of DNA functions (6). HMGB1 is released into the extracellular space during necrosis of all eukaryotic cells or via mitogen-activated protein kinase (MAPK) signaling pathways during macrophage responses to inflammatory stimuli such as endotoxin, tumor necrosis factor (TNF)-α and C-reactive protein (CRP) (7-9,12,25). HMGB1 acts as a potent proinflammatory cytokine through a multiligand receptor for advanced glycation end products (RAGE), toll-like receptor (TLR)-2, and TLR-4 (8,10). HMGB1 stimulates a number of cells to release cytokines such as TNF-α, interleukin (IL)-6, and IL-1ß. This suggests that extracellular HMGB1 plays a critical role in the development of several inflammatory diseases such as sepsis, rheumatoid arthritis (RA), disseminated intravascular coagulation (DIC), and periodontitis and in xenotransplantation (7,8,11,13,14). HMGB1 plays a role in the pathogenesis of plaque formation and in the progression of atherosclerotic lesions (9,15,16). However, it is widely accepted that endothelial dysfunction occurs early during the development of atherosclerosis (1,2,17). Nevertheless, the association between HMGB1 and ECs is poorly understood.

Vascular endothelial growth factor (VEGF) is an angiogenic factor which plays an important role in the progression of atherosclerotic lesions. Its expression is upregulated not only under hypoxic conditions but also in the presence of transforming growth factor-ß, angiotensin II, basic fibroblast growth factor (bFGF) and IL-1ß, which are expressed in newly formed atherosclerotic lesions. Activated macrophages play a central role in the development of atherosclerosis due to their ability to produce numerous cytokines and growth factors. A recent study revealed that angiogenesis and recanalization can be induced by VEGFs derived from activated macrophages, functioning in an autocrine or paracrine manner (18). HMGB1 is also an angiogenic factor because it induces bFGF via RAGE.

The present study aimed to investigate the effects of 5-HT on the secretion of HMGB1 by human umbilical vein endothelial cells (HUVECs) and to identify the underlying intracellular signaling pathway involved in these effects. Furthermore, we also investigated whether HMGB1 induced VEGF production in macrophage-like RAW264.7 cells.

## Materials and methods

*Antibodies.* The anti-HMGB1 antibody was obtained from Shino-Test Co. (Kanagawa, Japan). Anti-phosphorylated (p)-ERK1/2, anti-p-p38MAPK, anti-p-Jun-N-terminal kinase (JNK), and anti-ß-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA).

*Cell culture.* HUVECs were purchased from Takara Biomedical (Shiga, Japan). They were cultured in endothelial basal medium 2 (EBM2) supplemented with growth factors (Takara Biomedical) and in fetal bovine serum (FBS, Takara Biomedical) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown to 90% confluence, and further treatments were performed in EBM2 (Takara Biomedical).

Murine macrophage-like RAW264.7 cells were obtained from the American Type Cell Culture Collection (Manassas, VA) and were maintained in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% FBS and 2 mM glutamine.

*Stimulation of HUVECs with 5-HT.* The cultured HUVECs ($2x10^6$ cells per 6-cm dish) were starved for 2 h in a serum-free Opti-MEM-I medium (Invitrogen, Carlsbad, CA) and were stimulated with lipopolysaccharide (LPS)-free 5-HT (Sigma) in the medium. Following treatment, the HMGB1 levels in the culture media were analyzed by Western blot analysis.

*Stimulation of RAW264.7 cells with MAPK and PI3-kinase inhibitors.* Specific inhibitors of p38 MAPK (SB203580; Calbiochem, La Jolla, CA), MAP kinase (MEK1/2; U-0126, Calbiochem), and PI3 kinase (LY294002, Calbiochem) were used to evaluate the functional role of signaling pathways in 5-HT-induced HMGB1 release or HMGB1-induced VEGF production.

*Western blot analysis.* The HMGB1 levels in the culture supernatants were analyzed by Western blotting as described previously (12). In brief, each culture supernatant was incubated with 50 μl heparin-Sepharose 6B beads for 4 h. The heparin beads were then washed with 10 mM phosphate buffer (pH 7.0), mixed with 50 μl sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.002% bromophenol blue], and boiled for 5 min. These HMGB1 samples (40 μl) were subjected to 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the separated proteins were subsequently transferred onto a nitrocellulose membrane (GE Healthcare Bio-Sciences KK, Piscataway, NJ). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline (TBS, pH 7.4) containing 0.02% Tween-20 (TBST) at room temperature (RT) for 1 h and was then incubated with 2 μg/ml of the anti-HMGB1 antibody (Shino-Test) in TBST containing 1% non-fat dry milk at RT for 3 h. The membrane was washed and incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG polyclonal antibody (Invitrogen) diluted 1:3000 in TBST containing 2.5% non-fat dry milk at RT for 1 h. The membrane was washed once more, and the immunoreactive bands formed were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Bio-Sciences KK).

*Immunofluorescence microscopy.* Immunofluorescence microscopy was performed as described previously (12). In brief, $5x10^5$ HUVECs per well were cultured on 4-well BioCoat Collagen I culture slides (BD Biosciences, San Jose, CA). Following HUVEC stimulation as described above, the slides were washed with phosphate-buffered saline (PBS) and fixed with the OptiLyse C reagent (BD Biosciences) containing 0.1% Triton X-100 (Sigma). The slides were blocked with 1% bovine serum albumin (BSA) in PBS containing 0.1% Triton-X 100 (PBST) for 1 h, incubated with 1 μg/ml of the rabbit anti-HMGB1 antibody at RT for 1 h, and then washed with PBST. Further, they were incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (Invitrogen) for 1 h, washed with PBST, and finally labeled with 4',6-diamidino-2-phenylindole (DAPI;

