Proteolytic Cleavage of High Mobility Group Box 1 Protein by Thrombin–Thrombomodulin Complexes

Takashi Ito, Ko-ichi Kawahara, Kohji Okamoto, Shingo Yamada, Minetsugu Yasuda, Hitoshi Imaizumi, Yuko Nawa, Xiaojie Meng, Binita Shrestha, Teruto Hashiguchi, Ikuro Maruyama

Objective—High mobility group box 1 protein (HMGB1) was identified as a mediator of endotoxin lethality. We previously reported that thrombomodulin (TM), an endothelial thrombin-binding protein, bound to HMGB1, thereby protecting mice from lethal endotoxemia. However, the fate of HMGB1 bound to TM remains to be elucidated.

Methods and Results—TM enhanced thrombin-mediated cleavage of HMGB1. N-terminal amino acid sequence analysis of the HMGB1 degradation product demonstrated that thrombin cleaved HMGB1 at the Arg10-Gly11 bond. Concomitant with the cleavage of the N-terminal domain of HMGB1, proinflammatory activity of HMGB1 was significantly decreased \((P<0.01)\). HMGB1 degradation products were detected in the serum of endotoxemic mice and in the plasma of septic patients with disseminated intravascular coagulation (DIC), indicating that HMGB1 could be degraded under conditions in which proteases were activated in the systemic circulation.

Conclusions—TM not only binds to HMGB1 but also aids the proteolytic cleavage of HMGB1 by thrombin. These findings highlight the novel antiinflammatory role of TM, in which thrombin–TM complexes degrade HMGB1 to a less proinflammatory form. (Arterioscler Thromb Vasc Biol. 2008;29:1825-1830)

Key Words: high mobility group box 1 ■ sepsis ■ disseminated intravascular coagulation ■ thrombin ■ thrombomodulin

Sepsis is a life-threatening disorder that results from systemic inflammatory and coagulatory responses to infection.1 Hyperactivation of the inflammatory system is the most important feature of sepsis and has been the most common target of therapeutic strategies. So far, diverse therapies directed against proinflammatory mediators, such as tumor necrosis factor (TNF-\(\alpha\)) and interleukin (IL)-1, have revealed dramatic effects in animal models of sepsis.2-3 However, in humans, most of these strategies have failed to improve the survival of septic patients.4,5 In part, this is because classical proinflammatory mediators, such as TNF-\(\alpha\) and IL-1, are released within minutes of endotoxin exposure; thus, even a minimal delay in treatment may result in treatment failure.

High mobility group box 1 (HMGB1; also known as amphoterin), a mediator of endotoxin lethality, is a promising therapeutic target for sepsis. During septic conditions, serum HMGB1 levels are elevated in both humans and animals.6,7 The accumulation of HMGB1 in the systemic circulation occurs considerably later than that of classically early proinflammatory mediators such as TNF-\(\alpha\) and IL-1.6,8 and this delayed kinetics of HMGB1 makes it an attractive therapeutic target with a wider window of opportunity for treatment.

Indeed, blockade of HMGB1, even at later time points after onset of endotoxemia, has been shown to rescue animals from lethal endotoxemia.6,7,9,10 Disseminated intravascular coagulation (DIC) is found in 25% to 50% of patients with sepsis and seems to be a strong predictor of mortality.11,12 It has been shown that the plasma HMGB1 levels are increased in patients with DIC and that HMGB1 in the systemic circulation promotes development of DIC in rats.13,14 In a recent clinical trial, recombinant human soluble thrombomodulin (TM) significantly improved DIC.15 TM can bind to HMGB1 as well as thrombin, thereby dampening the inflammatory and coagulatory responses.10,16,17 However, the binding of TM to HMGB1 is reversible,10 and therefore may not permanently block inflammatory responses. In the present study, we examined the end results of this binding and found that thrombin–TM complexes efficiently cleave HMGB1 to a less proinflammatory form.

