Antimicrobial cathelicidin peptide CAP11 suppresses HMGB1 release from lipopolysaccharide-stimulated mononuclear phagocytes via the prevention of necrotic cell death

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Abstract. High mobility group box-1 (HMGB1) is extracellularly released from mononuclear phagocytes by lipopolysaccharide (LPS)-stimulation accompanied with cell death, and plays an important role in septic/endotoxin shock as a late phase mediator. Notably, CAP11 (cationic antibacterial polypeptide of 11-kDa), a member of cathelicidin family of antimicrobial peptides, has a potential to bind with LPS and neutralize the biological activity of LPS. In this context, we previously revealed that CAP11 can suppress the elevation of serum HMGB1 level in mouse endotoxin shock model and protect mice from endotoxin lethality. In the present study, to clarify the inhibitory mechanism of CAP11 on HMGB1 release, we evaluated the effect of CAP11 on the LPS-induced HMGB1 release and apoptotic/necrotic cell death using a murine macrophage cell line RAW264.7. The results indicated that LPS-stimulation induced the release of HMGB1 from RAW264.7 cells, accompanied with both apoptotic and necrotic cell death. Of interest, CAP11 markedly inhibited the binding of LPS to target RAW264.7 cells, and suppressed HMGB1 release as well as necrotic cell death; however, CAP11 could not affect the LPS-induced apoptotic cell death. These observations clearly indicate that CAP11 can efficiently abolish necrotic cell death via the inhibition of LPS-binding to target cells, thereby suppressing the release of HMGB1. Thus, CAP11 could be a therapeutic agent for septic/endotoxin shock, with a potential to regulate the release of HMGB1 from LPS-stimulated mononuclear phagocytes via the suppression of LPS-binding to target cells and prevention of necrotic cell death due to its potent LPS-binding activity.

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Introduction

Sepsis is caused by polymicrobial infections, and associated with severe systemic inflammatory response syndrome that leads to multiple organ failure (such as acute lung injury and renal failure) and septic shock (1-3). In septic shock (especially endotoxin shock), lipopolysaccharide (LPS), a major component of Gram-negative bacteria, plays a pivotal role by stimulating mononuclear phagocytes (macrophage and monocyte) to secrete various proinflammatory substances (e.g., cytokines, reactive oxygen species, arachidonic acid metabolites, NO and proteases) (1,2). Since these proinflammatory substances are overproduced and involved in the pathogenesis of septic shock, most therapeutic strategies have targeted the blockade of proinflammatory molecules; however, most of the strategies have been unsuccessful (4,5). Thus, the development of novel agents with therapeutic potential for sepsis/septic shock is explored.

High mobility group box-1 (HMGB1) is a highly conserved, non-histone nuclear protein with multiple functions. HMGB1 binds with DNA and participates in the gene transcription in the cells. Moreover, HMGB1 functions as a cytokine in the extracellular milieu (6-8); HMGB1 upregulates proinflammatory cytokines (e.g., TNF-α, IL-1ß and IL-8) in human mononuclear cells and neutrophils (9,10), and adhesion molecules (such as ICAM, intracellular cell adhesion molecule-1 and VCAM, vascular cell adhesion molecule-1) in endothelial cells (11). Furthermore, HMGB1 is suggested to play a crucial role in endotoxin shock as a late phase mediator, based on the findings that serum level of HMGB1 is increase in a late phase (8-32 h) after LPS exposure, and administration of anti-HMGB1 antibody attenuates the endotoxin lethality in mice (6).

Mammalian cells express a number of peptide antibiotics that function as effector components in the innate host defense system. Defensins and cathelicidins are the two major classes of antimicrobial peptides, which can kill a broad spectrum of invading micro-organisms, including both Gram-positive and Gram-negative bacteria, fungi and viruses (12-14). Previously, we revealed that CAP11 (cationic antibacterial polypeptide of 11-kDa), a member of cathelicidins isolated from guinea pig neutrophils, has potentials to not only kill

bacteria but also neutralize LPS; CAP11 can inhibit the binding of LPS to CD14-positive target cells and protect mice from endotoxin shock (15,16). Moreover, we have found that administration of CAP11 suppresses the increase of serum HMGB1 level in mouse endotoxin shock model (17), and HMGB1 is reported to be released from macrophages by LPS-stimulation, accompanied with cell death (18). Thus, we hypothesized that CAP11 may suppress the release of HMGB1 from mononuclear phagocytes by regulating cell death of target cells in endotoxin shock model. In this study, to test the possibility, we evaluated the effect of CAP11 on the LPS-induced HMGB1 release and apoptotic/necrotic cell death using a murine macrophage cell line RAW264.7 as a target cell.

