membrane, immunoreactive bands were visualized with an ECL detection system.

3. Results

3.1. HMGB1 is expressed by intimal VSMCs in human atherosclerotic lesions

In the current study, all CEA specimens were characterized by advanced fibroatheromas that were predominated by large eccentric atheromatous plaques, mainly composed of a lipid core (Fig. 1A). The region between the core and the endothelial luminal surface contained distinct clusters of foamy macrophages. Furthermore, closely layered VSMCs with lipid droplets were commonly observed on the lesion surface (Fig. 1B). Immunohistochemical staining of CEA specimens with anti-HMGB1 antibody showed that HMGB1-positive cells were exclusively localized in the intima of atherosclerotic plaques. HMGB1 immunoreactivity was abundant not only in macrophages but also in VSMCs (Fig. 1C). These HMGB1-positive cells were present in all samples examined in our study. In addition to carotid atherosclerotic lesions, HMGB1 expression was examined in human coronary atherosclerotic lesions obtained by DCA. DCA specimens were composed of both atherosclerotic and fibrous plaques. In atherosclerotic plaques, foamy macrophages and VSMCs with a variety of forms were observed. HMGB1 was conspicuously positive in both macrophages and spindle-shaped and foamy round VSMCs (Fig. 1D–F). HMGB1 immunoreactivity was observed in the cytoplasm (>85%) and nucleus (<5%) of these VSMCs (Fig. 1F). In contrast, HMGB1 expression was not detectable in atrophic VSMCs of fibrous plaques with scanty cellularity and normal medial VSMCs (Fig. 1G).

3.2. Myosin heavy-chain isoforms (SMembs) of HMGB1-positive VSMCs and coexpression of HMGB1 and RAGE by VSMCs in atherosclerotic plaques

To determine the characteristics of HMGB1-positive activated VSMCs, immunohistochemical analysis for myosin heavy-chain types of VSMCs was performed. The VSMCs distinctly expressed SMemb in atherosclerotic plaques, as well as HMGB1 (Fig. 2A). These VSMCs were also positive for SM1 immunoreactivity but were negative for SM2 (data not shown). RAGE is a specific HMGB1 receptor, and the interaction of HMGB1 with RAGE triggers cellular activation, resulting in the generation of key proinflammatory mediators [17]. Therefore, we examined whether these HMGB1-positive VSMCs expressed RAGE. As shown in Fig. 2B, most HMGB1-positive cells (80–90%) within the atherosclerotic intima also expressed RAGE.

3.3. Colocalization of IL-6, CRP, and NF-κB by HMGB1-positive VSMCs

A recent report has implicated that human VSMCs can produce CRP in response to inflammatory cytokines [11] and that these cytokines activate NF-κB followed by augmentation of IL-6, an instigator of CRP elaboration [18]. We examined the immunohistochemical detection of NF-κB, IL-6, and CRP in HMGB1-positive VSMCs. Double-labeled immunohistochemical results distinctly confirmed that significant numbers of HMGB1-positive VSMCs

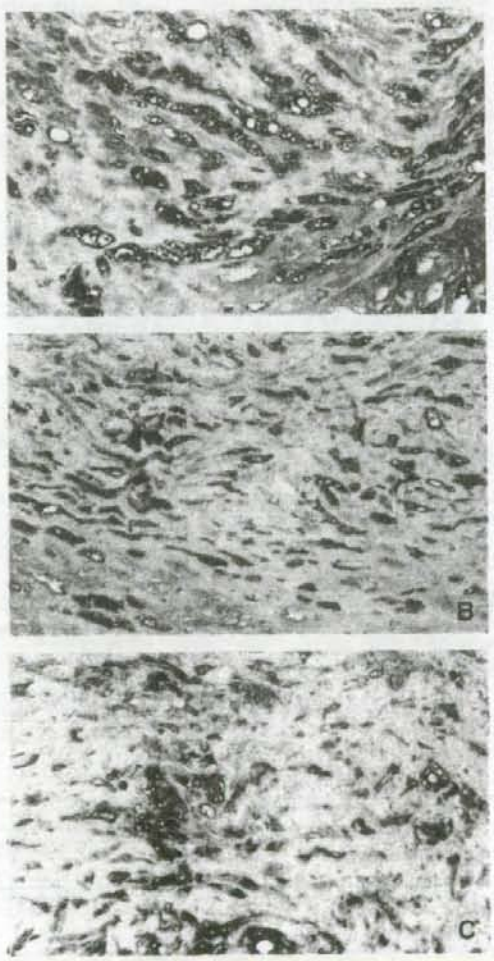

Fig. 3. Double immunohistochemical staining of serial sections of carotid atherosclerotic lesions for HMGB1 (brown) and NF-κB (blue) (A) (original magnification, ×120), IL-6 (blue) (B) (original magnification, ×80), and CRP (blue) (C) (Square c in Fig. 1A; original magnification, ×120). Many of the HMGB1-positive SMCs coexpress NF-κB, IL-6, and CRP.

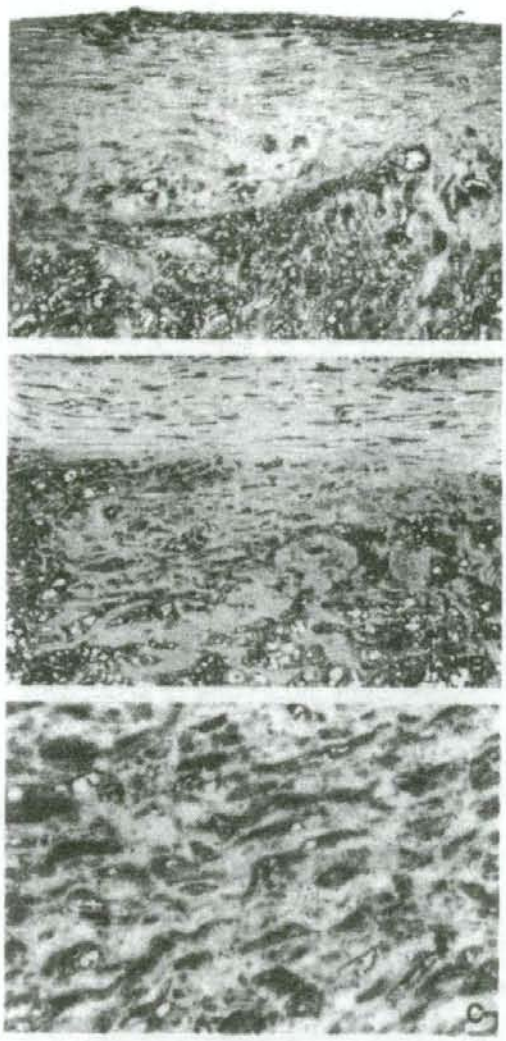

Fig. 4. Immunostaining of carotid atherosclerotic lesions for MMPs (MMP-2, MMP-3, and MMP-9). (A–C) Serial sections were stained with anti-MMP-3 (A) and anti-MMP-9 (B) antibodies (Square a in Fig. 1A; original magnification, ×60). Staining indicates that almost all VSMCs and macrophages contain immunoreactive MMPs. (C) Double immunostaining of the same intimal lesions for HMGB1 (blue) and MMP-2 (brown) (Square c in Fig. 1A; original magnification, ×120). Note the coexpression of HMGB1 and MMP-2 by these VSMCs.

coexpressed NF-κB (approximately 80%), IL-6 (70%), and CRP (80%) (Fig. 3A, B, and C).

3.4. Coexpression of HMGB1 and MMPs by VSMCs in atherosclerotic lesions

Expression of MMPs was examined in these VSMCs. As shown in Fig. 4, VSMCs and macrophages reacted with MMP-3 and MMP-9 antibodies in atheromatous plaques. Double immunostaining indicated coexpression of HMGB1 (blue) and MMP-2 (brown) (>65%) (Fig. 4C).

3.5. HMGB1 triggers CRP production and vice versa in VSMCs

To further confirm the above findings in vitro, we tested whether exogenous HMGB1 induced CRP production in cultured human VSMCs. For this purpose, cultured VSMCs were incubated with HMGB1 (1 μg/ml) or IL-6 (10 ng/ml) as a positive control for 48 h, and then CRP expression was examined in cultures at both mRNA and protein levels. As shown in Fig. 5A (upper panel), HMGB1 significantly stimulated CRP mRNA expression, as determined by the RT-PCR of cultured VSMCs. As expected, MMP-2 mRNA levels also increased as previously described [19]. Consistent with RT-PCR results, CRP protein levels, as determined by ELISA in culture medium, were also markedly increased in response to HMGB1 (Fig. 5B). We further investigated the possibility that a proinflammatory loop exists between

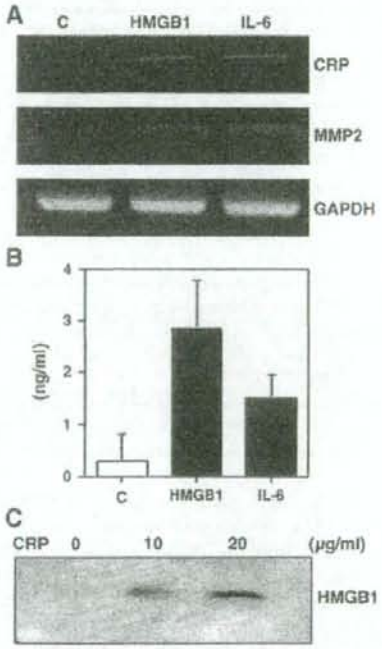

Fig. 5. HMGB1 induces CRP production and vice versa in cultured human VSMCs. VSMCs were incubated with HMGB1 (100 ng/ml) or IL-6 (10 ng/ml) for 48 h. mRNA expression of CRP or MMP-2 assayed by RT-PCR (A). Supernatants were analyzed for CRP protein levels by ELISA (B). *Statistically significant CRP production ($P<.05$, $n=3$) determined by Student's t test. Data shown are presented as mean±S.D. HMGB1 release by CRP treatment in VSMCs (C). VSMCs were incubated with CRP (0, 10, and 20 μg/ml) for 16 h. HMGB1 released in conditioned supernatant was detected by immunoblotting, as described in Methods.

