RESEARCH COMMUNICATION

HMGB1 Promotes the Synthesis of Pro-IL-1β and Pro-IL-18 by Activation of p38 MAPK and NF-κB Through Receptors for Advanced Glycation End-products in Macrophages

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Abstract

The high mobility group box-1 (HMGB1) protein and NALP3 inflammasome have been identified to play important roles in inflammation and cancer pathogenesis, but the relationships between the two and cancer remain unclear. The current study investigated the relationship between HMGB1 and the NALP3 inflammasome in THP-1 macrophages. HMGB1 was found unable to activate the NALP3 inflammasome and failed to induce the release of the IL-1β and IL-18 in THP-1 macrophages. HMGB1 was also found significantly enhanced the activity of ATP to induce IL-1β and IL-18 by the induction of increased expression of pro-IL-1β and pro-IL-18. This process was dependent on activation of RAGE, MAPK p38 and NF-κB signaling pathway. These results demonstrate that HMGB1 promotes the synthesis of pro-IL-1β and pro-IL-18 in THP-1 macrophages by the activation of p38 MAPK and NF-κB through RAGE. HMGB1 likely plays an important role in the first step of the release of the IL-1β and IL-18, preparing for other cytokines to induce excessive release of IL-1β and IL-18 which promote inflammation and cancer progression.

Keywords: High mobility group box 1 - NALP3 inflammasome - inflammation - cancer

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Introduction

Current estimates suggest that about 25% of cancers are associated with chronic inflammation (Mantovani et al., 2008). Many cytokines and mediators of inflammation are known to have an important role in cancer pathogenesis, but the underlying mechanisms are still not fully understood.

The high mobility group box 1 (HMGB1) protein was originally identified as a nuclear protein that can regulate transcription by changing DNA helical structure, recent studies suggest that the HMGB1 has been implicated in several disease states, including sepsis, arthritis, ischemia-reperfusion injury, and cancer (Sims et al., 2010; Tang et al., 2010). Necrotic Cancer cells can release HMGB1 into the local microenvironment. HMGB1 is also actively secreted by inflammatory cells, such as macrophages, acting as an endogenous danger signal and binding with high affinity to several receptors including the receptor for advanced glycation end products (RAGE), Toll-like receptors (TLR)-2, TLR-4 and TLR-9 (Park et al., 2006; Sims et al., 2010). Although a number of clinical studies have suggested that overexpression of HMGB1 is associated with cancer (Tang et al., 2010), but the mechanisms are still mysterious.

Recently many studies have demonstrated that IL-1β and IL-18 can attract and activate immune cells to induce inflammation and affect tumor progression, including cell survival and metastasis (Carrascal et al., 2003; Lewis et al., 2006). IL-1β and IL-18, derived from macrophage, are synthesized as proforms that require proteolytic maturation to become biologically active IL-1β and IL-18 are both cleaved by cysteine protease caspase-1, which must first be activated by inflammasomes. The “inflammasome” is a multi-protein complex whose assembly and activation are responsible for the recruitment and activation of caspases-1 and -5 in response to pathogen- and danger-associated molecular pattern (PAMP and DAMP) signals. The inflammasome is composed of three proteins: a nucleotide oligomerization domain (NOD)-like, leucine-rich repeat-containing receptor (NLR), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1. NALP3 inflammasome is the prototypical mode (Okamoto et al., 2010; Schroder et al., 2010).

Although HMGB1 and the NALP3 inflammasome are both related to the release of IL-1β and IL-18 in inflammation and cancer, there are few studies that have investigated the relationship between HMGB1 and the NALP3 inflammasome.

The current study demonstrated that HMGB1 does not appear capable of activating the NALP3 inflammasome...
and inducing the release of IL-1β and IL-18 in THP-1 macrophages. HMGB1, however, was shown to significantly enhance pro-IL-1β and pro-IL-18 expression by the activation of p38 MAPK and NF-κB through RAGE.

