[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

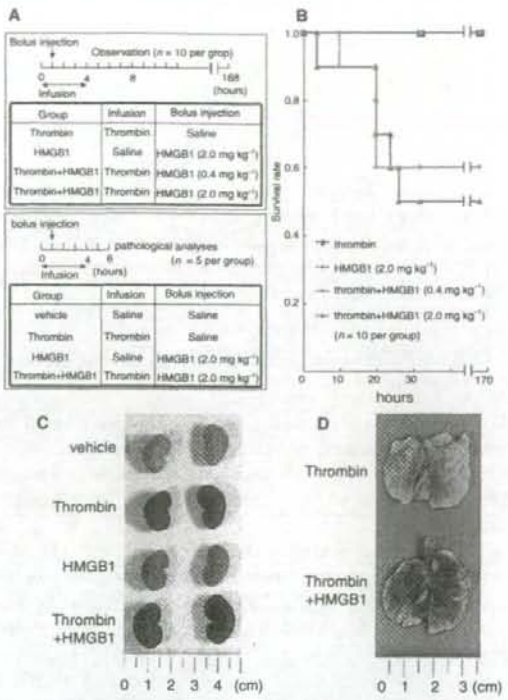

Fig. 1. High-mobility group box 1 protein (HMGB1) exacerbates renal and lung injuries and increases mortality in a thrombin-induced disseminated intravascular coagulation (DIC) model. (A) Experimental designs for survival analysis (upper panel) and pathologic analyses (lower panel) in rats. Thrombin 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.

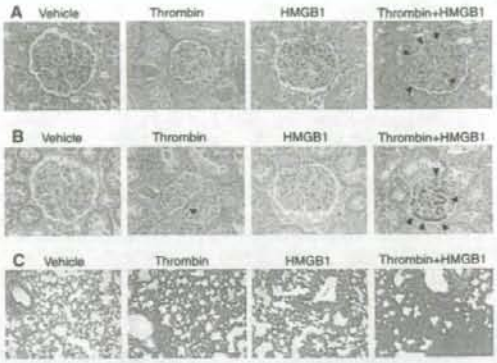

Fig. 2. High-mobility group box 1 protein (HMGB1) accelerates glomerular fibrin deposition, renal tubular degeneration and alveolar hemorrhage in a thrombin-induced disseminated intravascular coagulation model. (A) Hematoxylin and eosin (H&E) staining of kidney tissue sections in vehicle-treated, thrombin-treated, 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

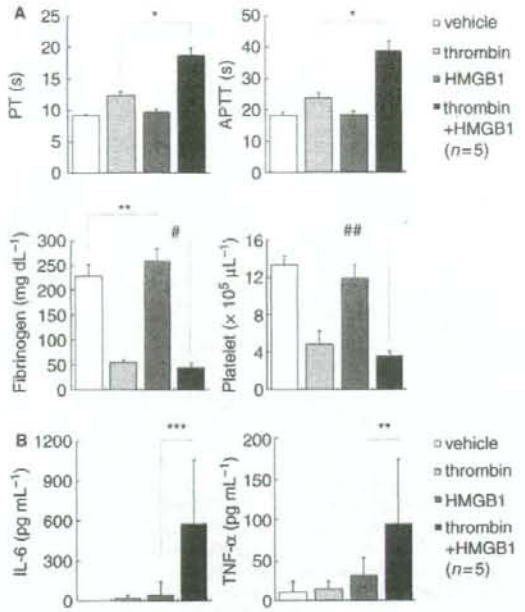

Fig. 3. Thrombin and high-mobility group box 1 protein (HMGB1) act synergistically to promote coagulation and inflammation *in vivo*. (A) Hemostatic profiles (prothrombin time, activated partial thromboplastin time, fibrinogen concentration, and platelet count) of rats treated with vehicle, thrombin, 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

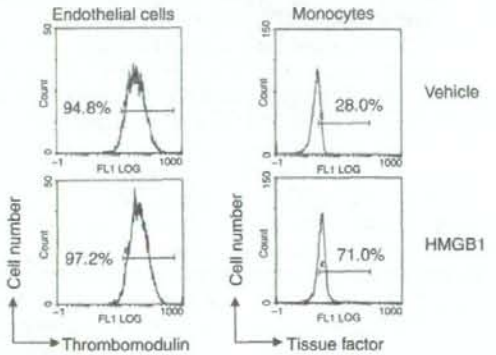

Fig. 4. High-mobility group box 1 protein (HMGB1) stimulates tissue factor expression *in vitro*. Representative data for thrombomodulin expression on HPAE-26 cells (left panel) and tissue factor expression on peripheral blood mononuclear cells (right panel) in the absence (upper panel) or presence (lower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

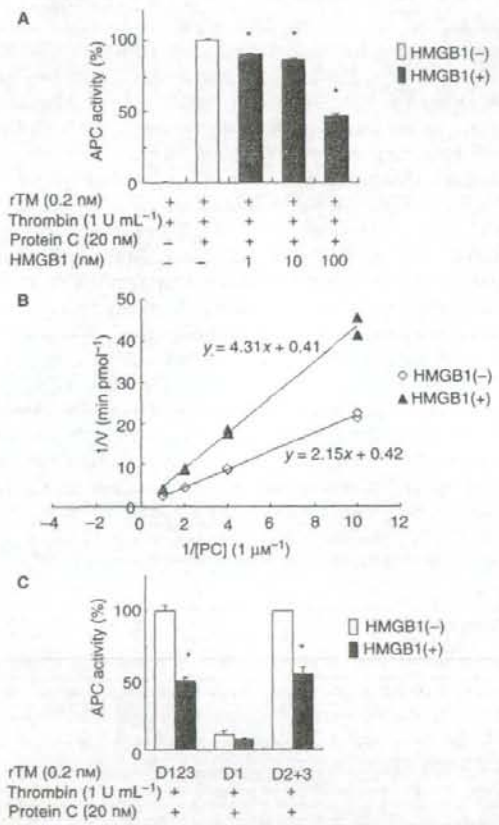

Fig. 5. High-mobility group box 1 protein (HMGB1) inhibits protein C activation *in vitro*. (A) Using a chromogenic substrate S-2238, we examined activated protein C (APC) activity in the absence (white bar) or presence (gray bar) of HMGB1. APC activity was expressed as percentages of that without HMGB1 (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.