HMGB1 and proinflammatory cytokines [5]. In this context, we examined whether CRP induces HMGB1 release in VSMCs. As shown in Fig. 5C, CRP stimulated HMGB1 release in a dose-dependent fashion. We repeated the experiments in triplicate and obtained the same positive results.

4. Discussion

It is especially noteworthy that HMGB1, which is a late mediator of inflammation, may play a crucial role in the progression of atherosclerosis. Recently, Kalinina et al. [9] reported that activated macrophages are a major source of HMGB1 in human atherosclerotic lesions. In that study, although VSMCs in fatty streaks and fibrofatty lesions of aorta showed HMGB1 immunoreactivity, the proportion of HMGB1-positive VSMCs was not significant, and its frequency was similar to that observed in normal aortic intima [9]. In contrast, we revealed that HMGB1 was highly expressed by intimal VSMCs in both carotid and coronary atherosclerotic arteries. The discrepancy of results may be due to the fact that specimens used in the present study had more advanced atherosclerotic lesions than those used by Kalinina et al. [9].

In the present study, the spindle-shaped and foamy round VSMCs clearly showed HMGB1 immunoreactivity virtually in the cytoplasm in the atherosclerotic arterial intima. These HMGB1-positive VSMCs expressed the nonmuscle-type SMemb. SMemb is predominantly expressed in undifferentiated VSMCs in the fetal stage and is reduced during vascular development [20]. Activated VSMCs in atherosclerotic plaques [20,21], as well as neointimal proliferative VSMCs after angioplasty [15], also express SMemb. Therefore, these results suggest that HMGB1-positive VSMCs are activated, possess features of a dedifferentiated state, and contribute to the development and progression of atherosclerogenesis.

In addition to these findings, most HMGB1-positive VSMCs expressed RAGE, NF-κB, IL-6, and CRP. HMGB1–RAGE interaction can trigger NF-κB activation [17], a transcription factor involved in the expression of genes encoding many proinflammatory functions of vascular cells [22], followed by induction of multiple cytokines, including IL-1β, IL-6, and TNF-α [23]. IL-6 is the principal stimulator of the production of acute-phase proteins of inflammation, such as CRP, in much larger quantities by hepatocytes [24]. Recently, we revealed that VSMCs in atherosclerotic plaques also express lymphotoxin-α (TNF-β) [12]. TNF-α can stimulate the production of HMGB1 in monocytes/macrophages [2,5], and HMGB1 can be a regulator of proinflammatory cytokine synthesis, including TNF-α and vice versa [4,5]. Thus, these results suggest that HMGB1 may play an important role in inflammatory cascade in activated VSMCs. Here, we demonstrated that HMGB1 induced CRP production by cultured VSMCs. Taken together, our findings may provide direct evidence that VSMCs are one of the sources of locally produced CRP in response to HMGB1 in human atherosclerotic plaques.

Several lines of evidence suggest that CRP may actively promote a proatherosclerotic and proinflammatory phenotype in the vascular wall [10] and also increase the vulnerability of atherosclerotic plaques toward rupture [25]. There is pathologic evidence that the immunoreactivity of CRP in coronary atheromatous plaques is increased in culprit lesions of unstable angina [26]. Balloon and Lozanski [27] demonstrated the potential existence of a positive feedback mechanism for the induction of several cytokines, such as IL-6 and TNF-α, by CRP in human monocytes/macrophages. Our results showed that CRP significantly induced HMGB1 release in VSMC culture medium. In the mechanism of HMGB1 release, acetylation by coactivators such as CREB-binding protein is required for HMGB1 release [28,29]. Thus, when HMGB1 is acetylated in the nucleus, HMGB1 function in the nucleus might be lost and may acquire cytokine function. We have previously demonstrated that hypernuclear acetylation (HNA) occurs in the nucleus of atherosclerotic VSMCs [16], suggesting that HMGB1 might be acetylated in atherosclerotic lesions. Furthermore, thrombin (a humoral factor known to cause the activation and proliferation of VSMCs) and serotonin (data not shown) strongly potentiated HNA via ERK1/2 in cultured VSMCs [16]. It is suggested that CRP might also induce HNA in atherosclerotic regions.

These findings indicate a possibility that a proinflammatory loop exists between HMGB1 and CRP via not only macrophages but also VSMCs in human atherosclerotic lesions.

Furthermore, the synthesis and release of MMPs by vascular cells have been pointed out as one of the important mechanisms in the development of atherosclerotic lesions [30]. It has been revealed that certain cytokines stimulate MMP production in human VSMCs [31]. MMPs secreted by VSMCs directly facilitate their migration [32], and HMGB1 has been documented to be a potent chemoattractant for VSMCs [33]. MMPs also play an important role in matrix degradation followed by rupture of arterial atherosclerotic plaques [34]. Increased expression of MMPs by VSMCs has been demonstrated in human vascular vulnerable plaques [35,36]. As shown in the present immunohistochemical study, atherosclerotic VSMCs coexpressed HMGB1 and MMP-2. In addition to morphological findings, we also showed that HMGB1 could stimulate the expression of MMP-2 mRNA by cultured VSMCs in vitro.

In summary, the present study provides preliminary evidence that HMGB1 is produced by VSMCs, especially those cells with a SMemb-positive dedifferentiated phenotype, and can directly stimulate the production of both CRP and MMP in VSMCs. In agreement with published studies, HMGB1 provides all the hallmarks of a molecule that can promote several of the processes that set the stage for atherosclerosis and plaque vulnerability. Thus, HMGB1

may serve as one potential target for therapeutic intervention in ischemic atherosclerotic diseases.

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Increased serum high mobility group box-1 level in Churg–Strauss syndrome

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Summary

Churg–Strauss syndrome (CSS) is a rare form of systemic vasculitis occurring in patients with asthma and hypereosinophilia; however, its mechanisms involved in the severe tissue inflammation with vasculitis are poorly understood. High mobility group box 1 (HMGB1) protein, originally identified as a DNA binding protein, also has potent pro-inflammatory and proangiogenic properties. In this study, we hypothesized that HMGB1 might be associated with CSS, and examined serum HMGB1 levels and compared those of asthma patients and healthy volunteers. We also investigated HMGB1 expression in the lesion, and eosinophil HMGB1 amount in CSS patients. We found that the serum HMGB1 levels in CSS patients were significantly higher than those of asthma patients and healthy volunteers. Eosinophils in the CSS lesion expressed HMGB1 and HMGB1 level in eosinophils from CSS patients was significantly higher than that of asthma patients, while there was no significant difference in HMGB1 levels in peripheral mononuclear cells. The serum HMGB1 level in CSS patients decreased after the steroid therapy, and showed significant positive correlations with several molecules, including soluble interleukin-2 receptor, soluble thrombomodulin, and eosinophil cationic protein in sera. We propose that HMGB1 might contribute to the pathogenesis of CSS.

Keywords: eosinophil, inflammation, vasculitis

Introduction

Churg–Strauss syndrome (CSS) is a primary systemic vasculitis of mainly small and intermediate vessels with a propensity for lung involvement [1]. Analysis of tissue biopsy specimens from patients with CSS shows an eosinophil-rich inflammatory infiltrate with granuloma formation in connective tissue [1,2]. It is commonly thought of as an autoimmune disease [3], but the mechanisms involved in the severe tissue inflammation with vasculitis are poorly understood [4].

High mobility group box-1 (HMGB1) is a widely expressed member of the HMGB family of chromosomal proteins [5]. It exerts nuclear functions by interacting with specific DNA structures or after recruitment by various DNA binding proteins [5]. Recent studies have demonstrated surprising cytokine-like roles for extracellular HMGB1 [6]. Indeed, HMGB1 released by injured or necrotic cells acts as a signalling molecule, inducing local inflammatory responses [7]. Also, HMGB1 is actively secreted by monocytes stimulated by cytokines and LPS [8]. In turn, extracellular HMGB1 regulates cytokine expression [9,10] and promotes inflammatory cell recruitment [7,10]. Moreover, HMGB1 stimulates the migration of adherent cells, such as fibroblasts and smooth muscle cells [11]. Thus, extracellular HMGB1 can be regarded as both a signal of tissue injury and a mediator of inflammation. In addition, HMGB1 exerts proangiogenic effects by inducing MAPK $ERK_{1/2}$ activation [12].

In this study, we investigated 18 patients with CSS and found increased serum levels of HMGB1 compared with asthma. HMGB1 might contribute to the pathogenesis of CSS.

Materials and methods

Patients

This study was reviewed and approved by the Kagoshima University Faculty of Medicine Committee on Human Research. We investigated 18 patients with CSS who were admitted to the Division of Respiratory Medicine,

Respiratory and Stress Care Centre, Kagoshima University Hospital, from 1995 to 2005. There were 8 men and 10 women whose mean age was 58·2 ± 18·2 years old (mean ± standard deviation). For comparison, we also investigated 19 patients with asthma (male : female = 8 : 11, 58·9 ± 15·3 years old), 12 healthy volunteers (male : female = 5 : 7, 58·9 ± 13·1 years old), and 8 patients with rheumatoid arthritis (RA) patients (male : female = 3 : 5, 53·5 ± 15·2 years old). The diagnosis of CSS was made according to the 1990 edition of CSS published by the American College of Rheumatology [13]. All patients with CSS fulfilled more than five criteria. We excluded patients with rheumatoid arthritis, diabetes mellitus, acute or chronic liver disease, and immunological abnormalities that predispose to opportunistic infection. Peripheral leucocyte counts before the start of therapy were also determined.