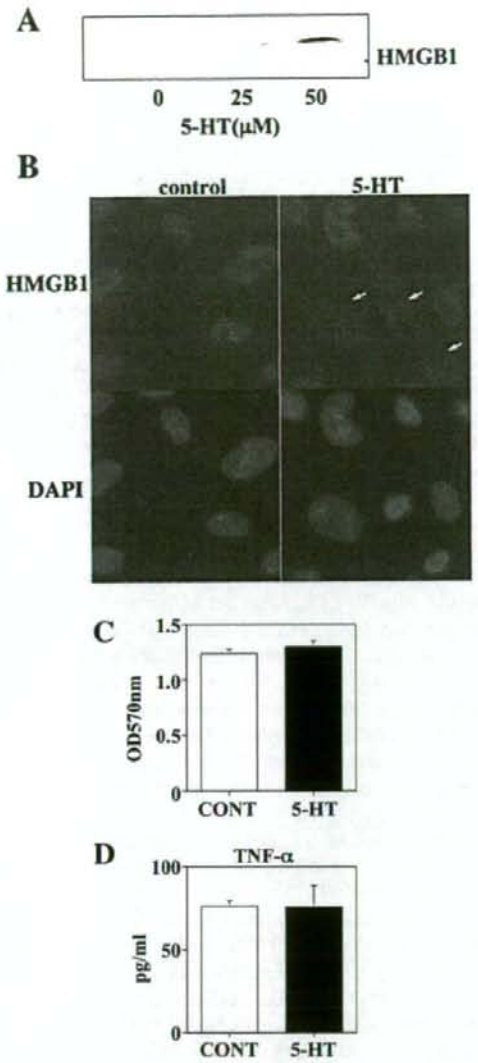


Figure 1. HMGB1 release from 5-HT-stimulated HUVECs. (A) Dose-dependent effect of 5-HT in HUVECs. HUVECs were incubated with 5-HT (0, 25, or 50 $\mu$M) for 16 h. The HMGB1 levels were analyzed by Western blotting. (B) Translocation of HMGB1 from the nucleus into the extracellular space in response to 5-HT treatment. HUVECs were incubated in the absence or presence of 5-HT (50 $\mu$M) for 16 h. The fixed cells were incubated with rabbit anti-HMGB1 polyclonal antibody, followed by incubation with FITC-labeled anti-rabbit IgG. The nuclei were labeled with DAPI. Original magnification, x400. Arrows indicate the translocation of HMGB1 from the nucleus. (C) Effects of 5-HT on cell viability. HUVECs were incubated with 5-HT (50 $\mu$M), and the cell viability was evaluated by an MTT assay. (D) Effects of 5-HT on TNF-α production. The HUVECs were treated with 5-HT (50 $\mu$M) for 16 h, following which the TNF-α levels in the culture supernatants were analyzed by performing ELISA. The values are presented as means ± SD. The data shown are the representative values for 3 independent experiments. *Statistically significant ($P<0.05$) change.

Wako Chemicals, Japan) to visualize the cell nuclei. Finally, the slides were washed and examined under an Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

*MTT assay*. Cell viability was analyzed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Wako Chemicals), as described previously (13). In brief, the cells were cultured in 96-well plates (100 $\mu$l of medium per well) in the absence or presence of 5-HT (50 $\mu$M) for 16 h. Subsequently, the cells were incubated with MTT (25 $\mu$g/ml per well) for 3 h. The formazan product was solubilized by treatment with 100 $\mu$l dimethyl sulfoxide for 16 h. The dehydrogenase activity was expressed as the ratio between the absorbance at the test wavelength of 570 nm and that at the reference wavelength of 630 nm.

*Enzyme-linked immunosorbant assay*. The VEGF and TNF-α levels in the culture supernatants were determined by using a commercial enzyme-linked immunosorbant assay (ELISA) kit specific for mouse VEGF and TNF-α (R&D Systems, MN). All experiments were performed in triplicate.

*RAGE small interfering RNA transfection analysis*. RAGE small interfering RNA (siRNA) transfection was carried out as described previously (12). In brief, RAW264.7 cells ($8x10^5$ cells/ml) were cultured in 6-cm dishes for 24 h; washed with the Opti-MEM-I medium; and transfected with RAGE siRNA (20 $\mu$M) or control siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 2 days, using Oligofectamine (Invitrogen). The transfected cells were treated with HMGB1 (10 $\mu$g/ml) for 20 h, and the VEGF levels in the medium were analyzed by performing ELISA.

*Statistical analysis*. Statistical analysis was performed using the Student's t-test. Statistical significance was accepted at $P\leq0.05$.

## Results

*HMGB1 release from 5-HT-stimulated HUVECs*. The aim of this study was to determine whether 5-HT induced HMGB1 release from HUVECs. The HUVECs were incubated with 5-HT (0-50 $\mu$M) for 16 h, and the culture supernatant was analyzed by performing Western blotting with anti-HMGB1 antibodies. As shown in Fig. 1A, there was a 5-fold increase in the HMGB1 levels in the medium containing 5-HT-stimulated HUVECs. We assessed whether HMGB1 was released from the HUVECs into the medium under the abovementioned conditions by performing microscopy. HUVECs were incubated in the absence or presence of 5-HT in 4-well slides for 16 h. As shown in Fig. 1B, HMGB1 disappeared from the nuclei (arrows). Next, we examined whether 5-HT treatment decreased the viability of the HUVECs. The cells were incubated in the absence or presence of 5-HT for 16 h, following which the MTT reagent was added to the medium. As shown in Fig. 1C, 5-HT did not affect cell viability. Since TNF-α, which induces HMGB1 expression, stimulates the active pathway for HMGB1 release, we examined whether 5-HT induced TNF-α production in the HUVECs. As shown in Fig. 1D, 5-HT had no effect on TNF-α production in the HUVECs.

*HMGB1 release from 5-HT-treated HUVECs via the p38MAPK pathway*. Next, we examined whether the presence

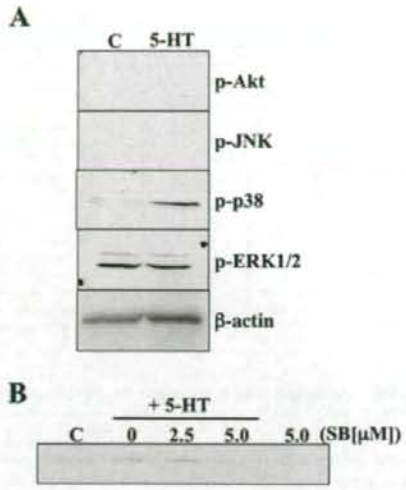


Figure 2. HMGB1 release in 5-HT-stimulated HUVECs via p38MAPK signaling. (A) 5-HT activates MAPKs. HUVECs were incubated with 5-HT (50 $\mu$M) for 30 min. The activation of p38MAPK, ERK1/2 and JNK was assayed by performing Western blot analysis with specific antibodies against p-p38MAPK, pERK1/2 and p-JNK1/2. (B) The HUVECs were pretreated with SB203580 (0, 2.5, and 5 $\mu$M) for 15 min, followed by incubation with 5-HT (50 $\mu$M) for 16 h. The HMGB1 levels in the culture supernatants were analyzed by using Western blotting. ß-actin was used as the loading control. Each test was performed in 3 independent experiments.

