Systemic inflammation and remote organ injury following trauma require HMGB1

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Toll-like receptors (TLR) are a family of evolutionarily conserved pattern recognition receptors known to drive the innate immune response to infection. Originally identified for its role in LPS recognition (33), TLR4 is now known to be involved in the systemic inflammatory response to sterile injury (18, 21). TLR4 signaling can be activated by multiple endogenous damage-associated molecular pattern molecules including the nuclear protein high-mobility group box 1 (HMGB1) (29, 30, 50).

Cytokine-like properties of HMGB1 were initially described in models of sepsis (44). HMGB1 is now known to be a key mediator of inflammation in models of sterile injury, including hemorrhagic shock and hepatic ischemia-reperfusion (16, 42, 48). The interaction of HMGB1 with TLR4 has been verified in cell lines (29, 30, 50). Thus, an HMGB1-TLR4 interaction could be a critical step in the initial inflammatory response to injury; however, the role of HMGB1 in the systemic inflammatory response following extremity fractures in the absence of shock is unknown.

Using a mouse model of bilateral femur fracture, we have shown that the systemic inflammatory response following peripheral tissue injury requires TLR4, but not TLR2 or cluster of differentiation (CD) molecule (CD14) (18). Accordingly, we carried out experiments to test the hypothesis that HMGB1 is a mediator of systemic inflammation and remote organ injury after peripheral tissue injury. Here, we show that neutralizing antibodies to HMGB1 prevents the early systemic inflammation and end-organ damage that follows bilateral femur fracture in TLR4-wild-type (TLR4-WT) mice; however, no further protection is seen in TLR4-mutant (TLR4-Mu) mice receiving anti-HMGB1. These studies underscore the profound importance of both TLR4 and HMGB1 in the initiation of systemic inflammation and remote organ responses following peripheral tissue injury.

MATERIALS AND METHODS

Reagents. All reagents were from Sigma (St. Louis, MO) unless otherwise indicated. Polyclonal antibodies against HMGB1 were prepared as described previously (46). Polyclonal antibodies against HMGB1 were raised in rabbits, and titers were determined by immunoblotting. Anti-HMGB1 antibodies were affinity-purified by using...
coated in carbonate buffer at 1
HMGB1 (cat. no. MAB1690; R&D Systems, Minneapolis, MN) was
inhibiting >80% of HMGB1-induced TNF release.

Animals. Mice used in the experimental protocols were housed in accordance with University of Pittsburgh and National Institutes of
HMGB1-stimulated macrophage cultures by assay of TNF release. In the
presence of anti-HMGB1 antibody, neutralizing was defined as
inhibiting >80% of HMGB1-induced TNF release.

Bone fracture model. This research protocol complied with the
regulations regarding the care and use of experimental animals published
by the NIH and was approved by the Institutional Animal Use
Circulating HMGB1 levels by ELISA.

RESULTS

Neutralizing antibodies to HMGB1 decrease systemic in-
flammation following bilateral femur fracture. Systemic levels
of IL-6 and IL-10 correlate with severity of injury as well as
both morbidity and mortality in human trauma patients (31, 40).
Experimental data have demonstrated an increase in these
cytokines within human fracture site hematomas, as well as in
the systemic circulation of patients sustaining fracture (9). We
therefore used levels of IL-6 and IL-10 as markers of the
systemic inflammatory response to fracture in vivo.

To determine whether endogenous HMGB1 contributes to
systemic inflammation and end-organ injury after peripheral
tissue injury, we administered neutralizing antibodies to
HMGB1 to C3H/HeOuJ (TLR4-WT) and C3H/HeJ (TLR4-Mu)
mice subjected to bilateral femur fracture. Animals recei-
ved either 600 μg of anti-HMGB1 antibody or irrelevant IgG
(Sigma-Aldrich) via intraperitoneal injection. MAP was main-
tained above 60 mmHg throughout the experiment with the adminis-
tration of lactated Ringers solution (Baxter, Deerfield, IL) through the
femoral cannula as needed in 0.1-ml boluses. This procedure served to
ensure the absence of fracture site hematomas. Serum from postmor-
tem blood samples was obtained for cytokine and blood chemistry
analysis. Organs were snap frozen in liquid nitrogen for molecular
analysis.

