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HMGB1 Develops Enhanced Proinflammatory Activity by Binding to Cytokines

Yonggang Sha, Jaroslaw Zmijewski, Zhiwei Xu, and Edward Abraham

High mobility group box 1 protein (HMGB1), originally characterized as a nuclear DNA-binding protein, has also been described to have an extracellular role when it is involved in cellular activation and proinflammatory responses. In this study, FLAG-tagged HMGB1 was inducibly expressed in the presence of culture media with or without added IL-1β, IFN-γ, or TNF-α. HMGB1 purified from cells grown in culture media alone only minimally increased cytokine production by MH-S macrophages and had no effect on murine neutrophils. In contrast, HMGB1 isolated from cells cultured in the presence of IL-1β, IFN-γ, and TNF-α had enhanced proinflammatory activity, resulting in increased production of MIP-2 and TNF-α by exposed cells. IL-1β was bound to HMGB1 isolated from cells cultured with this cytokine, and purified HMGB1 incubated with recombinant IL-1β acquired proinflammatory activity. Addition of anti-IL-1β Abs or the IL-1 receptor antagonist to cell cultures blocked the proinflammatory activity of HMGB1 purified from IL-1β-exposed cells, indicating that such activity was dependent on interaction with the IL-1 receptor. These results demonstrate that HMGB1 acquires proinflammatory activity through binding to proinflammatory mediators, such as IL-1β.


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3 Abbreviations used in this paper: HMGB1, high mobility group box protein 1; HA, hemagglutinin; IL-1Ra, IL-1R antagonist.

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Reagents and Abs

The RNAeasy kit was purchased from Qiagen, and ELISA kits were obtained from R&D Systems. Abs for β-actin, anti-mouse, or anti-rabbit HRP, as well as anti-IL-1β, anti-TNF-α, and anti-IFN-γ, were purchased from Santa Cruz Biotechnology. Anti-FLAG M2 affinity gel, FLAG peptide, TNF-α, IFN-γ, and IL-1β recombinant proteins as well as TRI reagent were obtained from Sigma-Aldrich. Ab against HMGB1 was obtained from Abcam. DNA ligase and pGEM-T Easy Vector were purchased from Promega, while Lipofectamine 2000 and pcDNA 4.0 were obtained from...
romycin for 2 wk, and a positive clone (MCF7-HMGB1-FLAG no. 2) was selected with blasticidin and puromycin (pcDNA4-HA-HMGB1-FLAG). Cells were selected with blasticidin and puromycin (pcDNA4-HA-HMGB1-FLAG). Samples were resolved using 11% SDS-PAGE followed by protein detection using Coomassie brilliant blue staining. HMGB1-FLAG protein was purified as a single band from the doxycycline-induced cells.

Expression and purification of HMGB1-FLAG from MCF7 cells. To induce HMGB1-FLAG expression, MCF7-HMGB1-FLAG cells (5 × 10⁶) were incubated with doxycycline (0 or 1 μg/ml) for 20 h and then protein extracts were resolved using 11% SDS-PAGE. A representative Western blot shows the level of endogenous and inducible expression of HMGB1-FLAG, with actin being used as a loading control.

Protein extracts obtained from doxycycline-treated or untreated cells were subjected to affinity purification with ANTI-FLAG M2 Affinity Gel. B, Protein extracts obtained from doxycycline-treated or untreated cells were subjected to affinity purification with ANTI-FLAG M2 Affinity Gel. Samples were resolved using 11% SDS-PAGE followed by protein detection using Coomassie brilliant blue staining. HMGB1-FLAG protein was purified as a single band from the doxycycline-induced cells.