Acknowledgements

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Disclosure of Conflict of Interests

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CARDIOVASCULAR PATHOLOGY

Original Article

CRP induces high-mobility group box-1 protein release through activation of p38MAPK in macrophage RAW264.7 cells☆

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Abstract

Background: C-reactive protein is widely used as a sensitive biomarker for inflammation. Increasing evidence suggests that C-reactive protein plays a role in inflammation. High-mobility group box-1, a primarily nuclear protein, is passively released into the extracellular milieu by necrotic or damaged cells and is actively secreted by monocytes/macrophages. Extracellular high-mobility group box-1 as a potent inflammatory mediator has stimulated immense curiosity in the field of inflammation research. However, the molecular dialogue implicated between C-reactive protein and high-mobility group box-1 in delayed inflammatory processes remains to be explored. **Methods and results:** The levels of high-mobility group box-1 in culture supernatants were determined by Western blot analysis and enzyme-linked immunosorbent assay in macrophage RAW264.7 cells. Purified C-reactive protein induced the release of high-mobility group box-1 in a dose- and time-dependent fashion. Immunofluorescence analysis revealed nuclear translocation of high-mobility group box-1 in response to C-reactive protein. The binding of C-reactive protein to the Fcγ receptor in RAW264.7 cells was confirmed by fluorescence-activated cell sorter analysis. Pretreatment of cells with IgG–Fc fragment, but not IgG–Fab fragment, efficiently blocked this binding. C-reactive protein triggered the activation of p38MAPK and ERK1/2, but not Jun N-terminal kinase. Moreover, both p38MAPK inhibitor SB203580 and small interfering RNA significantly suppressed the release of high-mobility group box-1, but not the MEK1/2 inhibitor U-0126. **Conclusion:** We demonstrated for the first time that C-reactive protein, a prominent risk marker for inflammation including atherosclerosis, could induce the active release of high-mobility group box-1 by RAW264.7 cells through Fcγ receptor/p38MAPK signaling pathways, thus implying that C-reactive protein plays a crucial role in the induction, amplification, and prolongation of inflammatory processes, including atherosclerotic lesions.

Keywords: HMGB1; CRP; Atherosclerosis; Fcγ receptor; p38MAPK

1. Introduction

C-reactive protein (CRP), so named for its capacity to precipitate the somatic C-polysaccharide of *Streptococcus pneumoniae*, has been described as a nonspecific acute-phase reactant protein and a sensitive marker of inflammation and

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tissue damage. Recently, an increased level of CRP has been described in the serum of patients suffering from cardiovascular events [1]. Recent studies have shown that the CRP protein is expressed in macrophages and vascular smooth muscle cells (VSMCs) in atheromatous plaques [2–5] and plays a role in the progression and vulnerability of atherosclerotic lesions [6–12]. Thus, therapeutic inhibition of CRP can represent a new approach to cardiovascular diseases [13]. However, little is known about whether and how CRP acts as a progressive and prolongation factor in cardiovascular disease.

We have recently shown that CRP is colocalized with high-mobility group box-1 (HMGB1) in atherosclerotic lesions [14]. The nuclear protein HMGB1 is present in many eukaryotic cells and has been identified as a late-phase mediator in septic shock [15,16]. HMGB1 consists of two tandem domains designated as HMG boxes A and B, and a highly acidic carboxyl-terminus. HMGB1 appears to have two distinct functions in cellular systems. First, it acts as an intracellular regulator of the transcription process and plays a crucial role in maintenance of DNA functions [17]. Second, HMGB1 is released into the extracellular space by all eukaryotic cells upon necrosis or by macrophages in response to inflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukin (IL) 1, and interferon-γ (IFN-γ) through mitogen-activated protein kinase (MAPK) signal transduction pathways and can act as a potent proinflammatory cytokine through a multiligand receptor for advanced glycation endproducts (RAGE) [18,19]. Through RAGE, HMGB1 stimulates macrophages to release cytokines such as TNF-α, IL-6, and IL-1β [19], suggesting that extracellular HMGB1 plays a critical role in several inflammatory diseases such as septic shock, lung inflammation, and rheumatoid arthritis [15,19,20]. Recently, it has been reported that macrophages are the major cell type responsible for HMGB1 production in human atherosclerotic lesions and that HMGB1 plays a role in the pathogenesis of plaque formation and progression [21]. Accumulating evidence indicates that CRP induces the expression of macrophage chemoattractant protein-1 (MCP-1), IL-6, IL-8, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [10,22,23]; however, to the best of our knowledge there have been no reports demonstrating a linkage between CRP (an acute-phase reactant) and HMGB1 (a late-phase mediator of inflammation).

The present study was undertaken to investigate the effect of CRP on the secretion of HMGB1 using a murine macrophage cell line, RAW264.7, and the underlying intracellular signal transduction pathways involved.

2. Materials and methods

2.1. Antibodies

Anti-HMGB1 antibody was obtained from SHINO-TEST (Kanagawa, Japan). Anti-phospho (p)-ERK1/2, anti-p-p38MAPK, anti-p-Jun N-terminal kinase (JNK), and anti-β-actin antibodies were purchased from Cell Signaling Technology (Beverly, MA).

2.2. MAPK inhibitors and p38MAPK small interfering RNA

Specific inhibitors of p38MAPK (SB203580; Calbiochem, La Jolla, CA) or MAPK kinase (MEK1/2) (U-0126; Promega, Madison, WI), and small interfering RNA (siRNA) for mouse p38MAPK (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were used to evaluate the functional role of MAPKs in CRP-induced HMGB1 release.

2.3. Purification of CRP

CRP (human) was purchased from Wako Chemicals (Kyoto, Japan). CRP was purified (to remove possible biologic contaminants such as sodium azide and LPS) as described previously [14]. In brief, CRP was filtered with Amicon-Ultra 4 (10,000 molecular weight cut off; Millipore Corporation, Bedford, MA) at 4°C and then washed twice with 20 ml of 0.9% NaCl solution in an intravenous solution (Otsuka, Tokushima, Japan). After CRP had been washed, its concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit specific for human CRP (Alpha Diagnostic International, Texas). The contents of LPS in purified CRP solutions (5, 10, 20, 40, and 80 μg/ml) were found to be <5 pg/ml, as determined by Limulus endotoxin assays. The purity of CRP preparations was determined by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). A 25-kDa single band corresponding to CRP was obtained by staining gels with sensitive silver staining and by Western blot analysis indicating the purity of CRP. In some experiments, the purified CRP was immunoprecipitated with anti-CRP antibody (Dako Cytomation, Denmark) or nonimmune control IgG (Dako Cytomation), followed by a 16-h incubation of protein G agarose (Santa Cruz Biotechnology, Inc.). The immunoprecipitated proteins were separated by centrifugation, and supernatants were collected for cell stimulation. Experiments were performed with the purified CRP, unless stated otherwise.

