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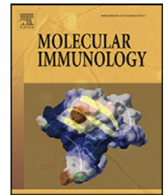

High mobility group box 1 protein synergizes with lipopolysaccharide and peptidoglycan for nitric oxide production in mouse peritoneal macrophages in vitro

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ABSTRACT

Extracellular high mobility group box 1 (HMGB1) protein and nitric oxide (NO) has been credited with multiple inflammatory functions using in vivo and in vitro systems. Therefore, delineating their regulation may be an important therapeutic strategy for the treatment of sepsis. In the present study, it is demonstrated that recombinant HMGB1 (rHMGB1) synergizes with sub threshold concentration of TLR2 agonist (PGN; 1 μg/ml) as well as with TLR4 agonist (LPS; 1 ng/ml) to induce NO release in mouse peritoneal macrophages. The enhanced iNOS expression was also observed at the transcription and translational level. Co-incubation of macrophages with rHMGB1 with either PGN or LPS showed enhanced expression of TLR2, TLR4 and RAGE. TLR2, TLR4 or RAGE knockdown macrophages effectively inhibited the rHMGB1 + PGN or LPS induced NO synergy. It was further observed that the JNK MAPK inhibitor SP600125 attenuated the PGN + rHMGB1 induced iNOS/NO synergy whereas p38 MAPK inhibitor SB908912 inhibited iNOS/NO synergy induced by LPS + rHMGB1. It was also observed that the activation of NF-κB is essential for the synergy as the pharmacological inhibition or siRNA knockdown of NF-κB (cRel) significantly reduced the rHMGB1 + PGN or rHMGB1 + LPS induced enhanced iNOS/NO expression. Altogether, the data suggests that the co-incubation of macrophages with rHMGB1 with either LPS or PGN induces the synergistic effect on iNOS expression and NO release by the upregulation of surface receptors (TLR2, TLR4 and RAGE) which in turn amplifies the MAPKs (p38 and JNK) and NF-κB activation and results in enhanced iNOS expression and NO production.

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1. Introduction

Sepsis is commonly initiated by an infection, but its pathogenesis is characterized by an overwhelming systemic inflammatory response and subsequent immune dysfunction, which can lead to lethal multiple organ failure (Lin and Yeh, 2005). Proinflammatory cells, primarily activated macrophages, are responsible for most of the cellular and molecular pathophysiology of sepsis, producing cytokines and other proinflammatory molecules such as platelet-activating factor, prostaglandins (PGs), enzymes, and free radicals (Moine and Abraham, 2004; Bianchi, 2007; Mantell et al., 2006). Recently, high-mobility group box 1 (HMGB1) has been reported to be an important proinflammatory mediator in the progression of sepsis (Wang et al., 2001).

HMGB1 protein was originally identified as an intranuclear DNA chaperon but lately it was identified as potent mediator of endotoxin lethality, local inflammation and macrophage activation when leaked out by necrotic cells or secreted by activated macrophages (Wang et al., 1999, 2001). HMGB1 can be secreted actively by macrophages and act as a cytokine in response to stress, exogenous and endogenous inflammatory stimuli such as UVB, endotoxin, CpG DNA, TNF-α, IL-1β (Chakraborty et al., 2012; Wang et al., 1999; Jiang et al., 2005; Lotze and Tracey, 2005).

The studies on cytokine-inducing properties of HMGB1 over the past years have been contradicting. We observed that rHMGB1 treated macrophages did not induce pro-inflammatory cytokines which were in consistent with the reports from other laboratories too (Rouhiainen et al., 2007; Sha et al., 2008; Tian et al., 2007; Ivanov et al., 2007; Cassetta et al., 2009). In 2007, two groups (Tian et al., 2007; Ivanov et al., 2007) reported that the activation of TLR9 by ssDNA is increased greatly in the presence of HMGB1 protein. HMGB1 also binds specifically to LPS and can catalytically disaggregate LPS from membranes and transfer it to soluble and membrane-bound CD14 in a dose-dependent manner, thereby enhancing the proinflammatory activity of LPS (Youn et al., 2008).

Abbreviations: HMGB1, high mobility group box 1; NO, nitric oxide; iNOS, inducible nitric oxide synthase; TLRs, toll like receptors; PGN, peptidoglycan; LPS, lipopolyssacride; RAGE, receptor for advanced glycan endproducts.

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HMGB1 has been reported to associates with IL-1β, and the complex has greater proinflammatory activity than IL-1β alone (Sha et al., 2008). In more general terms, HMGB1 can function in a synergistic fashion with multiple exogenous and endogenous ligands to induce pro-inflammatory cytokines. However, the synergistic role of HMGB1 in the induction of nitric oxide production has not been clearly investigated previously.

During the past two decades, nitric oxide (NO) has been recognized as one of the most versatile players in the immune system. Three isoforms of nitric oxide synthase have been identified and cloned (Knowles and Moncada, 1994; Nathan and Xie, 1994). The brain (type I) and endothelial (type III) enzymes are constitutively expressed and their enzymatic activity is regulated by changes in concentrations of free Ca^{2+}. The third member of the family is the inducible (type II) nitric oxide synthase (iNOS), which is expressed in many different cell types and produces high levels of NO (Moncada et al., 1991; Knowles and Moncada, 1994). NO is a product of macrophages activated by cytokines, microbial compounds or both, is derived from the amino acid L-arginine by the enzymatic activity of inducible nitric oxide synthase (iNOS or NOS2) and functions as a tumoricidal and antimicrobial molecule in vitro and in vivo (Nathan, 1992). Induced NO synthesis may play an important pathogenesis in infectious diseases, tumors, autoimmune processes and chronic degenerative diseases initiated by microbial products or autoimmune reactions (Bogdan, 2001).