To generate a construct that expressed human full-length HMGB1, which was hemagglutinin (HA) tagged at the N terminus and FLAG tagged at the C terminus, a cDNA fragment was amplified by PCR using human full-length HMGB1 cDNA in a pCMV-SPORT6 vector as a template (catalog no. MHS1010–57487, Open Biosystems). The primer sequences are: sense 5'-GAA TTC ACC ATG FAC TAC TAA TCC CTA TAC GAT GTT CCA GAT TAC GCT GTC GTC ATC CTT GTA ATC-3' and anti-sense 5'-GTC GTC ATC CTT GTA ATC ATC ATC ATC ATC TTT TTC TTT TTT TCT CTT ATC TTT TTT TCT TCT TCT-3'. The sequence encoding the HA or FLAG epitopes is listed in capital italics. The PCR product was cloned into pGEM-T Easy Vector, which was digested according to the manufacturer’s instructions with some minor modifications. Briefly, ~80% confluent MCF7-HMGB1-FLAG cells were treated with doxycycline (1 μg/ml) for 20 h. Bone marrow neutrophils were isolated as described previously (18).

Invitrogen. TaqMan reverse transcription reagents were from Roche. Restriction enzymes were purchased from New England Biolabs. MH-S cells and MCF7 cells were obtained from American Type Culture Collection. Recombinant human IL-1 receptor antagonist (IL-1Ra) was purchased from Cell Science.

FLAG- and HA-tagged HMGB1 construct

To generate a construct that expressed human full-length HMGB1, which was hemagglutinin (HA) tagged at the N terminus and FLAG tagged at the C terminus, a cDNA fragment was amplified by PCR using human full-length HMGB1 cDNA in a pCMV-SPORT6 vector as a template (catalog no. MHS1010–57487, Open Biosystems). The primer sequences are: sense 5'-GAA TTC ACC ATG FAC TAC TAA TCC CTA TAC GAT GTT CCA GAT TAC GCT GTC GTC ATC CTT GTA ATC-3' and anti-sense 5'-GTC GTC ATC CTT GTA ATC ATC ATC ATC ATC TTT TTC TTT TTT TCT CTT ATC TTT TTT TCT TCT TCT-3'. The sequence encoding the HA or FLAG epitopes is listed in capital italics. The PCR product was cloned into pGEM-T Easy Vector, which was digested according to the manufacturer’s instructions with some minor modifications. Briefly, ~80% confluent MCF7-HMGB1-FLAG cells were treated with doxycycline (1 μg/ml) for 20 h. In some experiments, HMGB1-FLAG was induced in MCF7 cells incubated with TNF-α (10 ng/ml), IFN-γ (10 ng/ml), or IL-1β (2.5 ng/ml) for 20 h. For cytokine expression studies, MH-S cells grown in RPMI 1640 media or isolated neutrophils were treated for 4 h with ANTI-FLAG M2 affinity agarose beads (Sigma, anti-IL-1 Ab, IgG, or albumin for 15 min before addition to cell culture. Purification of HMGB1-FLAG from MCF7 cells

HMGB1-FLAG protein was purified using ANTI-FLAG M2 Affinity Gel according to the manufacturer’s instructions with some minor modifications. Briefly, ~80% confluent MCF7-HMGB1-FLAG cells were treated with doxycycline (1 μg/ml) for 20 h, washed twice with ice-cold PBS, and then the cells were harvested and centrifuged (2,000 × g) for 10 min at 4°C. The cell pellet was suspended in lysis buffer (0.1% Nonidet P-40, 150 mM NaCl, 10 mM Na2HPO4, 2 mM EDTA), rotated for 60 min at 4°C. The cell pellet was suspended in lysis buffer (0.1% Nonidet P-40, 150 mM NaCl, 10 mM Na2HPO4, 2 mM EDTA), rotated for 60 min at 4°C. The cell pellet was then the cells were harvested and centrifuged (2,000 × g) for 10 min at 4°C. In some experiments, HMGB1-FLAG was induced in MCF7 cells incubated with TNF-α (10 ng/ml), IFN-γ (10 ng/ml), or IL-1β (2.5 ng/ml) for 20 h. For cytokine expression studies, MH-S cells grown in RPMI 1640 media or isolated neutrophils were treated for 4 h with ANTI-FLAG M2 affinity agarose beads (Sigma, anti-IL-1 Ab, IgG, or albumin for 15 min before addition to cell culture.