Measurement of serum HMGB1 and cytokines

In the patients with CSS, asthma and acute bronchitis, we measured serum levels of HMGB1 before the patients underwent therapy and 3 months after the start of therapy. All participants gave written consent to participate in this study. Also we measured serum soluble interleukin receptor-2 (sIL-2R), soluble thrombomodulin (sTM), and eosinophil cationic protein (ECP) levels before the start of therapy because these cytokines have been reported to be elevated in sera of CSS patients [14,15]. Enzyme-linked immunosorbent assay (ELISA) for HMGB1 in the sera was performed with the use of monoclonal antibodies to HMGB1 and with standardization to a curve of recombinant human HMGB1 as described previously [16]. Briefly, a polystyrene microtiter plate was coated with 100 µl of 3 mg/l anti-HMGB1 polyclonal antibody (Shino-TEST, Kanagawa, Japan) and incubated at 37 °C overnight. The unbound antibodies were removed by washing plates three times with PBS containing 0·05% Tween 20 (washing buffer) and the remaining binding sites in the wells were blocked by incubating the plates for 2 h with 400 µl/well PBS containing 1% BSA. After washing, 100 µl of each dilution of the standards and samples was added to the wells. The microtiter plates were incubated for 24 h at room temperature. After washing, 100 µl/well of anti-human HMGB1 peroxidase-conjugated monoclonal antibody (Shino-TEST) was added and the plate was incubated at room temperature for 2 h. After washing, 3,3,′,5,5′-tetramethylbenzide was added to the well. The enzyme reaction was allowed to proceed for 30 min at room temperature. The chromogenic substrate reaction was stopped by addition of stop solution (0·35 mol/l Na_2SO_4) and the absorbance was read at 450 nm. The serum levels of sIL-2R, sTM and ECP were measured using a commercial ELISA kit (sIL-2R, R & D Systems, Minneapolis, MN, USA; sTM, Tepnel Lifecodes Corp., Stamford, CT, USA; ECP, Medical & Biological Laboratories Co., Ltd, Nagoya, Japan) according to the manufacturer's protocols.

Immunohistochemical s taining for HMGB1

Immunohistochemical staining for HMGB1 was performed using a rabbit polyclonal antibody (Shino-TEST) employing the DAB method using the biopsy samples of five CSS patients as described previously [17]. Briefly, 4-µm thick sections were mounted on poly L-lysine-coated slides, dewaxed, and washed in Tris-buffered saline (pH 7·4) for 10 min. For optimal antigen retrieval, the sections were pressure cooked in 0·01 M citrate buffer (pH 6·0) for 90 s. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide solution in methanol for 10 min. Following two washes in phosphate buffered saline (PBS) containing 1% saponin, the blocking reaction was performed as reported previously [18]. The sections were incubated with a 1 : 400 dilution of the primary antibody solution for 2 h at room temperature. Negative control slides were incubated with rabbit IgG (R & D systems, Minneapolis, MN, USA). Secondary biotinylated anti-immunoglobulin antibodies (R & D systems) were added, and the mixture was incubated for 30 min at room temperature. Following washing, the sections were incubated with streptavidin conjugated to horseradish peroxidase (Amersham, Arlington Heights, IL, USA) and then rinsed with deionized water. DAB substrate solution was added, and the mixture was incubated for 10 min. A positive result was indicated by a brown colour reaction.

Preparation of cells and Western blotting for HMGB1

To access the amount of HMGB1 in eosinophils, peripheral eosinophils were selected from five different CSS patients and asthma patients using magnetic beads as described previously [19]. Briefly, human eosinophils were obtained from heparinized venous blood of the CSS and asthma patients before they underwent therapy. Heparinized venous blood was mixed with a quarter volume of 2% dextran solution (Sigma-Aldrich Corp., St Louis, MO, USA) to precipitate red blood cells. After incubation for 30 min at room temperature, the leucocyte-rich plasma was laid onto Histopaque (Sigma-Aldrich Corp., St Louis, MO, USA) and centrifuged at 800 × *g* for 20 min at room temperature. Granulocytes were separated from erythrocytes by lysis in 0·2% NaCl and washed in phosphate-buffered saline (PBS) three times at 4 °C; next, eosinophils were isolated by negative selection using magnetic beads (Eosinophil Isolation Kit; Miltenyi Biotec GmbH, Bergisch, Germany) according to the manufacturer's protocol. Eosinophils were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal calf serum (FCS) and streptomycin/penicillin (the complete medium). The purity of eosinophils was more than 99% by

morphological examination after staining with Diff-Quick (Wako, Tokyo, Japan). Also, peripheral blood mononuclear cells (PBMCs) were separated from heparinized venous blood of CSS and asthma patients by Histopaque gradient centrifugation as described previously [20].

Western blot analysis was performed as previously described [21,22]. Briefly, 1×10^6 eosinophils or 1×10^7 PBMCs were collected and lysed on ice for 20 min in 1 ml of lysis buffer containing 50 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulphonic acid (HEPES), 150 mM NaCl, 1% Triton X-100, 10% glycerol, and a cocktail of protease inhibitors (Roche, Indianapolis, IN, USA). The lysates were spun, and the 20-μl supernatants were collected and the same volume, i.e. 20 μl of double-strength sample buffer (20% glycerol, 6% sodium dodecyl sulphate (SDS), 10% 2-mercaptoethanol) was added. The samples were boiled for 10 min. Proteins were analysed on 12% polyacrylamide gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose membranes at 150 mA for 1 h by using a semi-dry system. The membranes were incubated with rabbit polyclonal anti-HMGB1 antibody (Shino-TEST) or mouse anti-human actin monoclonal antibody (Santa-Cruz Biotechnology Inc., Santa-Cruz, CA, USA) followed by a sheep anti-rabbit or mouse IgG coupled with horseradish peroxidase. Peroxidase activity was visualized by the Enhanced Chemiluminescence detection system (GE Healthcare, Little Chalfont, Bucks, UK).

Statistical analysis

We used one-way factorial analysis of variance (ANOVA) with the Bonferroni-Dunn test, Mann–Whitney test, or Pearson's correlation coefficient. A P-value below 0·05 was considered significant. Most values were expressed as mean ± standard deviation (s.d.).

Results

As shown in Fig. 1, the serum HMGB1 level was significantly higher than that in asthma patients, healthy volunteers and RA patients (CSS, 16·1 ± 8·65 pg/m; asthma, 0·44 ± 0·41 pg/ml; healthy volunteers, 0·46 ± 0·48 pg/ml; RA, 4·81 ± 3·53 pg/ml). There was no significant difference in serum HMGB1 levels among the asthma patients, healthy volunteers and RA patients. The sensitivity and specificity for the diagnosis of CSS was 94·4% and 100%, respectively (cut-off = 1·6 pg/ml, $P < 0·001$). The serum HMGB1 level in CSS patients significantly decreased 3 months after the start of therapy (Fig. 1b). The clinical symptoms of CSS patients improved with the use of corticosteroids. The serum HMGB1 level after the therapy in CSS patients was significantly higher than that of asthma patients (after the therapy, 5·41 ± 3·21 pg/ml; asthma, 0·44 ± 0·41 pg/ml; $P < 0·01$, Mann–Whitney test). In CSS, no patient suffered from infectious disease, including sepsis. Anti-neutrophil cytoplasmic antibody (ANCA) was positive in 10 CSS patients. The serum HMGB1 level in ANCA-positive CSS patients was significantly higher than that in ANCA-negative CSS patients ($P < 0·01$, ANCA-positive = 21·04 ± 6·77 pg/ml, ANCA-negative = 9·91 ± 6·65 pg/ml).

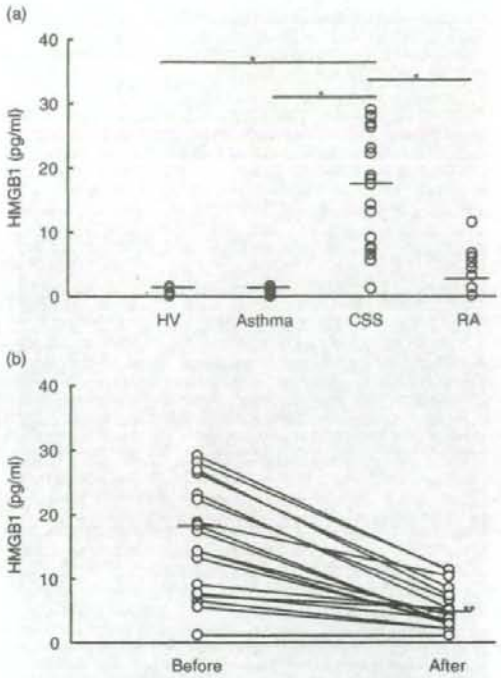

Fig. 1. (a) Comparison of serum HMGB1 level among four groups. The serum HMGB1 level in CSS patients was significantly higher than that in asthma patients, healthy volunteers and rheumatoid arthritis patients (HV, healthy volunteer; RA, rheumatoid arthritis; $^*P < 0·001$, Bonferroni-Dunn with one-way factorial ANOVA). (b) Change of serum HMGB1 level before and after the therapy in CSS patients. The serum HMGB1 level decreased significantly 3 months after the steroid therapy ($^{**}P < 0·01$, Mann–Whitney test).

In immunohistochemical analysis, tissue infiltrating inflammatory cells including eosinophils and blood vessel cells stained positive for HMGB1 (Fig. 2).

We compared other serological markers of CSS such as eosinophil counts, serum sIL-2R, serum sTM and ECP levels with serum HMGB1 levels. As shown in Fig. 3, peripheral eosinophil counts, serum sIL-2R level, serum sTM level and serum ECP level showed significant positive correlation with serum HMGB1 level in CSS patients. Serum HMGB1 level did not show significant correlation with peripheral lymphocyte counts ($r = 0·189$, $P = 0·22$) and peripheral neutrophil counts ($r = 0·213$, $P = 0·112$).