Materials and Methods

Reagents and antibodies

Inhibitors of extracellular regulated kinase (ERK; PD98059) and mitogen-activated protein kinase (MAPK) p38 (SB203580) were purchased from CalBiochem. The c-Jun N-terminal kinase (JNK) inhibitor (SP600125) and NF-κB inhibitor (Bay11–7085) were purchased from Sigma-Aldrich. Recombinant HMGB1 proteins were purchased from Sigma-Aldrich. Antibodies that block the Toll-like receptor 2 (TLR2) and TLR4 were purchased from eBioscience, and anti-RAGE Abs were purchased from Millipore. Anti-NALP3, anti-pro-IL-1β, anti-pro-IL-18, anti-ASC, anti-caspase-1, anti-pp38 and anti-p-p38 were purchased from Abcam. Anti-caspase-1 (20 kDa) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The 6-carboxy-2’,7’-dichlorodihydrofluorescein diacetate (DCFH-DA), was obtained from Beyotime Institute of Biotechnology (Haimen, China).

Cell culture and treatments

The human monocytic leukemia cell line, THP-1, was obtained from the ATCC and maintained in RPMI-1640 supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 25 mM HEPES, 100 U/mL penicillin, and 100 µg/mL streptomycin (standard THP-1 medium). To induce monocyte to macrophage differentiation, THP-1 cells were cultured for 48 h in standard culture medium supplemented with 0.1 µM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich).

THP-1 macrophages (5×10⁵ cells/well) were cultured on 6-well plates for 6 h at 37 °C at 5% CO₂ in a serum-free culture medium. Recombinant HMGB1 protein (100, 200, 400 and 600 ng/mL) was added to the incubation medium respectively. After incubation, culture media were collected for analysis of cytokines and the cells were washed with PBS and subjected to protein extraction or RNA isolation.

Analysis of cytokine secretion

Levels of IL-1β and IL-18 released from THP-1 macrophages into the culture medium were analyzed using commercially available ELISA kits (R&D Systems) according to the manufacturer’s protocols.

Western blotting

Protein concentrations were determined using the Bradford assay (Bio-Rad, Hercules, CA), and protein levels were adjusted to 22 mg/mL using buffer W. Lysates (10 µL) were aliquoted and placed at 30 °C to initiate processing and stopped by addition of an equal volume of 4× SDS-PAGE buffer. Lysates were analyzed by electrophoresis on 15% polyacrylamide gels and transferred to PVDF membranes (Millipore, Bedford, NH). The signal was detected using the ECL system (Pierce Chemical Company, Rockford, IL, USA) and quantified by densitometry as previously described (Wang et al., 2010).

RT-PCR

Total RNA was purified using TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from extracted RNA (0.25-0.5 µg) using MMLV reverse transcriptase and random hexamers (Promega, Madison, WI). The cDNA was amplified in duplicate using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) with gene-specific primers and probes using an ABI PRISM 7500 sequence detector system (Applied Biosystems). The data were collected and analyzed using Sequence Detector System software (version 1.4; Applied Biosystems) and the threshold value (Ct) of each sample was determined according to the manufacturer’s guidelines. The GAPDH housekeeping gene was used to normalize all tested genes, and quantification was performed using the ΔΔCT method. PCR primers: NF-κB forward, 5’-CTG CCG GGA TGG CTT CTA T-3’ and NF-κB reverse, 5’-CCG TCT CTT CAC ACA CTG GAT-3’; GAPDH forward, 5’-CTT CTC TGA TGA GCC CCA AG-3’ and GAPDH reverse, 5’-GCA GCA AAC TGG AAA GGA AG-3’.

Detection of intracellular reactive oxygen species (ROS)

Cells were treated with 5 mM ATP or 600 ng/mL HMGB1 for 0, 5, 15, 30 min respectively. Cells then were washed twice in phosphate-buffered saline (PBS pH =7.2), and incubated with 10 µM DCFH-DA at 37 °C for 20 min according to the manufacture’s instructions. A FACScan flow cytometer (FL-1, 530 nm) was used to detect the fluorescence.

Flow cytometric analysis

THP-1 macrophages (5×10⁵ cells/well) were incubated at room temperature for 30 min with fluorochrome-conjugated mAbs against RAGE (Millipore), TLR2, and TLR4 (eBioscience) or isotype control IgG in PBS containing 2% FBS. Cells were thoroughly washed and analyzed in a fluorescence-activated cell sorter scan (FACScan) system using CELLQuest software.

Statistics

Data are represented as the mean ±SEM from at least three independent experiments. Unpaired Student’s t-test was used when comparing two groups and ANOVA/Bonferroni test when comparing more than two groups. The differences were considered significant when p < 0.05. Statistical analysis was performed using SPSS (Version 17.0; IBM, Armonk, NY).