Cell culture and treatments

MH-S cells, MCF7 cells, and MCF7-HMGB1-FLAG cell lines were maintained in RPMI 1640 media, supplemented with 10% FBS (Atlanta Biologicals), penicillin (100 U/ml), and streptomycin (100 ng/ml) at 37°C, 5% CO₂. To induce expression of HMGB1-FLAG, ~80% confluent cells grown in 12-well plates were incubated with doxycycline (1 μg/ml) for 20 h. Bone marrow neutrophils were isolated as described previously (18).

Protein extracts obtained from doxycycline-treated or untreated cells were subjected to affinity purification with ANTI-FLAG M2 Affinity Gel. B, Protein extracts obtained from doxycycline-treated or untreated cells were subjected to affinity purification with ANTI-FLAG M2 Affinity Gel. Samples were resolved using 11% SDS-PAGE followed by protein detection using Coomassie brilliant blue staining. HMGB1-FLAG protein was purified as a single band from the doxycycline-induced cells.

FIGURE 1. Expression and purification of HMGB1-FLAG from MCF7 cells. To induce HMGB1-FLAG expression, MCF7-HMGB1-FLAG cells (5 × 10⁶) were incubated with doxycycline (0 or 1 μg/ml) for 20 h and then protein extracts were resolved using 11% SDS-PAGE. A, A representative Western blot shows the level of endogenous and inducible expression of HMGB1-FLAG, with actin being used as a loading control. B, Protein extracts obtained from doxycycline-treated or untreated cells were subjected to affinity purification with ANTI-FLAG M2 Affinity Gel.

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FIGURE 2. HMGB1 exhibits greater proinflammatory effect when purified from cytokine-treated MCF7-HMGB1-FLAG cells. MCF7-HMGB1-FLAG cells were incubated with doxycycline (1 μg/ml) alone or with TNF-α (10 ng/ml), IFN-γ (10 ng/ml), or IL-1β (2.5 ng/ml) for 20 h. HMGB1-FLAG was then purified and used to treat MH-S cells for 4 h. MIP-2 and TNF-α concentrations in the culture supernatants, as well as intracellular MIP-2 and TNF-α mRNA levels, were then determined. Protein levels (A and B) and mRNA for MIP-2 or TNF-α by MH-S cells (C and D) are shown. Means ± SD from five independent experiments are shown (*, p < 0.05, **, p < 0.01, and ***, p < 0.001 compared with untreated cells).

Neutrophils were cultured in RPMI 1640 media supplemented with FBS (0.5%). In some experiments, HMGB1-FLAG was induced in MCF7 cells incubated with TNF-α (10 ng/ml), IFN-γ (10 ng/ml), or IL-1β (2.5 ng/ml) for 20 h. For cytokine expression studies, MH-S cells grown in RPMI 1640 media or isolated neutrophils were treated for 4 h with ANTI-FLAG M2 affinity agarose beads (Sigma, anti-IL-1 Ab, IgG, or albumin for 15 min before addition to cell culture.

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Western blot analysis

Purified proteins or cell extracts were mixed with Laemmli sample buffer and boiled for 5 min. Equal amounts of protein were resolved by using 11%
SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). The membranes were probed with specific Ab to HMGB1, IL-1β, TNF-α, or IFN-γ followed by detection with HRP-conjugated anti-mouse or anti-rabbit IgG. Bands were visualized by ECL (ECL Plus, GE Healthcare) and quantified by using AlphaEaseFC software (Alpha Innotech).

**RT-PCR**

Total RNA was purified with TRI reagent and cDNA synthesized using TaqMan reverse transcription reagents. RT-PCR was performed using a Lightcycler 480 SYBR Green I Master system (Roche). The mouse TNF-α transcript was amplified using the following primers (XXIDT, Integrated DNA Technologies): forward, 5'-CCTCCCTCTCATCAGTTCTA-3' and reverse, 5'-CTTTGAGATCCATGCCG-3'. The mouse MIP-2 transcript was amplified using the following primers: forward, 5'-ACTGGTCCTGCTGCTGCT-3' and reverse, 5'-CAGTTCACTGGCCACAACA-3'. Mouse GAPDH transcript was used as an internal control: forward primer, 5'-TCACTGGCATGGCCTTCC-3' and reverse, 5'-GGCGGCACGTCAGATCC-3'.