2.4. Cell culture

Murine macrophage-like RAW264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum and 2 mM glutamine.

2.5. Stimulation of macrophage RAW264.7 cells with CRP, mouse IgG, Fc, and Fab fragments

Before stimulation with human-purified CRP, heat-inactivated CRP, a supernatant obtained by immunoprecipitation

(IP) of the purified CRP with anti-CRP antibody or nonimmune control IgG, mouse IgG–Fc fragment (1 μM), mouse IgG–Fab fragment (1 μM), and RAW264.7 cells (2×10^6 cells/6-cm dish) were starved for 2 h with serum-free Opti-MEM-I medium and then stimulated with the aforesaid stimulants in the presence of serum-free Opti-MEM-I medium. Following treatment, HMGB1 levels in culture media were analyzed by Western blot analysis and ELISA.

2.6. Flow cytometry analysis

To block the Fcγ receptor, RAW264.7 cells [5×10^5 cells/tube (100 μl)] were incubated with or without mouse IgG–Fc fragment (Jackson ImmunoResearch Laboratories, Inc., West Grove PA) and mouse IgG–Fab fragment (Jackson ImmunoResearch Laboratories, Inc.) for 30 min at room temperature (RT). Then cells were incubated with CRP (20 μg/ml) for 30 min at RT. Cells were fixed with OptiLyse C (250 μl; Immunotech, Marseille, France) for 15 min and then washed with phosphate-buffered saline (PBS). Washed cells were incubated with anti-CRP antibody (Dako Cytomation) diluted 1:100 with PBS for 60 min. After the cells had been washed, they were incubated with fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (Immunotech) diluted 1:50 with PBS for 30 min. Data were analyzed by flow cytometry (Beckman Coulter).

2.7. Preparation of HMGB1 samples for Western blot analysis

Preparations of HMGB1 samples were undertaken as described previously [14]. Following CRP treatment, the culture supernatant (2 ml) was incubated with 50 μl of heparin–Sepharose 6B (heparin beads) for 4 h and then washed thrice with 10 mM phosphate buffer (pH 7.0). Next, 50 μl of sample buffer [62.5 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, and 0.002% bromophenol blue] was added to the washed heparin beads and boiled for 5 min.

2.8. Western blot analysis

Western blot analyses were performed as described previously [19]. Briefly, cell lysates (50 μg) obtained from CRP-treated RAW264.7 cells or HMGB1 samples (40 μl) extracted from heparin beads were subjected to 12% SDS-PAGE, then separated proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline (pH 7.4) containing 0.02% Tween 20 (TBST) for 1 h at RT, then incubated with anti-HMGB1 antibody (2 μg/ml) in TBST containing 1% nonfat dry milk for 3 h at RT. After the membrane had been washed, it was incubated with horseradish-peroxidase-conjugated anti-rabbit IgG polyclonal antibody (Santa Cruz Biotechnology, Inc.) diluted 1:3000 in TBST containing 2.5% nonfat dry milk for 1 h at RT. The membrane was washed for a second time, then immunoreactive bands were visualized using an ECL detection system (Amersham Biosciences).

2.9. ELISA analysis

The levels of HMGB1 and TNF-α in cultured supernatants were determined using a commercial ELISA kit specific for human HMGB1 (SHINO-TEST) and TNF-α (R&D Systems, Minnesota), respectively. All experiments were performed in triplicate.

2.10. Annexin V analysis

RAW264.7 cells (2×10^6 cells/6-cm dish) were cultured for 24 h. Cells were then washed with Opti-MEM-I medium (Gibco BRL) and stimulated with or without CRP (20 μg/ml) for 24 h. The cells were incubated with FITC-labeled Annexin V (MBL, Nagoya, Japan) for 5 min. Data were analyzed by flow cytometry (Beckman Coulter).

2.11. Immunofluorescence analysis

To investigate the translocation of HMGB1, RAW264.7 cells were cultured on Lab-Tek chamber slides (Nalge Nunc International, Cambridge, MA) and incubated with CRP (20 μg/ml) for 16 h. Following stimulation, cells were fixed with 2% paraformaldehyde containing 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO) for 15 min. Slides were then blocked in 1% bovine serum albumin in PBS containing 0.1% Triton X-100 (PBST) for 1 h and incubated with rabbit anti-HMGB1 polyclonal antibody (1 μg/ml) or normal rabbit IgG as a control for 30 min at RT. Slides were then washed with PBST and incubated with FITC-conjugated anti-rabbit IgG (Immunotech) diluted 1:50 in PBST for 20 min at RT. Finally, cell nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI; Nakalai Tesque, Kyoto, Japan). Slides were then washed and examined using an Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

2.12. MTT assay

Cell viability was analyzed by mitochondrial respiratory activity measured using MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenol tetrazolium bromide] assay (Wako Chemicals), as described previously [24]. Briefly, cells were cultured in 96-well plates (with 100 μl/well medium) in the absence or in the presence of CRP (20 μg/ml) for 16 h. Then cells were incubated with MTT (20 μl of 2.5 μg/ml per well) for 3 h. Formazan product was solubilized by the addition of 100 μl of dimethyl sulfoxide for 16 h. Dehydrogenase activity was expressed as absorbance at a test wavelength of 570 nm and at a reference wavelength of 630 nm.