Serum aminotransferase assay. To assess hepatocellular injury
following bilateral femur fracture, serum alanine aminotransferase
(ALT) levels were measured by using the Opera Clinical Chemistry
System (Bayer, Tarrytown, NY).

Serum IL-6 and IL-10 assay. Serum IL-6 and IL-10 levels were
used as a means of evaluating systemic inflammation and were quantified with ELISA kits (R&D Systems, Minneapolis, MN) carried
out according to the manufacturer’s instructions.

EMSA. NF-κB DNA binding activity was measured by EMSA by
using nuclear extracts prepared from liver and ileal gut mucosa tissue.
Livers and ileal gut mucosa harvested at the conclusion of the
experimental protocol were snap frozen in liquid nitrogen and stored
at −80°C. Preparation of nuclear extracts and performance of EMSA
were carried out as previously described (18).

Circulating HMGB1 levels by ELISA. Mouse monoclonal anti-
HMGB1 (cat. no. MAB1690; R&D Systems, Minneapolis, MN) was
coated in carbonate buffer at 1 μg/ml in a 50 μl/well volume in
96-well Nunc Maxisorp plates (Nalge Nunc International, Rochester,
NY) and allowed to incubate overnight at 4°C. The plates were
blocked with 4% BSA in PBS overnight at 4°C in a volume of 200
μl/well. Human purified recombinant HMGB1 produced in Esche-
richia coli was used as a standard. Blocked plates were incubated with
a final volume of 50 μl/ml of standards, and samples were diluted in
PBS plus 4% BSA at room temperature for 1 h on a shaking platform
to allow capture of the HMGB1. Plates were washed three times in
PBST (PBS plus 0.2% Tween-20) for 10 min each in 200 μl. The
detection antibody is a peptide polyclonal antibody raised in rabbits
to the human HMGB1 peptide sequence 5′-KPDAAKKGVVKAEEKS-3′
and was incubated at 0.5 μg/ml for 1 h at room temperature in PBS plus
4% BSA. The samples were then washed three times with PBST for
10 min each in 200 μl. Donkey anti-rabbit horseshadish peroxidase
(Jackson ImmunoResearch, West Grove, PA) was used as the final step
for detection of the rabbit anti-HMGB1 and was incubated for 1 h at
room temperature with shaking in a final volume of 50 μl in PBS plus
4% BSA. Plates were washed again for three times for 10 min each in
200 μl of PBST and then developed with 3,3′,5,5′-tetramethyl benz-
diene peroxidase substrate (Pierce Endogen, Rockford, IL) according to
the manufacturer’s instructions. The signal was read at 450 nm by
using a Safire plate reader (Tecan, Durham, NC). Sample concentra-
tions were determined using the freeware ELISA analysis program
Titr, version 5.04.

Statistical analysis. Results are expressed as means ± SE. Group
comparisons were assessed using the Student’s t-test or Mann-
Whitney’s rank sum test. The null hypothesis was rejected for P <
0.05. Data were analyzed using SigmaStat version 3.1 (SPSS, Chi-
ago, IL). For comparisons between C3H/HeOuJ and C3H/HeJ mice,
the sample size was six animals per group.
Neutralizing antibodies to HMGB1 in the central organ response to peripheral traumatic injury.