Materials and methods

Reagents. Lipopolysaccharide (LPS, 0111:B4) was purchased from Sigma Chemical (St. Louis, MO); Alexa Fluor-488 conjugated LPS from Molecular Probes (Eugene, OR); rabbit anti-HMGB1 polyclonal antibody from Abcam (Cambridge, UK). A 43-mer peptide of CAP11, (G^{1}LRKKFRKTRKR IQKLGRKIGKTGRKVWKAWREYGQIPYPCRI^{43}) was synthesized by a solid phase method on a peptide synthesizer (model PSSM-8; Shimadzu, Kyoto, Japan) by fluorenyl-methoxycarbonyl chemistry, and purified, as described previously (16,19).

Cell culture. A RAW264.7 murine macrophage cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown and maintained in Dalbeco's modified Eagle's medium (DMEM: Sigma) containing 10% fetal calf serum (FCS, endotoxin level <10 EU/ml; Cell Culture Technologies, Herndon, VA), penicillin (100 U/ml) and streptomycin (0.1 mg/ml) at 37˚C in a 5% CO_2 incubator. Cells were detached from monolayer with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2PO_4, 1.5 mM KH_2PO_4, pH 7.4)-0.05% EDTA for passage.

Measurement of HMGB1 release. Release of HMGB1 from RAW264.7 cells was measured by Western blotting. RAW264.7 cells were cultured in a 48-well cell culture plate (10^5 cells/300 μl/well; 60-70% confluent) in DMEM-10% FCS for 20 h. Thereafter, cells were stimulated with LPS (10-500 ng/ml) for 24 h. In some of the experiments, to examine the effect of CAP11, cells were incubated with LPS in the absence or presence of CAP11 (0.1 and 1 μg/ml). After stimulation, culture supernatants were recovered and centrifuged (100 x g, 4 min). Furthermore, cells were recovered in 300 μl lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton-X 100, 0.5% NP-40, pH 7.4) and sonicated (model UD-201 ultrasonic disruptor, Tomy, Tokyo, Japan; output 10 W, duty 10 for 10 times). Culture supernatants or cell lysates were mixed with 4X SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) sample buffer (250 mM Tris-HCl, pH 6.8, 40% glycerol, 8% SDS, 20% ß-mercaptoethanol, 0.02% bromophenol blue), and boiled for 3 min. Aliquots (10 μl; ~$3x10^3$ cell equivalent supernatants and cell lysates) were subjected to 12% SDS-PAGE. After electrophoresis, the proteins were electro-transferred to Immobilon-P polyvinylidene difuoride membrane (Millipore, Bedford, MA) using a Trans-Blot SD apparatus (Bio-Rad, Hercules, CA). The blots were blocked for 1 h with 5% skim milk in Tris-buffered saline (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, pH 7.6)-0.1% Tween-20 (TBST) at room temperature. The blots were washed with TBST, probed for overnight at 4˚C with rabbit anti-HMGB1 antibody (500-fold dilution; Abcam). After extensive washing, the blots were further probed with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (5000-fold dilution; Chemicon International, Temecula, CA), and HMGB1 was finally detected with SuperSignal® West Pico Chemiluminescent substrate (Pierce, Rockford, IL). The detected bands were quantified by a Fujifilm Luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan). The extracellular release of HMGB1 was calculated using the following formula: HMGB1 release (%) = [HMGB1 contents in the supernatants/ HMGB1 contents in the supernatants and cell lysates] x100.

