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H. 知的財産権の出願・登録状況

特になし。

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分担研究報告書

HMGB1 の細胞外放出の分子機構とその制御法の研究

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研究要旨

血管内皮細胞およびマクロファージにおける HMGB1 の放出機構について検討するとともに、その制御法について検討した。その結果、リポ多糖（LPS）およびリポタイコ酸（LTA）の刺激による HMGB-1 の放出には、能動的放出のほかに壊死による放出が含まれていることが明らかになった。また、Gp96 は HMGB1 の放出を制御している可能性が示唆された。

A. 研究目的

敗血症性ショック時における HMGB1 放出の制御は、ショックや多臓器不全の救命的医療において極めて重要である。本研究では、細菌のエンドトキシン刺激による血管内皮細胞およびマクロファージの HMGB1 放出について解析するとともに、その制御の可能性について検討した。

B. 研究方法

（1）エンドトキシン刺激による HMGB1 放出の解析

ヒト大動脈由来血管内皮細胞（HAEC）およびマウスマクロファージ系細胞株 RAW264.7(RAW) 細胞を細菌由来エンドトキシン（LPS、LTA）で刺激し、経時的に培養上清を回収した。その後、培養上清を回収し、ELISA で HMGB1 濃度を測定した。また、同時に、細胞の生存率を MTT 法で測定した。

（2）Gp96 の knock-down による HMGB1 制御の試み

RAW 細胞に Gp96 の siRNA を導入し、Gp96 mRNA の knock-down を試みた。その後、LPS 刺激による HMGB1 放出の程度を検討した。

C. 研究結果

（1）エンドトキシン刺激による HMGB1 放出

血管内皮細胞を LPS および LTA で刺激すると 6 時間後から HMGB1 の放出が認められ、48 時間後にピークを迎えた。RAW 細胞培養系でも同様の傾向がみられたが、その量は血管内皮のそれより 10 倍程度多かった。それらの細胞の生存率を調べると 24 時間後から細胞の viability 低下が確認された。

（2）Gp96 siRNA による HMGB1 放出制御

Raw 細胞に gp96 siRNA を導入し、24 時間後その発現レベルを調べた。その結果、その発現は 80%程度抑止されることが確認された。次いで、同細胞を LPS で刺激し、24 時間後の HMGB1 放出量を調べた。その結果、gp96 の knock-down によって HMGB1 の放出が 50%程度抑制された。

D. 考察

本研究の結果から、細菌由来のエンドトキシン (LPS,LTA)によって血管内皮細胞およびマクロファージからの HMGB1 の放出が誘導されたが、細胞死も同時に誘導されていることから、それは能動的な分泌誘導とともに細胞からの溢出の総和であることが示唆された。また、ER シャペロンの一種である gp96 は LPS による HMGB1 の放出

を制御することが明らかになった。Gp96 は LPS や LTA の受容体である TLRs の発現を制御して、エンドトキシンに対する細胞応答を制御している可能性が考えられる。

E. 結論

Gp96 は HMGB1 放出制御のための標的分子として有用である可能性が示唆された。

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Ⅲ. 研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