Methods

In Vitro HMGB1 Degradation Assays

HMGB1 (Shino-Test Corporation, Sagamihara, Japan) was prepared from bovine thymus by the method of Sanders, as described...
Figure 1. Proteolytic cleavage of HMGB1 by thrombin. A, Silver-staining (left) and immunoblot (right) analyses of HMGB1 and its degradation product. HMGB1 (400 nmol/L) was incubated with thrombin (0, 5, 10, or 20 U/mL) for 4 hours. B, N-terminal amino acid sequencing of the HMGB1 degradation product yielded a sole peptide beginning from Gly11 of HMGB1.

Previously,18,19 HMGB1 has 99% amino acid sequence identity among all mammals.20 Protein purity was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by silver-staining (supplemental Figure I, available online at http://atvb.ahajournals.org). HMGB1 (400 nmol/mL) was incubated with 0 to 20 U/mL of bovine thrombin (Mochida Pharmaceutical) or human thrombin (Sigma-Aldrich) with or without 400 nmol/L recombinant soluble TM (Asahi Kasei Pharma Corporation), TM-derived peptide D1, D23, or D1 + D23 for 15 to 240 minutes at 37°C in 50 mmol/L Tris-HCl (pH 8.0), 2 mmol/L CaCl2, and 0.1 mol/L NaCl in a total volume of 50 mL. Bovine and human thrombin cleaved HMGB1 in the same way (Figure 1 and supplemental Figure II), and we used bovine thrombin in most experiments unless otherwise indicated. These samples were then assessed by SDS-PAGE followed by silver-staining or immunoblot as described previously.21 A rabbit polyclonal antibody against peptide Lys167-Lys180 of HMGB1 (Shino-Test Corporation) was used as a primary antibody for immunoblot analyses. This antibody recognizes human, rabbit, bovine, pig, rat, and mouse HMGB1 but not HMGB2. Densitometry was performed using National Institutes of Health (NIH) Image software.

Analysis of Proinflammatory Activity of the HMGB1 Degradation Product

The HMGB1 degradation products were subjected to SDS-PAGE, transferred to a polyvinylidene fluoride (PVDF) membrane, and analyzed on an automated amino acid sequencer, Procise 494 HT Protein Sequencing System (Applied Biosystems).

[Supplemental Figure I]

RT-PCR

Total RNA was extracted from cells using the RNAqueous kit (Ambion Inc). RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Relative expression levels of mRNA were determined using an Applied Biosystems 7300 Real-Time PCR System with a TaqMan Universal PCR Master Mix (Applied Biosystems) and gene-specific primers (TNF: Mn00443258_m1, NOS2: Mn00440485_m1). Expression levels were calculated as the ratio of mRNA level for a given gene relative to the mRNA level for glyceraldehyde-3-phosphate dehydrogenase (Mm99999915_g1) in the same cDNA sample.

Patients

In total, 8 septic patients with DIC, 4 colon cancer patients with metastasis, and 8 healthy volunteers were enrolled in this study. Diagnosis of sepsis was made according to the guideline of the Society of Critical Care Medicine Consensus Conference Committee, and diagnosis of DIC was made according to the criteria established by the Japanese Ministry of Health and Welfare. None of the patients with DIC had taken anti-DIC or anticoagulant therapy before blood sampling. All the colon cancer patients had received chemotherapy but had not undergone operations for at least 3 months before blood sampling. Plasma or serum samples were obtained at the University of Occupational and Environmental Health, Sapporo Medical University, Fuji Hospital and Kagoshima University according to the recommendations of the Medical Ethics Committees of each institution. All patients and healthy volunteers gave informed consent for participation in this study.

Systemic Endotoxin Challenge in Mice

This animal study was approved by the Institutional Animal Care and Use Committee of Kagoshima University. Male C57BL/6J mice were divided into 3 groups: a saline group (n = 2, body weight = 32.4 ± 2.8 g), a lipopolysaccharide (LPS) group (n = 5, body weight = 32.1 ± 4.3 g), and an LPS + TM group (n = 4, body weight = 32.3 ± 2.2 g). Mice in the LPS and LPS + TM groups were exposed to LPS (E. coli serotype O111:B4, 5 mg/kg, intraperitoneally) in the absence or presence, respectively, of recombinant soluble TM (100 nmol/kg, intraperitoneally at 0 and 12 hours after LPS exposure). Sixteen hours after LPS exposure, blood samples were collected from ether-anesthetized mice.