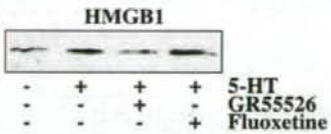


Figure 3. HMGB1 release via the 5-HT1B receptor in 5-HT-stimulated HUVECs. The HUVECs were incubated with GR55526 or fluoxetine for 2 h, following which 5-HT was added to the medium and maintained for 16 h. The HMGB1 levels were analyzed by Western blotting. Each test was performed in 3 independent experiments.

of 5-HT treatment activated MAPKs (ERK1/2, p38MAPK and JNK) or Akt in the HUVECs. The cells were incubated in the absence or presence of 5-HT for 30 min, following which the cell lysates were analyzed by performing Western blotting with anti-p-ERK1/2, anti-p-p38MAPK, anti-pJNK, or anti-pAkt antibodies. As shown in Fig. 2A, 5-HT activated the phosphorylation of p38MAPK, but not the other kinases in the HUVECs. Moreover, we examined whether the 5-HT-induced HMGB1 release in the HUVECs was mediated via p38MAPK expression. The HUVECs were incubated with 5-HT alone or supplemented with SB203580 (0-5.0 $\mu$M), which is a specific p38MAPK inhibitor, for 16 h. As shown in Fig. 2B, SB203580 treatment markedly inhibited HMGB1 release in 5-HT-stimulated HUVECs.

*Inhibition of HMGB1 release from the HUVECs following treatment with 5-HT1B antagonists.* We examined whether treatment with a 5-HT receptor (5-HT1B) antagonist GR55526 or selective 5-HT reuptake inhibitors (SSRIs) such as fluoxetine inhibited HMGB1 release from 5-HT-stimulated HUVECs. HUVECs were preincubated with GR55526 or fluoxetine, following which 5-HT was added to the medium containing the HUVECs. After incubation for 16 h, the HMGB1 levels in the medium were analyzed by Western blotting. As shown in Fig. 3, GR55526 but not fluoxetine markedly inhibited HMGB1 release.

*VEGF induction in HMGB1-treated RAW264.7 cells.* HMGB1 is reported to be an angiogenic factor that might be correlated with angiogenesis (19), which is an important process involved in the development of atherosclerosis. We hypothesized that HMGB1 stimulates VEGF protein production in RAW264.7 cells. As shown in Fig. 4A, HMGB1 but not heat-treated HMGB1 significantly stimulated VEGF production in a dose-dependent manner. Further, HMGB1 treatment did not induce VEGF production in the RAGE knock-down cells. Moreover, the mechanism of VEGF production by HMGB1 was found to be mediated via the Akt signaling pathway (Fig. 4B). Since RAGE is a specific HMGB1 receptor, we examined whether cells in which RAGE expression was knocked down by siRNA transfection produced VEGF following stimulation with HMGB1. As shown in Fig. 4C, under our experimental conditions, RAGE protein expression in the siRNA-treated cells was reduced to 75% as compared with the control cells (100%).

## Discussion

The present study is the first to demonstrate that 5-HT-treated HUVECs can actively and selectively release HMGB1 via the 5-HT1B receptor and that this release is specifically mediated by the p38MAPK signaling pathway. Furthermore, HMGB1 treatment triggered VEGF production via Akt signaling in RAW264.7 cells; this suggests that HMGB1 may play an important role in the development of atherosclerosis.

Platelet activation associated with EC dysfunction is the earliest event that occurs during the development of atherosclerosis. It is predicted that EC injury due to increased platelet activation causes HMGB1 release via a passive pathway. However, it is also possible that HMGB1 is released via an active pathway due to EC dysfunction. In fact, endotoxin is reported to induce HMGB1 release via an active pathway in ECs (17).

The present study shows that in HUVECs, 5-HT triggered HMGB1 release in an active rather than a passive manner. We believe that HMGB1 release triggered by 5-HT treatment occurs via an active pathway. Our findings can be summarized as follows. First, under our experimental conditions, 5-HT treatment did not affect cell proliferation, cell death, and TNF-α production in the HUVECs. Second, treatment with the pharmacological inhibitor SB203580 but not U-0126 significantly inhibited HMGB1 release; this suggests that the activation of p38MAPK but not other kinases plays a crucial role in 5-HT-induced HMGB1 release. p38MAPK induces the production of key inflammatory mediators including TNF-α and IL-1ß, and HMGB1 which plays an important role in promoting inflammatory diseases (12,20). Our findings