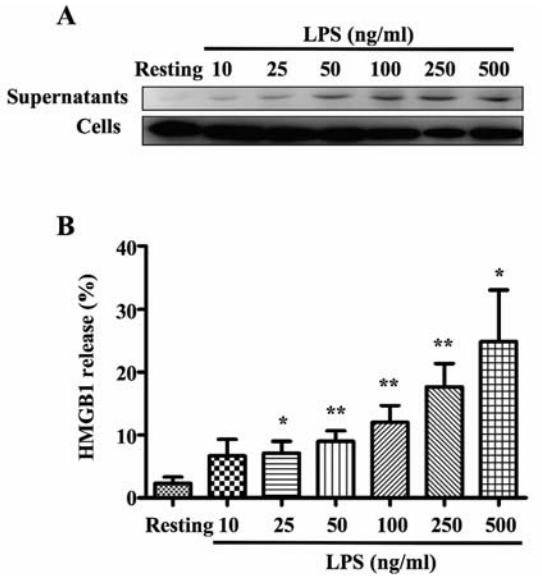

Figure 1. Effect of LPS on HMGB1 release from RAW264.7 cells. RAW264.7 cells were incubated without (Resting) or with LPS (10-500 ng/ml) for 24 h at 37˚C. (A) Culture supernatants and cell lysates were prepared and analyzed on 12% SDS-PAGE for detection of HMGB1 using rabbit anit-HMGB1 antibody and HRP-conjugated goat anti-rabbit IgG. (B) HMGB1 release was calculated using the following formula: HMGB1 release (%) = [HMGB1 contents in the supernatants/HMGB1 contents in the supernatants and cell lysates] x100. Data represent the mean ± SE of seven independent experiments. Values were compared between Resting (without LPS) and LPS-stimulation. $^{*}P<0.05$, $^{**}P<0.01$.

Evaluation of apoptotic and necrotic cell death. Cell death of RAW264.7 was evaluated by flow cytometry using a commercially available Annexin-V-Flous staining kit (Roche Diagnostics, Penzberg, Germany). Cells were incubated in a 12-well cell culture plate ($4x10^5$ cells; 60-70% confluent) with LPS (10-250 ng/ml) in the absence or presence of CAP11 (1 μg/ml) for 24 h. After stimulation, cells were harvested with PBS-0.05% EDTA and incubated with a mixture of Flous-Annexin V and propidium iodide (PI) in binding buffer (10 mM HEPES-NaOH, 140 mM NaCl, 5 mM $CaCl_2$, pH 7.4) at room temperature for 15 min in the dark. After incubation, cells were washed with PBS containing 1 mM $CaCl_2$ and analyzed with FACSCan® flow cytometer and

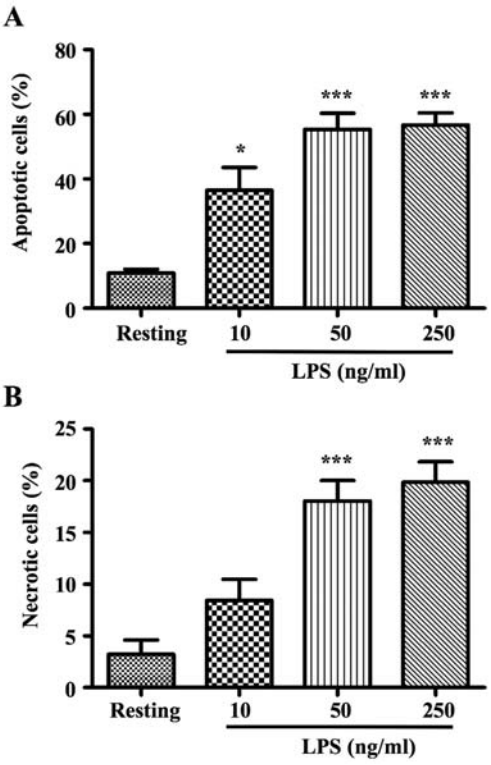

Figure 2. Effect of LPS on apoptotic and necrotic cell death of RAW264.7 cells. RAW264.7 cells were incubated without (Resting) or with LPS (10-250 ng/ml) for 24 h at 37˚C. After incubation, the cells were harvested and analyzed by flow cytometry after annexin V- and PI-staining. Apoptotic cells (A) were defined as annexin V-positive but PI-negative cells, and necrotic cells (B) as annexin V-positive and PI-positive cells. Data represent the mean ± SE of four independent experiments. Values were compared between Resting (without LPS) and LPS-stimulation. $^{*}P<0.05$, $^{***}P<0.001$.