Immunoblot Analyses of the HMGB1 Degradation Product in Critically Ill Patients and in Endotoxemic Mice

Plasma samples from septic patients and serum samples from endotoxemic mice, cancer patients, and healthy volunteers were analyzed by immunoblot. HMGB1 in plasma and serum (250 µL) was immunoprecipitated using ExactaCruz F (Santa Cruz Biotechnology) and a rabbit polyclonal antibody against peptide Lys167-Lys180 of HMGB1. After that, immunoprecipitated HMGB1 was analyzed by immunoblot as described previously.22 Whether we used plasma or serum made little or no difference to the immunoblot analysis (data not shown). The positive control (PC) consists of quantitative reverse-transcription polymerase chain reaction (RT-PCR).

N-Terminal Amino Acid Sequence Analysis of the HMGB1 Degradation Product

The HMGB1 degradation products were subjected to SDS-PAGE followed by silver-staining or immunoblot as described previously.21 Whether we used plasma or serum made little or no difference to the immunoblot analysis (data not shown). The positive control (PC) consists of HMGB1 (400 nmol/L) was incubated with thrombin (0, 5, 10, or 20 U/mL) for 4 hours. B, N-terminal amino acid sequencing of the HMGB1 degradation product yielded a sole peptide beginning from Gly11 of HMGB1.
bovine HMGB1 (175 ng/mL) mixed in vitro with whole blood from a healthy volunteer. Of the 8 healthy volunteers examined, results from 2 representative volunteers are shown in Figure 5A. Of the eight septic patients examined, results from 3 representative patients are shown in Figure 5B.

Statistical Analyses
Data are presented as means±SD. Statistical analyses were performed using Student t test.

Results
First, we examined whether thrombin could cleave HMGB1. A computational analysis identified a possible thrombin cleavage site (Arg10-Gly11) in HMGB1. Thrombin displays a marked preference for certain amino acid side chains of substrates, including an arginine at the P1 position (nomenclature of Schechter and Berger22), a proline at P2, a hydrophobic residue at P4, and a small residue at P122,23,24 Pro9, Arg10, and Gly11 of HMGB1 seem to be applicable to thrombin’s preferred P2, P1, and P1’ amino acids, respectively, although Lys7 of HMGB1 is not hydrophobic. To confirm whether thrombin cleaves HMGB1, we performed in vitro experiments. As shown in Figure 1A, thrombin cleaved HMGB1 in a dose-dependent manner. N-terminal amino acid sequence analysis of the HMGB1 degradation product demonstrated that thrombin cleaved HMGB1 at the Arg10-Gly11 bond (Figure 1B). The cleavage of HMGB1 depended on the proteolytic activity of thrombin, since the reaction was completely blocked in the presence of antithrombin (supplemental Figure IIIA).

Although thrombin-mediated cleavage of HMGB1 did occur, it required rather high doses of thrombin (5 to 20 U/mL) and a long duration of time (4 hours). This led us to speculate that there could be a cofactor in this reaction. We recently reported that TM, a cofactor for thrombin-mediated activation of anticoagulant protein C,16 bound to HMGB1 via its lectin-like domain.10 Therefore, we examined whether TM also acted as a cofactor for thrombin-mediated cleavage of HMGB1. As shown in Figure 2 and supplemental Figure II, TM at a physiologically relevant concentration found in capillaries25 significantly enhanced thrombin-mediated cleavage of HMGB1. Neither TM per se nor activated protein C (APC) cleaved HMGB1 at all (supplemental Figure IIIB). The cleavage of HMGB1 by thrombin–TM complexes is Ca++ independent (supplemental Figure IIIB). Kinetic analysis showed that the estimated Km and kcat of thrombin–TM for the cleavage of HMGB1 were 1.89 μmol/L and 2.28 minutes⁻¹, respectively (supplemental Figure IV). The kcat/Km of thrombin–TM for the cleavage of HMGB1 was approximately equivalent to or within one order of magnitude lower than that for protein C activation, using the same recombinant soluble TM.26 Cofactor activity assays using various TM-derived peptides revealed that both the lectin-like domain (D1) and the epidermal growth factor (EGF)-like domain plus the proteoglycan-like domain (D23) of TM were essential for efficient cleavage (Figure 3). These findings are consistent with previous reports that the lectin-like domain binds to HMGB1 and the EGF-like domain binds to thrombin,10,23 and indicate the possibility that the lectin-like domain of TM binds to HMGB1, positioning HMGB1 so that thrombin–TM complexes can effectively degrade it.