**Cytokine ELISA**

Levels of TNF-α or MIP-2 released from MH-S cells or neutrophils into culture media were determined using commercially available ELISA kits (R&D Systems).

**Statistical analyses**

For each experiment, data are presented as mean ± SD for each experimental group. One-way ANOVA, the Tukey-Kramer multiple comparisons test (for multiple groups), or Student's t test (for comparisons between two groups) were used (p < 0.05 was considered to be significant).

**Results**

**Expression and purification of FLAG-tagged HMGB1**

FLAG-tagged HMGB1 was purified, using ANTI-FLAG M2 Affinity Gel, from stably transfected cells induced with doxycycline for a 20-h period. During the induction period, the media was either left unaltered or was supplemented with IL-1β, IFN-γ, or A

**FIGURE 3.** HMGB1 binds to IL-1β and acquires enhanced proinflammatory activity during cell culture. A, HMGB1-FLAG was induced in MCF7-HMGB1-FLAG cells treated with doxycycline with or without IL-1β (2.5 ng/ml) for 20 h. Protein extracts were subjected to FLAG-affinity purification, and the resulting HMGB1-FLAG cells were analyzed by Western blot using Abs to IL-1β or HMGB1. IL-1β recombinant protein (rIL-1β, 10 ng) was used as a positive control. A representative experiment is shown. Two additional experiments provided similar results. B and C, MH-S cells were incubated for 4 h with HMGB1 (100 ng/ml) or IL-1β (5 ng/ml) alone, or with purified HMGB1-IL-1β complex consisting of 5 ng/ml IL-1β bound to 100 ng/ml HMGB1. Levels of MIP-2 (B) and TNF-α (C) in the culture media were determined using ELISA. Means ± SD from three independent experiments are shown (*, p < 0.05 or ***, p < 0.001 compared with untreated cells).

**FIGURE 4.** Anti-IL-1β Abs and IL-1Ra inhibit the proinflammatory activity of HMGB1 purified from IL-1β-exposed cells. HMGB1-FLAG protein was purified from MCF7-HMGB1-FLAG cells cultured with IL-1β during the 20-h doxycycline induction period and was then used to treat MH-S cells in the presence of anti-IL-1β Ab (1 μg/ml) or control IgG (1 μg/ml) (A and B) or IL-1Ra (0.1 μg/ml) or albumin (Alb, 0.1 μg/ml) (C and D) for 4 h. MIP-2 and TNF-α concentrations in the culture supernatants were determined by ELISA. Means ± SD from three to five independent experiments are shown (*, p < 0.05 and ***, p < 0.001 compared with untreated cells and ****, p < 0.001 compared with cells treated with HMGB1-IL-1β complex).
TNF-α. Control cultures included media alone or media supplemented with cytokines, but without doxycycline. As shown in Fig. 1A, FLAG-tagged HMGB1 migrated as a band with higher molecular weight than endogenous HMGB1 in doxycycline-induced cells. After purification, HMGB1-FLAG appeared as a single band from the doxycycline-induced cultures, but was not present in extracts from cells that were not exposed to doxycycline (Fig. 1B). Approximately equivalent amounts of FLAG-tagged HMGB1 were induced and purified from cells with or without addition of cytokines to the culture media.

Activity of HMGB1 purified from cytokine-exposed and unexposed cells

In previous studies, exposure of macrophages to recombinant HMGB1 isolated from E. coli or eukaryotic HMGB1 obtained from pig or calf thymus resulted in release of proinflammatory cytokines (10, 19–22). However, while LPS concentrations in such HMGB1 preparations were low or undetectable, the association of HMGB1 with other potentially proinflammatory mediators was not completely ruled out. Therefore, to determine whether highly purified HMGB1 has proinflammatory properties, we examined cytokine expression by MH-S macrophages exposed to HMGB1-FLAG isolated from cells exposed to media and doxycycline alone, or from cells cultured with media and doxycycline supplemented with IL-1β, IFN-γ, or TNF-α. The concentration of HMGB1 used in the present experiments (100 ng/ml) has previously been demonstrated to potently activate macrophages to produce proinflammatory cytokines (20, 23).