In the present study, it is reported that the combination treatment of rHMGB1 with sub threshold concentration of LPS or PGN induces synergistic effect on iNOS/NO expression, by the upregulation of macrophage surface receptors (TLR2, TLR4 and RAGE). This enhanced expression of surface receptors synergistically activates the MAPKs (p38 and JNK) which in turn induce NF-κB activation and its translocation to nucleus, resulting in enhanced expression of iNOS and nitric oxide production in mouse peritoneal macrophages.

2. Materials and methods

2.1. Ethics statement

Studies presented in this manuscript were approved by the Scrutiny Committee of School of Biotechnology, Banaras Hindu University, as per University directive no. R/Dev/Project 1987/dt. 31.11.1987.

2.2. Mice

Inbred strains of BALB/c mice of either sex at 8–10 weeks of age were used for obtaining peritoneal macrophages.

2.3. Isolation of macrophages and culture conditions

Macrophage monolayers were prepared as described previously (Bhatt et al., 2012). Peritoneal exudates cells were harvested from peritoneal lavage using chilled serum-free RPMI 1640 medium (Sigma–Aldrich Chemicals, St. Louis, MO, USA) supplemented with 10% fetal calf serum (Biological Industries, Israel), penicillin (100 U/ml), streptomycin (100 U/ml) and gentamycin (20 mg/ml) and were added to wells of 24-well tissue culture plates (Nunc, Denmark). After 2 h incubation at 37 °C atmosphere of 5% CO_2 in air in a CO_2 incubator, the nonadherent cells were removed by vigorous washing (thrice) with warm serum-free medium, and the adherent cells were incubated overnight in complete medium to form macrophage monolayer. More than 95% of the adherent cell population was macrophages as determined by morphology and nonspecific esterase staining.

2.4. Reagents

PGN (*S. aureus*), ultrapure LPS (*E. coli*), recombinant HMGB1, glycyrrhizin, polymixin B, TRI reagent, TLR2 and RAGE siRNA and most of the other reagents were obtained from Sigma–Aldrich Chemicals, St. Louis, MO. TLR4 siRNA and scrambled siRNA were purchased from Ambion, Austin, TX, USA. MAPK inhibitors PD98059 (catalog no. 513000); p38mapk inhibitor, SB202190 (catalog no. 559388); JNK inhibitor, SP600125 (catalog no. 420119) were purchased from Calbiochem, La Jolla, CA, USA. Mouse monoclonal anti-HMGB1 (sc-56698), anti-NOS2 (sc-7271), anti-p-JNK (sc-12882), anti-p-ERK (sc-16982), anti-p-p38 (sc-17852), anti-actin (sc-1615), anti-RAGE (80313), anti-TLR2, anti-TLR4 and all secondary antibodies and ECL reagent were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. All of the reagents were endotoxin-free as determined by the Limulus lysate assay (sensitivity limit, 0.1 ng/ml).

2.5. Inhibitor studies

Macrophage monolayers were cultured in serum-free medium in 24-well culture plates (10^6 cells/well) with SP600125 (50 μM), PD98059 (50 μM), or SB202190 (10 μM) or a vehicle control (dimethyl sulfoxide at a concentration of 0.1%) for 30 min. The monolayers were then washed twice with warm incomplete medium, followed by the treatment of LPS or PGN alone or in combination with rHMGB1 and incubate in a CO_2 incubator for 3 or 24 h. Glycyrrhizin (HMGB1 inhibitor) was diluted fresh to a concentration of 1 mg/ml in triple distilled tissue culture grade water at 37 °C and the pH was adjusted to pH 7.4 using 1 M Tris–HCl. The solution was then filtered through a 0.22 μ syringe pump filter. Glycyrrhizin and polymixin B were co-incubated along with the combined treatment of rHMGB1 with either LPS or PGN in macrophage culture for 3 or 24 h. All inhibitors were used at the generally recommended concentrations (Davies et al., 2000; Mollica et al., 2007). After inhibitor treatment, cell death was checked by using an MTT [3(4,5)-dimethylthiazol-2,5-diphenyltetrazolium bromide] assay (Mizel, 1982).

2.6. Gene knockdown studies

Macrophages were cultured overnight at a density of 1×10^6 cells/well in 12 well tissue culture plate. TLR2, TLR4, RAGE or scrambled siRNA were used for gene knockdown. Transfection was performed using N-TER Nanoparticle siRNA Transfection System (Sigma–Aldrich; N2913, USA) according to manufacturer's instructions. Final siRNA concentration was 100 nM. Transfection was performed for 5 h. After 5 h medium containing siRNA-complex was removed and replaced by RPMI 1640 supplemented with 10% FCS. Gene knockdown was confirmed after 24 h with real time RT-PCR and after 72 h with western blot analysis.

2.7. Measurement of nitrite production

The concentration of nitrite in the supernatants, the stable end product of NO, was determined on the basis of the Griess reaction (Ding et al., 1988). Nitrite content was quantified by extrapolation from the sodium nitrite standard curve in each experiment.

2.8. Real time RT-PCR analysis

Total RNA was isolated from the macrophages by TRI reagent according to suppliers' instructions. Real time RT-PCR was done using single step real time RT-PCR kit (Qiagen, Hilden, Germany) in Bio-Rad iQ5 real time PCR machine (Bio-Rad Laboratories, Hercules,

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