Next, we examined the functional consequences of HMGB1 degradation. HMGB1 incubated in the thrombin–TM column (TTM column) was degraded (Figure 4A). Because the N terminus of HMGB1 contains a consensus sequence for heparin-type glycan recognition,27 we examined the binding affinity of HMGB1 to heparin. As shown in supplemental Figure V, the HMGB1 degradation product showed reduced heparin-binding affinity. Concomitant with the degradation and the loss of heparin-binding affinity, the proinflammatory activity of HMGB1 was significantly decreased (Figure 4B and 4C, P<0.01). A minor fraction of TM, which could be contaminated in HMGB1 samples from the TTM column, was not responsible for the decrease in proinflammatory activity, because (1) HMGB1 samples from the thrombin column, which did not contain TM at all,
exhibited decreased proinflammatory activity if sufficient thrombin to degrade HMGB1 was conjugated to the column; and (2) an abundant supply of TM (400 nmol/L) to HMGB1 samples from the control column did not cause a decrease in proinflammatory activity (relative expression of TNF-α mRNA in RAW 264.7 cells stimulated by HMGB1 from control columns /H11005 4.22 /H11006 0.34; stimulated by HMGB1 from thrombin columns /H11005 1.72 /H11006 0.04; stimulated by HMGB1 from control columns plus 400 nmol/L TM /H11005 4.16 /H11006 0.19). The inhibitory effect of thrombin–TM complexes on HMGB1-mediated inflammation was completely diminished in the presence of a serine protease inhibitor (supplemental Figure VI), suggesting that the inhibitory effect was dependent on the proteolytic activity of thrombin. The binding affinity of the HMGB1 degradation product to macrophages (RAW 264.7) was similar to that of intact HMGB1 (supplemental Figure VII). The binding affinity to receptor for advanced glycation end-products receptor (RAGE, a receptor for HMGB1) was also similar between the HMGB1 degradation product and intact HMGB1 (supplemental Figure VIII). These findings suggest that the decreased proinflammatory activity of the HMGB1 degradation product was attributable not to decreased binding affinity for its receptor, but rather to decreased signaling after binding to its receptor. To confirm this idea, we analyzed proinflammatory signaling pathways in RAW 264.7 cells. HMGB1 signaling through RAGE leads to activation of the nuclear factor-kB pathway, as well as to signal transduction through extracellular signal regulated kinase (ERK) and p38, which promotes cytokine production.9 HMGB1 activated these signaling pathways within 30 minutes, and the activation was sustained for at least 2 hours (supplemental Figure IX and data not shown). However, the HMGB1 degradation product did not activate the proinflammatory signaling pathways in RAW 264.7 cells (supplemental Figure IX). Thus, thrombin–TM complexes can alter the function of HMGB1, including proinflammatory activity and heparin-binding affinity, through the proteolytic cleavage of HMGB1.