ELSEVIER

Cardiovascular Pathology xx (2007) xxx – xxx

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

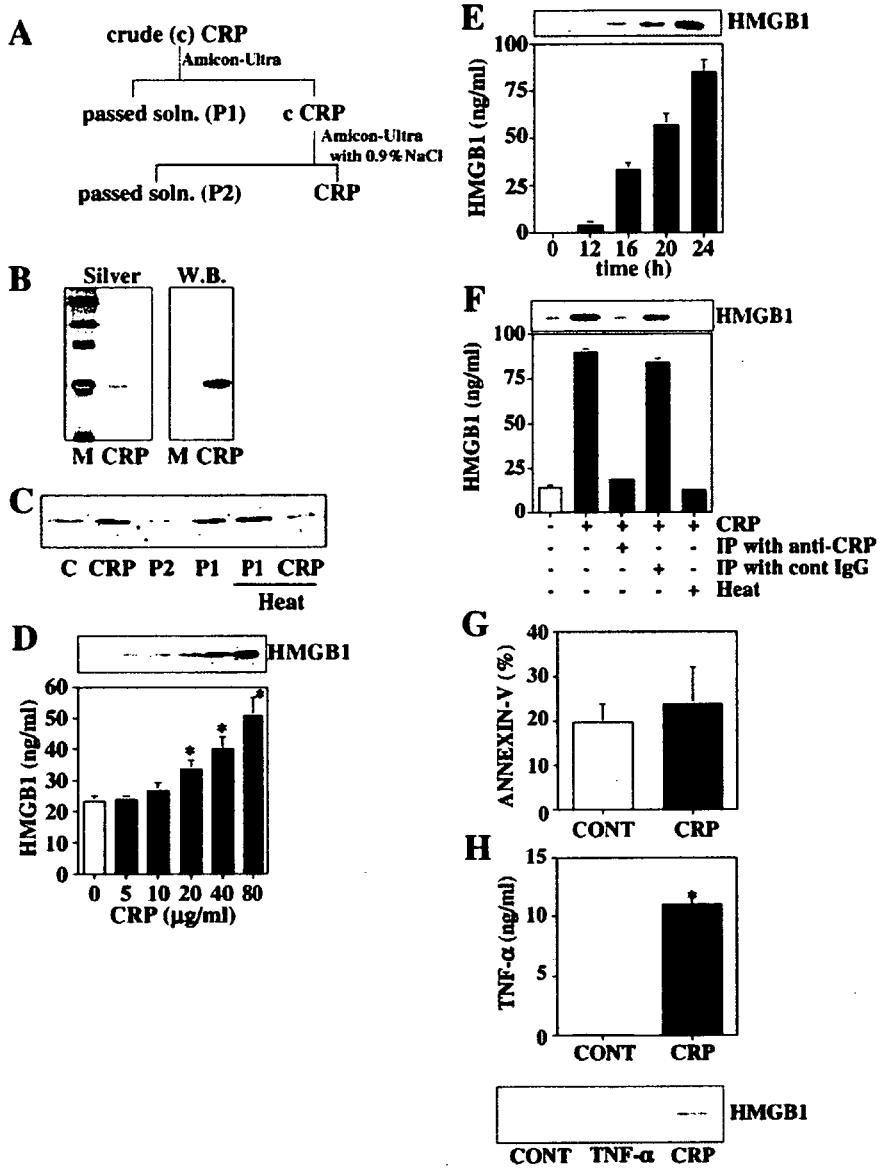

Fig. 1. CRP-induced HMGB1 release by RAW264.7 cells. (A) Schematic presentation of CRP purification with Amicon-Ultra. cCRP was separated with Amicon-Ultra, and the first-pass solution (soln.) was designated as P1. cCRP was washed with LPS-free 0.9% NaCl, and the second-pass solution was designated as 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

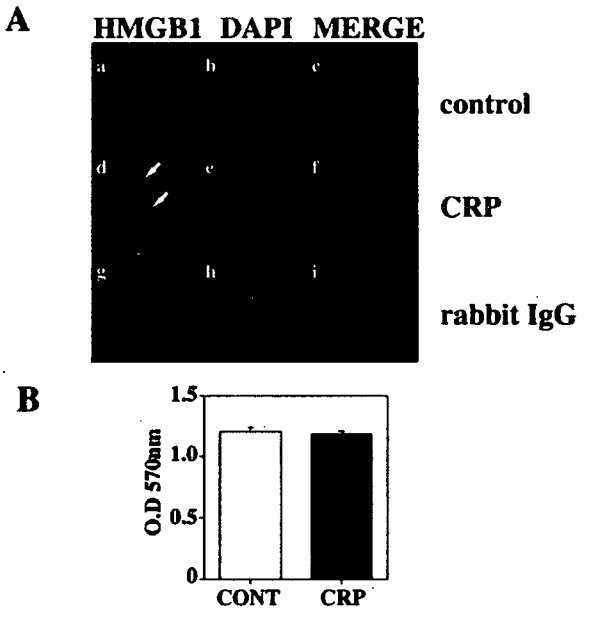

Fig. 2. Nuclear translocation of HMGB1 in response to CRP. (A) RAW264.7 cells were incubated with or without CRP (20 μg/ml) for 16 h. Nontreated (a–c) and CRP-treated (d–f; arrow) cells were incubated with rabbit anti-HMGB1 polyclonal antibody, then with FITC-labeled anti-rabbit IgG as a secondary antibody. 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