2.13. p38MAPK siRNA transfection analysis

RAW264.7 cells (8×10^5 cells/ml) cultured in 6-cm dishes for 24 h were washed with Opti-MEM-I medium (Gibco BRL) and then transfected with siRNA (20 μM) or control siRNA (Santa Cruz Biotechnology, Inc.) using oligofectamine (Gibco BRL) for 2 days. Transfected cells were treated with CRP (20 μg/ml) for 20 h, and culture

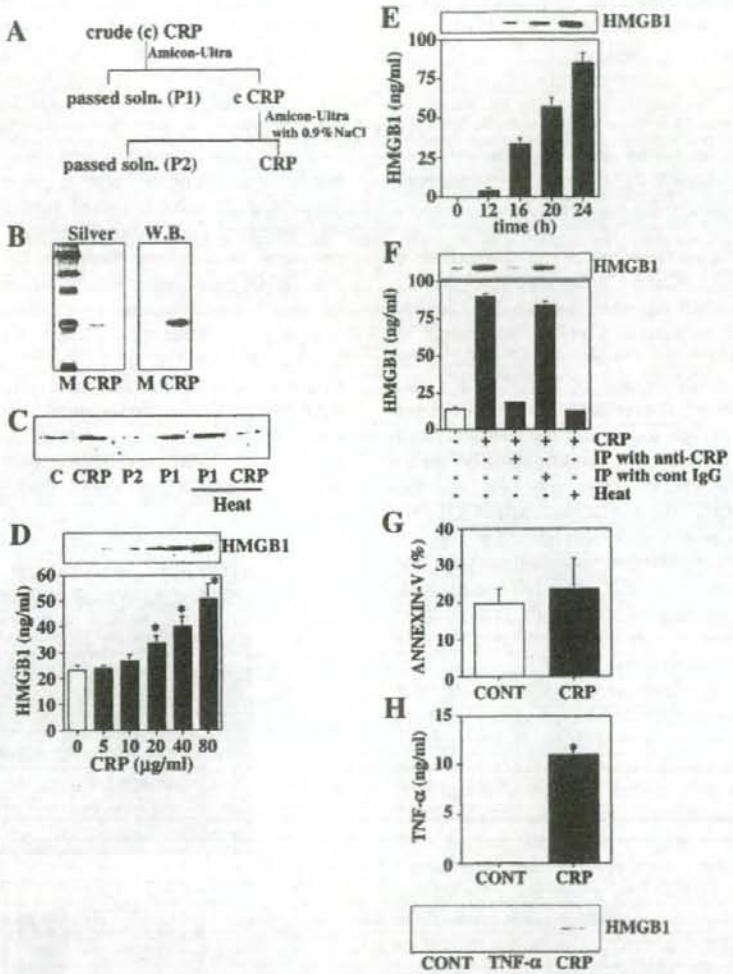

Fig. 1. CRP-induced HMGB1 release by RAW264.7 cells. (A) Schematic presentation of CRP purification with Amicon-Ultra. cCRP was separated with Amicon-Ultra, and the first-pass solution (soln.) was designated as P1. cCRP was washed with LPS-free 0.9% NaCl, and the second-pass solution was designated as P2. (B) Purified CRP was separated with 12% SDS-PAGE. The gels were stained with silver staining (Silver; left) and Western blot analysis (WB; right). (C) The purified CRP induced HMGB1 release. RAW264.7 cells were incubated with pCRP, P1, P2, heated CRP, and heated P1, and the levels of HMGB1 in the supernatants were analyzed for HMGB1 levels by Western blot analysis (upper panel). (D) Dose-dependent effect of CRP. RAW264.7 cells were incubated with CRP (0, 5, 10, 20, 40, and 80 μg/ml) for 20 h. The levels of HMGB1 in the supernatants were analyzed using Western blot analysis (upper panel) and ELISA (lower panel). (E) Time-course effect of CRP. RAW264.7 cells were incubated with CRP (20 μg/ml) for 12, 16, 20, or 24 h. At the end of the treatment, HMGB1 levels were analyzed with Western blot analysis (upper panel) and ELISA (lower panel). (F) RAW264.7 cells were incubated with purified 80 μg/ml CRP (Lane 2); supernatants were obtained by IP with anti-CRP antibody (Lane 3; IP with anti-CRP) or control IgG (Lane 4; IP with control IgG) and heat-inactivated purified CRP (Lane 5) for 24 h; and the levels of HMGB1 in the supernatants were analyzed by Western blot analysis (upper panel) and ELISA (lower panel). (G) Annexin V staining of CRP-treated RAW264.7 cells. RAW264.7 cells were treated with 80 μg/ml CRP for 24 h. Then cells were stained with FITC-labeled Annexin V and analyzed by flow cytometry. (H) Effects of HMGB1 release by TNF-α. RAW264.7 cells were treated with 20 μg/ml CRP for 20 h, and then the TNF-α of supernatants was analyzed by ELISA. RAW264.7 cells were incubated with 50 ng/ml TNF-α or 20 μg/ml CRP for 20 h, and then the HMGB1 of the supernatants was analyzed by Western blot analysis. *Statistically significant ($P<.05$) changes. Note that HMGB1 was released by CRP in RAW264.7 cells, but not TNF-α, under our conditions.

supernatants were analyzed for HMGB1 levels by Western blot analysis.

2.14. Statistical analysis

Statistical analysis was performed using Student's t test. Statistical significance was set at P=.05.

3. Results

3.1. CRP dose dependently and time dependently triggers the active release of HMGB1 by murine macrophage RAW264.7 cells

Macrophage activation is central to the progression of multiple diseases via the release of inflammatory mediators, including cytokines. CRP has been suggested to directly induce inflammatory responses; therefore, we sought to investigate whether CRP, a biomarker of acute-phase inflammation, triggers the release of HMGB1, a potent late-phase mediator of inflammation by macrophage RAW264.7 cells. However, to rule out the possibility that the effects of CRP were due to biologically active contaminants such as sodium azide and LPS, we first proceeded to purify CRP. The purification steps of CRP are shown schematically in Fig. 1A. As presented in Fig. 1B, crude CRP (cCRP), after filtration with Amicon-Ultra and 12% SDS-PAGE, showed a 25-kDa single band corresponding to CRP after the staining of the gels with sensitive silver staining (left panel) and Western blot analysis (right panel), thus clearly indicating the purity of the CRP.