Finally, we compared the HMGB1 level in eosinophils and PBMCs between CSS patients and asthma patients. As shown

Fig. 2. Immunohistochemical staining for HMGB1. Eosinophils and vascular endothelial cells stained intense positive for HMGB1 (×300 original magnification; (a) hematoxilin eosin staining; (b) HMGB1 staining; (c) negative control). Representative data from five different CSS patients.

in Fig. 4, the HMGB1 level in eosinophils from CSS patients was significantly higher than that in eosinophils from asthma patients. However, the HMGB1 level in PBMCs of CSS patients was not different from that in PBMCs from asthma patients.

Discussion

To our knowledge, this is the first report describing HMGB1 in CSS. HMGB1 appears to have two distinct functions in cellular systems. First, it has been shown to have an intracellular role as a regulator of transcription [23] and, second, an extracellular role in which it promotes tumour metastasis [24] and inflammation [25]. HMGB1 acts as a late mediator of lethal endotoxaemia [25], a mediator of acute lung injury [9] and as a pro-inflammatory cytokine [10]. Serum levels of HMGB1 have been directly associated with mortality in patients with lethal sepsis [25], suggesting that HMGB1 may be a crucial member of the uncontrolled pro-inflammatory response associated with fatal outcome. HMGB1 can be released from cells of the macrophage/monocyte lineage following activation by pro-inflammatory stimuli [26] and has been reported to be involved in the inflammation of autoimmune disease such as rheumatoid arthritis [27], systemic lupus erythematosus [28], and ulcerative colitis [29]. HMGB1 is a possible target antigen of ANCAs [30] and its binding with the receptor for advanced glycation end products acts as a potent angiogenic molecule [12]. In our study, serum HMGB1 level was significantly higher than that of asthma patients even after the therapy. The serum HMGB1 in ANCA-positive CSS patients was significantly higher than those in ANCA-negative CSS patients, and serum HMGB1 level showed significant positive correlation with several molecules that have been reported to have association with the pathogenesis of CSS [14,15].

Prominent eosinophilia is one of the defining features of CSS [31]. Its magnitude commonly reflects clinical disease activity and in many situations eosinophil suppression results in clinical improvement [31–33]. CSS can affect virtually any organ system in the body [34,35], and therefore, systemic symptoms are prominent in CSS [1,2]. Analysis of tissue biopsy specimens from patients with CSS shows an eosinophil-rich inflammatory infiltrate with granuloma formation in connective tissue and blood-vessel walls [1,2]. Thus, eosinophils play an important role in the pathogenesis of CSS. In our study, eosinophils stained positive for HMGB1 and serum HMGB1 level showed significant positive correlation with peripheral eosinophil counts. In addition, the HMGB1 level in eosinophils from CSS patients was significantly higher than that in eosinophils from asthma patients, while there was no significant difference in HMGB1 level in PBMCs between CSS patients and asthma patients. HMGB1 is one of the candidates that can induce eosinophil chemotaxis [36]. HMGB1 also can bind interleukin-5 promoter [37] and induce the secretion of interleukin-5 [38], which play a pivotal role in the control of eosinophils [39]. Taken together, we think it might be possible that HMGB1 can work as an autocrine factor regulating eosinophil function, and therefore, we propose that HMGB1 might contribute to the pathogenesis of CSS. HMGB1 antagonists provide a relatively wide window for therapeutic intervention in some experimental disease models such as sepsis [40]. Further studies addressing HMGB1 in CSS might bring a new insight to clarify the pathogenesis of CSS.

Churg–Strauss syndrome (CSS) is a rare disorder that is characterized by asthma, hypereosinophilia, and evidence

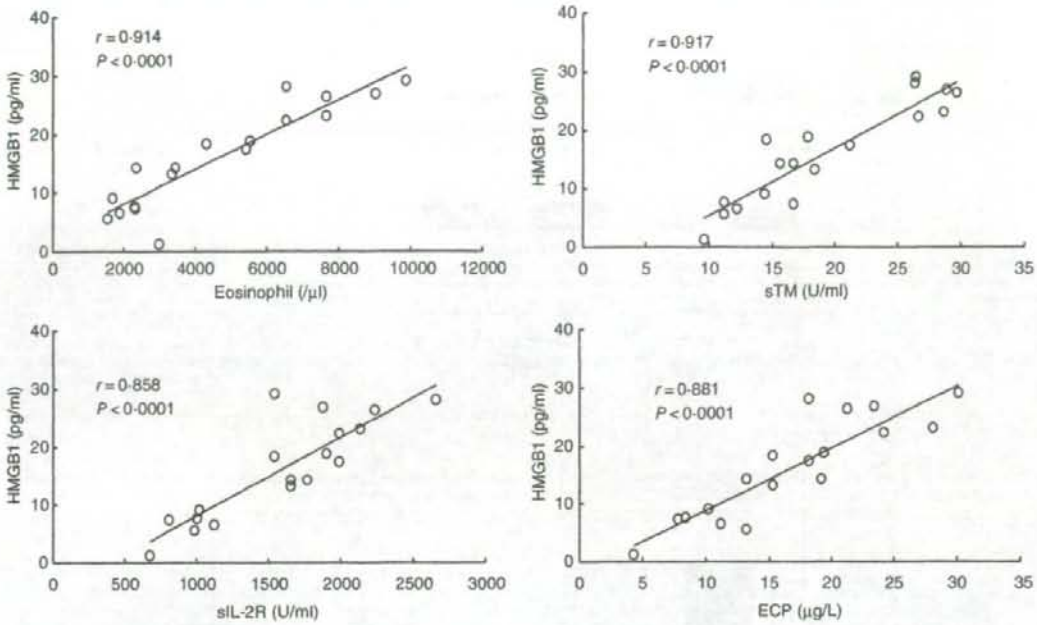

Fig. 3. Correlation between serum HMGB1 level with serum cytokine levels in CSS patients. The serum HMGB1 level showed significant positive correlation with peripheral eosinophil counts, serum soluble interleukin-2 receptor (sIL-2R) levels, serum soluble thrombomodulin (sTM) levels, and serum eosinophil cationic protein (ECP) levels.

of vasculitis with massive infiltration of eosinophils affecting a number of organs [1]. The prevalence is of the order of 1·3/100 000 in the general population, compared with 3·3 for polyarteritis nodosa and 5·3 for Wegener's granulomatosis [41,42]. Successful treatment of this rare syndrome needs prompt differentiation of CSS from asthma alone [1,2]; however, there have been few serological markers to distinguish CSS from asthma. Therefore, we examined the clinical value of serum HMGB1 measurement to distinguish CSS from asthma. The sensitivity and specificity of serum HMGB1 levels of 1·6 pg/ml to distinguish CSS from asthma were 94·4% and 100%, respectively. Abnormal laboratory findings in patients with CSS include increased peripheral blood eosinophil count and a raised ESR [1,43]. However, it is sometimes difficult to distinguish CSS from asthma using these markers because of the following reasons: (i) rarely, eosinophilia is not present and wide-ranging and rapid changes in eosinophil counts happen in CSS [31]; (ii) use of corticosteroids to treat asthma may result in failure to detect eosinophilia in patients with undiagnosed CSS; (iii) increase of ESR occurs in other disorders such as infection. Other serological markers, ANCA, are present in 44% to 66% of CSS patients, with the most common pattern being perinuclear [32,44,45]. In addition, ANCA-positivity needs to be confirmed by demonstration of myeloperoxidase in serum [2]. In our study, the serum HMGB1 level of CSS patients after the therapy was significantly higher than those of asthma patients even after the therapy. Therefore, we propose that measurement of serum VEGF might become one of the useful serum markers to distinguish CSS from asthma, as a positive result greatly increases the likelihood of CSS. However, our study is too small and short to draw definitive conclusions. We propose that larger and longer-scale studies addressing this point are necessary to judge its diagnostic value.

In conclusion, we propose the possible involvement of HMGB1 in the pathogenesis of CSS. However, the origin of HMGB1 is not clear from the results of our study, and it is curious that the serum HMGB1 level is increased in other systemic vasculitis conditions such as microscopic angitis and Wegener's granulomatosus. Our next goal is to clarify these points.

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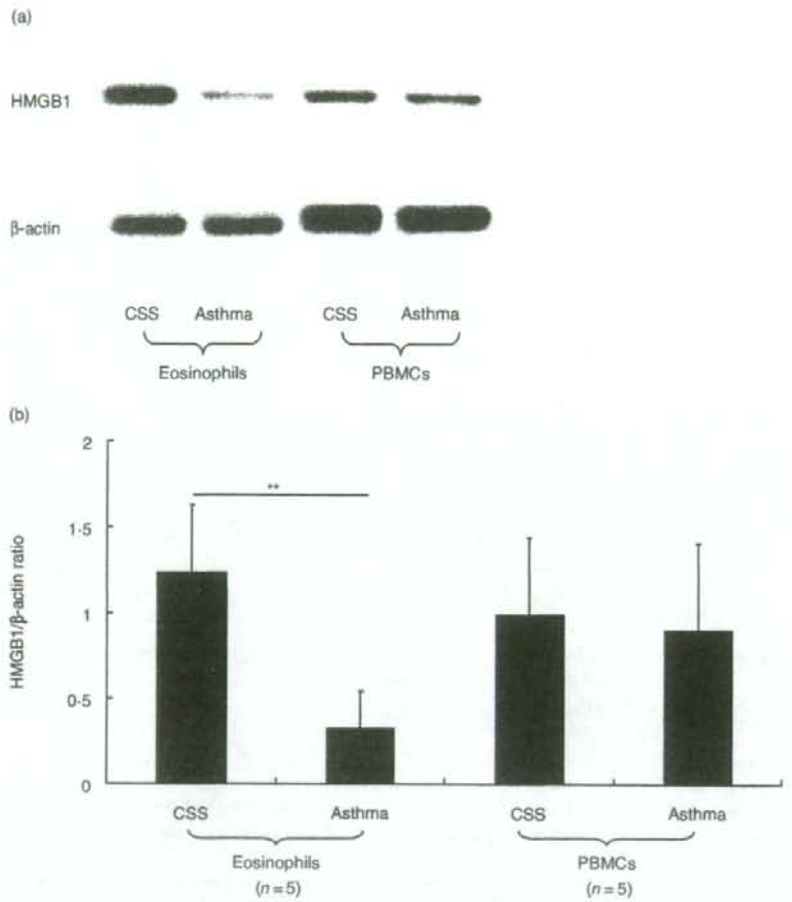

Fig. 4. Comparison of HMGB1 amount in eosinophils and PBMCs between CSS patients and asthma patients. The HMGB1 amount in eosinophils from CSS patients was significantly higher than that of asthma patients. However, there was no significant difference in HMGB1 level in PBMCs between CSS patients and asthma patients (a, representative result; b, data from five different patients in each group; $^{**}P < 0{\cdot}01$, Bonferroni-Dunn test with one-way factorial ANOVA).