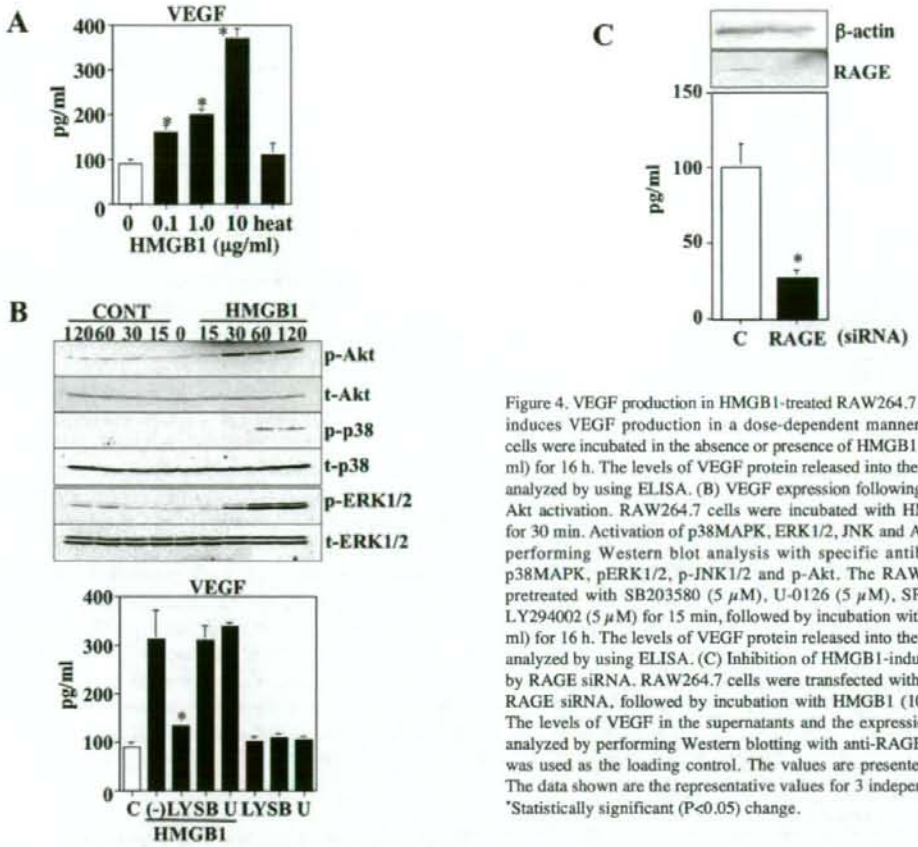


Figure 4. VEGF production in HMGB1-treated RAW264.7 cells. (A) HMGB1 induces VEGF production in a dose-dependent manner. The RAW264.7 cells were incubated in the absence or presence of HMGB1 (0, 0.1, and 10 μg/ml) for 16 h. The levels of VEGF protein released into the supernatants were analyzed by using ELISA. (B) VEGF expression following HMGB1-induced Akt activation. RAW264.7 cells were incubated with HMGB1 (10 μg/ml) for 30 min. Activation of p38MAPK, ERK1/2, JNK and Akt was assayed by performing Western blot analysis with specific antibodies against p-p38MAPK, pERK1/2, p-JNK1/2 and p-Akt. The RAW264.7 cells were pretreated with SB203580 (5 μM), U-0126 (5 μM), SP60025 (5 μM) or LY294002 (5 μM) for 15 min, followed by incubation with HMGB1 (10 μg/ml) for 16 h. The levels of VEGF protein released into the supernatants were analyzed by using ELISA. (C) Inhibition of HMGB1-induced VEGF release by RAGE siRNA. RAW264.7 cells were transfected with control siRNA or RAGE siRNA, followed by incubation with HMGB1 (10 μg/ml) for 20 h. The levels of VEGF in the supernatants and the expression of RAGE were analyzed by performing Western blotting with anti-RAGE antibody. ß-actin was used as the loading control. The values are presented as means ± SD. The data shown are the representative values for 3 independent experiments. *Statistically significant ($P<0.05$) change.

are consistent with those of a previous study which reported that HMGB1 release is mediated via the p38MAPK signaling pathway (12). Recent studies have suggested that HMGB1 release can occur through the activity of ERK1/2 or JNK rather than p38MAPK (9,17). The discrepancy between these results and those of our study might be attributed to the differences in cell types and the stimulants examined. Third, HUVECs have 5-HT1B and transporter (21). In the HUVECs, the 5-HT-induced HMGB1 release was inhibited by the 5-HT1B antagonist GR55526, but not fluoxetine; this suggests that 5-HT directly triggers HMGB1 release via the active pathway in these cells.

Recent studies have demonstrated the presence and distribution of VEGF in the macrophages of atherosclerotic arteries (22). In non-atherosclerotic arteries, only weakly positive VEGF staining was observed in the intima of some sections and in the adventitia. In newly formed atherosclerotic lesions, VEGF staining was frequently observed in the subendothelial regions abundant in macrophages; this indicates that macrophages function as a potential source of VEGF (22).

We also demonstrated that HMGB1 induces VEGF mRNA expression (data not shown) and enhances VEGF protein levels in the culture supernatants of macrophage-like RAW264.7 cells via the Akt signaling pathway. Our data appear to support previous evidence in this regard (22,23). First, as mentioned above, HMGB1 treatment directly induces VEGF production in RAW264.7 cells but not in both HUVECs and VSMCs (data not shown). Second, to confirm the specificity of direct HMGB1 stimulation, we examined the effects of heat-treated HMGB1 and found that it did not induce VEGF production in RAW264.7 cells. Third, HMGB1 treatment did not induce VEGF production in cells wherein RAGE, a specific HMGB1 inhibitor, was knocked down. This suggests that HMGB1 directly induces VEGF production, mainly by macrophages. Fourth, VEGF production is mediated via the MAPK or Akt signaling pathway. Although in the inhibitor experiments, we found that HMGB1 activates both the MAPK and Akt signaling pathways, it mediates VEGF production via the Akt signaling pathway. Our data are consistent with a recent report stating that HMGB1 acts as a proangiogenic factor due to the induction of fibroblast growth factor (FGF), and that the inhibition of RAGE expression by siRNA eliminates VEGF production (19,24). Thus, HMGB1 may contribute to the development of atherosclerosis by enhancing VEGF production.

To the best of our knowledge, this is the first study to demonstrate that 5-HT triggers active HMGB1 release via the 5-HT1B and p38MAPK signaling pathways in ECs. Our findings suggest that 5-HT may play an important role in inducing, amplifying, and prolonging the inflammatory processes, including atherosclerosis, by inducing the release of

a key inflammatory mediator, HMGB1. Moreover, HMGB1 treatment induced VEGF production via the RAGE and Akt signaling pathways in RAW264.7 cells. This suggests that cross-talk between 5-HT and HMGB1 may be involved in the development and progression of atherosclerosis.

**Acknowledgements**

We thank Nobue Uto, Tomomi Morizono and Tomoka Nagasato for their excellent technical assistance. This study was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by Grants-in-Aid nos. 19791618 (Y.M.) and 18791341 (T.I.), and by a Health and Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare (I.M.).