CellQuest software (Becton Dickinson, San Jose, CA). Apoptotic cells were defined as annexin V-positive but PI-negative cells, and necrotic cells as annexin V-positive and PI-positive cells (20).

Measurement of LDH release. Release of lactate dehydrogenase (LDH) from RAW264.7 cells was measured by assessment of LDH activity. RAW264.7 cells were cultured in a 48-well cell culture plate (10^5 cells/300 μl/well; 60-70% confluent) in DMEM-10% FCS for 20 h, and then stimulated with LPS (10-500 ng/ml) for 24 h. In some experiment, to examine the effect of CAP11, cells were incubated with LPS in the absence or presence of CAP11 (1 μg/ml). After stimulation, culture supernatants were recovered and centrifuged (100 x g, 4 min). Furthermore, cells were recovered in 300 μl LDH substrate solution (626 μM sodium pyruvate, 45 mM K_2HPO_4, 7.3 mM KH_2PO_4, 1% Triton-X 100) and sonicated. Aliquots (50 μl; ~$1.5x10^4$ cell equivalent supernatants and cell lysates) were incubated with 1.5 ml of LDH substrate solution for 5 min at room temperature. Then, 25 μl NADH solution (13 mM NADH; ß-nicotinamide adenime dinucleotide reduced form in 119 mM $NaHCO_3$) was added, and LDH activities were determined by monitoring the absorbance at 340 nm (oxidation of NADH) in a spectrophotometer for 4 min at room temperature. The release of LDH was calculated using the following formula: LDH release (%) = [LDH activities in the supernatants/LDH activities in the supernatants and cell lysates] x100.

Caspase 3 assay. Caspase 3 activity was assayed using CaspACE™ assay system, Colorimetric (Promega, Madison, WI). RAW264.7 cells were stimulated with LPS (10-250 ng/ml) for 24 h in a 35 mm cell culture dish ($2x10^6$ cells; 60-70% confluent). In some of the experiments, to examine the effect of CAP11, cells were incubated with LPS in the absence or presence of CAP11 (1 μg/ml). After incubation, cells were harvested with PBS/0.05% EDTA and lysed in 50 μl of Cell lysis buffer (Promega). To prepare cytoplasmic protein, cell lysates were sonicated and centrifuged for 20 min at 15000 x g. Protein contents of the supernatants were determined with a BCA™ protein assay kit (Pierce). Extracted protein (100 μg) was incubated with 1 mM acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (a caspase 3-specific substrate) in the absence or presence of 20 μM Z-VAD-FMK (benzyloxycarbonylvalyl-alanyl-aspartyl fluoromethyl ketone), a pan-caspase inhibitor, at 37˚C for 4 h in a total volume of 100 μl of assay buffer. Caspase 3 activity was measured at 405 nm in a model 3550-UV microplate reader (Bio-Rad), and expressed as pmol of *p*-nitroanilide liberated/h/μg protein.

LPS-binding assay. RAW264.7 cells (10^6 cells/ml) were incubated in DMEM-10% FCS with Alexa 488-conjugated LPS (100 ng/ml) at 37˚C for 15 min in the absence or presence of CAP11 (0.1 or 1 μg/ml) or a neutralizing anti-CD14 monoclonal antibody (4C1; a gift from Dr Y. Adachi, Tokyo University of Pharmacy and Life Sciences, 4 μg/ml). Cells were then washed twice with ice-cold PBS, and the LPS-binding was analyzed by flow cytometry (16).

Statistical analysis. The data are expressed as mean ± SE. Statistical analysis was performed by using Student's t-test or one-way ANOVA followed by Bonferroni's multiple comparison analysis. $P<0.05$ was considered as significant.

Results

Effect of LPS on HMGB1 release and cell death. We first examined the effect of LPS on HMGB1 release from RAW264.7 macrophage-like cells. LPS-stimulation dose-dependently induced the extracellular release of HMGB1 from RAW264.7 cells (Fig. 1), and the effects were significant ($P<0.05$) at >25 ng/ml LPS.