Results

HMGB1 alone cannot activate the NALP3 inflammasome nor induce the secretion of IL-1β and IL-18
As shown in the current study, many substances including some DAMPs, such as adenosine triphosphate (ATP), can activate the NALP3 inflammasome (Figure 1C). HMGB1 is regarded as one of these DAMPs (Klune et al., 2008), but it is unclear whether HMGB1 can activate the NALP3 inflammasome. To determine whether HMGB1 can activate the NALP3 inflammasome, we examined the expression of cleaved caspase-1, but it was not detected in the current study after the addition of 100, 200, 400 and 600 ng/mL HMGB1 for 24 h respectively (Figure 1C). Moreover, no significant secretion of IL-1β and IL-18 was detected after stimulation with HMGB1 alone (Figure 1A). However, we detected cleaved caspase-1 and significant secretion of IL-1β and IL-18 in THP-1 cells after treatment with 5 mM ATP (Figure 1 A, C). Recent studies showed that the NALP3 inflammasome may be activated by reactive oxygen species (ROS) generated in the cell (Martinon, 2010; Tschopp and Schroder, 2010). Thus, we detected ROS in THP-1 cells after treatment with HMGB1 and found no ROS were generated. But we detected ROS in THP-1 cells after treatment with ATP (Figure 1 D, E). So we speculated that HMGB1 cannot activate the NALP3 inflammasome and cannot induce the secretion of IL-1β and IL-18 because of no ROS production in THP-1 cells.

In the current study we found the secretion of IL-1β and IL-18 in response to the addition of 200 ng/mL HMGB1 plus 5 mM ATP was greater than for 5 mM ATP alone(Figure 1B). The secretion of IL-1β and IL-18 consists of two steps, the synthesis of pro-IL-1β, pro-IL-18 and inflammasome activation. Previous results showed that 5 mM ATP can induce the secretion of IL-1β and IL-18 but HMGB1 cannot, so we detected pro-IL-1β and pro-IL-1β by western blot analysis. The results showed that HMGB1 promoted the synthesis of pro-IL-1β and pro-IL-18 in THP-1 macrophages (Figure 2). The expression of pro-IL-1β and pro-IL-18 was significantly over-expressed at 200 ng/mL HMGB1 and significantly increased in a dose-dependent manner, but expression of NALP3, ASC and caspase-1 did not increase (Figure 2). These data suggest that HMGB1 significantly enhances the activity of ATP to induce IL-1β and IL-18 in vitro by enhancing pro-IL-1β and pro-IL-18.

Since it was found that HMGB1 led to the increased synthesis of pro-IL-1β and pro-IL-18 in THP-1 macrophages, further analysis of the signaling pathway was performed. HMGB1 was reported to bind to and signal through the receptor RAGE, as well as TLR2 and TLR4 (Park et al., 2006; Luan et al., 2010). In the current study, we found that RAGE was significantly up-regulated, but TLR2 and TLR4 were not (Figure 3 A, B). To further assess which receptor was involved in the synthesis of pro-IL-1β and pro-IL-18 protein in response to HMGB1, blocking antibodies (anti-TLR2, anti-TLR4, and RAGE-Fc) were added 30 min before the addition of HMGB1 to the medium. The expression of pro-IL-1β and pro-IL-18 were analyzed by western blot, respectively. It was revealed that treatment of macrophages with RAGE-Fc almost completely inhibited the synthesis of pro-IL-1β and pro-IL-18.
D (A)THP-1 macrophages were after THP-1 macrophages were pretreated with blocking antibodies (anti-TLR2, anti-TLR4, and RAGE-Fc) 30 min before stimulation with 200 ng/mL HMGB1, and (C, D) protein levels for pro-IL-1β and pro-IL-18 were determined. *p<0.05 and **p<0.01. Data represent mean ± SD of three different experiments and pro-IL-18, whereas treatment with anti-TLR2 or anti-TLR4 antibodies had no effect (Figure 3 C, D). Therefore, it seems that HMGB1 can promote the synthesis of pro-IL-1β and pro-IL-18 in THP-1 macrophages through RAGE receptors, but not TLR2 and TLR4.