Finally, we examined the expression pattern of HMGB1 in critically ill patients and in endotoxemic mice. In critically ill patients, including patients with advanced cancer and patients with DIC, a lower molecular-weight HMGB1 band was detected along with the conventional HMGB1 band (Figure 5). In endotoxemic mice, a lower molecular-weight HMGB1 band was also detected (supplemental Figure X). A similar, lower molecular-weight HMGB1 band has also been found in the synovial fluid of patients with rheumatoid arthritis,21 in which high levels of thrombin and TM are present.28,29 It seems highly probable that these bands correspond to HMGB1 degradation products, rather than homologous proteins of the HMG protein family, because we used an antibody against the Lys167-Lys180 sequence, which represents the most distinctive feature of HMGB1. Collectively, HMGB1 could be degraded in critically ill patients and in endotoxemic mice, indicating a possible relevance of HMGB1 degradation in clinical settings.

Discussion

Our results, in combination with those of previous studies,10,17 suggest that the lectin-like domain of TM binds to HMGB1, positioning HMGB1 so that thrombin–TM complexes can effectively degrade it to a less proinflammatory form. These findings highlight the novel antiinflammatory role of TM, in which TM can sequester and degrade HMGB1, thereby preventing HMGB1 from exacerbating inflammation. In a series of these TM-mediated HMGB1 inactivation steps, the first step may be binding of TM to HMGB1.10 By binding patients 1 through 4; S1 through S3: patients with sepsis and DIC 1 through 3. Deg: HMGB1 degradation product.
to HMGB1, TM can suppress the proinflammatory effects of HMGB1 in a thrombin-independent manner. However, the binding is noncovalent and reversible, causing insufficient suppression of HMGB1 in some cases. In such conditions, thrombin–TM complex-mediated HMGB1 degradation may be important for the inactivation of HMGB1. Because TM expression is decreased on the endothelium of patients with sepsis, its replacement with recombinant TM could aid in the inactivation of HMGB1 and offer a therapeutic value for sepsis or DIC. In the present study, however, the reason why HMGB1 lacking its N-terminal domain lost its proinflammatory activity was unclear. One possibility is that HMGB1 lacking its N-terminal domain is unable to bind to heparin-type glycans, such as syndecans, leading to the loss of its proinflammatory activity. HMGB1/ampthoterin was reported to bind to the heparan sulfate side chains of syndecan-1, a transmembrane proteoglycan. Syndecans bind to some growth factors and growth factor receptors, and thereby regulate their assembly into signaling complexes. Similarly, syndecan-HMGB1-RAGE binding may also be important in formation of proinflammatory signaling complex. Because HMGB1 lacking its N-terminal domain lost binding affinity to heparin, it might no longer induce formation of the intracellular signaling complex, thereby losing its proinflammatory activity.

It will be important to address the mechanism by which TM enhances thrombin-mediated cleavage of HMGB1. In the cofactor activity assay using various TM-derived peptides (Figure 3), both the lectin-like domain (D1) and the EGF-like domain plus the proteoglycan-like domain (D23) of TM were essential for the efficient cleavage of HMGB1. Furthermore, compared with D23 peptide alone, addition of D1 peptide to D23 peptide tended to promote the thrombin-mediated cleavage of HMGB1, although this did not reach statistical significance. These findings indicate the possibility that D1-bound HMGB1 may be cleaved by thrombin associated with adjacent TM (trans), rather than with the same TM (cis) to which the HMGB1 is simultaneously bound. It is not yet known whether endogenous cell-associated TM can enhance HMGB1 cleavage as effective as recombinant soluble TM, however, HMGB1 was degraded in endotoxemic mice (supplemental Figure X) and in patients with advanced cancer and DIC (Figure 5), suggesting that endogenous TM might also play a role in enhancing the cleavage of HMGB1.

Another important feature of this study is that HMGB1 could be degraded in DIC patients. During DIC, thrombin generation would be expected to be increased, whereas TM expression would be decreased. In such conditions, HMGB1 might be insufficiently degraded (Figure 5), presumably because of a lack of sufficient TM on endothelial cells, and replacement with recombinant TM would promote the degradation of HMGB1 (supplemental Figure X). It is probable that not only thrombin–TM complexes, but also other proteases, may contribute to the degradation of HMGB1 in these patients and animals. For example, plasmin can degrade HMGB1/ampthoterin in the filopodia of neuroblastoma cells, suggesting that plasmin can also degrade HMGB1 in the systemic circulation.