**References**

1. Ross R: Atherosclerosis - an inflammatory disease. N Engl J Med 340: 115-126, 1999.
2. Golino P, Crea F and Willerson JT: How to study the effects of platelet aggregation and thrombosis on coronary vasomotion and their clinical relevance. Ital Heart J 3: 220-225, 2002.
3. Nemecek GM, Coughlin SR, Handley DA and Moskowitz MA: Stimulation of aortic smooth muscle cell mitogenesis by serotonin. Proc Natl Acad Sci USA 83: 674-678, 1986.
4. Liu Y, Suzuki YJ, Day RM and Fanburg BL: Rho kinase-induced nuclear translocation of ERK1/ERK2 in smooth muscle cell mitogenesis caused by serotonin. Circ Res 95: 579-586, 2004.
5. Kawano H, Tsuji H, Nishimura H, *et al*: Serotonin induces the expression of tissue factor and plasminogen activator inhibitor-1 in cultured rat aortic endothelial cells. Blood 97: 1697-1702, 2001.
6. Lu J, Kobayashi R and Brill SJ: Characterization of a high mobility group 1/2 homolog in yeast. J Biol Chem 271: 33678-33685, 1996.
7. Wang H, Bloom O, Zhang M, *et al*: HMG-1 as a late mediator of endotoxin lethality in mice. Science 285: 248-251, 1999.
8. Taniguchi N, Kawahara K, Yone K, *et al*: High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. Arthritis Rheum 48: 971-981, 2003.
9. Kalinina N, Agrotis A, Antropova Y, *et al*: Increased expression of the DNA-binding cytokine HMGB1 in human atherosclerotic lesions: role of activated macrophages and cytokines. Arterioscler Thromb Vasc Biol 24: 2320-2325, 2004.
10. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A and Abraham E: Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. J Biol Chem 279: 7370-7377, 2004.
11. Ito T, Kawahara K, Nakamura T, *et al*: High-mobility group box 1 protein promotes development of microvascular thrombosis in rats. J Thromb Haemost 5: 109-116, 2007.
12. Kawahara K, Kamal KB, Unoshima M, *et al*: CRP induces high mobility group box-1 protein release through a p38 MAPK in the macrophage cell line RAW264.7 cells. Cardiovasc Pathol 17: 129-138, 2008.
13. Morimoto Y, Kawahara K, Tancharoen S, *et al*: Tumor necrosis factor-α stimulates gingival epithelial cells to release high mobility-group box 1. J Periodontal Res 43: 76-83, 2008.
14. Kawahara K, Setoyama K, Kikuchi K, *et al*: HMGB1 release in co-cultures of porcine endothelial and human T cells. Xenotransplantation 14: 636-641, 2007.
15. Inoue K, Kawahara K, Biswas KK, Ando K, Mitsudo K, Nobuyoshi M and Maruyama I: HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerosis plaques. Cardiovasc Pathol 16: 136-143, 2007.
16. Porto A, Palumbo R, Pieroni M, *et al*: Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. FASEB J 20: 2565-2566, 2006.
17. Treutiger CJ, Mullins GE, Johansson ASM, *et al*: High mobility group 1 B-box mediates activation of human endothelium. J Intern Med 254: 375-385, 2003.
18. Inoue M, Itoh H, Ueda M, *et al*: Vascular endothelial growth factor (VEGF) expression in human coronary atherosclerotic lesions: possible pathophysiological significance of VEGF in progression of atherosclerosis. Circulation 98: 2108-2116, 1998.
19. Mitola S, Belleri M, Urbinati C, *et al*: Cutting edge: extracellular high mobility group box-1 protein is a proangiogenic cytokine. J Immunol 176: 12-15, 2006.
20. Kuldo JM, Westra J, Asgeirsdóttir SA, *et al*: Differential effects of NF-{kappa}B and p38 MAPK inhibitors and combinations thereof on TNF-{alpha}- and IL-1{beta}-induced pro-inflammatory status of endothelial cells in vitro. Am J Physiol Cell Physiol 289: 1229-1239, 2005.
21. Ullmer C, Schmuck K, Kalkman HO and Lübbert H: Expression of serotonin receptor mRNAs in blood vessels. FEBS Lett 370: 215-221, 1995.
22. Ramos MA, Kuzuya M, Esaki T, *et al*: Induction of macrophage VEGF in response to oxidized LDL and VEGF accumulation in human atherosclerotic lesions. Arterioscler Thromb Vasc Biol 7: 1188-1196, 1998.
23. Salomonsson L, Svensson L, Pettersson S, Wiklund O and Ohlsson BG: Oxidised LDL decreases VEGFR-1 expression in human monocyte-derived macrophages. Atherosclerosis 169: 259-267, 2003.
24. Yamamoto Y, Yonekura H, Watanabe T, *et al*: Short-chain aldehyde-derived ligands for RAGE and their actions on endothelial cells. Diabetes Res Clin Pract 77: 30-40, 2007.
25. Scaffidi P, Misteli T and Bianchi ME: Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418: 191-195, 2002.

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[112] Lipids
[16] Myocardial cardiomyopathy disease
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[114] Pericardial disease
[17] Peripheral vascular disease
[18] Pulmonary circulation and disease
[115] Remodeling
[116] Restenosis
[19] Valvular heart disease
[20] Other etiology

**Treatment**

[22] Ablation/ICD/surgery
[117] Behavioral/psychosocial - treatment
[25] CPR and emergency cardiac care
[118] Cardiovascular Pharmacology
[23] Catheter-based coronary and valvular interventions: other
[24] Catheter-based coronary interventions: stents
[119] Compliance/Adherence
[26] Exercise/exercise testing/rehabilitation
[120] Pacemaker
[121] Primary prevention
[123] Restenosis
[122] Secondary prevention
[27] Other Treatment

**Diagnostic Testing**

[30] CT and MRI
[124] Cardiovascular imaging agents/Techniques
[29] Coronary imaging: angiography/ultrasound/Doppler/CC
[31] Echocardiography
[171] Electrocardiology
[106] Electrophysiology
[125] Exercise testing
[32] Nuclear cardiology and PET
[33] Other diagnostic testing

**Cardiovascular (CV) Surgery**

[126] Behavioral/psychosocial - CV surgery
[37] CV surgery: transplantation, ventricular assistance, cardiomyopathy
[35] CV surgery: aortic and vascular disease
[36] CV surgery: coronary artery disease
[38] CV surgery: valvular disease
[39] CV surgery: other
[40] Cardiovascular Nursing
[41] Pediatric and congenital heart disease, including cardiovascular surgery

**Stroke**

[43] Acute Cerebral Hemorrhage
[44] Acute Cerebral Infarction
[45] Acute Stroke Syndromes
[46] Behavioral Changes and Stroke
[127] Behavioral/psychosocial - stroke
[47] Brain Circulation and Metabolism
[49] Carotid Stenosis
[48] Carotid and Vertebral A. Dissection
[50] Cerebral Aneurysm, AVM, & Subarachnoid hemorrhage
[51] Cerebral Lacunes
[52] Cerebral Venous Thrombosis
[53] Embolic stroke
[54] Emergency treatment of Stroke
[55] Genetics of Stroke

**Imaging of the brain and arteries**

[57] Angiography
[58] Computerized tomography and Magnetic Resonance Imaging
[59] Doppler ultrasound, Transcranial Doppler etc.
[60] PET and SPECT
[61] Other imaging

[62] Intracerebral Hemorrhage
[63] Pathology of Stroke
[64] Primary and Secondary Stroke Prevention
[65] Rehabilitation, Stroke
[66] Risk Factors for Stroke
[67] Spinal Cord Vascular Disease
[68] Stroke in Children and the Young

**Stroke Treatment - Medical**

[70] Anticoagulants
[71] Antiplatelets
[72] Neuroprotectors

- [73] Thrombolysis
- [74] Other Stroke Treatment - Medical

**Stroke Treatment - Surgical**

- [76] Carotid endarterectomy
- [77] By-pass procedures
- [78] Angioplasty and Stenting
- [79] Aneurysm, AVM, hematoma
- [80] Other Stroke Treatment - Surgical

- [81] Transient Ischemic Attacks
- [82] Vasculitis Brain Arteries
- [83] Other Stroke

**Basic Science Research**

- [128] ACE/Angiotension receptors
- [129] Angiogenesis
- [130] Animal models of human disease
- [131] Apoptosis
- [132] Arrythmias-basic studies