As shown in Fig. 2, there was minimal or no significant increase in mRNA levels or secreted protein for TNF-α or MIP-2 by macrophages cultured with HMGB1 that had been isolated from doxycycline-induced cells grown in media without any supplementary cytokines. In contrast, HMGB1 purified from cells cultured in the presence of IL-1β, IFN-γ, and TNF-α induced significant increases of both protein and mRNA expression of TNF-α and MIP-2. Although coculture of the transfected cells with IL-1β, IFN-γ, or TNF-α during the 20-h induction period resulted in HMGB1 acquiring proinflammatory properties, MIP-2 and TNF-α expression by macrophages was consistently greater after culture with HMGB1 obtained from IL-1β-exposed cells, as compared with cells cultured with either IFN-γ or TNF-α.

IL-1β bound to HMGB1 contributes to macrophage and neutrophil activation

To determine whether HMGB1 isolated from IL-1β-exposed cells acquired proinflammatory activity through association with IL-1β, we performed Western blotting with HMGB1-FLAG purified from MCF7 cells cultured with IL-1β during the 20-h induction period and then stained for IL-1β. As shown in Fig. 3A, IL-1β was associated with HMGB1 purified from IL-1β-exposed cells, but not with HMGB1 isolated from cells induced in media lacking IL-1β.

To determine whether association with HMGB1 resulted in enhanced proinflammatory properties for IL-1β, we cultured MH-S cells with equivalent amounts (5 ng/ml) of free IL-1β or IL-1β bound to HMGB1 (Fig. 3, B and C). The amount of IL-1β associated with HMGB1 was determined by using Western blot analysis and sequential dilution of IL-1β protein as a standard. IL-1β in complex with HMGB1 (i.e., 5 ng/ml IL-1β associated with 100 ng/ml HMGB1) significantly increased production of MIP-2 and TNF-α, whereas IL-1β alone (5 ng/ml) did not affect cytokine expression.

IL-1β induces cellular activation through interaction with the IL-1 receptor complex (24, 25). To determine whether the acquired proinflammatory activity of HMGB1 purified from IL-1β exposed cells was due to associated IL-1β, we exposed MH-S cells to HMGB1-FLAG isolated from cells cultured with IL-1β during the 20-h doxycycline induction period. In these experiments the media was supplemented with either anti-IL-1β Abs or control rabbit IgG. The addition of anti-IL-1β Abs, but not control Abs, to the cultures diminished the ability of HMGB1 to induce MIP-2 and TNF-α expression (Fig. 4, A and B). Addition of the IL-1Ra, but not albumin, to the cultures abolished HMGB1-IL-1β-mediated activation of MH-S cells (Fig. 4, C and D). The proinflammatory effects of the HMGB1-IL-1β complex were not limited only to macrophages, as similar induction of cytokine production was found with bone marrow neutrophils (Fig. 5). Similar to results with macrophages, the proinflammatory properties of HMGB1-IL-1β on neutrophil activation were ablated by inclusion of anti-IL-1β Abs or the IL-1Ra in the cultures (Fig. 5).

**FIGURE 5.** Inclusion of anti-IL-1β Abs or IL-1Ra in neutrophil cultures inhibits the proinflammatory activity of HMGB1 purified from IL-1β-exposed cells. HMGB1-FLAG protein was purified from MCF7-HMGB1-FLAG cells cultured with or without IL-1β during the 20-h doxycycline induction period and was then used to treat murine bone marrow neutrophils in the presence of anti-IL-1β Abs (1 μg/ml), control IgG (1 μg/ml), IL-1Ra (0.1 μg/ml), or albumin (Alb, 0.1 μg/ml) for 4 h. MIP-2 (A) and TNF-α (B) concentrations in the culture supernatants were determined by ELISA. Means ± SD from three independent experiments are shown (***, p < 0.001 compared with untreated cells and ++++, p < 0.001 compared with cells treated with HMGB1-IL-1β alone).
are cultured with IL-1β. However, these studies do not demonstrate whether HMGB1 can directly associate with IL-1β or whether intermediate intracellular steps are necessary for this process to occur. To investigate this issue, we incubated purified HMGB1-FLAG with IL-1β or PBS for 4 h at room temperature, and then used anti-FLAG M2 Affinity Gel and extensive washing to repurify the HMGB1-FLAG. As shown in Fig. 6, exposure of HMGB1 to IL-1β in a cell-free environment was sufficient for HMGB1 to acquire proinflammatory activity. The association of HMGB1 with IL-1β in the cell-free environment was confirmed by immunoprecipitation with anti-FLAG Abs, followed by Western blotting and staining for IL-1β and HMGB1 (Fig. 7A).