Our findings indicate the need for caution in interpreting the significance of the HMGB1 degradation product in clinical samples. HMGB1 can be degraded under critically ill conditions, such as DIC or other conditions in which proteases are systemically activated. Therefore, HMGB1 degradation products may reflect poor prognosis attributable to deleterious protease activation, even though the degradation of HMGB1 itself may be protective. In contrast, if the degradation of HMGB1 is achieved by replacement with recombinant TM, it may indicate improved prognosis. Recombinant TM can suppress inflammatory and coagulatory responses at least in part through sequestration of thrombin and HMGB1 and subsequent degradation of HMGB1 by thrombin–TM complexes, and should be a promising therapeutic option against DIC or sepsis.

Serum samples from patients with advanced cancer contain many protein fragments and could be a rich source of cancer-specific diagnostic information. The terms “peptidome” and “degradome” are now proposed to describe the low molecular weight range of the circulatory proteome. The HMGB1 fragment found in this study in patients with advanced cancer or DIC may be one aspect of the degradome. However, in vitro experiments suggested that this fragment would probably be more meaningful than a diagnostic marker, because the function of HMGB1 could be changed through the proteolytic cleavage. HMGB1 lost its proinflammatory activity and heparin-binding ability. It is not yet known whether HMGB1 gains new functions via this proteolytic cleavage. Further investigations are therefore required to determine the physiological and pathological importance of HMGB1 degradation. These studies will provide new insights into how HMGB1 is metabolized and how the roles of HMGB1 are regulated.

Recent studies have implicated HMGB1 as a possible contributor to atherogenesis. Endothelial cell–associated TM might play an important role in regulating atherogenesis through promoting proteolytic cleavage of HMGB1 by thrombin.

Sources of Funding
This study was supported in part by a Research Grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid 18791341 to T.I.) and a Health and Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare (to I.M.).

Disclosures
None.

References


Proteolytic Cleavage of High Mobility Group Box 1 Protein by Thrombin-Thrombomodulin Complexes
Takashi Ito, Ko-ichi Kawahara, Kohji Okamoto, Shingo Yamada, Minetsugu Yasuda, Hitoshi Imaizumi, Yuko Nawa, Xiaojie Meng, Binita Shrestha, Teruto Hashiguchi and Ikuro Maruyama

Arterioscler Thromb Vasc Biol. 2008;28:1825-1830; originally published online July 3, 2008; doi: 10.1161/ATVBAHA.107.150631

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/28/10/1825

Data Supplement (unedited) at:
http://atvb.ahajournals.org//subscriptions/

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Figure I. Ito et al.

Figure I. Silver-staining analyses of purified HMGB1. M: protein marker, H: 75 ng of purified HMGB1
Figure II. Proteolytic cleavage of HMGB1 by human thrombin-TM complexes. Silver-staining analysis of HMGB1 and its degradation product. (A) HMGB1 was incubated with human thrombin (0, 2, 5, or 10 U/ml) for 4 h. (B) HMGB1 was incubated with human thrombin (2 U/ml) in the absence or presence of TM (100 nM) for 0, 15, 30, 60, and 120 min at 37°C.
Supplemental Figure III. Ito et al.