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ORIGINAL ARTICLE

High-mobility group box 1 protein promotes development of microvascular thrombosis in rats

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Summary. *Background:* Sepsis is a life-threatening disorder resulting from systemic inflammatory and coagulatory responses to infection. High-mobility group box 1 protein (HMGB1), an abundant intranuclear protein, was recently identified as a potent lethal mediator of sepsis. However, the precise mechanisms by which HMGB1 exerts its lethal effects in sepsis have yet to be confirmed. We recently reported that plasma HMGB1 levels correlated with disseminated intravascular coagulation (DIC) score, indicating that HMGB1 might play an important role in the pathogenesis of DIC. *Objectives:* To investigate the mechanisms responsible for the lethal effects of HMGB1, and more specifically, to explore the effects of HMGB1 on the coagulation system. *Methods:* Rats were exposed to thrombin with or without HMGB1, and a survival analysis, pathologic analyses and blood tests were conducted. The effects of HMGB1 on the coagulation cascade, anticoagulant pathways and surface expression of procoagulant or anticoagulant molecules were examined *in vitro*. *Results:* Compared to thrombin alone, combined administration of thrombin and HMGB1 resulted in excessive fibrin deposition in glomeruli, prolonged plasma clotting times, and increased mortality. *In vitro*, HMGB1 did not affect clotting times, but inhibited the anticoagulant protein C pathway mediated by the thrombin–thrombomodulin complex, and stimulated tissue factor expression on monocytes. *Conclusions:* These findings demonstrate the procoagulant role of HMGB1 *in vivo* and *in vitro*. During sepsis, massive accumulation of HMGB1 in the systemic circulation would promote the development of DIC.

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Introduction

Sepsis is a complex clinical syndrome resulting from systemic inflammatory and coagulatory responses to infection [1,2]. 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 have revealed dramatic effects in animal models of sepsis. However, in humans, most of these strategies have not improved survival of septic patients [2]. Coagulopathy is another important feature of sepsis, and 30–50% of patients show the most severe clinical form, called disseminated intravascular coagulation (DIC) [3]. Intravascular fibrin deposition leads to obstruction of the microvascular bed, resulting in development of multiple organ dysfunction syndrome (MODS), including renal insufficiency and acute respiratory distress syndrome [4]. As DIC and MODS are associated with poor outcomes, anticoagulant therapy during sepsis seems to be a valuable option. Activated protein C (APC), an anticoagulant with a broad spectrum of anti-inflammatory effects, led to improved survival in patients with severe sepsis [5]. However, because of bleeding complications, the use of APC therapy needs to be carefully considered in relation to risks and benefits [6]. Thus, it is highly desirable to identify novel targets for the treatment of sepsis.

High-mobility group box 1 protein (HMGB1) is one promising therapeutic target for the treatment of sepsis. Blockade of HMGB1, even at later time points after onset of infection, was shown to rescue mice from lethal sepsis [7,8]. HMGB1 has pleiotropic effects both inside and outside the cell. In the cell nucleus, HMGB1 bends DNA, and promotes protein assembly on DNA targets [9]. When released from necrotic or activated cells, extracellular HMGB1 triggers inflammation, immune responses, and tissue regeneration

[10–13]. HMGB1 also acts as a lethal mediator in conditions such as sepsis, where serum HMGB1 levels are significantly increased [7,8]. However, the precise mechanisms by which HMGB1 exerts its lethal effects in sepsis have yet to be confirmed.

HMGB1 may induce lethality through multiple mechanisms. Proinflammatory activity of HMGB1 is one possible mechanism [14,15]. Procoagulant effects may be another mechanism. We recently reported that plasma HMGB1 levels correlated with DIC score and sepsis-related organ failure assessment score [16], indicating that HMGB1 might play a role in the pathogenesis of DIC and MODS. To confirm this idea, we explored the effects of HMGB1 in a thrombin-induced DIC rat model. We found that HMGB1 promoted development of microvascular thrombosis, and increased the rate of mortality.

Materials and methods

In vivo *DIC model*

Experiments involving animals were approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Kagoshima, Japan, and were conducted according to the National Institute of Health guidelines. Male Sprague–Dawley rats, each weighing 190–230 g, were used for all experiments. Before use, animals were randomly divided into groups, as indicated in Fig. 1A, were fed with regular chow, and had free access to drinking water for more than 1 week. Bovine thrombin (Mochida Pharmaceutical, Tokyo, Japan) was administered in one of the tail veins by continuous infusion at a rate of 1250 U kg^{-1} h^{-1} for 4 h. This infusion rate, which induced organ dysfunction in a reversible manner but did not affect survival, was determined in a preliminary experiment. One hour after treatment initiation, 0.4 mg kg^{-1} or 2 mg kg^{-1} HMGB1, prepared from calf and porcine thymus (a gift from Shino-Test Corporation, Sagamihara, Japan) [17], was administered as a bolus injection. The theoretical maximum HMGB1 concentration in plasma was 53 μg mL^{-1} when 2 mg kg^{-1} HMGB1 was administered as a bolus injection; however, the actual plasma HMGB1 concentrations at 5 h after administration were as low as 1.06 ± 0.68 ng mL^{-1}. It is likely that plasma HMGB1 levels of these rats are comparable with those of DIC or septic patients [7,16,17]. As a control, an equal volume of physiologic saline was administered instead of thrombin and/or HMGB1. Survival was monitored for up to 1 week (n = 10 per group). Blood tests and pathologic analyses were performed 6 h after treatment initiation (n = 5 per group).

Blood tests and pathologic analyses

Six hours after treatment initiation, blood was collected from ether-anesthetized rats, and was anticoagulated with either sodium citrate or EDTA. Anticoagulated blood with sodium

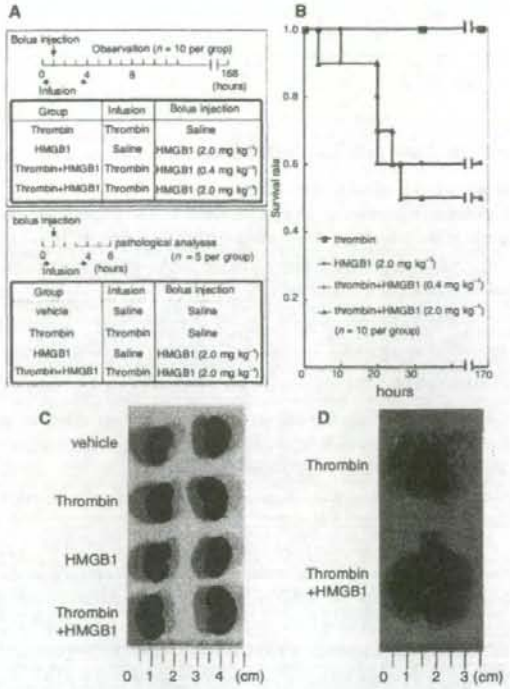

Fig. 1. High-mobility group box 1 protein (HMGB1) exacerbates renal and lung injuries and increases mortality in a thrombin-induced disseminated intravascular coagulation (DIC) model. (A) Experimental designs for survival analysis (upper panel) and pathologic analyses (lower panel) in rats. Thrombin and HMGB1 were administered i.v. by continuous infusion and by a bolus injection, respectively. As control, an equal volume of saline was administered instead of thrombin and/or HMGB1. Survival was monitored for up to 1 week. Pathologic analyses were performed 6 h after treatment initiation. (B) Survival curves of rats treated with thrombin, HMGB1 (2.0 mg kg^{-1}), thrombin plus HMGB1 (0.4 mg kg^{-1}), and thrombin plus HMGB1 (2.0 mg kg^{-1}). n = 10 per group. (C) Representative appearances of kidneys in each group. (D) Representative appearances of lungs in the thrombin-treated group and the thrombin plus HMGB1-treated group.

citrate (3.8% w/v) was centrifuged immediately for 15 min at 1710 × g, and the plasma supernatant was separated. Prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen were measured by standard assays in an automatic coagulometer (CA-5000; Sysmex Corporation, Kobe, Japan). Complete blood counts were performed with EDTA-anticoagulated blood, using an automated counting device (ADVIA120; Bayer Diagnostics, Dublin, Ireland).

After collection of blood samples, histopathologic analyses were performed. Firstly, organ appearance was examined macroscopically. Then, sections of formaldehyde-fixed and paraffin-embedded organs were examined microscopically. The sections were stained with either hematoxylin and eosin or phosphotungstic acid hematoxylin. Fibrin deposition was semiquantitated and given a score of 0–4 as follows: 0, no

fibrin deposition; 1, up to 25% of glomerular cross-section positive for fibrin deposition; 2, 25–50%; 3, 50–75%; and 4, more than 75%. Each group contained five rats, and 40 glomeruli per rat were evaluated in a blinded fashion.

Measurement of inflammatory cytokines

Interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) concentrations in rat plasma, collected 6 h after treatment initiation, were determined using ELISA kits for rat IL-6 and rat TNF-α respectively, as recommended by the manufacturer (BIOSOURCE, Camarillo, CA, USA).