As shown in Fig. 7A–C, binding of cytokines to HMGB1 does not appear to be a generalized phenomenon, as the degree of association of TNF-α and IFN-γ with HMGB1 was less than that found with IL-1β. The binding of IL-1β with HMGB1 was reversible, as disassociation occurred after incubation with detergent (Triton X-100) or SDS (Fig. 7D).

Discussion

Although HMGB1 has been proposed to potently induce acute inflammatory processes, the mechanisms through which it activates cells and increases expression of proinflammatory mediators, including cytokines and reactive oxygen and nitrogen species, have not been completely characterized. Several studies indicated that HMGB1 could interact with multiple receptors, including TLR2, TLR4, the receptor for advanced glycation end products, and, most recently, TLR9 (10, 17, 19, 20, 26). Similarly, varying intracellular signaling pathways, involving p38 MAP, ERK, JNK, and other kinases, were reported to be activated in HMGB1-exposed cells (10, 11, 19–22, 27–32).

The HMGB1 used to stimulate cells in previous studies was generally either recombinant bacterially produced or eukaryotically derived protein. Although the concentrations of LPS in the preparations of HMGB1 used in cell-activation studies were low or nondetectable, there was only limited effort made to identify other potential contaminants that might contribute to proinflammatory activity. However, affinity chromatography has recently shown that bacterially derived material, including lipids, tightly bind to HMGB1 and contribute to the proinflammatory activity of recombinant HMGB1 obtained from E. coli (33). Similarly, DNA-containing immune complexes can be associated with extracellular HMGB1 and contribute to the ability of HMGB1 to activate B cells through pathways involving TLR9 and MyD88 (17).

In the present studies, indusly expressed HMGB1 obtained from eukaryotic cells cultured with media that only contained...
doxycycline induced minimal cytokine production by macrophages or neutrophils. In contrast, HMGB1 that had been purified following addition of IL-1β, IFN-γ, or TNF-α to the culture media during the 20-h induction period demonstrated enhanced proinflammatory activity as shown by its ability to significantly increase release of TNF-α and MIP-2 from macrophages as well as neutrophils. The acquired proinflammatory properties of HMGB1 appeared to be due to binding of cytokines present in the culture media during the induction and purification periods. In particular, IL-1β was demonstrated to be bound to HMGB1 purified from cells cultured with this cytokine, and also was found to associate with the HMGB1-IL-1 inflammatory complex. Although the sites for interaction between HMGB1 and IL-1 cytokines and other proinflammatory mediators, is not an indiscriminant event. Although there was minimal or no association of TNF-α and IFN-γ with HMGB1, HMGB1 obtained from cell cultures that included these cytokines induced greater cytokine expression by MH-S cells than did HMGB1 alone. A possible mechanism for this finding is that cellular exposure to TNF-α or IFN-γ during the culture period induced production of IL-1 and perhaps other proinflammatory mediators, which then became bound to HMGB1, thereby enhancing its activity. The association of HMGB1 with IL-1β enhanced the proinflammatory properties of IL-1β, as cellular activation induced by the HMGB1-IL-1β complex was significantly greater than that found with equivalent amounts of IL-1β alone. These results show that HMGB1 itself has only minimal proinflammatory properties, but it acquires much greater activity through its role as a carrier protein for cytokines or other mediators capable of inducing cell activation.

HMGB1 has previously been shown to bind in the intracellular milieu to a wide range of proteins, including transcriptional factors, steroid receptors, and viral proteins (34). The present findings, as well as those of other laboratories (16, 17), demonstrate that extracellular proteins, such as cytokines and DNA-containing immune complexes, can also bind to HMGB1, creating a proinflammatory complex. Although the sites for interaction between HMGB1 and IL-1β were not examined in these experiments, previous studies have shown that the A and B boxes, and possibly the highly acidic C terminal domain, are involved in HMGB1 binding to proteins (34).