Neutralizing antibodies to HMGB1 prevent activation of gut NF-κB following bilateral femur fracture. Intestinal epithelial inflammation and dysfunction have been implicated in the development of multiple organ dysfunction in trauma patients (8, 32). We and others have demonstrated that TLR4 is present on the apical surface of intestinal cells (1, 4, 24). To determine whether gut epithelial cell inflammation occurs after femur fracture in a TLR4-dependent manner, we analyzed jejunal and ileal mucosal tissue for evidence of NF-κB activation. Mucosal scrapings from the jejunum and ileum of mice subjected to femur fracture were harvested for analysis of NF-κB DNA binding. Similar to what was seen in the liver, NF-κB activa-
tion was increased in TLR4-WT mice subjected to femur fracture and receiving control IgG (Fig. 3). TLR4-Mu mice subjected to a similar injury did not demonstrate increased NF-κB activation. TLR4-WT mice receiving anti-HMGB1 prior to fracture, however, demonstrate levels of NF-κB activation similar to those seen in TLR4-Mu mice. These data suggest that the local intestinal inflammatory response to injury is dependent on both TLR4 and HMGB1.

HMGB1 is elevated in the serum early after peripheral tissue injury in both TLR4-WT and TLR4-Mu mice. Having shown that both the systemic inflammatory response and the end-organ postinjury responses to femur fracture are dependent on HMGB1, we assessed HMGB1 levels in the systemic vascular compartment. Using a specific ELISA, we found an increase in serum HMGB1 levels in both TLR4-WT and TLR4-Mu mice at 1 h postinjury (Fig. 4A). Levels were significantly increased from those seen in sham-treated animals in both TLR4-WT and TLR4-Mu mice. No significant differences were seen between TLR4-WT and TLR4-Mu mice subjected to fracture. Interestingly, a small but significant increase in serum HMGB1 levels was noted after sham operation in TLR4-WT mice compared with controls. No such increase was noted in TLR4-Mu mice. This finding may indicate that a lower threshold of injury is required for HMGB1 release in TLR4-Mu mice. The increase in HMGB1 levels after fracture was not seen at the 6 h time point (Fig. 4B).

**DISCUSSION**

HMGB1 is a 30-kDa DNA-binding protein present within the nuclei of most eukaryotic cells (19). Based on the seminal work by Wang and colleagues (44) and Andersson et al. (2), HMGB1 is now known to embody many of the attributes of a proinflammatory cytokine. As with many damage-associated molecular pattern molecules, a large intracellular reservoir of HMGB1 exists within the cell, only to be released during periods of cellular damage or necrosis, stimulating an inflammatory cascade. Our laboratory and others have demonstrated a key role for HMGB1 in the initiation and propagation of inflammation and organ injury in settings of sterile inflammation involving ischemic insults (16, 26, 42, 48). Unlike ischemia-reperfusion injury and hemorrhagic shock, however, femur fracture does not appear to involve a period of either local or systemic hypoperfusion. Although small transient perfusion deficits may occur, these stresses alone seem unlikely to drive the robust systemic inflammatory response that follows injury. The purpose of this study was to test the hypothesis that HMGB1 is an early mediator of systemic inflammation and end-organ injury after peripheral tissue injury. We found that neutralization of HMGB1 using a polyclonal antibody prevents systemic and remote inflammation as well as organ injury after bilateral femur fracture. We also found that transient elevations in serum HMGB1 occur within 1 h following bilateral femur fracture.

Long bone fractures with associated soft tissue injury contribute to the systemic inflammatory response following trauma...
(22). Isolated peripheral tissue injury stimulates a local inflammatory response but also sets in motion a systemic inflammatory cascade leading to remote organ injury (23). This injury response is mediated by the release of proinflammatory cytokines and chemokines as well as hepatic acute-phase proteins, arachidonic acid metabolites, complement components, reactive oxygen species, proteolytic enzymes, and neuroendocrine hormones (15, 36, 49). Clinical studies have demonstrated that levels of many of these inflammatory mediators, such as IL-6, IL-8, and IL-10, correlate closely with severity of injury and complication rates (5, 10, 20, 25, 38). The traumatic injury response may lead to the systemic inflammatory response syndrome or overwhelming multiple organ dysfunction syndrome. Neutralizing HMGB1 almost completely blocks the systemic and local manifestations of inflammation in our femur fracture model as measured by IL-6 and IL-10 levels. Whether the production of other mediators known to be elevated early after injury is simultaneously affected is unknown. These observations do suggest that the release of HMGB1 either from the site of injury or from remote sites, such as the liver or gut, are proximal events in the systemic response to severe peripheral tissue injury.