It has been previously reported that HMGB1 is extracellularly released from various types of cells accompanied with cell death (18,21-23). Thus, we determined whether LPS-stimulation also induces apoptotic and/or necrotic cell death in RAW264.7 cells. Of importance, similar to the effects on HMGB1 release, LPS dose-dependently elicited both apoptotic (annexin V-positive but PI-negative) and necrotic (annexin V-positive and PI-positive) cell death in RAW264.7 cells, as assessed by flow cytometry (Fig. 2).

Further, we assessed the LPS-induced apoptosis and necrosis, using caspase 3 (a key executor for apoptotic cell death) (24), and LDH (a cytoplasmic enzyme extracellularly released from necrotic cells) (25), as alternative cell death markers. Similar to the results with flow cytometry (Fig. 2),

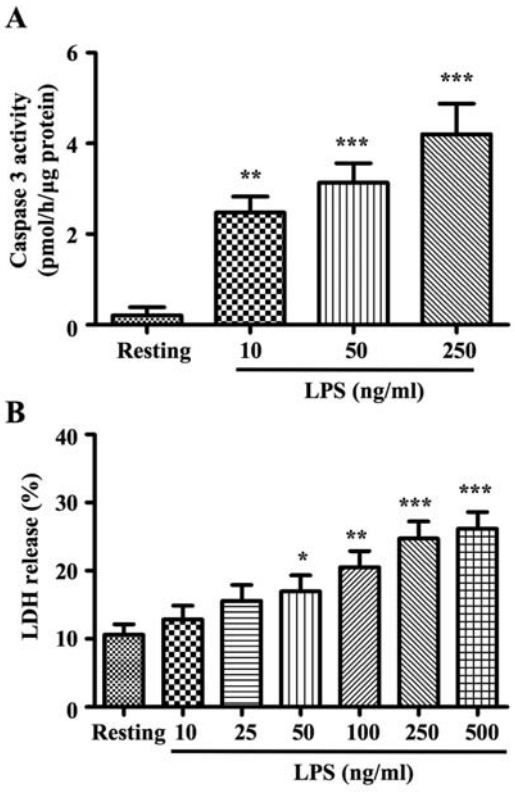

Figure 3. Effect of LPS on caspase 3 activation and LDH release. RAW264.7 cells were incubated without (Resting) or with LPS (10-500 ng/ml) for 24 h at 37˚C. (A) Caspase 3 activity was measured by incubating cell lysates with a caspase 3-specific substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide in the absence or presence of Z-VAD-FMK (a pan-caspase inhibitor) at 37˚C for 4 h. Caspase 3 activity is expressed as pmol of *p*-nitroanilide liberated/h/μg protein. (B) LDH release was measured by assessment of LDH activities in the culture supernatants and cell lysates using sodium pyruvate and NADH. LDH release was calculated using the following formula: LDH release (%) = [LDH activities in the supernatants/LDH activities in the supernatants and cell lysates] x100. Data represent the mean ± SE of six to seven independent experiments. Values were compared between Resting (without LPS) and LPS-stimulation. *P<0.05, **P<0.01, ***P<0.001.

LPS dose-dependently induced caspase 3 activation and LDH release (Fig. 3). Collectively, these observations indicate that LPS-stimulation induces not only HMGB1 release but also apoptotic/necrotic cell death in RAW264.7 cells.

Effect of CAP11 on LPS-induced HMGB1 release and LPS-induced cell death. We next investigated the effect of CAP11 on LPS-induced HMGB1 release from RAW264.7 cells. CAP11 (1 μg/ml) markedly suppressed LPS (250 ng/ml)-induced HMGB1 from the cells (P<0.05); however, 0.1 μg/ml CAP11 did not essentially affect the HMGB1 release (Fig. 4).

Further, we evaluated the effects of CAP11 on the LPS-induced cell death in RAW264.7 cells. Of note, flow cytometric analysis indicated that CAP11 (1 μg/ml) significantly inhibited the LPS (250 ng/ml)-induced necrotic cell death (annexin V-positive and PI-positive) (P<0.01) but not apoptotic cell death (annexin V-positive but PI-negative) (Fig. 5). Consistent with this finding, enzymatic analysis also indicated that CAP11 (1 μg/ml) suppressed LPS (250 ng/ml)-induced necrotic cell death (assessed by LDH release) (P<0.01) but not apoptotic cell death (assessed by caspase 3 activation) (Fig. 6).

