA) and subsequent isopropanol—ethanol precipitation as described previously [18,25].

Real-time quantitative reverse transcriptase polymerase chain reaction was carried out as described previously [18,25]. Gene expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. Monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, CD68, and GAPDH assays were purchased as pre-optimized kits ("TaqMan" probe; Applied Biosystems, Carlsbad, CA, USA: Catalogue number, Mm99999056_m1 [MCP-1], Mm00443258_m1 [TNF-α], Mm00838636_g1 [CD68]).

**Zymography**

Equal amounts of 10 μg of protein were electrophoresed on 10% Zymogram (gelatin) Gel (Invitrogen). After electrophoresis, the gels were renatured in renaturing buffer; 50 mM Tris—HCl containing 100 mM NaCl and 2.5% Triton X-100. They were then incubated with developing buffer; 50 mM Tris—HCl containing 10 mM CaCl2. The gels were stained with Coomassie Brilliant Blue (Invitrogen), and destained in water.

**Statistical analyses**

Categorical variables were compared between groups using chi-squared test (with Yates' continuity correction). All continuous data are expressed as mean value ± SD unless otherwise stated. Statistical significance of differences between multiple groups was determined using ANOVA and post hoc analysis with Bonferroni test. Statistical significance was defined as a p value of < 0.05. All statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

**Results**

**HMGB1 expression in human AAA**

HE staining showed extensive atherosclerotic plaque and marked inﬁltration of inﬂammatory cells in human AAA samples compared with control samples (Fig. 1A, D, and G). EVG staining showed that AAA walls had medial disruption or attenuation and degradation of the wavy structure of elastin (Fig. 1B, E, and H). Immunohistochemical staining for HMGB1 revealed that HMGB1 expression was enhanced in all layers of the AAA aortic wall, especially in the media and adventitia (Fig. 1C, F, and I). HMGB1 was highly expressed in inﬂammatory cells within the adventitia (Fig. 1J), media, and atherosclerotic plaque (Fig. 1K). HMGB1 was also expressed in SMCs and endothelial cells in AAA tissue (Fig. 1L and M).

Serum HMGB1 level was signiﬁcantly higher in patients with ruptured AAA than in those with nonruptured AAA (4.9 ± 3.4 ng/ml vs. 1.9 ± 2.9 ng/ml, p < 0.01). There were no signiﬁcant differences in age (65 ± 13 years vs. 64 ± 13 years, p = 0.63), sex (male/female, 6/0 vs. 31/3, p = 0.45), smoking (50% vs. 38%, p = 0.35), hypertension (83% vs. 65%, p = 0.11), dyslipidemia (33% vs. 41%, p = 0.96), and diabetes mellitus (17% vs. 21%, p = 0.98) between patients with ruptured AAA and nonruptured AAA. The maximum AAA diameter was greater in ruptured AAA than in nonruptured AAA (6.9 ± 1.0 mm vs. 5.8 ± 1.2 mm, p = 0.03).

**HMGB1 expression and MMP activity in experimental AAA**

In an experimental model of AAA, HMGB1 expression was increased in aneurysmal aorta relative to non-aneurysmal sham (Fig. 2A). MMP-2 and MMP-9 activity also increased in aneurysmal tissue (Fig. 2B) and showed a significantly positive correlation with HMGB1 level (Fig. 2C). In AAA mice, immunohistochemical staining of HMGB1 was enhanced in inﬁltrating inflammatory cells, SMCs, and endothelial cells (Fig. 2D–G).

**Effect of HMGB1 blockade in experimental AAA**

Morphometric analyses of the aorta showed that the maximum aortic diameter was signiﬁcantly smaller in AAA/anti-H compared with AAA/C (Fig. 3A–D). HE staining showed that inﬁltration of inﬂammatory cells in the aortic wall was more marked in AAA/C than in sham-operated mice. However, it was attenuated by HMGB1 blockade (Fig. 4A–C). EVG staining showed that the aorta of AAA/C had thinning of the medial layer and disruption of the elastic lamellae, which showed a ﬂattened appearance instead of the wavy appearance observed in sham. Neutralizing anti-HMGB1 antibody treatment partially restored the wavy structure of the
elastin lamellae (Fig. 4D–F). The increased number of Mac-1-positive macrophages in aneurysmal tissue was attenuated by HMGB1 blockade (Fig. 4G–J). Neutralizing anti-HMGB1 antibody treatment diminished AAA-induced mRNA expression of TNF-α and CD68 in aneurysmal tissue. Although the difference was not statistically significant, mRNA expression of MCP-1 in AAA/anti-H was less than half that in AAA/C (Fig. 5A–C). Zymographic activity of MMP-2 and MMP-9 was elevated in AAA/C compared to sham. Mice in AAA/anti-H exhibited significantly lower MMP-2 and MMP-9 activity compared with that in AAA/C (Fig. 5D and E).