**HMGB1 induced synthesis of pro-IL-1β and pro-IL-18 is dependent on activation of MAPK p38 and NF-κB.**

Previous studies have demonstrated that RAGE can lead to the activation of MAPK p38 and NF-κB after HMGB1 binding (Luan et al., 2010). In the current study, we found that MAPK p38 and NF-κB were significantly activated (Figure 4 C, D). Thus, we next studied whether MAPK p38 and NF-κB were involved in the synthesis of pro-IL-1β and pro-IL-18 in response to HMGB1. Inhibitors of ERK (PD98059), MAPK p38 (SB203580), JNK (SP600125) and NF-κB (Bay11–7085) were added 60 min before the addition of HMGB1 to the medium and expression of pro-IL-1β and pro-IL-18 were analyzed by western blot, respectively. It was revealed that treatment of macrophages with Inhibitors of MAPK p38 (SB203580) and NF-κB (Bay11–7085) almost completely inhibited the synthesis of pro-IL-1β and pro-IL-18, whereas treatment with ERK (PD98059) or JNK (SP600125) Inhibitors had not effect (Figure 4 A, E and F). Therefore, it seems that HMGB1 can promote the synthesis of pro-IL-1β and pro-IL-18 in THP-1 macrophages depending on activation of MAPK p38 and NF-κB.

**Discussion.**

Chronic inflammation is a common etiologic factor for cancer. A wide variety of cytokines and mediators in chronic inflammation contribute to carcinogenesis including IL-1β, IL-18 and HMGB1. It is reported that IL-1β is the primary mediator of tumor angiogenesis, invasiveness and metastasis in experimental tumor models and neutralization of IL-1β of host origin reduces tumor angiogenesis and invasiveness. IL-1β expression is elevated in human breast, colon, lung, head and neck cancers, and melanomas, and patients with IL-1β producing tumors have generally bad prognoses (Lewis et al., 2006). IL-18 is known as an immune activator. Activated NK or T cells by IL-18 eliminate spontaneous cancer or pathogen infected cells. Contrary to the anti-cancer effect of IL-18, its pro-cancerous effect has been recently suggested in gastric cancer patients, the analysis of the expression pattern of IL-18 and tumor progression revealed that IL-18 was expressed highly in the tumor region in comparison with non-tumor region and related with distant metastasis (Ye et al., 2007). Eissa et al. (2005) and Merendino et al. (2001) have reported that IL-18 is also an important marker of breast cancer progression because higher IL-18 levels were detected in serum from breast cancer patients with metastasis compared to healthy volunteers and breast cancer patients without metastasis.

These researchs showed that the increased secretion of IL-1β and IL-18 may contribute to uncontrol inflammation and tumorigenesis. IL-1β and IL-18, are synthesized as proforms that require proteolytic maturation to become biologically active. IL-1β and IL-18 are both cleaved by cysteine protease caspase-1, which must first be activated by inflammasomes. Many substances including some DAMPs, such as adenosine triphosphate (ATP), can activate the NALP3 inflammasome. In our study we found that ATP can activate the NALP3 inflammasome.
and promote the release of IL-1β and IL-18. HMGB1 is recognized as the prototypical DAMP (Yang et al., 2007) and functions as an extracellular signaling molecule during inflammation, cell differentiation, cell migration, and tumor metastasis (Lotze and Tracey, 2007).

Previous studies have reported that HMGB1 induced the secretion of proinflammatory cytokines, such as IL-1β, from macrophages in vitro (Andersson et al., 2000). However, recent studies have shown that HMGB1 had little or no effect on macrophages unless bound to DNA-containing immune complexes or to DAMPs, such as IL-1β and LPS (Sha et al., 2008). These data are consistent with the findings of the current study that also found that HMGB1 alone cannot promote the release of IL-1β and IL-18.

Recent studies showed that the NALP3 inflammasome may be activated by ROS generated in the cell (Martinon, 2010; Tschopp and Schroder, 2010). The current study found that no ROS were generated by THP-1 macrophages cultured with HMGB1, and HMGB1 can not activate the NALP3 inflammasome. On the other hand, we found that ROS was significantly increased in THP-1 macrophages cultured with ATP. Taken together, these data indicated that HMGB1 alone cannot activate the NALP3 inflammasome and, therefore, cannot promote the release of IL-1β and IL-18.