**Atherosclerosis**

- [134] Pathophysiology
- [135] Risk Factors
- [144] Other arteriosclerosis

- [85] Autonomic, reflex, and neurohumoral control of circulation
- [136] Calcium cycling/excitation-contraction coupling
- [137] Cell biology/structural biology
- [138] Cell signalling/signal transduction
- [87] Coronary circulation
- [139] Developmental biology
- [140] Energy metabolism
- [141] Functional genomics
- [142] Gene expression
- [143] Gene regulation
- [88] Gene therapy
- [145] Genetically altered mice
- [89] Genetics of cardiovascular disease
- [146] Genomics
- [147] Growth factors/cytokines
- [148] Heart failure - basic studies
- [149] Hypertension - basic studies
- [150] Imaging
- [153] Information technology
- [152] Ion channels/membrane transport
- [151] Ischemic biology - basic studies
- [90] Lipid and lipoprotein metabolism

**Myocardial biology**

- [104] Structure
- [105] Contractile function
- [107] Biochemistry and metabolism
- [108] Other myocardial biology

- [154] Myogenesis
- [101] Nutrition
- [91] Oxidant stress
- [155] Physiological and pathological control of gene expression
- [156] Pulmonary biology and circulation
- [157] Quantitative modeling
- [93] Receptor pharmacology
- [163] Resource utilization

**Thrombosis**

- [172] Arterial thrombosis
- [173] Deep vein thrombosis
- [92] Platelets
- [174] Coagulation
- [86] Coagulation and fibrinolysis
- [160] Fibrinolysis
- [175] Fibrinogen/fibrin
- [176] Thrombin
- Platelet adhesion
  - [177] Secretion
  - [178] Aggregation
  - [179] Morphology
  - [180] Metabolism
  - [181] Signal transduction
- [182] Coagulation inhibitors
- Anticoagulants
  - [183] Heparin
  - [184] Coumarins
  - [185] Other anticoagulants
- [159] Anticoagulant mechanisms
- [186] Platelet function inhibitors
- [187] Thrombophilia
- [188] Thrombosis risk factors

- [161] Transplantation

**Vascular biology**

- [95] Endothelium/vascular type/nitric oxide
- [96] Mechanism of atherosclerosis/growth factors
- [162] Smooth muscle proliferation and differentiation
- [97] Other Vascular biology

- [98] Other Research

**Ethics and Policy**

- [100] Health policy and outcome research
- [102] Other Ethics and Policy

**Diabetes**

- [189] Type 1 diabetes
- [190] Type 2 diabetes
- [191] Other diabetes
- [192] Glucose intolerance

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# 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;29:000-000**)

**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

Original received June 28, 2007; final version accepted June 18, 2008.

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DOI: 10.1161/ATVBAHA.107.150631

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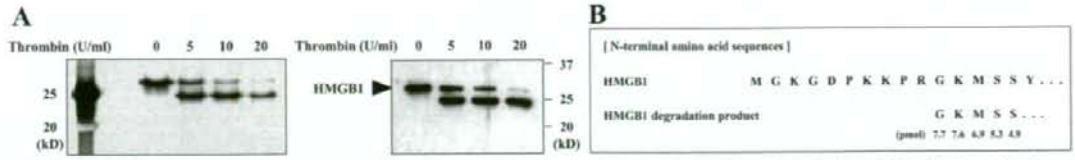


**Figure 1.** Proteolytic cleavage of HMGB1 by thrombin. A, Silver-staining (left) and immunoblot (right) analyses of HMGB1 and its degradation product. HMGB1 (400 nmol/L) was incubated with thrombin (0, 5, 10, or 20 U/mL) for 4 hours. B, N-terminal amino acid sequencing of the HMGB1 degradation product 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/L) 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 $CaCl_2$, and 0.1 mol/L NaCl in a total volume of 50 μL. 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.

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## N-Terminal Amino Acid Sequence Analysis 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).

## Analysis of Proinflammatory Activity of the HMGB1 Degradation Product

HMGB1 preparations were tested for endotoxin content by the Limulus assay, and confirmed to contain 40 pg or less of endotoxin per microgram of bovine thymus HMGB1. This dose of endotoxin was completely neutralized by 10 μg/mL of polymyxin B sulfate in the culture media (data not shown). Endotoxin-free bovine serum albumin (BSA, Calbiochem) and thrombin were coupled to separate HiTrap NHS-activated HP 1-mL columns (GE Healthcare) according to the manufacturer's recommendations. Next, recombinant TM was added to the thrombin-conjugated columns and incubated for 30 minutes at 37°C. After washing each column with 8 column volumes of washing buffer (0.05 mol/L phosphate buffer, pH 7), they were designated as control columns (BSA-conjugated columns) and TTM columns (thrombin-conjugated columns in which 9.14 nmol TM was noncovalently bound to thrombin). HMGB1 (100 μg in 1 mL of saline) was incubated in control and TTM columns for 15 minutes at 37°C, before samples were collected by injection of another 1 mL of saline. The protein concentrations of HMGB1 samples from each column were adjusted to 100 μg/mL, and these samples were individually added to RPMI-1640 medium (1% fetal bovine serum, 10 μg/mL polymyxin B sulfate) at a dilution of 1:40 (100 nmol/L), and were used to stimulate RAW 264.7 cells ($4\times10^5$ cells per well). The TNF-α concentrations in the cell supernatants after 15-hour stimulation were determined using an ELISA kit for mouse TNF-α (R&D Systems). The messenger RNA (mRNA) levels of TNF-α and nitric oxide synthase 2 (NOS2; also known as iNOS) in RAW 264.7 cells after 3 hours of stimulation were determined by real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR).

## 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: Mm00443258_m1, NOS2: Mm00440485_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±1.6), a lipopolysaccharide (LPS) group (n=5, body weight=32.1±4.4), and an LPS + TM group (n=4, body weight=32.3±2.2). 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.[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

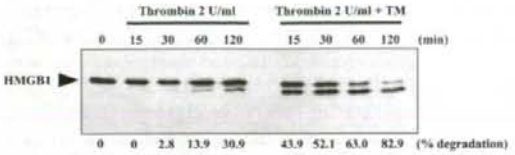


**Figure 2.** Thrombomodulin enhances thrombin-mediated cleavage of HMGB1. HMGB1 (400 nmol/L) was incubated with thrombin (2 U/mL) in the absence or presence of TM (400 nmol/L) for 15, 30, 60, or 120 minutes. Then, samples were analyzed by immunoblot. The numbers at the bottom of the lanes refer to the relative percentages of the degraded form.