Discussion

In this study, we demonstrated that HMGB1 expression was increased in human aneurysmal tissue. In an experimental study using a mouse CaCl₂-induced-AAA model, we also found increased aneurysmal HMGB1 expression, which was positively correlated with MMP-2 and MMP-9 activity. Blockade of HMGB1 by neutralizing anti-HMGB1 antibody attenuated the development of AAA, in association with reduced infiltration of macrophages and MMP activity. These findings suggested that HMGB1 plays a significant role in the pathogenesis of AAA by affecting vascular inflammation and ECM degradation.

HMGB1 was recently shown to act as a danger signal, mediating crosstalk among injured cells, activated inflammatory cells, and relatively healthy cells around damaged tissue, which leads to the initiation and perpetuation of inflammation [10–13, 23]. Previous studies showed that HMGB1 contributes to inflammation in atherosclerosis [15–17]. Kalinina et al. demonstrated that the number of HMGB1-producing macrophages increased during the development and progression of atherosclerotic lesions [15]. Although chronic inflammation contributes to the pathogenesis of both atherosclerosis and AAA, it has become clear that aneurysms, once thought to be a form of atherosclerosis, have a distinct pathogenesis from that of stenotic vascular disease. Atherosclerotic plaques are primarily characterized by intimal infiltration of inflammatory cells. In contrast, aneurysmal segments are characterized by inflammatory cell accumulation primarily in the media and adventitia at all stages of disease progression [3, 4]. We showed that HMGB1 expression was increased in macrophages within atherosclerotic plaque. However, regardless of atherosclerosis severity, intense HMGB1 staining in inflammatory cells and SMCs within the media and adventitia was observed in aneurysmal tissue. Therefore, it
Figure 2  Expression of high-mobility group box 1 protein (HMGB1) (A) and activity of matrix metalloproteinase (MMP)-2 and MMP-9 (B) in aneurysmal tissue in mouse abdominal aortic aneurysm (AAA) model. Correlation between HMGB1 expression and MMP activity (C). Open circles, sham; closed circles, AAA. Representative low-power micrographs showing HMGB1 immunohistochemical staining in mouse AAA model (D). HMGB1 staining showed that HMGB1 protein was seen in infiltrating inflammatory cells (E), smooth muscle cells (F), and endothelial cells (G) in mouse AAA tissue (high-power views of the portion indicated by square in [D]). Scale bar indicates 100 μm (D) and 20 μm (E–G). Data are mean ± SEM. *p < 0.05 vs. Sham.

Figure 3  Effects of high-mobility group box 1 protein (HMGB1) blockade on development of abdominal aortic aneurysm (AAA). Representative macroscopic appearance of the aorta in sham-operated mouse (Sham: A) and AAA mouse treated with control (AAA/C: B) or neutralizing anti-HMGB1 antibodies (AAA/anti-H: C). Graph shows the diameter of the abdominal aorta 6 weeks after operation (D). Data are mean ± SEM. *p < 0.05 vs. Sham. †p < 0.05 vs. AAA/C.
is likely that increased HMGB1 expression in the media and adventitia might be related to the provocation of inflammatory response that is required for the development of AAA.

Although nonsurgical treatment for AAA is much awaited, few options are available because its molecular pathogenesis remains elusive [2,24]. We showed that HMGB1 blockade resulted in attenuation of AAA progression. Interestingly, the

Figure 4  Hematoxylin–eosin (HE), elastic van Gieson (EVG), and Mac-3 staining of aortic wall of sham-operated mice (Sham: A, D, and G) and abdominal aortic aneurysm (AAA) mice treated with control (AAA/C: B, E, and H) or neutralizing anti-high-mobility group box 1 protein antibodies (AAA/anti-H: C, F, and I). Graph shows Mac-3-positive cells in a field (J). Scale bar indicates 50 μm. Data are mean ± SEM. *p < 0.05 vs. Sham. †p < 0.05 vs. AAA/C.