The results of these experiments provide a possible mechanism for the observed interaction of HMGB1 with multiple TLR/IL-1 receptors (TLR/IL-1R) that is independent of direct engagement of HMGB1 with these receptors (10, 17, 19, 20). Association of bacterial products, cytokines, DNA, or other mediators of inflammation with HMGB1 would result in interaction of HMGB1 with TLR/IL-1R in its role as a carrier protein that presents these molecules to their specific receptors. Similar functions have been demonstrated for heat shock proteins, such as heat shock protein 70, which induce activation of macrophages through TLR4 only through their association with LPS (35, 36). In the present studies, addition of IL-1Ra inhibited most, but not all, of the proinflammatory effects of the HMGB1-IL-1 complex, indicating that the IL-1Ra was primarily responsible for such actions. However, because residual activity in inducing cytokine expression continued to be present when IL-1Ra was included in the cultures, it remains possible that HMGB1 bound to IL-1 also signals through other receptors, such as the receptor for advanced glycation end products, TLR2, or TLR4.

In acute inflammatory states, such as sepsis, plasma concentrations of HMGB1 are elevated for prolonged periods of days to weeks, and they are present even as the patient’s condition is improving (15, 37). Tissue concentrations of HMGB1 secreted by activated cells and released during apoptosis and necrosis are likely to be even higher in such acute inflammatory states. In pathophysiological conditions associated with severe infection, there are transient elevations in circulating and tissue levels of bacterial products and cytokines, such as LPS and IL-1β, as well as other proinflammatory mediators (38). As shown in the present experiments, relatively short periods of interaction between HMGB1 and extracellular proteins are sufficient to result in binding. The concentrations of HMGB1 used in our studies (100 ng/ml) are often exceeded for prolonged periods in plasma during severe infection (15, 37). It is therefore possible that HMGB1 binds to cytokines and other proinflammatory mediators, including LPS, during the early stages of sepsis, pneumonia, and conditions associated with severe infection or organ dysfunction. HMGB1-mediator complexes may then function to potentiate inflammatory processes in settings associated with critical illness through prolonging the proinflammatory properties of bacterial products, chemokines, and cytokines. As the pathophysiological processes and organ dysfunction resolve, and levels of proinflammatory mediators decrease, cytokines and other molecules that induce inflammation may no longer be bound to HMGB1, so that even though circulating HMGB1 levels remain elevated, the proinflammatory properties present at earlier time points are lost.

Abs to HMGB1 are protective in models of endotoxemia, sepsis, hemorrhage, ventilator-induced acute lung injury, ischemia-reperfusion, immune complex-mediated arthritis, and other inflammatory conditions that result in organ dysfunction and mortality (9, 12, 14, 22, 39–42). The present results, showing that HMGB1 functions as a carrier protein, are still consistent with the demonstrated benefits of HMGB1 blockade in pathophysiological situations associated with acute inflammation. Binding of proinflammatory mediators, such as LPS or IL-1β, to HMGB1 may potentiate their activity and half-life. Diminishing the interaction of these HMGB1-mediator complexes with TLR/IL-1R and other receptors through administration of anti-HMGB1 Abs or nonbinding HMGB1 domains, such as the A box, would diminish cellular activation driven by HMGB1 complexes containing proinflammatory mediators.

Results from previous studies that reported interaction of HMGB1 with TLR/IL-1R and other receptors, as well as experiments describing the effects of HMGB1 on cellular activation, may have reflected contamination of HMGB1 with proteins and pathogen-associated molecular patterns (36). The present experiments emphasize the need to be aware of the ability of HMGB1 to bind cytokines and other proinflammatory mediators. Given the findings of this study, showing that HMGB1 itself has only minimal proinflammatory activity in terms of inducing macrophage- and neutrophil-associated cytokine expression, it will be important to reexamine the putative inflammatory and pathogenic actions of HMGB1 to ensure that such properties are due to HMGB1 and not to associated molecules.

Disclosures

The authors have no financial conflicts of interest.

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