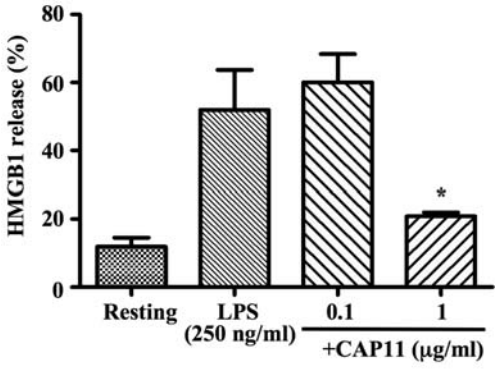

Figure 4. Effect of CAP11 on LPS-induced HMGB1 release from RAW264.7 cells. RAW264.7 cells were incubated with LPS (250 ng/ml) in the absence or presence of CAP11 (0.1 or 1 μg/ml; +CAP11) for 24 h at 37˚C. RAW264.7 cells were also incubated without LPS and CAP11 for 24 h at 37˚C (Resting). After incubation, HMGB1 release was analyzed and calculated as described above. Data represent the mean ± SE of three to four independent experiments. Values were compared between the incubation with LPS alone (LPS) and in the presence of CAP11 (+CAP11). *P<0.05.

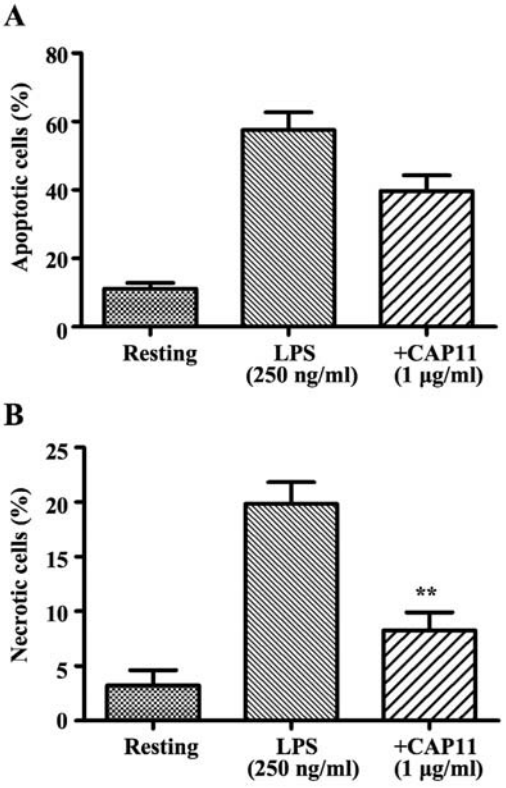

Figure 5. Effect of CAP11 on LPS-induced cell death of RAW264.7 cells analyzed by flow cytometry. RAW264.7 cells were incubated with LPS (250 ng/ml) in the absence or presence of CAP11 (1 μg/ml; +CAP11) for 24 h at 37˚C. RAW264.7 cells were also incubated without LPS and CAP11 for 24 h at 37˚C (Resting). After incubation, apoptotic cell death (A) and necrotic cell death (B) were analyzed by flow cytometry as described above. Data represent the mean ± SE of three to four independent experiments. Values were compared between the incubation with LPS alone (LPS) and in the presence of CAP11 (+CAP11). **P<0.01.

Effect of CAP11 on LPS-binding to RAW264.7 cells. To clarify the inhibitory mechanism of CAP11 on the LPS-induced