Figure III. Thrombomodulin enhances thrombin-mediated cleavage of HMGB1, whereas antithrombin inhibits this process. (A) HMGB1 (400 nM) was incubated with thrombin (2 U/ml) for 30 min in the absence or presence of 400 nM recombinant soluble TM and/or antithrombin (4 U/ml). These samples were then assessed by immunoblot. (B) HMGB1 (400 nM) was incubated with thrombin (2 U/ml) and/or TM (100 or 400 nM) for 30 min in the presence of Ca^{++} (2 mM) or EDTA (5 mM). HMGB1 was also incubated with activated protein C (APC, 200 nM) instead of thrombin-TM. These samples were then assessed by immunoblot.
Figure IV. Kinetic analysis of thrombin-TM-mediated HMGB1 degradation. (A) HMGB1 (100, 200, 400 or 1000 nM) was incubated with thrombin (20 nM) and TM (400 nM) for 30 min. These samples were then assessed by immunoblot. The amounts of the HMGB1 degradation product formed during the course of the reaction were determined by densitometrical analysis. Lineweaver-Burk plots are shown. The $K_m$ and $k_{cat}/K_m$ are 1.89 μM and 1.21 x 10^6 M^{-1} min^{-1}, respectively. (B) Kinetic analysis was performed as above, except that HMGB1 was incubated with thrombin (20 nM) alone for 30 min. The $K_m$ and $k_{cat}/K_m$ are 10.7 μM and 4.13 x 10^4 M^{-1} min^{-1}, respectively.
Supplemental Figure V. Ito et al.

Figure V. The HMGB1 degradation product loses binding affinity for heparin.

Immunoblot analyses of the HMGB1 degradation product before and after adsorption by heparin beads. (A) Schematic representation of the experimental design. (B) After a 30-min incubation with or without thrombin-TM complexes, HMGB1 samples were captured by HiTrap Heparin HP beads (GE Healthcare, Piscataway, NJ). Then, samples were analyzed by immunoblot. The numbers in the right panel are the relative percentages of the adsorbed HMGB1 species, which were calculated as a ratio of the density of each band after adsorption to before adsorption (c/a and d/b). Densitometry was performed using the NIH Image software. The symbols a-d in (B) correspond to those in (A). A result representative of two experiments is shown.
Figure VI. The inhibitory effect of thrombin-TM complexes on HMGB1-mediated inflammation is dependent on the proteolytic activity of thrombin. HMGB1 (1 μg/ml), thrombin (10 U/ml), TM (800 nM), and 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF, 250 μM) were added to Opti-MEM-I medium. These media were pre-incubated for 30 min, and were used to stimulate RAW 264.7 cells (2 x 10^5 cells/well). The tumor necrosis factor (TNF)-α concentrations in the cell supernatants after 12 h stimulation were determined using an ELISA kit for mouse TNF-α. n = 4
**Supplemental Figure VII. Ito et al.**

**A**

<table>
<thead>
<tr>
<th>HMGB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control column</td>
</tr>
<tr>
<td>TTM column</td>
</tr>
</tbody>
</table>

RAW 264.7

**B**

- Without HMGB1
- HMGB1 from control column (Alexa 488)
- HMGB1 from TTM column (Alexa 488)

**Figure VII. Binding affinity of the HMGB1 degradation product for macrophages is not different from that of intact HMGB1.** (A) HMGB1 was incubated in control or TTM columns as in Figure 4, and then labeled with Alexa Fluor 488 (Invitrogen, Eugene, OR) according to the manufacturer’s recommendations. RAW 264.7 cells (2 x 10^4 cells/well) were incubated with labeled HMGB1 (200 nM) or its degradation product (200 nM) for 60 min in BD BioCoat 4-well Culture Slide (BD Biosciences, Bedford, MA). After washing with phosphate buffer and fixing with OptiLyse C (Beckman Coulter, Marseille, France), cell nuclei were stained with DAPI. Images were analyzed using Axio Vision 4.5 software (Carl Zeiss, Tokyo, Japan). A result representative of three experiments is shown. Scale bars, 10 μm. (B) RAW 264.7 cells (6 x 10^5 cells/60 mm dish) were incubated with labeled HMGB1 (200 nM) or its degradation product (200 nM) for 60 min. After washing with phosphate buffer and fixing with OptiLyse C, cell fluorescence was measured using an Epics XL flow cytometer equipped with System II software (Beckman Coulter).
Figure VIII. Binding affinity of the HMGB1 degradation product for RAGE is not different from that of intact HMGB1. RAGE-transfected or mock-transfected COS-7 cells, prepared as described previously (Abeyama et al. J Clin Invest. 2005;115:1267-1274), were incubated with labeled HMGB1 (200 nM) or its degradation product (200 nM) for 60 min, as in Figure VII. Cell fluorescence was analyzed by microscopy (A) and flow cytometry (B). A result representative of three experiments is shown. Scale bars, 10 μm.
Figure IX. HMGB1, but not the HMGB1 degradation product, can activate proinflammatory signaling pathway. HMGB1 degradation products were obtained from TTM columns as in Figure 4. RAW264.7 cells (2 × 10⁶ cells/60 mm dish) were starved for 120 min in serum-free Opti-MEM-I medium, and then stimulated with 1 μg/ml of intact HMGB1 or the HMGB1 degradation product. After 60 min stimulation, cell lysates were harvested. (A) These samples (10 μg) were then assessed by SDS-PAGE followed by immunoblot using a rabbit polyclonal antibody (Cell Signaling Technology, Beverly, MA) against total ERK1/2 (t-ERK 1/2) or phospho ERK1/2 (p-ERK 1/2). (B) Nuclear extracts were prepared as described previously (Kawabata et al. J Biol Chem 2002;277:8099-8105). NF-κB activation was then determined using a commercial ELISA kit for NF-κB p50/p65 transcription factor assay (Chemicon Temecula, CA).
**Supplemental Figure X. Ito et al.**