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 Berger[22]), a proline at P2, a hydrophobic residue at P4, and a small residue at P1′.[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 capillaries[25] 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 *K*m and *k*cat of thrombin–TM for the cleavage of HMGB1 were 1.89 μmol/L and 2.28 minutes$^{-1}$, respectively (supplemental Figure IV). The *k*cat/*K*m 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,

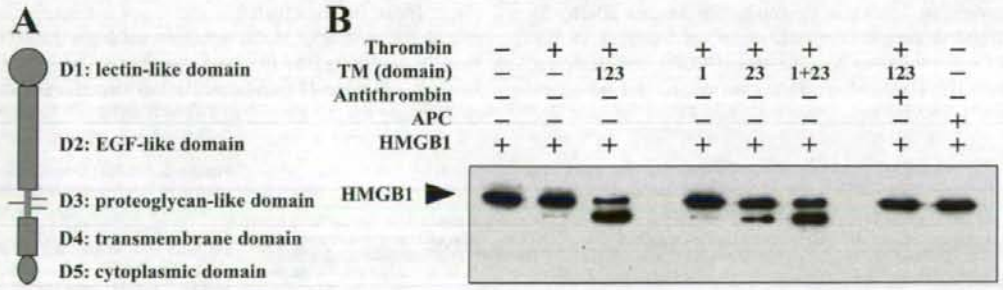


**Figure 3.** Both D1 and D23 of thrombomodulin are essential for efficient cleavage of HMGB1. A, The domain structure of TM (D1 to D5). B, HMGB1 was incubated with thrombin (2 U/mL) in the absence or presence of 400 nmol/L TM (D123), TM-derived peptide D1, D23, or D1 + D23 for 30 minutes. These samples were then assessed by immunoblot.

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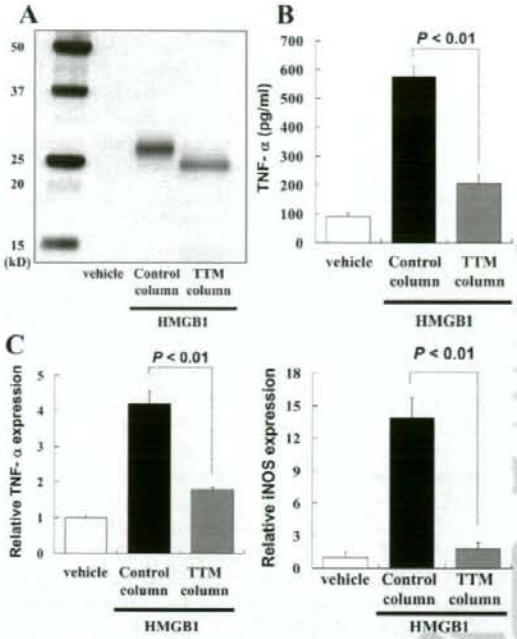


**Figure 4.** Thrombin-TM complexes degrade HMGB1 to a less proinflammatory form. A, Silver-staining analyses of HMGB1 that passed through the columns. Control column: BSA column; TTM column: thrombin–TM column. B, TNF-α secretion by RAW 264.7 cells. C, Relative expression levels of TNF-α mRNA and iNOS mRNA in RAW 264.7 cells. n=3.

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=4.22±0.34; stimulated by HMGB1 from thrombin columns=1.72±0.04; stimulated by HMGB1 from control columns plus 400 nmol/L TM=4.16±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-κB 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 3-hydroxy-3-methylglutaryl (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. F5

## 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

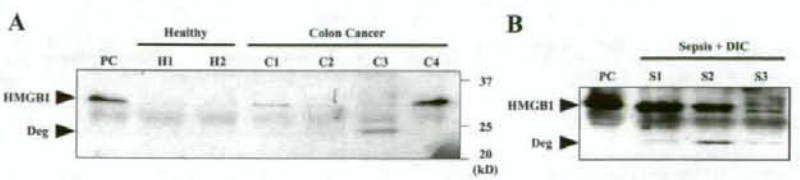


**Figure 5.** HMGB1 degradation products in critically ill patients. Immunoblot analyses of serum and plasma samples from patients with advanced cancer (A) and patients with sepsis and DIC (B). H1 and H2: healthy volunteers 1 and 2; C1 through C4: colon cancer patients 1 through 4; S1 through S3: patients 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.[10] However, the binding is noncovalent and reversible, causing insufficient suppression of HMGB1 in some cases. In such conditions, thrombin–TM complex-mediated HMGB1degradation may be important for the inactivation of HMGB1. Because TM expression is decreased on the endothelium of patients with sepsis,[30] its replacement with recombinant TM could aid in the inactivation of HMGB1 and offer a therapeutic value for sepsis or DIC.[15] 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/amphoterin was reported to bind to the heparan sulfate side chains of syndecan-1, a transmembrane proteoglycan.[31] Syndecans bind to some growth factors and growth factor receptors, and thereby regulate their assembly into signaling complexes.[32,33] Similarly, syndecan-HMGB1-RAGE binding may also be important in formation of proinflammatory signaling complex.[27] 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/amphoterin in the filopodia of neuroblastoma cells,[34] 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.[35–37] 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.[38–40] 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. AQ: 1

## References

1. Cohen J. The immunopathogenesis of sepsis. *Nature*. 2002;420:885–891.
2. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature*. 1987;330:662–664.
3. Ohlsson K, Bjork P, Bergenfeldt M, Hageman R, Thompson RC. Interleukin-1 receptor antagonist reduces mortality from endotoxin shock. *Nature*. 1990;348:550–552.
4. Reinhart K, Karzai W. Anti-tumor necrosis factor therapy in sepsis: update on clinical trials and lessons learned. *Crit Care Med*. 2001;29: S121–S125.
5. Fisher CJ Jr, Dhainaut JF, Opal SM, Pribble JP, Balk RA, Slotman GJ, Iberti TJ, Rackow EC, Shapiro MJ, Greenman RL, et al. Recombinant human interleukin 1 receptor antagonist in the treatment of patients with