Purified CRP (20 μg/ml) induced a significant release of HMGB1 by macrophage RAW264.7 cells (Fig. 1C, upper panel, Lane 2) compared to control (Lane 1), although the effect was not observed with heated CRP (Lane 6). On the other hand, Amicon-Ultra passed Solution-1-induced (P1; Lane 4), but not Solution-2-induced (P2; Lane 3), HMGB1 release. These findings suggested that the purified CRP (without either sodium azide or LPS) could induce a significant release of HMGB1 by macrophage RAW264.7 cells. A previous study has demonstrated that extracellular HMGB1 originates from activated monocytes or macrophages and necrotic cells [15]. Next, we examined whether the purified CRP, within a pathophysiological range of concentrations, stimulated macrophage RAW264.7 cells to release HMGB1. For this, we treated macrophage 264.7 cells with various concentrations (5, 10, 20, 40, and 80 μg/ml) of the purified CRP for 20 h. HMGB1 was not detected in the cultured medium in the absence of CRP, whereas at a concentration of as low as 5 μg/ml, CRP triggered a marked increase in HMGB1 levels that was significantly up-regulated at ≥20-μg/ml concentrations of CRP, as determined by Western blot analysis (Fig. 1D, upper panel) and ELISA (Fig. 1D, lower panel). Next, we evaluated the time-course effects of CRP stimulation on HMGB1 release. HMGB1 was detected in the cultured medium after 12 h and continued to increase up to 24 h in response to 20 μg/ml CRP (Fig. 1E). We further confirmed the specific action of CRP on the release of HMGB1 by stimulating cells with heat-inactivated purified CRP or supernatants that were obtained by IP of the purified CRP with anti-CRP antibody or control IgG for 24 h. As shown in Fig. 1F, IP or heat inactivation of the purified CRP caused a marked abrogation of HMGB1 release (control, Lane 1, 13.7±1.7 ng/ml; CRP, Lane 2, 89.7±1.6 ng/ml; CRP+anti-CRP (IP), Lane 3, 13.8±1.7 ng/ml; CRP+control IgG (IP), Lane 4, 84.7±2.2 ng/ml; heat-inactivated CRP, Lane 5, 12.2±0.9 ng/ml), whereas nonimmune control IgG exhibited no loss of CRP activity (Lane 4, 84.7±2.2 ng/ml). Since HMGB1 can be released passively from the nucleus into the cytosol and extracellular space following necrotic cell death, we examined whether the CRP-induced release of HMGB1 originated from necrotic cells. As shown in Fig. 1G, no significant cell death was observed by fluorescence-activated cell sorter (FACS) analysis using Annexin V staining after exposure to CRP (up to 80 μg/ml) for 24 h. To further confirm that the CRP-induced release of HMGB1 by RAW264.7 cells was accompanied by activation of the cells, we evaluated the release of cytokine TNF-α in culture supernatants of CRP-treated RAW264.7 cells by ELISA. As shown in Fig. 1H (upper panel), CRP induced a significant

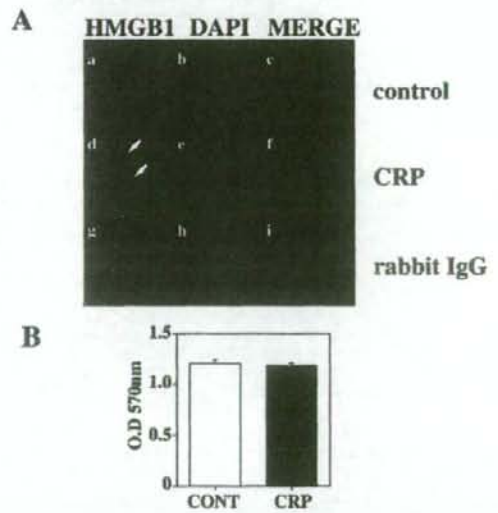

Fig. 2. Nuclear translocation of HMGB1 in response to CRP. (A) RAW264.7 cells were incubated with or without CRP (20 μg/ml) for 16 h. Nontreated (a–c) and CRP-treated (d–f; arrow) cells were incubated with rabbit anti-HMGB1 polyclonal antibody, then with FITC-labeled anti-rabbit IgG as a secondary antibody. As a control IgG, cells were treated with normal rabbit IgG (g–i). Nuclei were labeled with DAPI (original magnification ×400). Arrows indicate nuclear translocation of HMGB1. (B) Effects of CRP on cell viability. Cells were incubated with CRP (20 μg/ml), and the viability of the cells was evaluated by MTT assay. Values are presented as mean±S.D. The data shown are representative of three independent experiments.

release of TNF-α (10±2 ng/ml) compared to control (in the absence of CRP). However, 50 ng/ml TNF-α (fivefold of CRP-induced levels) failed to induce the release of HMGB1 by RAW264.7 cells under the same experimental conditions (Fig. 1H, lower panel). Consistent with this result, anti-TNF-α antibody failed to significantly suppress CRP-induced HMGB1 release (data not shown). These results collectively indicated that CRP stimulated the active release of HMGB1 by RAW264.7 cells and that this was not mediated by cytokine elaboration (at least not through a TNF-α-dependent manner, although we cannot rule out the possible involvement of other cytokines).

3.2. Nuclear translocation of HMGB1 in response to CRP

We next investigated whether CRP stimulation influenced HMGB1 cellular localization. Immunofluorescence analysis revealed that CRP (20 μg/ml) stimulation caused a distinct translocation of nuclear HMGB1 to the cytoplasm (Fig. 2A, d–f, arrow), whereas HMGB1 was observed only in the nucleus in controls (without CRP stimulation) (Fig. 2A, a–c). As expected, no nuclear translocation of HMGB1 was observed in CRP-treated cells that were stained with control

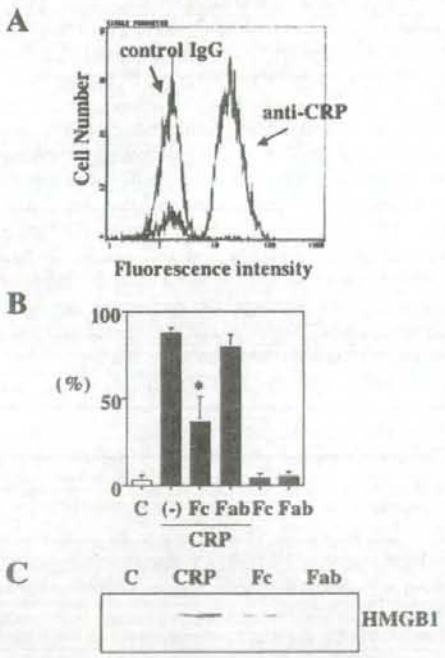

Fig. 3. CRP induced HMGB1 release through the Fcγ receptor. (A) Direct binding of CRP with RAW264.7 cells. The cells were exposed to CRP (20 μg/ml) for 2 h, then incubated with anti-CRP antibody (anti-CRP) or rabbit IgG (control IgG). Next, the cells were incubated with FITC-labeled anti-rabbit IgG antibody and analyzed by flow cytometry. (B) Inhibition of CRP binding by IgG–Fc fragment. Cells were pretreated with Fc fragment (Fc; 1 μM) or Fab fragment (1 μM) for 30 min, then incubated with CRP for 30 min. Cells were then incubated with FITC-labeled anti-CRP rabbit IgG antibody and analyzed by flow cytometry. *Statistically significant ($P<.05$) changes (significantly decreased compared to CRP; Lane 2). (C) HMGB1 release by Fc. CRP was incubated with or without Fc or Fab in RAW264.7 cells, and supernatants were analyzed for HMGB1 levels by Western blot analysis.