The release of the IL-1β and IL-18 consists of two steps, the first step is synthesis of pro-IL-1β and pro-IL-18, which increased expression is likely to lead to the release of more IL-1β and IL-18 (Schröder et al., 2010). In the current study, we found that although HMGB1 alone failed to promote the release of IL-1β and IL-18, the secretion of IL-1β and IL-18 in THP-1 macrophages cultured with HMGB1 plus ATP showed significantly increased expression. Further, we also determined that pro-IL-1β and pro-IL-18 expression was increased in THP-1 macrophages cultured with HMGB1. Therefore, we speculated that HMGB1 increased the synthesis of pro-IL-1β and pro-IL-18 in THP-1 macrophages. These data suggest that HMGB1 significantly enhances the activity of ATP to induce IL-1β and IL-18 in vitro by enhancing pro-IL-1β and pro-IL-18. Thus, HMGB1 likely plays an important role in the first step of the release of the IL-1β and IL-18 by enhancing pro-IL-1β and pro-IL-18, preparing for other cytokines to induce excessive release of IL-1β and IL-18. The increased secretion of IL-1β and IL-18 may contribute to uncontrolled inflammation and tumorigenesis.

The biological effects of HMGB1 are proposed to be mediated by RAGE, TLR2, or TLR4 (Park et al., 2006; Sims et al., 2010). However, the mechanisms through which receptors and signal pathway HMGB1 activates cells and increases the expression of proinflammatory cytokines have not been completely characterized. Currently, conflicting data suggest that this may be related to different cell types and different stimulators. The findings reported here indicated that the expression of RAGE was significantly higher than TLR2 and TLR4 in THP-1 macrophages cultured with HMGB1. To further explore which receptor is involved in the increased expression of pro-IL-1β and pro-IL-18 cultured with HMGB1, we used receptor blocking antibodies. Anti-TLR2 and anti-TLR4 antibodies did not result in a change in the production of pro-IL-1β and pro-IL-18. However, THP-1 macrophages cultured with the RAGE-Fc resulted in the significantly decreased production of pro-IL-1β and pro-IL-18. Taken together, these data indicated that HMGB1 promotes the synthesis of pro-IL-1β and pro-IL-18 through RAGE in THP-1 macrophages.

The activation of RAGE relays cell surface signals to various intracellular pathways, including the NF-xB pathway, that are responsible for a number of transcriptional pathways leading to the production of target proteins (Yang et al., 2011). In addition to NF-xB, the known pathways that can be activated by RAGE are mitogen-activated protein kinase (MAPKs), including JNK, p38, and ERK (Bauerfeind et al., 2009; Yang et al., 2011). To explore the signal pathways of the RAGE in THP-1 macrophages cultured with HMGB1, we investigated the effects on NF-xB and MAPKs. It was revealed that treatment of macrophages with Inhibitors of MAPK p38 (SB203580), activation of MAPK p38 was decreased and almost completely inhibited the synthesis of pro-IL-1β and pro-IL-18, whereas treatment with ERK (PD98059) or JNK (SP600125) Inhibitors had not effect. And NF-xB mRNA levels were shown to be significantly increased after culturing with HMGB1. Moreover, upon the addition of the NF-xB inhibitor, Bay11–7085, resulted in abolished production of pro-IL-1β and pro-IL-18. Recently, some studies have suggested that other pathways, such as the phosphoinositide 3-kinases (PI3K)/Akt pathway (Bauerfeind et al., 2009; Yang et al., 2011), that can be activated by RAGE. Therefore, the pathway involved in HMGB1 promoting the synthesis of pro-IL-1β and pro-IL-18 may be complex and likely to involve cross-talk between pathways. Future studies will explore these mechanisms and potential interplay between these in more detail. Although the signal pathway is complicated, the findings presented in this report at least indicate that activation of p38 MAPK and NF-xB is an important component in the synthesis of pro-IL-1β and pro-IL-18 initiated by HMGB1 in THP-1 macrophages.

In conclusion, our data indicate that HMGB1 failed to activate the NALP3 inflammasome and cannot induce the release of the IL-1β and IL-18 in THP-1 macrophages, but can significantly enhance pro-IL-1β and pro-IL-18. HMGB1 significantly enhances the activity of ATP to induce IL-1β and IL-18 in vitro by enhancing pro-IL-1β and pro-IL-18, signals through RAGE, and enhances the phosphorylation of MAPK p38 and the activation of NF-xB. Thus, HMGB1 likely plays an important role in the first step of the release of the IL-1β and IL-18 by enhancing pro-IL-1β and pro-IL-18, preparing for other cytokines to induce excessive release of IL-1β and IL-18. The increased secretion of IL-1β and IL-18 may contribute to uncontrolled inflammation and tumorigenesis. We postulate that these results may provide the potential future targets for the prevention of cancer.

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References