sepsis syndrome. Results from a randomized, double-blind, placebo-controlled trial. Phase III rhIL-1ra Sepsis Syndrome Study Group. *JAMA*. 1994;271:1836–1843.
6. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ. HMG-1 as a late mediator of endotoxin lethality in mice. *Science*. 1999;285:248–251.
7. Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, Susarla SM, Ulloa L, Wang H, DiRaimo R, Czura CJ, Roth J, Warren HS, Fink MP, Fenton MJ, Andersson U, Tracey KJ. Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A*. 2004;101:296–301.
8. Wang H, Yang H, Czura CJ, Sama AE, Tracey KJ. HMGB1 as a late mediator of lethal systemic inflammation. *Am J Respir Crit Care Med*. 2001;164:1768–1773.
9. Lotze MT, Tracey KL. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat Rev Immunol*. 2005;5:331–342.
10. Abeyama K, Stern DM, Ito Y, Kawahara K, Yoshimoto Y, Tanaka M, Uchimura T, Ida N, Yamazaki Y, Yamada S, Yamamoto Y, Yamamoto H, Iino S, Taniguchi N, Maruyama I. The N-terminal domain of thrombomodulin sequesters high-mobility group-B1 protein, a novel antiinflammatory mechanism. *J Clin Invest*. 2005;115:1267–1274.
11. Bakhtiari K, Meijers JC, de Jonge E, Levi M. Prospective validation of the International Society of Thromb Haemost scoring system for disseminated intravascular coagulation. *Crit Care Med*. 2004;32:2416–2421.
12. Zeerleder S, Hack CE, Wuillemin WA. Disseminated intravascular coagulation in sepsis. *Chest*. 2005;128:2864–2875.
13. Hatada T, Wada H, Nobori T, Okabayashi K, Maruyama K, Abe Y, Uemoto S, Yamada S, Maruyama I. Plasma concentrations and importance of High Mobility Group Box protein in the prognosis of organ failure in patients with disseminated intravascular coagulation. *Thromb Haemost*. 2005;94:975–979.
14. Ito T, Kawahara K, Nakamura T, Yamada S, Abeyama K, Hashiguchi T, Maruyama I. High-mobility group box 1 protein promotes development of microvascular thrombosis in rats. *J Thromb Haemost*. 2007;5:109–116.
15. Saito H, Maruyama I, Shimazaki S, Yamamoto Y, Aikawa N, Ohno R, Hirayama A, Matsuda T, Asakura H, Nakashima M, Aoki N. Efficacy and safety of recombinant human soluble thrombomodulin (ART-123) in disseminated intravascular coagulation: results of a phase III, randomized, double-blind clinical trial. *J Thromb Haemost*. 2007;5:31–41.
16. Esmon CT. The regulation of natural anticoagulant pathways. *Science*. 1987;235:1348–1352.
17. Esmon C. Do-all receptor takes on coagulation, inflammation. *Nat Med*. 2005;11:475–477.
18. Sanders C. A method for the fractionation of the high-mobility-group non-histome chromosomal proteins. *Biochem Biophys Res Commun*. 1977;78:1034–1042.
19. Yamada S, Inoue K, Yakabe K, Imaizumi H, Maruyama I. High mobility group protein 1 (HMGB1) quantified by ELISA with a monoclonal antibody that does not cross-react with HMGB2. *Clin Chem*. 2003;49:1535–1537.
20. Erlandsson Harris H, Andersson U. Mini-review: The nuclear protein HMGB1 as a proinflammatory mediator. *Eur J Immunol*. 2004;34:1503–1512.
21. Taniguchi N, Kawahara K, Yone K, Hashiguchi T, Yamakuchi M, Goto M, Inoue K, Yamada S, Ijiri K, Matsunaga S, Nakajima T, Komiya S, Maruyama I. High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum*. 2003;48:971–981.
22. Schechter I, Berger A. On the size of the active site in proteases. I. Papain. *Biochem Biophys Res Commun*. 1967;27:157–162.
23. Huntington JA. Molecular recognition mechanisms of thrombin. *J Thromb Haemost*. 2005;3:1861–1872.
24. Page MJ, Macgillivray RT, Di Cera E. Determinants of specificity in coagulation proteases. *J Thromb Haemost*. 2005;3:2401–2408.
25. Esmon CT. The protein C pathway. *Chest*. 2003;124:26S–32S.
26. Suzuki K, Hayashi T, Nishioka J, Kosaka Y, Zushi M, Honda G, Yamamoto S. A domain composed of epidermal growth factor-like structures of human thrombomodulin is essential for thrombin binding and for protein C activation. *J Biol Chem*. 1989;264:4872–4876.
27. Huttunen HJ, Rauvala H. Amphoterin as an extracellular regulator of cell motility: from discovery to disease. *J Intern Med*. 2004;255:351–366.
28. Furmaniak-Kazmierczak E, Cooke TD, Manuel R, Scudamore A, Hoogendorn H, Giles AR, Nesheim M. Studies of thrombin-induced proteoglycan release in the degradation of human and bovine cartilage. *J Clin Invest*. 1994;94:472–480.
29. Conway EM, Nowakowski B. Biologically active thrombomodulin is synthesized by adherent synovial fluid cells and is elevated in synovial fluid of patients with rheumatoid arthritis. *Blood*. 1993;81:726–733.
30. Faust SN, Levin M, Harrison OB, Goldin RD, Lockhart MS, Kondaveeti S, Laszik Z, Esmon CT, Heyderman RS. Dysfunction of endothelial protein C activation in severe meningococcal sepsis. *N Engl J Med*. 2001;345:408–416.
31. Salmivirta M, Rauvala H, Elenius K, Jalkanen M. Neurite growth-promoting protein (amphoterin, p30) binds syndecan. *Exp Cell Res*. 1992;200:444–451.
32. Rapraeger AC. Syndecan-regulated receptor signaling. *J Cell Biol*. 2000;149:995–998.
33. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*. 2007;446:1030–1037.
34. Parkkinen J, Rauvala H. Interactions of plasminogen and tissue plasminogen activator (t-PA) with amphoterin. Enhancement of t-PA-catalyzed plasminogen activation by amphoterin. *J Biol Chem*. 1991;266:16730–16735.
35. Petricoin EF, Belluco C, Araujo RP, Liotta LA. The blood peptidome: a higher dimension of information content for cancer biomarker discovery. *Nat Rev Cancer*. 2006;6:961–967.
36. Villanueva J, Shaffer DR, Philip J, Chaparro CA, Erdjument-Bromage H, Olshen AB, Fleisher M, Lilja H, Brogi E, Boyd J, Sanchez-Carbayo M, Holland EC, Cordon-Cardo C, Scher HI, Tempst P. Differential exoprotease activities confer tumor-specific serum peptidome patterns. *J Clin Invest*. 2006;116:271–284.
37. Petricoin EF, Ardekani AM, Hitt BA, Levine PJ, Fusaro VA, Steinberg SM, Mills GB, Simone C, Fishman DA, Kohn EC, Liotta LA. Use of proteomic patterns in serum to identify ovarian cancer. *Lancet*. 2002;359:572–577.
38. Inoue K, Kawahara K, Biswas KK, Ando K, Mitsudo K, Nobuyoshi M, Maruyama I. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerosis plaques. *Cardiovasc Pathol*. 2007;16:136–143.
39. Porto A, Palumbo R, Pieroni M, Aprigliano G, Chiesa R, Sanvito F, Maseri A, Bianchi ME. Smooth muscle cells in human atherosclerotic plaques secrete and proliferate in response to high mobility group box 1 protein. *Faseb J*. 2006;20:2565–2566.
40. Kalinina N, Agrotis A, Antropova Y, DiVitto G, Kanellakis P, Kostolias G, Ilyinskaya O, Tararak E, Bobik A. Increased expression of the DNA-binding cytokine HMGB1 in human atherosclerotic lesions: role of activated macrophages and cytokines. *Arterioscler Thromb Vasc Biol*. 2004;24:2320–2325.

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