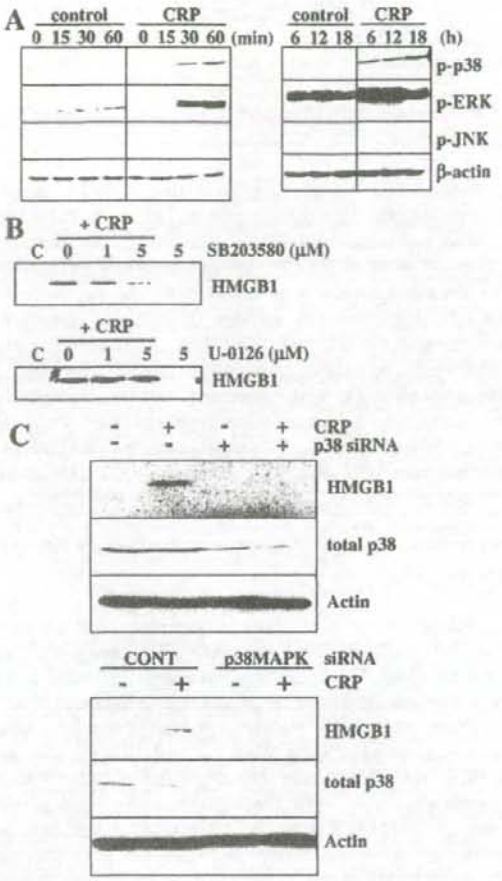

Fig. 4. CRP-induced HMGB1 release mediated by p38MAPK activation. (A) CRP triggered the activation of MAPKs. RAW264.7 cells were incubated with CRP (20 μg/ml) for 0–18 h. Activation of p38MAPK, ERK1/2, and JNK was assayed by Western blot analysis with antibodies specific for p-p38MAPK, pERK1/2, and p-JNK1/2 as, described in Materials and Methods. β-Actin was used as a loading control. Each test represents three separate experiments. (B) RAW264.7 cells were pretreated with SB203580 (1 and 5 μM) or U-0126 (1 and 5 μM) for 15 min, and then incubated with CRP (20 μg/ml) for 20 h. The levels of HMGB1 released into the supernatants were analyzed by Western blot analysis. (C) p38MAPK siRNA inhibited CRP-induced HMGB1 release and p38MAPK expression. RAW264.7 cells were transfected with siRNA for p38MAPK (upper panel) or control (lower panel), and then incubated with CRP (20 μg/ml) for 20 h. The levels of HMGB1 in the supernatants and the expression of p38MAPK were analyzed by Western blot analysis with anti-HMGB1 antibody and anti-total p38MAPK antibody, respectively. Actin was used as a loading control. The data shown are representative of three independent experiments.

IgG instead of HMGB1 (Fig. 2, g–i). Furthermore, as shown Fig. 2B, CRP (20 μg/ml) did not significantly induce cell death as evaluated by MTT assay.

3.3. CRP-induced HMGB1 release is mediated through the Fcγ receptor

Human CRP has been shown to bind directly to murine macrophages through the Fcγ receptor [10,14,25–28], which is also expressed in RAW264.7 cells [29]. We therefore examined whether the CRP-stimulated release of HMGB1 by RAW264.7 cells was mediated by the Fcγ receptor through flow cytometry analysis. CRP was found to bind directly to RAW264.7 cells (Fig. 3A), and this binding was significantly suppressed (about 40% compared to nontreated cells) when cells were pretreated with mouse IgG–Fc fragment (Fc), but not IgG–Fab fragment (Fab) (Fig. 3B). Furthermore, Fc, but not Fab, significantly induced HMGB1 release from cells (Fig. 3C), suggesting that CRP bound directly to the RAW264.7 cells through the Fcγ receptor.

3.4. CRP triggers the activation of ERK1/2 and p38MAPK, but not JNK

Recent studies have demonstrated that CRP activates MAPKs (ERK1/2, JNK, and p38MAPK) through the Fcγ receptor [30,31]. We examined whether CRP stimulation accompanied the activation of MAPKs in RAW264.7 cells by Western blot analysis. CRP stimulation caused a marked activation of MAPKs, p38MAPK, and ERK1/2, but not JNK, in RAW264.7 cells (Fig. 4A, left panel). In parallel, β-actin was used as a loading control in Western blot analysis. p38MAPK and ERK1/2 activation was detected within 30 min, dramatically increased for 1 h, and was thereafter sustained for 18 h (right panel). However, a weak activation of ERK1/2, but not p38MAPK, was observed in nonstimulated cells (control). These results raise the possibility that CRP-induced HMGB1 release may be accompanied by the activation of ERK1/2 and p38MAPK.

3.5. Involvement of p38MAPK in CRP-induced HMGB1 release

We then evaluated the role of the activation of MAPKs p38MAPK and ERK1/2 in HMGB1 release by examining whether specific inhibitors for p38MAPK (SB203580) and ERK1/2 (U-0126) could suppress CRP-induced HMGB1 release by RAW264.7 cells. Cells were treated with SB203580 (1 or 5 μM) or U-0126 (1 or 5 μM) for 60 min prior to CRP stimulation and were then left in the culture until harvest at 20 h poststimulation. It was found that SB203580 (dose dependently), but not U-0126, significantly suppressed CRP-induced HMGB1 release (Fig. 4B). We further confirmed the results by knocking down p38MAPK in RAW264.7 cells using specific siRNA of p38MAPK. RAW264.7 cells transfected with p38MAPK siRNA showed marked suppression of p38MAPK expression and complete inhibition of CRP-induced HMGB1 release (Fig. 4C, upper panel)—effects that were not exhibited by RAW264.7 cells that had been transfected with control siRNA (Fig. 4C, lower panel).