<table>
<thead>
<tr>
<th></th>
<th>C57BL/6J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>+ + + + +</td>
</tr>
<tr>
<td>LPS (5 mg/kg)</td>
<td>- - + + +</td>
</tr>
<tr>
<td>TM (100 nmol/kg)</td>
<td>- - - - -</td>
</tr>
</tbody>
</table>

Figure X. HMGB1 degradation products in mice with endotoxemia. Male C57BL/6J mice were divided into three groups: a saline group (n = 2), an LPS group (n = 5), and an LPS + TM group (n = 4). Mice in the LPS and LPS + TM groups were exposed to LPS O111:B4 (5 mg/kg, intraperitoneally) in the absence or presence, respectively, of recombinant soluble TM (100 nmol/kg, twice at 0 and 12 hours after LPS exposure). Two mice in the LPS group died within 16 hours, whereas no mice in the saline or LPS + TM groups died. Sixteen hours after LPS exposure, blood samples were collected from ether-anesthetized mice. HMGB1 in serum (250 μl) was immunoprecipitated using ExactaCruz F and a rabbit polyclonal antibody against peptide Lys166-Lys179 of HMGB1. Immunoprecipitated HMGB1 was then analyzed by immunoblot. HMGB1 degradation products were detected in a mouse in the LPS group and in a mouse in the LPS + TM group. Deg: HMGB1 degradation product.
Supplemental Figure XI. Ito et al.

Amino acid sequence of HMGB1

[Homo Sapiens]
mgkgdpkkpr_gkmssyaffv qtcreehkkk hpdasvnfse fskkcservwk tmsakekgkf edmakadkar yeremktyip pkgetkffk dpnapkrppl afflfcseyr pkikgehpql sigdvakklg emwnntadd kqpyekkaak lkekyekdia ayrakgkpda_akkqvvkaek skkkkeeed eedeedeeed

[Bos Taurus]
mgkgdpkkpr_gkmssyaffv qtcreehkkk hpdasvnfse fskkcservwk tmsakekgkf edmakadkar yeremktyip pkgetkffk dpnapkrppl afflfcseyr pkikgehpql sigdvakklg emwnntadd kqpyekkaak lkekyekdia ayrakgkpda_akkqvvkaek skkkkeeed eedeedeeed

[Mus Musculus]
mgkgdpkkpr_gkmssyaffv qtcreehkkk hpdasvnfse fskkcservwk tmsakekgkf edmakadkar yeremktyip pkgetkffk dpnapkrppl afflfcseyr pkikgehpql sigdvakklg emwnntadd kqpyekkaak lkekyekdia ayrakgkpda_akkqvvkaek skkkkeeeddd eedeedeeed

Differences

Thrombin-cleavage site

Antibody-recognition site