TLRs are a family of molecules that link innate and adaptive immunity (13, 39) and have been implicated in a range of human pathologic conditions including sepsis, atherosclerosis, asthma, and autoimmune diseases (3, 28, 34, 45). TLR2, TLR4, and TLR9 may be unique among TLR family members in their ability to recognize both exogenous and endogenous ligands (17, 29, 30, 35, 50). In addition to LPS (33), TLR4 recognizes heparan sulfate (14), heat shock proteins (27), and HMGB1 (29, 30, 50) among other endogenous substances. Recent evidence has demonstrated that TLR4 signaling plays a key role in tissue injury (21). In vivo studies involving hemorrhagic shock (3, 34) as well as cardiac, (28) renal, (45) and hepatic ischemia/reperfusion injury (42) have implicated TLR4 signaling in the inflammatory and organ damage responses to these diverse stresses. These studies have reported reduced tissue damage in TLR4−/− animals compared with TLR4 WT mice. Interestingly, neutralizing antibodies to HMGB1 mimic the TLR4-deficient/mutant state in both hemorrhagic shock (16) and hepatic I/R (42). These findings, combined with data showing a TLR4-HMGB1 interaction (29, 30, 50), suggest that HMGB1 released from injured or stressed cells plays a key role in the systemic inflammatory response following extremity fracture. Our studies do not address how HMGB1 may lead to TLR4 signaling nor are the anatomic sites of this interaction identified. Studies using TLR4 chimeric mice have shown that TLR4 on both parenchymal and bone marrow-derived cells is required for full activation of the systemic inflammatory response to bilateral femur fracture (Mollen KP, Levy RM, Prince JM, Hoffman RA, Scott MJ, Kaczorowski DJ, Vallabhani R, Vodovotz Y, Billiar TR, unpublished observation). Thus, numerous levels of interaction may take place.

Recently published data suggest that very highly purified HMGB1 has only minimal cytokine-like activity (37). Since HMGB1 avidly interacts with numerous other substances, including bacterial substances, prokaryotic and eukaryotic DNA, and phospholipids, it seems plausible to suggest that the inflammatory trigger associated with femur fracture might not be HMGB1 per se but rather one or more complexes of HMGB1 with other endogenous substances released as a result of bony and soft tissue injury. It is also plausible to suggest that HMGB1 interacts with bacterial LPS, facilitating its proinflammatory activity in the setting of sterile injury. It has been hypothesized that bacterial translocation may provide a source for systemic LPS in the setting of trauma (32). However, our own studies using germ-free mice demonstrated that the presence or absence of intestinal flora does not impact the level of systemic inflammation in the setting of femur fracture (Levy RM, Prince JM, Mollen KP, Kaczorowski DJ, Liu S, Fink MP, Vodovotz Y, Billiar TR, unpublished results). These data are consistent with earlier results using gnotobiotic mice in the setting of hemorrhagic shock (47).

In summary, this study demonstrates for the first time a significant contribution of HMGB1 in the initiation and propagation of systemic inflammation and end-organ injury following peripheral tissue trauma. Further work is necessary to understand the nature and site of interaction between HMGB1 and TLR4 after injury. Our data indicate that neutralizing antibodies to HMGB1 are profoundly protective in our injury model and that systemic levels of HMGB1 are elevated in both TLR4−/− and TLR4−/− animals as early as 1 h after injury. This finding suggests that HMGB1 is released systemically, allowing it to act as an early mediator of sterile inflammation after injury. Our work provides evidence that interventions to inhibit HMGB1 release or its interaction with TLR4 may be effective strategies to limit systemic inflammatory response syndrome following traumatic injury. Understanding how HMGB1 both recruits and activates innate immune effectors as well as how it is released from stressed cells are areas of active investigation in our laboratories (6, 12, 43).

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DISCLOSURES

M. P. Fink and K. J. Tracey are cofounders of Critical Therapeutics, which is developing therapeutic agents targeting HMGB1.

REFERENCES