4. Discussion

In this study, we have demonstrated that purified CRP (sodium azide and LPS free) induced an active release of HMGB1 in a time- and dose-dependent manner by macrophage RAW264.7 cells through the Fcγ receptor. This induction of HMGB1 release was completely abrogated by heat inactivation or IP of the purified CRP with anti-CRP antibody, again confirming that this effect of CRP was not caused by LPS contamination. CRP at concentrations of >5 pg/ml has been shown to stimulate cultured human monocytes to release the inflammatory cytokines IL-1β, TNF-α, and IL-1β—an effect that is unaffected by polymyxin B but is cancelled by boiling CRP [32], thus implying that the purified CRP can trigger the release of inflammatory cytokines. Moreover, no loss of cell viability was observed in response to CRP (up to 80 μg/ml) for 24 h, as judged by MTT assay and FACS analysis using Annexin V staining. CRP was also found to stimulate the cells to induce a significant release of TNF-α (an inflammatory cytokine) and to slightly express PGE_2(120±10 pg/ml). On the other hand, CRP did not induce matrix metalloproteinases 2 and 9 in RAW264.7 cells (data not shown). Although CRP induced a significant release of TNF-α, anti-TNF-α antibody failed to significantly suppress CRP-induced HMGB1 release (data not shown). These findings thus further confirm that CRP-induced release of HMGB1 was due to the activation, but not the death, of cells and further indicated that the effect of CRP was not mediated by cytokine elaboration, at least not in a TNF-α-dependent manner, although we cannot rule out the possible involvement of other cytokines.

The concentrations of CRP (5–80 μg/ml) used in the present study are equivalent to those observed in obesity and cardiovascular diseases [33–36]. The finding of the present study—that pathophysiological ranges of CRP induced a significant release of HMGB1 by macrophages—thus highlights an important pathophysiological role for CRP in many inflammatory systems.

The p38MAPK signaling pathway plays an important role in promoting inflammatory diseases [37–39]. Activation of p38MAPK induces the production of key inflammatory mediators, including TNF-α, IL-1β, and HMGB1 [38–40], suggesting that p38MAPK is an obvious therapeutic target for chronic inflammatory diseases. In the current study, we observed that CRP triggered the activation of ERK1/2 and p38MAPK, which was sustained up to 18 h. We examined the functional relationship between MAPK activity and the HMGB1-releasing process. We found that p38MAPK, but not ERK1/2 MAPK, activity played a crucial role in CRP-induced HMGB1 release, as SB203580 (the pharmacological

inhibitor and siRNA of p38MAPK), but not U-0126, significantly abrogated HMGB1 release. This suggested the involvement of p38MAPK in CRP-induced HMGB1 release by RAW264.7 cells. Our findings are consistent with a previous report demonstrating that HMGB1 release is mediated through the p38MAPK signaling pathway [40]. However, it has also been reported that HMGB1 release can occur through the activity of MAPKs other than p38MAPK [21,28]. This discrepancy of results might be due to differences in the cell types and stimulants examined.

The level of CRP, a key inflammatory cytokine, is a strong predictor of cardiovascular events [1]. CRP has a prognostic value for predicting the activity and vulnerability of atheromatous plaque rupture [31,41–43]. Highly elevated CRP in atherosclerosis patients not only serves as a biomarker for cardiovascular disease risk but also functions as an active mediator of atherosclerosis by promoting arterial endothelial activation and macrophage recruitment [44]. Recent studies have also shown that CRP is expressed in macrophages and VSMCs in atheromatous plaques [2–5] and plays a role in the progression and vulnerability of atherosclerotic lesions. Several investigators have suggested that therapeutic inhibition of CRP is a new approach for the treatment of cardiovascular diseases [13,14]. Atherosclerosis is now considered a chronic inflammatory disease of the arterial system. Although endothelial injury and dysfunction remain central to the initiation and pathogenesis of the disease, accumulating evidence suggests that inflammation evoked by injury plays a pathogenic role in all stages of the disease, from initiation to plaque rupture and associated thrombotic complications. On the other hand, HMGB1, which is released from macrophage lineage cells in response to the inflammatory cytokines TNF-α, IL-1β, IFN-γ, and transforming growth factor-β of acute-phase inflammation, plays a pivotal role in chronic inflammatory diseases and also acts as a late-phase lethal mediator in endotoxin shock [15,19,20,27,40,45,46]. HMGB1 acts on its receptor, RAGE, and activates NF-κB signaling, resulting in the expression of proinflammatory cytokines, including IL-1 and TNF-α. HMGB1 has also been shown to stimulate human umbilical vascular endothelial cells, thereby up-regulating adhesion molecules such as ICAM-1, VCAM-1, and E-selectin, inducing granulocyte colony-stimulating factor expression and IL-8 release [45]. CRP induces the expression of ICAM-1, VCAM-1, and E-selectin, in addition to the chemokine MCP-1 [10,47]. These findings suggest that CRP promotes endothelial cell activation and dysfunction, indicating that CRP may enhance and amplify atherosclerosis by promoting the inflammatory component of atherosclerosis by both activated macrophages. We have recently demonstrated that CRP is colocalized with the proinflammatory cytokine HMGB1 in macrophages and VSMCs in atherosclerotic lesions [14]. Kalinina et al. [21] have recently reported that HMGB1 expression levels are up-regulated in atherosclerotic lesions and that macrophages are the major cell type responsible for the production of HMGB1. They also suggested that HMGB1 plays a role in the pathogenesis of plaque formation and progression [21]. Thus, others, as well as our previous reports and the findings of the present study, provide evidence for potential links among CRP, HMGB1, and atherosclerosis. In the current study, we used a murine macrophage cell line, RAW264.7, the cells of which are often used as fair substitutes for macrophages in analyzing the production of inflammatory mediators/cytokines, including HMGB1, in response to various inflammatory stimuli [48,49]. It has been reported that murine macrophage RAW 264.7 cells, human or mouse alveolar macrophages, and monocytes, when differentiated into macrophages, exhibit almost similar patterns of proinflammatory mediator production [50–52]. Similar levels of HMGB1 release by LPS or IFN-γ-activated macrophage RAW264.7 cells and human peripheral blood monocytes have also been reported [45,53]. However, we cannot rule out the possibility that CRP may exhibit some influence on human monocytes/macrophages to release HMGB1. Further study will be needed to clarify this important issue. Indeed, we hope to continue our investigations into the release of HMGB1 by human and animal monocytes/macrophages (by in vitro and in vivo studies) in response to the purified CRP.