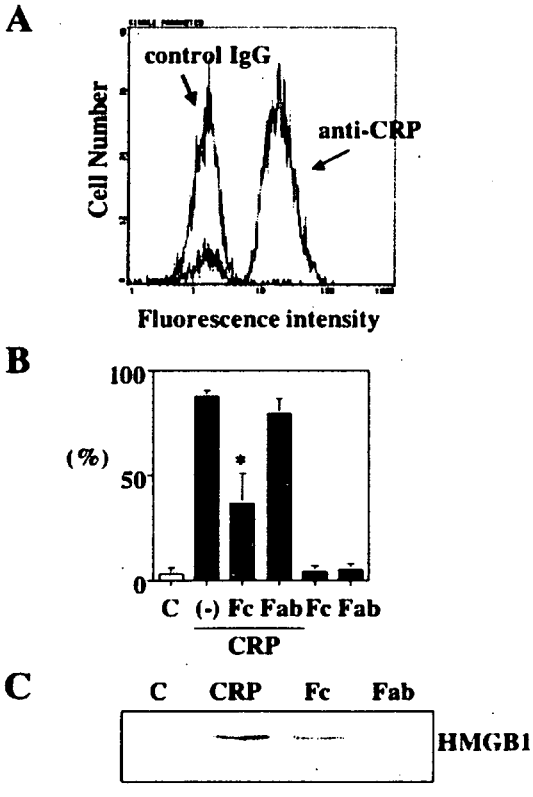

Fig. 3. CRP induced HMGB1 release through the Fcγ receptor. (A) Direct binding of CRP with RAW264.7 cells. The cells were exposed to CRP (20 μg/ml) for 2 h, then incubated with anti-CRP antibody (anti-CRP) or rabbit IgG (control IgG). Next, the cells were incubated with FITC-labeled anti-rabbit IgG antibody 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.

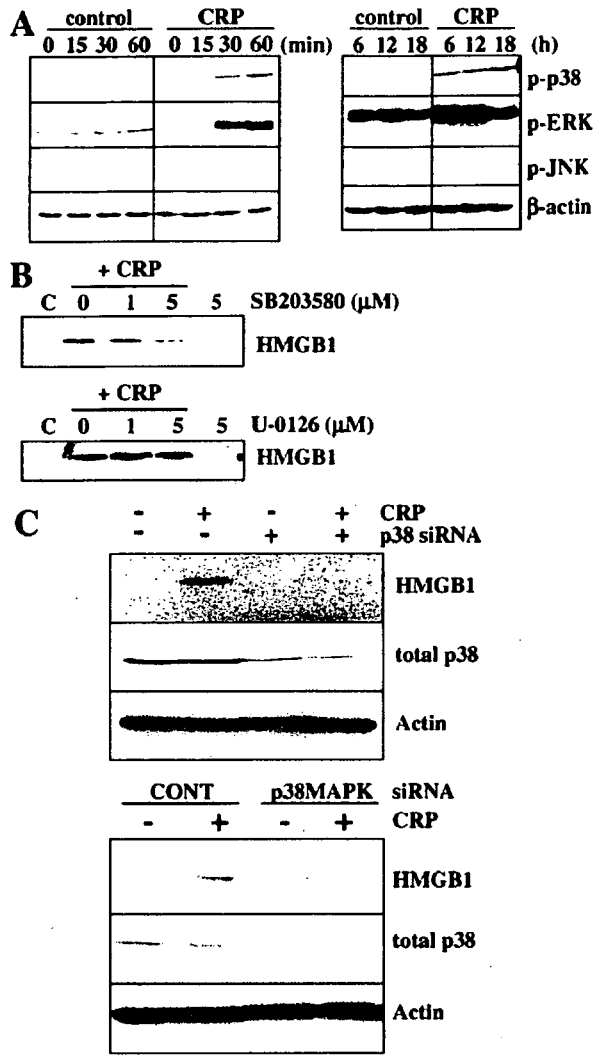

Fig. 4. CRP-induced HMGB1 release mediated by p38MAPK activation. (A) CRP triggered the activation of MAPKs. RAW264.7 cells were incubated with CRP (20 μg/ml) for 0–18 h. Activation of p38MAPK, ERK1/2, and JNK was assayed by Western blot analysis with antibodies specific for p-p38MAPK, pERK1/2, and p-JNK1/2 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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