Effects of HMGB1 on clotting time and on thrombomodulin (TM) function

For the *in vitro* clotting assay, we pooled plasma taken from five healthy volunteers, who had given their informed consent. Thrombin time (TT) and PT were measured by standard assays using an automatic coagulometer (KC1 Delta; Trinity Biotech, Bray, Ireland).

The protein C-activating cofactor activity of TM was evaluated by the modified method of Suzuki *et al.* [18], in the presence or absence of HMGB1. Briefly, recombinant human soluble TM, TM-derived peptide P-D1, or TM-derived peptide $P\text{-}D_{2+3}$ (final concentration 0.2 nM), prepared as described previously [18,19], was incubated with HMGB1 (final concentration, 0, 1, 10 or 100 nM) at 37 °C for 30 min in a mixture of 50 mM Tris–HCl, 2 mM $CaCl_2$ and 0.1 M NaCl (pH 8.0), containing 0.1% bovine serum albumin. Then, thrombin (final concentration, 1 U mL^{-1}) and protein C (final concentration, 20 nM; donated from Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan) were added, and the mixture was incubated at 37 °C for 60 min. Activation of protein C was terminated by adding antithrombin (Sigma-Aldrich, St Louis, MO, USA) and heparin (Mochida Pharmaceutical, Tokyo, Japan). The amount of APC formed during the reaction was then determined with a second assay using a chromogenic substrate (300 μg mL^{-1} S-2238; Chromogenix, Milan, Italy) in a mixture of 20 mM Tris–HCl and 0.1% NaCl (pH 7.4), containing 0.1% bovine serum albumin. Thrombin-mediated cleavage of S-2238 was blocked by antithrombin and heparin in all experiments, and residual thrombin-mediated cleavage of S-2238 was defined as 0 U in this assay. As a positive control, the activity of the ready-made APC (donated from Chemo-Sero-Therapeutic Research Institute) was measured in the presence or absence of HMGB1 (100 nM). The experiment was performed in triplicate. The endothelium-mediated protein C activation assay was performed as above, except that 0.05×10^6 human umbilical vein endothelial cells (HUVECs; Cambrex, Walkersville, MD, USA) per well in a 48-well plate were used instead of recombinant human soluble TM. Kinetic analyses were performed by measuring the activation of protein C in the presence of varying concentrations of protein C (0.1–1 μM), and fixed concentrations of TM (0.2 nM) and thrombin (0.5 U mL^{-1}), with or without HMGB1 (1 μM). The amount of APC generation was measured as above.

Thrombin-activatable fibrinolysis inhibitor (TAFI) activation was determined using a plasma-based chromogenic assay (Pefakit TAFI; Pentapharm, Basel, Switzerland). The experiment was performed in triplicate.

In vitro assays of tissue factor and TM expression

Peripheral blood mononuclear cells (PBMCs) from healthy volunteers (as mentioned above) were isolated using Mono-Poly Resolving Medium as recommended by the manufacturer (Dainippon Pharmaceutical, Suita, Japan). PBMCs were stimulated with HMGB1 (100 nM), heat-inactivated HMGB1 (100 nM), or vehicle in RPMI-1640 medium with 1% fetal bovine serum (FBS) and 5 μg mL^{-1} polymyxin B sulfate. The polymyxin B dose was that needed to neutralize 100 pg mL^{-1} lipopolysaccharide (LPS) from *Escherichia coli* 055:B5. Purified HMGB1 contained < 100 pg mL^{-1} LPS as assessed by a kinetic–turbidimetric assay. Following 6 h of incubation, PBMCs were harvested by gentle scraping, and were incubated for 15 min with either fluorescein isothiocyanate (FITC)-conjugated non-immune mouse IgG or an FITC-conjugated mouse monoclonal antibody against human tissue factor (American Diagnostica Inc., Stanford, CT, USA). Then, PBMCs were fixed in OptiLyse C (Beckman Coulter, Tokyo, Japan) for 10 min. Cell fluorescence was measured using an Epics XL flow cytometer equipped with the SYSTEM II software (Beckman Coulter). Monocytes were gated by forward-scatter and side-scatter properties, and were confirmed by expression of CD45 and CD14 antigens in separate experiments. Cells were defined as positive using gate settings, which excluded 99% of cells treated with FITC-conjugated non-immune immunoglobulins.

Human pulmonary artery endothelial HPAE-26 cells (American Type Culture Collection, Manassas, VA, USA) were grown and maintained in F-12K nutrient mixture medium (Gibco BRL, New York, NY, USA) supplemented with 10% FBS, 0.03 mg mL^{-1} endothelial cell growth supplement, and 10 U mL^{-1} heparin. HPAE-26 cells were stimulated with 100 nM HMGB1 or vehicle in F-12K nutrient mixture medium with 10% FBS and 5 μg mL^{-1} polymyxin B sulfate for 16 h. Then, HPAE-26 cells were harvested by gentle scraping, and were incubated for 15 min with either non-immune rabbit IgG or a rabbit antibody against human TM [20]. FITC-conjugated goat anti-(rabbit IgG) (ICN Biomedical, Aurora, OH, USA) was then added. Following 15 min of incubation, HPAE-26 cells were fixed in OptiLyse C. Cell fluorescence was measured using an Epics XL flow cytometer.

Statistical analyses

Data were presented as means ± SD. Statistical analyses were performed using analysis of variance (ANOVA) followed by the protective least significant difference Fisher's test. A probability of < 0.05 was considered significant.

Results

HMGB1 increases thrombin-induced mortality

Using the thrombin-induced DIC rat model, we investigated the effects of HMGB1 on the coagulation system. Six-week-old male rats were randomly divided into groups, as indicated in Fig. 1A. In this model, the thrombin-treated rats all developed reddish urine and dyspnea. However, these signs were reversible, and all rats in the thrombin-alone group survived (Fig. 1B). In contrast, combined administration of thrombin and HMGB1 caused severe reddish urine and dyspnea, and half of the rats were dead within 2 days. No apparent changes were observed in the HMGB1-alone group during the 1-week observation period. These findings suggested that HMGB1 did not act as a lethal mediator by itself, but increased thrombin-induced mortality.

To investigate the potential cause of death, we performed pathologic analyses. In the vehicle group and the HMGB1-alone group, no gross abnormalities were detected in organ morphology, including kidneys and lungs (Fig. 1C, and data not shown). In the thrombin-alone group, kidneys were enlarged and dark in color, and lungs were discolored with brown spots (Fig. 1C,D). These findings were much more severe in the thrombin plus HMGB1 group. Taken together, these observations indicated that HMGB1 increased susceptibility to acute renal and lung injuries in the thrombin-induced DIC rat model.

HMGB1 accelerates glomerular fibrin deposition, renal tubular degeneration and alveolar hemorrhage in a thrombin-induced DIC model

To further explore the effects of HMGB1 on thrombin-induced organ failure, we analyzed histologic changes in each group. As shown in Fig. 2A,B, administration of thrombin resulted in a small amount of fibrin deposition in renal glomeruli and degeneration in renal tubules. Compared to thrombin-treated rats, thrombin plus HMGB1-treated rats had significantly increased fibrin deposition in the glomeruli (fibrin deposition scores 1.2 ± 1.6 and 3.4 ± 1.3, respectively, $P < 0.05$). Administration of HMGB1 alone elicited no changes in glomeruli and tubules (fibrin deposition scores 0). Thus, HMGB1 promoted the development of microvascular thrombosis in the kidneys of thrombin-induced DIC rats.

We also analyzed histologic changes in lungs. Administration of thrombin, HMGB1 or thrombin plus HMGB1 increased alveolar wall thickness, interstitial edema, and cell infiltration (Fig. 2C). In addition, administration of thrombin plus HMGB1 caused alveolar hemorrhage (Figs 1D and 2C). In this study, we could not detect fibrin deposition in the lungs of any rats. No other organs, including liver, spleen, and brain, had apparent abnormalities in each group (data not shown). These results suggested that HMGB1 acted as a lethal mediator in the thrombin-induced DIC model, at least in part through acceleration of microvascular thrombosis and subsequent renal and respiratory failure.

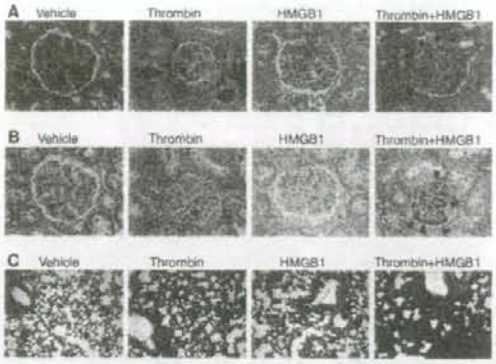

Fig. 2. High-mobility group box 1 protein (HMGB1) accelerates glomerular fibrin deposition, renal tubular degeneration and alveolar hemorrhage in a thrombin-induced disseminated intravascular coagulation model. (A) Hematoxylin and eosin (H&E) staining of kidney tissue sections in vehicle-treated, thrombin-treated, HMGB1-treated and thrombin plus HMGB1-treated rats 6 h after treatment initiation. Arrowheads indicate fibrin deposition. Scale bar: 10 μm. (B) Phosphotungstic acid hematoxylin staining of kidney tissue sections. Arrowheads indicate fibrin fibers stained in dark blue. Scale bar: 10 μm. (C) H&E staining of lung tissue sections. Scale bar: 50 μm.

Thrombin and HMGB1 act synergistically to promote coagulation in vivo

The observation that HMGB1 accelerated fibrin deposition in the thrombin-induced DIC model suggested that HMGB1 might affect hemostatic profiles. As shown in Fig. 3A, the PT and APTT of thrombin-treated rats were prolonged 1.3-fold, compared to those of vehicle-treated rats. HMGB1 treatment did not affect PT or APTT. Interestingly, the PT and APTT of thrombin plus HMGB1-treated rats were prolonged more than 2-fold compared to those of vehicle-treated rats, and more than 1.5-fold compared to those of thrombin-treated rats ($P < 0.001$ and $P < 0.001$, respectively). These results suggested that HMGB1 promoted thrombin-induced consumption of coagulation factors. Thrombin plus HMGB1-treated rats showed significantly lower fibrinogen and platelet concentrations than vehicle-treated or HMGB1-treated rats. They tended to show lower fibrinogen and platelet levels than thrombin-treated rats, although these differences were not significant. Taking into consideration that fibrinogen levels of HMGB1-treated rats were significantly higher than those of vehicle-treated rats ($P < 0.05$), consumption of fibrinogen in thrombin plus HMGB1-treated rats might be canceled out by the upregulation of fibrinogen induced by HMGB1.