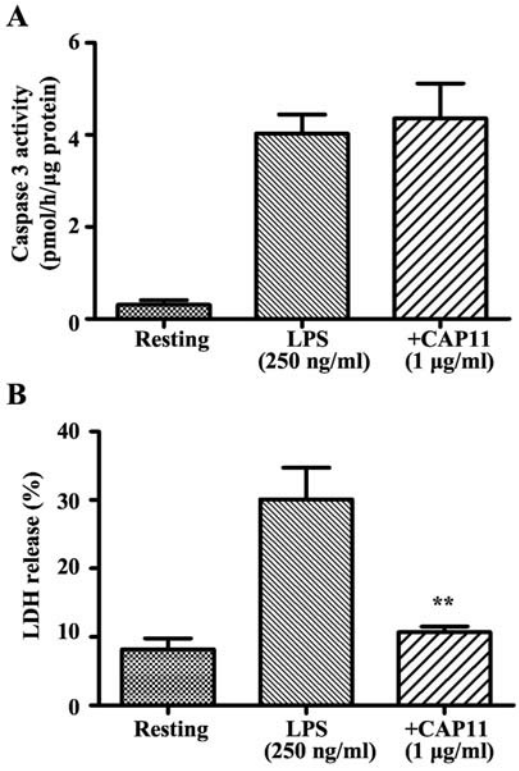

Figure 6. Effect of CAP11 on LPS-induced cell death of RAW264.7 cells analyzed by caspase 3 activation and LDH release. RAW264.7 cells were incubated with LPS (250 ng/ml) in the absence or presence of CAP11 (1 μg/ml; +CAP11) for 24 h at 37˚C. RAW264.7 cells were also incubated without LPS and CAP11 for 24 h at 37˚C (Resting). After incubation, caspase 3 activation (A) and LDH release (B) were analyzed by enzymatic activities as described above. Data represent the mean ± SE of five to eleven independent experiments. Values were compared between the incubation with LPS alone (LPS) and in the presence of CAP11 (+CAP11). **P<0.01.

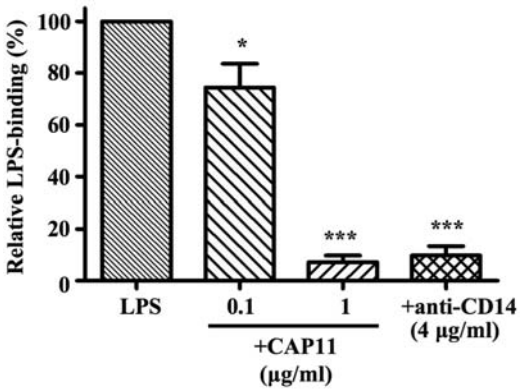

Figure 7. Effect of CAP11 on the LPS-binding to RAW264.7 cells. RAW264.7 cells were incubated with Alexa488-conjugated LPS (100 ng/ml) at 37˚C for 15 min in the absence or presence of CAP11 (0.1 or 1 μg/ml) or a neutralizing anti-CD14 monoclonal antibody (4C1, 4 μg/ml). Cells were then washed twice with PBS, and the LPS-binding was analyzed by flow cytometry. LPS-binding was expressed as a ratio relative to the incubation with LPS alone. Data represent the mean ± SE of three independent experiments. Values were compared between the incubation with LPS alone (LPS) and in the presence of CAP11 (+CAP11) or anti-CD14 monoclonal antibody (+anti-CD14). *P<0.05, ***P<0.001.

HMGB1 release from RAW264.7 cells and cell death in RAW264.7 cells, we finally examined the effect of CAP11 on LPS-binding to target cells. Of note, CAP11 dose-dependently suppressed the LPS binding to RAW264.7 cells; 1 μg/ml CAP11 strikingly (~90%) inhibited the binding, whereas 0.1 μg/ml CAP11 did so only partially (20-30%) (Fig. 7). We confirmed that the LPS binding is primarily caused by CD14 (an LPS receptor), since a neutralizing anti-CD14 (4C1) antibody markedly suppressed the LPS-binding.

Discussion

Sepsis is an uncontrolled fatal condition that arises from harmful or damaging host response to infections. Components of innate immune response that are normally concerned with host defense against infection can, under circumstances, cause cell and tissue damage (1-3). Macrophages play a central role in innate immune system by not only phagocytosing invading pathogens but also by secreting proinflammatory substances (such as cytokines, reactive oxygen species, arachidonic acid metabolites, NO and proteases), thereby transmitting danger signals (1). Excess production of proinflammatory mediators leads to the amplified systemic inflammation, tissue injury, organ failure and septic shock (1-3). Based on these findings, therapeutic strategies have mainly targeted the blockade of proinflammatory mediators; however, most of the strategies have been unsuccessful (4,5).