Figure 5  Effect of high-mobility group box 1 protein (HMGB1) blockade on tumor necrosis factor (TNF)-α (A), monocyte chemoattractant protein (MCP)-1 (B), and CD68 gene expression (C), and matrix metalloproteinase (MMP)-2 (D) and MMP-9 activity (E) in aortic wall of sham-operated mice and abdominal aortic aneurysm (AAA) mice treated with control (AAA/C) or neutralizing anti-HMGB1 antibodies (AAA/anti-H). Data are mean ± SEM. *p < 0.05 vs. Sham. †p < 0.05 vs. AAA/C.
expression of an HMGB1 receptor, receptor for advanced glycation end products (RAGE), was also upregulated in AAA tissue, and RAGE gene knockout reduced the incidence of AAA through inhibition of MMP-9 activity [26]. Additionally, HMGB1-induced signaling through RAGE led to activation of the nuclear factor (NF)-κB pathway, and inhibition of NF-κB also attenuated aneurysmal dilatation [10,27]. Taking these findings together, HMGB1 could be an upstream target for therapeutic intervention in AAA.

Degradation of ECM by MMPs has been reported to be an important contributor to the pathogenesis of AAA [1–3,7–9]. Macrophage-derived MMP-9 and interstitial cell-derived MMP-2 work in concert toward the development of AAA [8]. We showed that HMGB1 was localized in inflammatory cells and SMCs in aneurysmal tissue, similar to the localization of MMPs. We also showed that HMGB1 expression was positively correlated with MMPs activity, and HMGB1 blockade attenuated increased activity of MMPs in an experimental AAA model, suggesting that HMGB1 may affect AAA formation, at least in part, by activation of MMPs. Although this study does not clarify the precise mechanism of MMPs activation by HMGB1, there might be two possible mechanisms. The first is that HMGB1 could enhance MMP activity in aneurysmal tissue through increased macrophage recruitment. The second is that HMGB1 can directly upregulate MMP expression. Recent studies showed that addition of HMGB1 causes MMP-2 and MMP-9 upregulation in vitro [17,22,23]. Advanced glycation end products and S100A12, another RAGE ligand, also directly induce the production of MMP-9 in macrophages and that of MMP-2 in SMCs, respectively [26,28]. HMGB1 might activate MMPs in aneurysmal tissue through several mechanisms.

Elevated levels of various plasma markers of inflammation have been reported in patients presenting with ruptured AAA as compared with nonruptured AAA [5]. We demonstrated that the serum HMGB1 level was elevated in patients with ruptured AAA. The design of our studies cannot exclude the possibility that the increase in HMGB1 level is a consequence, rather than a predictor, of rupture. However, their use in conjunction with clinical evaluation may prove a valuable indicator of impending or established aneurysm rupture.

The present study has some limitations. First, the number of clinical study participants was limited; therefore, the statistical power might not be adequate to detect any negative data. Second, we do not have any data of serum HMGB1 level in healthy controls. A large number of circulating markers of inflammation have been assessed for association with cardiovascular diseases including AAA [2,29–31]. A recent clinical study also showed that the aneurysm itself is a source of elevated proinflammatory cytokines in serum [5,6]. To investigate whether serum HMGB1 could be elevated in patients with AAA compared to control subjects might be worthwhile, and further studies are needed to clarify the possibility of serum HMGB1 level as a diagnostic marker of AAA development and progression.

In conclusion, the present study demonstrated elevated HMGB1 expression in human and mouse experimental AAA. HMGB1 blockade attenuated AAA progression through a decrease in macrophage infiltration and MMP activity, suggesting that HMGB1 acts as a critical mediator of inflammation and connective tissue remodeling in AAA. The identification of involvement of HMGB1 in the pathogenesis of AAA might provide a new target for therapeutic intervention.

Conflict of interest

None declared.

Acknowledgments

This study was supported in part by a grant from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (20790548 to T.K.), a Medical School Faculty and Alumni Grant from the Keio University Medical Science Fund (to T.A.), and a grant from the Ministry of Health, Labor and Welfare (Research Group of Intractable Vasculitis) Japan (to Y.O.).

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