Fibrin deposition in blood vessels might cause red blood cell fragmentation and hemolysis. We tested whether these phenomena also occurred in our experimental model. Red blood cell fragmentation and hemolysis were observed in thrombin-treated rats, and to an even greater extent in thrombin plus HMGB1-treated rats (data not shown). It is conceivable that hemolysis may further exacerbate vasculopathy [21]. Thus, not

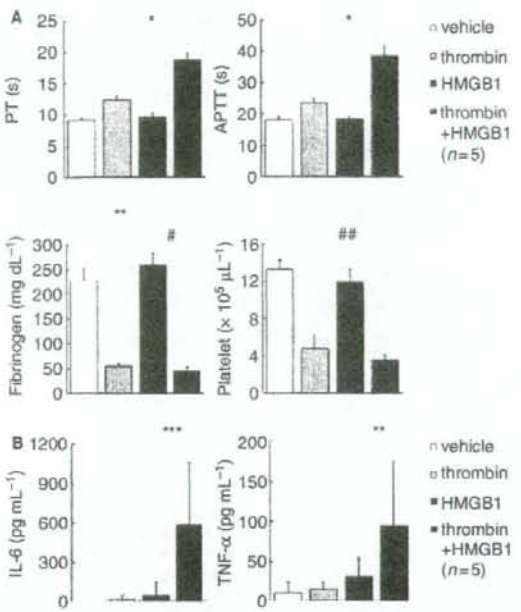

Fig. 3. Thrombin and high-mobility group box 1 protein (HMGB1) act synergistically to promote coagulation and inflammation *in vivo*. (A) Hemostatic profiles (prothrombin time, activated partial thromboplastin time, fibrinogen concentration, and platelet count) of rats treated with vehicle, thrombin, HMGB1 and thrombin plus HMGB1 6 h after treatment initiation. $n = 5$ per group. (B) Plasma levels of interleukin-6 (left panel) and tumor necrosis factor-α (right panel) 6 h after treatment initiation in each group. $n = 5$ per group. $^{*}P < 0.001$; $^{**}P < 0.05$; $^{***}P < 0.01$; $P = 0.42$; $^{\#\#}P = 0.12$.

only the histologic findings but also the coagulation parameters supported the concept that HMGB1 exacerbated DIC *in vivo*.

Thrombin and HMGB1 act synergistically to promote inflammation in vivo

Thrombin, a coagulation protease, can evoke an inflammatory response through protease-activated receptors [22]. HMGB1 can evoke an inflammatory response through the receptor for advanced glycation end-products and possibly through Toll-like receptors 2 and 4 [14,15]. In our *in vitro* experiments, thrombin and HMGB1 were capable of stimulating proinflammatory cytokine production in murine macrophage-like RAW 264.7 cells (data not shown). In our experiment with rats, thrombin or HMGB1 also induced proinflammatory cytokines such as IL-6 and TNF-α (Fig. 3B). Interestingly, rats stimulated by both thrombin and HMGB1 exhibited more than tenfold higher levels of IL-6 than rats stimulated by thrombin or HMGB1 alone, which exhibited a rather mild inflammatory response ($P = 0.002$ and $P = 0.004$, respectively). Taken together, thrombin and HMGB1 acted synergistically to promote coagulation and inflammation, leading to multiple organ failure (Figs 2 and 3).

HMGB1 stimulates tissue factor expression on monocytes

To identify the mechanisms whereby HMGB1 promotes development of microvascular thrombosis, we examined the effects of HMGB1 on the coagulation system *in vitro*. Neither TT nor PT was affected by HMGB1 *in vitro* (TT 14.8 ± 0.3 s without HMGB1, compared to 14.6 ± 0.2 s with 100 nM HMGB1, and PT 13.2 ± 0.1 s without HMGB1, compared to 13.2 ± 0.1 s with 100 nM HMGB1).

The prothrombotic effects of HMGB1 in rats might be associated with receptor-mediated cellular responses, such as upregulation of procoagulant molecules or downregulation of anticoagulant molecules. We examined the effects of HMGB1 on cell surface expression of tissue factor and TM by flow cytometry (Fig. 4). Although TM expression on endothelial cells did not change, tissue factor expression on monocytes was increased by HMGB1 stimulation. Contaminating LPS was not responsible for the stimulatory effect of the HMGB1 preparation, because: (i) heat-treated HMGB1 did not induce tissue factor expression at all; and (ii) contaminating LPS was < 100 pg mL^{-1}, and this concentration of LPS did not induce tissue factor expression under our experimental conditions with 5 μg mL^{-1} polymyxin B (data not shown). Thus, HMGB1-induced tissue factor expression on monocytes might be one mechanism responsible for the prothrombotic effects.

HMGB1 inhibits protein C activation in vitro

The prothrombotic effects of HMGB1 in rats might be due to inhibitory effects on the anticoagulant system. We first examined the effects of HMGB1 on antithrombin, and found that HMGB1 did not affect antithrombin binding to thrombin (data not shown). We recently reported that TM bound HMGB1, thereby suppressing induction of proinflammatory events [23]. This raised the question of whether HMGB1, in

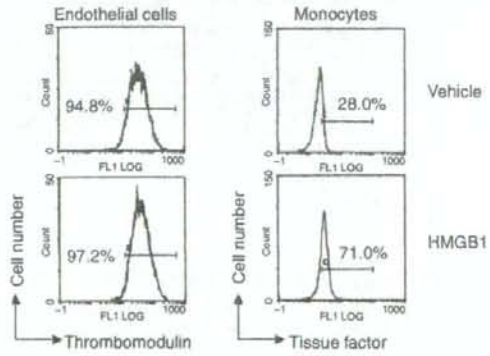

Fig. 4. High-mobility group box 1 protein (HMGB1) stimulates tissue factor expression *in vitro*. Representative data for thrombomodulin expression on HPAE-26 cells (left panel) and tissue factor expression on peripheral blood mononuclear cells (right panel) in the absence (upper panel) or presence (lower panel) of HMGB1 stimulation. Percentages indicate fractions of antigen-positive cells.

turn, would suppress TM function. As TM acts as a cofactor for thrombin-mediated activation of protein C and TAFI [24], we examined the effects of HMGB1 on the cofactor activity of TM. As shown in Fig. 5A, HMGB1 dose-dependently inhibited activation of protein C mediated by the thrombin–TM complex. In kinetic analyses, HMGB1 exhibited no change in the V_{max} and a 2.1-fold augmentation in the K_m for protein C (Fig. 5B). HMGB1 had little effect on the activity of ready-made APC (data not shown), indicating that HMGB1 inhibited conversion of protein C to APC. Also, HMGB1 had no effect on protein C activation mediated by snake venom protein (data not shown), indicating that HMGB1 specifically inhibited protein C activation mediated by the thrombin–TM complex. In addition, HMGB1 had no effect on TAFI activation mediated by the thrombin–TM complex (1 ± 0.06 activity without HMGB1 compared to 0.99 ± 0.08 activity with 100 nM HMGB1), indicating that HMGB1 specifically inhibited TM cofactor activity for protein C activation.

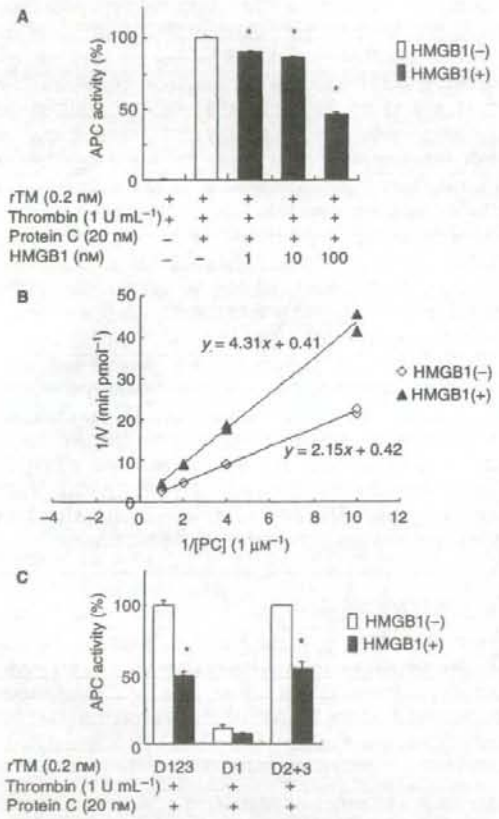

Fig. 5. High-mobility group box 1 protein (HMGB1) inhibits protein C activation *in vitro*. (A) Using a chromogenic substrate S-2238, we examined activated protein C (APC) activity in the absence (white bar) or presence (gray bar) of HMGB1. APC activity was expressed as percentages of that without HMGB1 (white bar). The experiment was performed in triplicate. *P < 0.001 compared to APC activity without HMGB1 (white bar). rTM, recombinant thrombomodulin. (B) Lineweaver–Burk plots for thrombin–thrombomodulin-mediated protein C activation in the absence or presence of HMGB1. Enzyme velocity (V = molecules of protein C activated min^{-1}) was calculated, and 1/[PC] vs. $1/V$ was then plotted. PC, protein C. (C) APC activity was measured in the absence (white bar) or presence (gray bar) of 100 nM HMGB1 with various TM-derived peptides: full-length TM (D123), P-D1, and P-D_{2+3}. *P < 0.001 compared to APC activity without HMGB1 (white bar).