High mobility group box-1 (HMGB1) is extracellularly released from mononuclear phagocytes and plays a crucial role in septic/endotoxin shock by functioning as a late phase mediator (7,26). In this context, it has been reported that serum HMGB1 levels are highly elevated in septic patients who succumbed (non-survivors), compared to patients with nonlethal infection (survivors); moreover, serum HMGB1 level is increased in a late phase (8-32 h) in mouse endotoxin shock model, and administration of anti-HMGB1 antibody attenuates the endotoxin lethality in mice (6). Thus, HMGB1, a late phase mediator, is recognized as a potential therapeutic target for treatment of endotoxin/septic shock (27).

We previously revealed that a cathelicidin peptide CAP11 (originally isolated from guinea pig neutrophils) potently binds with LPS and neutralizes the biological activity of LPS (16). Of interest, CAP11 can suppress the elevation of serum HMGB1 level in mouse endotoxin shock model and protect mice from endotoxin lethality (16,17). Moreover, HMGB1 is reported to be extracellularly released, accompanied with cell death (18,21-23). Thus, to clarify the inhibitory mechanism of CAP11 on HMGB1 release, we evaluated the effect of CAP11 on the LPS-induced HMGB1 release and apoptotic/necrotic cell death using a murine macrophage cell line RAW264.7 as a target cell. The results obtained indicated that LPS-stimulation induced the release of HMGB1 from RAW264.7 cells, accompanied with apoptotic and necrotic cell death assessed by flow cytometric and enzymatic analyses (Figs. 1-3). Of interest, CAP11 markedly inhibited the binding of LPS to target RAW264.7 cells and suppressed HMGB1 release as well as necrotic cell death induced by LPS; however, CAP11 did not affect the LPS-induced apoptotic cell death (Figs. 4-7). These observations clearly indicate that CAP11 can efficiently abolish necrotic cell death via the inhibition of LPS-binding to target cells due to its potent ability to bind with LPS (16), thereby suppressing the release of HMGB1.

The present findings that CAP11 suppressed the LPS-induced HMGB1 release and necrotic cell death but not apoptotic cell death, apparently suggest that HMGB1 is mostly released from LPS-stimulated necrotic RAW264.7 cells. In contrast, Jiang *et al* argued that HMGB1 is released from apoptotic RAW264.7 cells (assessed by caspase 3 activation), when stimulated with LPS or polyinosinic-polycytidylic acid (18). However, in their experiments LDH (a necrotic marker) was also extracellularly released under the stimulation conditions, and the release was correlated with HMGB1 release. Moreover, Lim *et al* reported that ethyl pyruvate, a stable pyruvate derivative, suppresses the glucose deprivation-induced HMGB1 release from A549 lung adenocarcinoma cells by preventing necrotic cell death and switching cell death mode to apoptosis (23). In addition, Scaffidi *et al* revealed using HeLa cells that HMGB1 is extracellularly released from necrotic but not apoptotic cells after treatment with various cytotoxic substances (21). These observations obviously support our finding that HMGB1 is mainly released from LPS-stimulated necrotic cells.

Of note, CAP11 did not affect the LPS-induced apoptosis of RAW264.7 cells (assessed by flow cytometry and caspase 3 activation) even at 1 μg/ml, although CAP11 markedly suppressed LPS-binding, and concomitantly prevented necrotic cell death and HMGB1 release at the same concentration. This could be explained by the facts that LPS was able to induce apoptosis of RAW264.7 cells at lower concentrations (>10 ng/ml, $P<0.05$) compared to necrosis (>50 ng/ml LPS, $P<0.001$) and HMGB1 release (>25 ng/ml LPS, $P<0.05$), and that the inhibition of LPS-binding to target cells was not complete (~90%) at 1 μg/ml CAP11. Probably, even when the LPS-binding is considerably suppressed, low doses of LPS are available and can efficiently activate cells to induce apoptotic cell death.

In summary, the present study has revealed that CAP11 can suppress the LPS-induced HMGB1 release as well as necrotic cell death via the inhibition of LPS-binding to target RAW264.7 cells. Furthermore, we have already indicated that CAP11 suppresses the elevation of serum HMGB1 level in mouse endotoxin shock model and protects mice from endotoxin lethality (16,17). Thus, CAP11 could be a therapeutic agent for septic/endotoxin shock with a potential to regulate the release of HMGB1 from LPS-stimulated mononuclear phagocytes via the suppression of LPS-binding to target cells and prevention of necrotic cell death due to its potent LPS-binding activity.

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