Taken together, to the best of our knowledge, this is the first study to have demonstrated that CRP triggers an active release of HMGB1 by macrophage RAW264.7 cells through the Fcγ receptor and p38MAPK signal transduction pathways. Our findings suggested that CRP plays a potentially important role in the induction, amplification, and prolongation of inflammatory processes, including atherosclerosis, by inducing the release of the key inflammatory mediator HMGB1 and thus presents a potential target for the treatment of cardiovascular diseases.

5. Summary

The interaction between proinflammatory cytokines, CRP, and HMGB1 is unclear. Here, we show that CRP induced HMGB1 release by macrophage (RAW264.7) cells through the Fcγ receptor and the activation of p38MAPK, suggesting that CRP plays an important role in the propagation and prolongation of inflammation.

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Saturdays at 12.10pm, repeated Mondays at 7.10pm
with Robyn Williams

Print

Bite of the Komodo Dragon

Saturday 3 December 2005

Summary

Until Melbourne researchers published their findings in November this year, it was thought that toxic mouth bacteria were responsible for the affects of being bitten by a Komodo Dragon. But lead researcher Brian Fry says these large monitor lizards actually carry venom and around 200 million years ago they shared a common ancestor with venomous snakes.

Program Transcript

Chris Smith: For years it was thought that the seething mass of bacteria living in the mouths of these lizards were responsible for the unpleasant effects of being bitten. But in a landmark study in this week's edition of *Nature* , University of Melbourne research Brian Fry and his colleagues show that these and other lizards are actually venomous and that about two hundred million years ago they shared a common ancestor with the venomous snakes.

Brian Fry: In the Komodo dragons people have long thought that they've actually got this toxic bacteria in their mouth but it turns out that this is actually wrong. When people get bitten or when the animals get bitten they get sick far too quickly for it to be a bacteria, so we actually had a look. And lo and behold it turns out that they actually have venom.

Chris Smith: What sort of time scale does the venom act over then?

Brian Fry: When an animal like this bites you a human can have effects within minutes, rapid swelling of finger and then bleeding that can last for an hour or two as well as shooting pains that can extend up the entire arm. So the effects are quite rapid in their development, far too quickly for a bacteria to work.

Chris Smith: So how does this fit into our understanding of where they got this venom?

Brian Fry: Well, what we did was we had a look at not just giant dragons but also their closest relatives and what we actually found out was that the Komodo dragons and the other monitor lizards share an ancestor with the venomous lizards like the Gila monsters and the beaded lizards, but then it got even more surprising that we were actually able to trace it all the way back to where these venomous lizards shared a common venomous ancestor with the snakes. So what this means is that snakes were venomous from their very beginning and that the snakes that we have nowadays that don't have venom, like the powerful constricting pythons and such, actually have lost their venom after they developed a much more effective prey capture strategy for killing giant gazelles and such.

Chris Smith: But these animals have got pretty big jaws, haven't they? Is it not good enough to just grab something and hang

onto it?

Brian Fry: Well, with prey capture it's more than just having venom or it's more than just having very large sharp teeth, it's all this working together where you're building an arsenal. So it's kind of interesting, for example, to look at the monitor lizards where they have very large teeth but the heads are pretty elegant looking. But if you compare them to the South American Tegus lizard that don't have the venom, they are a much more muscular animal, they have very, very strong jaws, much more powerful crushing jaws than you get in a monitor lizard. So it shows that venom has shaped not just the skull morphology where they don't need to be as powerful in their bite but also it's allowed them to radiate and diversify. So now that we've discovered they're venomous we've got to go back and re-evaluate the entire life history of these animals. All this stuff about toxic bacteria we've got to just throw away and just start with a fresh piece of paper and re-evaluate what's going on.

Guests on this program:

Dr Bryan Fry
Australian Venom Research Unit
Department of Pharmacology
University of Melbourne
Parkville Victoria
http://www.avru.unimelb.edu.au/avruweb/staff.htm

Chris Smith
Presenter, *Nature* podcasts
http://www.nature.com/nature/podcast/index.html

Presenter: Robyn Williams
Producer: Polly Rickard and David Fisher

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High Mobility Group Box Chromosomal Protein 1 in Patients with Renal Diseases

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Key Words
Vasculitis · Inflammation · IgA nephropathy · Cytokines · Crescentic glomerulonephritis · Henoch-Schönlein purpura · Antineutrophil cytoplasmic antibody

Abstract
Background/Aim: The high mobility group box chromosomal protein 1 (HMGB1), a nuclear DNA-binding protein, has recently been recognized as a new proinflammatory cytokine. The purpose of this study was to examine the significance of HMGB1 in patients with renal diseases. ***Methods:*** HMGB1 concentrations in sera were measured by enzyme-linked immunosorbent assay, and antibodies against HMGB1 were examined by Western blotting in patients who underwent renal biopsies and in healthy controls. Immunohistochemistry for HMGB1 was also performed. ***Results:*** Serum HMGB1 was more likely to be positive in patients who underwent renal biopsies as compared with the controls. Patients with anti-neutrophil cytoplasmic antibody-related glomerulonephritis (ANCA-GN) and those with Henoch-Schönlein purpura nephritis showed a significantly higher tendency to be HMGB1 positive. The presence of anti-HMGB1 antibody was not associated with the presence of serum HMGB1. Immunohistochemistry revealed that HMGB1 was expressed in mononuclear cells in the interstitium or in the glomeruli of some patients with ANCA-GN or IgA nephropathy (IgAN). Subanalysis demonstrated that among patients with IgAN, those who had crescent formation showed a higher tendency to be HMGB1 positive than those who did not. ***Conclusions:*** HMGB1 was expressed in the sera of patients with renal diseases who underwent renal biopsies, especially among those who had vasculitis including ANCA-GN, Henoch-Schönlein purpura nephritis, and IgAN with glomerular crescents.

Introduction

The high mobility group box chromosomal protein 1 (HMGB1; previously called HMG1) was originally discovered as a nuclear DNA-binding protein. It is a ubiquitous protein present in eukaryotic cells with highly conserved amino acid sequences among species [1–3]. As a nuclear protein, HMGB1 stabilizes the nucleosomal structure, enables the binding of transcription factors to their cognate DNA sequences, and facilitates gene transcription [4–7].

HMGB1 is also expressed in the extracellular space. Relatively recent studies have demonstrated that it functions as a potent proinflammatory mediator [8–12].

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