Previous studies have demonstrated that D1 (lectin-like domain) of TM has anti-inflammatory properties and is essential for HMGB1 binding [23,25], and D2 (endothelial growth factor-like domain) of TM is essential for thrombin binding and protein C activation [19]. To examine whether binding of HMGB1 to TM was involved in the inhibitory effects on protein C activation, we used a D1 deletion mutant of TM (P-D_{2+3}) for the protein C activation assay. As shown in Fig. 5C, HMGB1 inhibited protein C activation mediated by P-D_{2+3} to the same extent as that mediated by full-length TM (D123). These results indicated that binding of HMGB1 to TM was not involved in the inhibitory effects on protein C activation. Considering that the concentrations of HMGB1 (100 nM) were much higher than those of TM (0.2 nM) in the assays, unbound HMGB1 might be responsible for these effects. Finally, we examined the effects of HMGB1 on cell surface protein C activation, and found that HMGB1 significantly inhibited protein C activation mediated by HUVECs (1 ± 0.01 APC activity without HMGB1, compared to 0.79 ± 0.03 APC activity with 100 nM HMGB1, P < 0.001). Thus, HMGB1 inhibited protein C activation mediated by the thrombin–TM complex *in vitro*.

Discussion

Recent studies have identified HMGB1 as a lethal mediator of sepsis, as well as a promising therapeutic target for sepsis [2,7,8]. Defining the roles of HMGB1 during experimental sepsis in greater detail is now important for understanding the pathogenesis of sepsis and designing novel therapeutic strategies. Here, we have shown that HMGB1 promotes the development of microvascular thrombosis, and increases mortality in a thrombin-induced DIC model. In combination with 1250 U kg^{-1} h^{-1} thrombin, HMGB1 administration at 0, 0.4, 2 and 5 mg kg^{-1} to rats resulted in survival rates of 100%, 60%, 50%, and 50%, respectively (Fig. 1B and data not shown). In contrast, when the dose of thrombin was increased to 2000 U kg^{-1} h^{-1}, the survival rate decreased to 20%. Although these results indicate that HMGB1 is indeed a lethal mediator in the DIC model, they also indicate that 5 mg kg^{-1} HMGB1 may be redundant, and that the lethal activity of HMGB1 may be dependent on other factors such as thrombin.

Thrombin, the principal procoagulant enzyme generated at sites of injury, is converted to an anticoagulant enzyme at distant sites through its interaction with the endothelial cell protein TM, and subsequent generation of APC [20,26]. During sepsis, this anticoagulant mechanism is compromised

[27]. In part, this is due to proinflammatory mediators, such as TNF-α, that reduce TM expression on endothelial cells [28]. In our *in vitro* experiments, HMGB1 reduced the activity of thrombin–TM complexes. The percentage inhibition of APC generation in the presence of 1, 10 and 100 nM HMGB1 was 10%, 14%, and 54%, respectively. As the plasma or serum levels of HMGB1 in DIC or septic patients have been reported to range from 0 to 10 nM [7,16,17], the percentage inhibition of APC generation in such patients may be about 10%. Considering that 40% inhibition of APC generation or heterozygous deletion of TM was previously reported to cause thrombosis in animal models [29,30], HMGB1 in septic patients at a concentration of about 1 nM may not be sufficient to cause thrombosis through inhibition of APC generation alone. It is therefore possible that other mechanisms, such as increased tissue factor expression, are also important in the aggravation of DIC by HMGB1. The molecular balance between HMGB1 and TM may also be important. On the one hand, TM sequesters HMGB1, while on the other hand, HMGB1 inhibits the activity of thrombin–TM complexes. We examined the effects of HMGB1 with 0.2 nM TM, as higher concentrations of TM might sequester 1 nM HMGB1 completely. In our *in vitro* experiments, the percentage inhibition of APC generation decreased when the TM dose was increased, possibly because the TM-unbound fraction of HMGB1 was decreased (data not shown). In our *in vivo* experiments, fibrin deposition was mainly detected in renal glomeruli. Considering that glomerular capillary loops express less TM [31], the relatively high concentration of HMGB1 might promote the development of thrombosis in glomeruli. In addition, it is conceivable that the preceding administration of thrombin might decrease the levels of TM expressed on endothelial cells [32].

Previous studies and our present results suggest possible mechanisms whereby HMGB1 exerts its lethal effects under septic conditions. HMGB1 promotes inflammatory responses by acting on monocytes, endothelial cells, and other types of cell [10,11,23,33,34]. Therefore, massive accumulation of HMGB1 in the systemic circulation would lead to systemic inflammatory response syndrome, an important feature of sepsis. In addition, our results suggest that HMGB1 in the systemic circulation promotes the development of DIC. These dysregulated inflammatory and coagulatory responses may be related to the lethal activity of HMGB1 in sepsis. We recently reported that TM interacts with HMGB1, and protects mice against lethal endotoxemia [23]. TM also interacts with thrombin, resulting in inhibition of the enzyme's procoagulant activity [26]. Binding of HMGB1 and thrombin by TM provides a mechanism for damping the amplification of inflammatory and coagulatory responses. Under septic conditions, HMGB1 and thrombin present in the circulation would propagate inflammatory and coagulatory responses to remote organs [1,34]. However, once an adjacent portion of the vessel wall with intact endothelial cells is encountered, TM-bearing cells can sequester HMGB1 and thrombin, thereby preventing them from reaching remote organs. Replacement with recombinant TM will offer therapeutic value in sepsis, as the expression of endothelial TM is impaired [27].

Some open questions remain. For example, it is important to define the exact mechanisms involved in the aggravation of DIC by HMGB1 *in vivo*, including the involvement of the protein C pathway. It is also important to elucidate the amount of HMGB1 that binds to TM *in vivo*. In our experimental model, i.v. administered HMGB1 at a dose of 2 mg kg^{-1} (theoretical plasma HMGB1 level of 53 μg mL^{-1}) was rapidly cleared from the circulation, and the plasma HMGB1 levels at 5 h after administration were as low as 1.06 ± 0.68 ng mL^{-1}. Binding of HMGB1 to TM might be one mechanism responsible for the rapid clearance of HMGB1 from the circulation. Such phenomena can be seen in clinical settings, suggesting that HMGB1 levels in plasma are lower than those at local injured sites. Another question is whether HMGB1 acts as a lethal mediator by itself. None of 10 rats died after i.v. HMGB1 administration at 5 mg kg^{-1} in our study. Three of five mice died after intraperitoneal administration of 0.5 mg of HMGB1/mouse in a previous study [7]. In contrast, administration of 0.4 mg kg^{-1} of HMGB1 in our study or 0.05 mg HMGB1/mouse in reference 7 could be lethal in DIC or septic conditions, in which other mediators, such as thrombin or LPS, exist. These findings indicate that HMGB1 may be a promoter, rather than an initiator, of DIC or sepsis.

At injured sites, hemostasis, immune responses and subsequent tissue regeneration are necessary. Recent observations have suggested that HMGB1, which is released by necrotic and inflammatory cells at sites of injury, plays important roles in local immune responses and tissue regeneration [12,13]. In addition, our present results suggest that HMGB1 may play a role in hemostasis. All these findings suggest that HMGB1 is a type of general organizer in postinjury wound healing.

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Disclosure of Conflict of Interests

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Inhibition of HMGB1 by deep ocean water attenuates endotoxin-induced sepsis

Recent studies have clarified the mechanism of development and progression in sepsis [1]. However, the sepsis remains a serious problem in critically ill patients. The mortality in patients with sepsis is caused by bacterial endotoxin. Endotoxin stimulates macrophages/monocytes to release high mobility group box 1 (HMGB1), a chromatin protein with a cytokine function. The extracellular HMGB1 acts as a pro-inflammatory cytokine following releases from necrotic cells or macrophages activated by tumor necrosis factor (TNF)-α or interferon (IFN)-γ through the toll-like receptor -2 or -4 and receptor for advanced glycation end-products (RAGE), suggesting "cytokine loops" in inflammatory disease such as rheumatoid arthritis (RA) and atherosclerosis [2,3]. Furthermore, injection of recombinant HMGB1 to mice demonstrated severe clinical signs of sepsis, including fever, allodynia, derangement of intestinal barrier function, lung injury, and lethal multiple organ failure. Anti-HMGB1 antibodies significantly protect mice against endotoxin-induced acute lung injury [1]. Taken together, it may be realistic to expect that HMGB1 is a "magic bullet" targeting for inflammatory disease especially in late phase sepsis. Therefore, suppression of HMGB1 release may lead to novel therapeutic approaches for progressive sepsis.

Recent studies have shown that natural products such as green tea, herbal extract can suppress inflammation, suggesting that they are useful compounds as anti-inflammatory agents [4]. They might suppress acute inflammatory disease (e.g. sepsis) as well as chronic inflammatory diseases such as rheumatoid arthritis (RA), Crohn's disease (CD), and ulcerative colitis (UC). Moreover, natural foods suppressed endotoxin-induced HMGB1 release in vitro and in vivo [4].

The surface of about 97% of the earth is covered with deep ocean water (DOW). DOW is approximately 200-m deep or deeper under the surface of the ocean. DOW is expected for food, resources, energy, agriculture, marine, and chemical fields. Especially, DOW is very well nourished, mineralized, therefore, it is expected for medical purpose as its function in the inflammatory suppressive effect (e.g., atopic dermatitis), suggesting the possibility of DOW as a powerful anti-inflammatory agent [5]. In this study, we reviewed that DOW (Koshiki kaiyo-shinso-sui, Kagoshima, Japan): Japanese name meaning "motherly water" could suppress endotoxin-induced HMGB1 release in murine macrophage-like RAW264.7 cells. Thus, we hypothesize that DOW might be able to reduce the mortality rate of lethal sepsis induced by endotoxin. To support our hypothesis, further exciting trials in pre-clinical animal models and human clinical studies need to be investigated.

References

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